Antitumor Activity of Human Papillomavirus Type 16 E7–Specific T Cells against Virally Infected Squamous Cell Carcinoma of the Head and Neck

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Abstract

Human papillomavirus (HPV)–associated squamous cell carcinoma of the head and neck (SCCHN) seems to be a suitable target for cancer vaccination. HPV-encoded oncoproteins, such as E7, are promising tumor-specific antigens and are obligatory for tumor growth. Because few immunologic studies have analyzed the endogenous HPV-specific immune response in this subset of SCCHN patients, we studied T-cell frequencies against HPV-16 E711-20 or E786-93 in tumor-bearing, human leukocyte antigen (HLA)-A*0201+ SCCHN patients, whose tumors were either HPV-16+ or HPV-16−. In HPV-16+ SCCHN patients, frequencies of T cells against either peptide were significantly elevated (P < 0.005) compared with HPV-16− patients or healthy volunteers. Tetramer+ T cells showed evidence of terminally differentiated phenotype (CD15RA−CCR7+) and an elevated level of CD107a staining for degranulation. Despite detectable expression of the restricting HLA class I allele, HLA-A*0201-E711-20– or HLA-A*0201-E786-93–specific CTL obtained by in vitro stimulation of healthy donor peripheral blood mononuclear cells only recognize a naturally HPV-16-transformed, HLA-A*0201+ SCCHN cell line after pretreatment with IFN-γ. This cell line had little or no expression of LMP2, TAP1, and tapasin, critical components of the HLA class I antigen-processing machinery, which were up-regulated by IFN-γ treatment. Immunohistochemistry of HPV-16+ SCCHN tumors showed that these antigen-processing machinery components are down-regulated in tumors in vivo compared with adjacent normal squamous epithelium. Thus, immunity to HPV-16 E7 is associated with the presence of HPV-16 infection and presentation of E7-derived peptides on SCCHN cells, which show evidence of immune escape. These findings support further development of E7-specific immunotherapy and strategies for up-regulation of antigen-processing machinery components in HPV-associated SCCHN. (Cancer Res 2005; 65(23): 11146-55)

Introduction

Oncogenic human papillomavirus (HPV) subtypes (16 and 18) seem to play a role in the etiology of a subset of squamous cell carcinomas of the head and neck (SCCHN), particularly those arising in the oropharynx (1, 2). HPV infection has been detected in 20% to 30% of tumors located in all head and neck anatomic subsites and ~50% of tonsil squamous cancers. The pathogenesis of head and neck cancer may differ from that of cervical cancer and immunologic studies of these patients are necessary. The lower rate of carcinogenic risk factors and p53 mutations and a younger patient population suggests that unique factors are associated with viral entry, propagation/transformation, and immune evasion in HPV-associated SCCHN patients (1, 3). However, few studies have characterized endogenous T-cell immunity to HPV-encoded oncoproteins, E6 or E7, in SCCHN patients, to facilitate immunotherapeutic targeting of these antigens. In patients with cervical carcinoma (a disease more frequently associated with HPV), circulating HPV E7 tetramer+ T cells were detectable (4). Determining whether infection with HPV-16 significantly alters the frequency and functional capacity of virus-specific T cells in SCCHN patients and defining the immunogenicity of HPV-16-encoded antigens are factors critical to developing vaccine-based strategies for enhancing antitumor immunity in patients with HPV+ tumors.

In addition to the presence of HPV-specific effector T cells, successful tumor elimination requires that HPV-infected tumor cells function as appropriate targets for CTL recognition and elimination. However, until now, no study has shown whether naturally HPV-16-transformed SCCHN cells endogenously process and present HPV-encoded peptides for T-cell recognition (5, 6). We used HPV-16 E711-20 or E786-93 peptide-loaded tetramers to compare the frequencies, phenotypic attributes, and functional attributes of CD8+ T cells from human leukocyte antigen (HLA)-A*0201+ HPV-16+ and HPV-16− SCCHN patients compared with those from healthy HLA-A*0201+ control subjects. Our data provide evidence that endogenous E7-specific immunity exists even in the presence of ongoing virus-associated malignancy, perhaps due to immune escape of tumor cells from CTL recognition by down-regulation of some antigen-processing machinery component expression. E7-derived peptides may be useful vaccine targets to facilitate HPV-specific immunotherapy of SCCHN, and strategies to reverse antigen-processing machinery component defects should be considered.

Materials and Methods

Patients and peripheral blood mononuclear cells. Consecutive patients with oropharyngeal or laryngeal SCCHN (n = 18) were seen in the outpatient clinics at the University of Pittsburgh Cancer Institute. Peripheral blood mononuclear cells (PBMC) from eight healthy HLA-A*0201+ donors were also collected. All subjects signed written, informed Institutional Review Board–approved consent, and SCCHN patients...
included primary tumor sites as follows: tonsil (n = 10), base of tongue (n = 4), and larynx (n = 4). Peripheral venous blood (30-40 mL) was drawn into heparinized tubes and immediately delivered to the laboratory for lymphocyte recovery on Ficoll-Hypaque gradients. The elapsed time between phlebotomy and PBMC staining for flow cytometry was 1 to 2 hours. The HPV-16, HLA-A*0201 SCCHN cell line, UPEC5SCC90 (hereafter SCC90), derived from a patient treated at the University of Pittsburgh for a T2N1 squamous cell carcinoma of the base of tongue, has been recently characterized (7, 8).

Human papillomavirus-16 determination. Briefly, expression of the type-specific HPV-16 E6 and E7 genes was investigated by reverse transcription-PCR (RT-PCR) analysis as described (7). RNA was isolated using ULTRASPEC RNA isolation system (Biotecx, Houston, TX) according to the protocol of the manufacturer. The cDNA synthesis was done at 37°C for 1 hour in a final volume of 20 μL using 1 μg of total RNA template, 0.5 μg of oligo(dT)12-18, 10 mMol/L deoxyribonucleotide triphosphate (dNTP), 30 units of RNase inhibitor, and 200 units of Moloney murine leukemia virus reverse transcriptase. Expression of the HPV-16 E6 and E7 genes was studied using the primer pair as described (7, 9). The expression of the cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a control. PCR was done in a 50 μL volume containing 50 mMol/L KCl, 10 mMol/L Tris (pH 8.3), 1.5 mMol/L MgCl2, 0.01% gelatin, 200 μMol/L dNTP mix, 0.4 μMol/L of each primer, and 2.5 units of the Taq DNA polymerase. The DNA was denatured at 94°C for 5 minutes, followed by 40 PCR amplification cycles that consisted of denaturation (94°C, 1 minute), annealing (55°C-60°C, 1 minute), and extension (72°C, 2 minutes). An additional extension step of 72°C for 5 minutes was included at the end of the reaction. The PCR products were analyzed by electrophoresis on 1% agarose gels. HPV-16 tumors were also found to be negative by consensus primer PCR, using M09/M11 primer pairs that amplify an ~450 bp conserved region of the L1 gene of all HPVs (10). Quantitative RT-PCR for E7 expression was done as described (7). The relative expression was calculated according to that of the endogenous housekeeping gene, β-glucuronidase, using the 2-ΔΔCt calculation.

Cytokines. Granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1β, IL-4, and tumor necrosis factor-a were purchased from R&D Systems (Minneapolis, MN). IFN-γ was purchased from Intermune (Brisbane, CA).

Antibodies. The monoclonal antibodies (mAb) used for surface staining of CD8 and CD45RA were purchased from Becton Dickinson, Inc. (San Jose, CA). Anti-CD166a (LAMP1, clone H4A3) was purchased from Research Diagnostics (Concord, MA) and mAb to CCR7 was from R&D Systems. Isotype control antibodies were purchased from Beckman Coulter, Inc. (Fullerton, CA). All patients were initially phenotyped for HLA-A2 by flow cytometry of their PBMCs using mAbs BB7.2 and MA2.1 (produced by hybridomas obtained from American Type Culture Collection, Manassas, VA), confirmed in some patients by DNA-based PCR methods.

HLA-A-, HLA-B-, and HLA-C-specific mAb W6/32, HLA-A2- and HLA-A68-specific mAb BB7.2, hμ-microglobulin-specific mAb L368, HLA-DR, HLA-DQ, HLA-DP-specific mAb LGII-612-14, δ-specific mAb SY-4, MB-1-specific mAb SJ3, low-molecular-weight protein (LMP) 2–specific mAb SY-1, LMP7-specific mAb SY-3, LMP10-specific mAb TO-7, calnexin-specific mAb TO-5, calreticulin-specific mAb TO-11, ERp57-specific mAb TO-2, and tapasin-specific mAb TO-3 were developed and characterized as described (11-15). The TAP1-specific mAb NOB-1 and the TAP2-specific mAb NOB-2 are secreted by hybridomas derived from the fusion of murine myeloma cells P3-X63-Ag8.653 with spleenocytes from BALB/c mice immunized with partial length TAP1 recombinant protein (amino acids 434-735) and a keyhole limpet hemocyanin-conjugated TAP1 peptide (amino acids 717-735) and with partial length TAP2 recombinant protein (amino acids 316-703), respectively. The specificity of the mAb was assessed by their reactivity with molecules with the size corresponding to the immunizing TAP1 and TAP2 when tested in Western blotting with a lysate of lymphoid cells that express TAP1 and TAP2 and by the lack of reactivity with a lysate of the T2 cell line, which does not express these molecules (16, 17).

Peptides and tetrarmers. HPV-16 E711-20 and E786-93 were synthesized at the University of Pittsburgh Peptide Synthesis Facility, using f-moc technology. The Influenza Matrix58,68 peptide was synthesized by Commonwealth Biotechnology (Cambridge, MA). All peptides were purified >90%, confirmed by mass spectrometry, and stored lyophilized until use, when they were dissolved at 1 mg/mL in PBS. The APC-labeled HPV-E711-20 and E786-93 and the phycocerythrin-labeled HIV-1 reverse transcriptase peptide (pOL476-483) ILK3VH4G tetrarmers were obtained through the National Institute of Allergy and Infectious Diseases Tetrarmer Facility (Atlanta, GA). Specificity of E7 peptide-specific tetrarmers was done by flow cytometric detection of HLA-A*0201–restricted CD8+ T cells generated by vaccination of HLA-A*0201–transgenic mice (Jackson Laboratories, Bar Harbor, ME). Two groups of HLA-A*0201–restricted murine T cells, pooled from spleens of three mice immunized i.p. with dendritic cell loaded with each peptide, were stained using the HPV-16 E711-20 tetrarm. For control staining, the E711-20, tetrarmer was used to detect nonspecific binding in mice immunized against HLA-A*0201-E786-93 and the HLA-A*0201-E786-93 tetrarm was used to detect nonspecific binding in mice immunized against E711-20 (data not shown). Titration of tetrarmers and specificity assays were previously described (4), and the same procedures were followed in this study.

Flow cytometry of antigen–specific T cells. For tetrarm flow cytometry of human PBMC, a combination of tetrarm, CD8, CD45RA, and CCR7 was used to determine the differentiation of antigen-specific CD8+ T cells (18). Negative control staining was done as described (19). To quantify antigen-specific cytotoxic CD8+ T cells, PBMC were surface stained with CD8, tetrarm, and CD107a as described by Betts et al. (20), except that monensin was not used. Nonspecific background CD107a staining of tetrarm+ T cells was determined using isotype-matched control mAb as well as by staining healthy control PBMC. Briefly, for tetrarm staining, 15 μL aliquots of diluted tetrarm stock (1/100) were added directly to cell pellets (5×10^6 cells) and cells were labeled for 30 minutes at room temperature in the dark. Next, 5 μL aliquots of the labeled mAbs for surface staining were added, and cells were incubated for 30 minutes at 4°C in the dark. Cells were washed twice with flow buffer and fixed by adding 300 μL of a 2% paraformaldehyde solution. The samples were analyzed within 12 hours using a Becton Dickinson FACScan/ibur. List mode files were analyzed using EXPO32 ADC software (Beckman-Coulter). At least 2 million events were acquired for each sample. To determine the threshold over background staining, the appropriate fluorescence minus one control (the sample was stained with all, except the reagent of interest) or isotype controls were done. Background staining of patient PBMC was determined using empty tetrarm of the HIV-1 reverse transcriptase peptide (pol 476-483), ILK3VH4G-loaded (E7575-5840).

Generation of murine dendritic cell from bone marrow and immunization of A2.1-k+B transgenic mice. Dendritic cells from femurs and tibiae of male, 7- to 8-week-old HLA2A2 transgenic mice were prepared as previously described (19). Briefly, bone marrow cells were flushed with PBS using a 21 G needle. After clusters within the marrow suspension were disintegrated by vigorous pipetting and washing in PBS, the suspension of leukocytes was cultured in bacteriologic 100 mm Petri dishes (Falcon No. 1029/Becton Dickinson, Heidelberg, Germany) at 2×10^9 cells in 10 mL culture medium. Cell culture medium was RPMI 1640 (Life Technologies, Eggenstein, Germany) supplemented with penicillin (100 units/mL, Sigma, St. Louis, MO), streptomycin (100 μg/mL, Sigma), l-glutamine (2 mMol/L, Sigma), 2-mercaptoethanol (50 mMol/L, Sigma), 10% heat-inactivated and filtered FCS (Mediatech, Herndon, VA), 10 ng/mL of murine GM-CSF, and 5 ng/mL of murine IL-2 were added to the dishes. At day 3, another 10 mL culture medium containing 10 ng/mL GM-CSF and 5 ng/mL IL-2 was added to the dishes. At day 6, half of the culture supernatant was collected, centrifuged, and the cell pellets were resuspended in 10 mL fresh medium containing 10 ng/mL GM-CSF and 5 ng/mL IL-2. At day 9, 10 ng/mL HPV-E711-20 peptide was added and cells were incubated overnight. At day 10, dendritic cells were harvested and resuspended in fresh medium. Immunizations were done by i.p. injection of 0.5× 10^6 dendritic cells to each mouse.

Enzyme-linked immunospot assays. IFN-γ enzyme-linked immunospot (ELISPOT) assays were done as described previously (21). All experiments used negative and positive controls, consisting of T2 cells
Figure 1. Elevated frequencies of HPV-16 E7–specific T cells in HPV-16+ SCCHN patients compared with HPV-16−/C0 SCCHN patients or healthy controls. Summary of quantitative analysis of HPV-16 E711-20–specific (A) and HPV-16 E786-93 specific (B) T-cell frequencies in healthy control PBMC (HD, n = 8), HPV-16+ SCCHN patients (n = 12), and HPV-16−/C0 SCCHN patients (n = 6). Box and whisker plots show median (white bar), interquartile range (box), and farthest point not exceeding 1.5 times the interquartile range (whiskers). C, mean CD8+ tetramer+ T-cell reciprocal frequencies. Differences were statistically significant between HPV-16+ SCCHN patients and HPV-16−/C0 SCCHN patients and healthy donors (P < 0.001). Negative control tetramer consisted of HLA-A2 tetramer loaded with an irrelevant peptide (HIV-1 reverse transcriptase) or no peptide (D) and positive control E711-20–specific tetramer staining (D) of an HLA-A2 E711-20–specific CTL line. E and F, representative flow cytometry plots for HLA-A2 tetramers loaded with E711-20 or E786-93, respectively.
Figure 2. Phenotypic characterization of HPV-16 E7–specific tetramer+ T cells. Flow cytometry for phenotypic and functional markers expressed by HPV-16 E711-20–specific (A) and E786-93–specific (B) CD8+ T cells in healthy donors (n = 8), HPV-16+ (n = 12), and HPV-16– (n = 6) SCCHN patients. The mean frequencies of terminally differentiated/lytic T-lymphocyte tetramer+ (CD45RA+CCR7−) and circulating memory (CD45RA+CCR7+) T cells specific for E711-20 (A) is depicted as described in Materials and Methods.

C, a representative flow cytometry experiment from an HPV-16+ SCCHN patient, indicating phenotypic analysis and results obtained. D, significantly greater CD107a binding of E711-20 or E786-93 tetramer+ cells in HPV-16+ SCCHN patients compared with bulk CD8+ T cells from these patients (P < 0.01). E, representative staining of an HPV-16+ SCCHN patient’s PBMC in bulk CD8+ CTL (left and middle, top) compared with E711-20 or E786-93 tetramer+ cells (right, top). An example of an HPV-16+ SCCHN patient’s PBMC stained for CD107a in bulk CD8+ CTL (left and middle, bottom) compared with E711-20 or E786-93 tetramer+ cells (right, bottom). Lower total spots are observed in the HPV-16+ SCCHN patient’s PBMC due to significantly lower frequency of E7 tetramer+ cells.

<table>
<thead>
<tr>
<th>Phenotypic Characterization</th>
<th>HPV-16 E711-20</th>
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<tr>
<td>CD45RA+CCR7− tetramer+ T cells in healthy donors (n = 8)</td>
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<td>28%</td>
</tr>
<tr>
<td>CD45RA+CCR7+ T cells in healthy donors (n = 8)</td>
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<td>27%</td>
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Enrichment of CD107a+ tetramer+ cells in HPV+ SCCHN vs HPV− individuals

<table>
<thead>
<tr>
<th>HPV− mean</th>
<th>HPV+ mean</th>
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</thead>
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<tr>
<td>% CD107a+ cells of tet 11-20 CD8+</td>
<td>61.0</td>
</tr>
<tr>
<td>% CD107a+ cells of tet 86-93 CD8+</td>
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<tr>
<td>% CD107a+ of total CD8+</td>
<td>1.2</td>
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CD107a expression on E7-tetramer+ CD8+ T cells

- Gated on CD8 and HPV11,20-A2 Tetrimer
- Gated on CD8 and HPV86,93-A2 Tetrimer
- Gated on CD8

HPV− patient

<table>
<thead>
<tr>
<th>CD107a</th>
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<tr>
<td>55.9%</td>
<td>46.1%</td>
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HPV+ patient

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<tr>
<th>CD107a</th>
<th>CD107a</th>
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<tr>
<td>35.3%</td>
<td>40.4%</td>
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without peptide, with negative control influenza matrix58-66 peptide, for minimum IFN-γ spots or pulsed with tumor antigen peptide (1 μmol/L for 2 hours at room temperature) or phorbol 12-myristate 13-acetate/ionomycin-treated CTL for maximum spots, respectively. T2 cells loaded with tumor antigen peptide were incubated with either W6/32, BB7.2, or L243 (mAb directed against HLA-DR) as an irrelevant antibody. These mAbs were incubated at 10 μg/mL for 30 minutes to confirm HLA class I and HLA-A*0201 antigen–restricted, but not HLA class II antigen–restricted T-cell activity. The plates were read using a Carl Zeiss VISION ELISPOT reader using standard settings. Background was considered as the number of spots secreted by the CD8+ T cells alone. T-cell reactivity as measured by the ELISPOT assay was considered positive if test wells were significantly greater than background wells when using a one-tailed permutation test at \( z < 0.05 \).

**Immunohistochemistry.** Immunostaining of HPV-16+ SCCHN tumors and surrounding normal specimens was done by the avidin-biotin-peroxidase method using the same cohort of patients from whom PBMC were studied. The color reaction was developed in diaminobenzidine solution, and counterstaining was done with Mayer’s hematoxylin solution (22). Tissues were stained for antigen-processing machinery and HLA using the mAb as described (12, 13). Antibody titrations and isotype control antibodies were used to determine optimal staining conditions. A semiquantitative analysis was done, assessing percentage of tissue (normal and tumor) with staining and the intensity of the staining on a scale of 0 (negative staining) to 3 (strong staining). A composite score multiplying these features was developed for data analysis.

**Statistical methods.** Nonparametric tests were used for data analysis as needed. Data generated by an experiment to test tetramer specificity in murine splenocytes were analyzed by the double permutation test. The Kruskal-Wallis test was applied to the comparison of tetramer frequency and phenotypes among the three groups—HPV+ patients, HPV- patients, and HLA-A*0201 healthy controls. Pairwise contrasts were tested with the Wilcoxon-Mann-Whitney test. Because tetramer+ T cells were characterized by a series of four mutually exclusive and exhaustive phenotypes, the joint distribution of the four phenotypes among the three groups was compared with the Puri and Sen I. statistic.

**Results**

Elevated frequencies of HLA-A*0201-E711-20 and HLA-A*0201-E786-93 peptide–specific T cells in HPV-16+ squamous cell carcinoma of the head and neck patients. Having validated the specificity of tetramer staining for HLA-A*0201+E711-20 or HLA-A*0201+E786-93 as described in Materials and Methods, we used these reagents to stain human PBMC for HPV-16 HLA-A*0201+E7–specific T cells using multicolor flow cytometry. PBMC were obtained from individuals with SCCHN determined to be HPV-16+ or HPV-16− or from healthy HLA-A*0201 donors. Our *ex vivo* tetramer analysis showed that a higher frequency of E7–specific precursor CTL was detected in the PBMC from HPV-16+ SCCHN patients, with transcriptionally active HPV-16+ infection in their tumors (Fig. 1). Patients with HLA-A*0201 HPV-16+ SCCHN possessed significantly higher E711-20 and E786-93–specific tetramer+ T cells than PBMC from HPV-16− SCCHN patients or from healthy control HLA-A*0201 donors. HPV-16+ patients (\( n = 12 \)) were found to have mean CD8+ T-cell frequencies specific for these two peptides (E711-20 and E786-93), of 1/5,068 and 1/2,408 cells in the peripheral blood, respectively. In contrast, HPV-16− patients (\( n = 6 \)) were found to have substantially lower mean CD8+ T-cell frequencies specific for E711-20 and E786-93 of 1/16,612 and 1/5,305 cells in the peripheral blood, respectively (\( P < 0.005 \)). Interestingly, healthy control donors (\( n = 8 \)) were found to have mean CD8+ T-cell frequencies specific for E711-20 and E786-93 of 1/16,661 and 1/4,254 cells in the peripheral blood, respectively, which was similar to the frequency detected in HPV-16− patients’ PBMC.

Although evidence for a low (2–4%) frequency of sporadic oral HPV infection in the general population has been reported (23), this would constitute a minute contribution to our healthy control population. Furthermore, we did not observe any differences in tetramer+ frequencies between HPV-16− SCCHN patients, documented to be HPV−, compared with our healthy controls (Fig. 1A-B).

Phenotypic and functional markers were analyzed on E711-20 or E786-93–specific T cells (Fig. 2A-C). A relatively high proportion of terminally differentiated/lytic phenotype (CD45RA+CCR7−) of antigen-specific T cells in the peripheral circulation was present (24). This population was more prominent in our data set with a mean value of 30% (Fig. 2A), but it did not reach statistical significance over the values found in the healthy donor (22.6%) or HPV-16+ SCCHN patient population (21.6%). This difference was confirmed using E786-93–specific tetramer staining of these cohorts of PBMC (Fig. 2B). Based on these findings, E711-20− and E786-93–specific tetramer+ populations were further characterized for the apparent lytic phenotype, using CD107a staining as a measure of degranulation (Fig. 2D). We found that a significantly higher proportion of tetramer+ T cells (against E711-20 or E786-93) were stained positively for CD107a than bulk population of CD8+ T cells, which was most prominent in the HPV-16+ cohort of subjects (Fig. 2D).

CD8+ T-cell recognition of SCC90 cells requires IFN-γ pretreatment. To explain the presence of significantly elevated frequencies of CD8+ T cells specific for HPV-16 E711-20 or E786-93 in patients with transcriptionally active, HPV-16-infected tumors, we examined whether HPV-16 E7–derived peptides are endogenously processed and presented. An HPV-16 E711-20–specific T cell line was generated by *in vitro* stimulation of PBMC from an HLA-A*0201+ healthy control (Fig. 3A). This CTL line was found to be reactive against T2 cells loaded with E711-20 but not irrelevant peptides (influenza MA58-66 or E786-93) or against T2 cells alone. Titration experiments were done using human CD8+ CTL generated by *in vitro* stimulation of human PBMC from a healthy donor. We evaluated the avidity of these T cells using serial dilutions of E711-20 versus control tetramer (Fig. 3B) and peptide titration assays, indicating a peptide concentration of half-maximal reactivity to be ∼50 to 100 ng/mL (Fig. 3C).

The HLA-A*0201 SCC90 cell line, a naturally HPV-16-transformed SCCHN cell line, expresses high levels of HPV-16 E6 and E7 (7). CTL recognition of SCC90 cells was examined by IFN-γ ELISPOT assay, using CTL specific for HLA-A*0201-E711-20 generated by *in vitro* stimulation of PBMC from a healthy HLA-A*0201+ donor (see Fig. 3). A representative ELISPOT experiment is shown for HLA-A*0201 SCC90 cells (Fig. 4A), showing that significant E711-20–specific CTL recognition of SCC90 cells was observed only after IFN-γ pretreatment with 100 IU/mL for 72 hours at 37°C (\( P < 0.05 \)). This recognition was inhibited using blocking mAb against HLA-A, HLA-B, and HLA-C (W6/32) or HLA-A2 and HLA-A68 (BB7.2), but was not affected by treatment with mAb against HLA class II (anti-HLA-DR) antigens. The experiments described above, leading to CTL recognition of a naturally HPV-16-transformed SCCHN cell line, raised the question as to the mechanism of IFN-γ induction of CTL recognition of SCC90 cells, which could include alterations in level of expression of HPV-16 E7, costimulatory molecules, or antigen-processing machinery.
Lack of detectable changes in tumor antigen expression or costimulatory molecule expression in squamous cell carcinoma of the head and neck cells after IFN-γ treatment. One hypothesis to explain the restoration of recognition by HLA–tumor antigen peptide–specific CTL after IFN-γ treatment is up-regulation of the tumor antigen. However, such treatment of these cells (100 units/mL for 72 hours) resulted in no increase in expression over baseline of HPV-16 E7 (using quantitative

Figure 3. Specificity of human CTL recognition against HLA-A*0201-E7-11-20-CTL reactivity (A) against T2 cells were incubated with no peptide, E7-86-93 peptide, or E7-11-20 peptide before addition of HLA-A*0201-E7-11-20–specific CTL obtained by in vitro stimulation from healthy HLA-A*0201+ donor PBMC. HLA-A*0201-restricted CTL activity was determined by incubating the target cells with no blocking mAb (dark gray columns), HLA-A2-specific mAb (BB7.2 at 10 μg/mL for 30 minutes, white columns), or irrelevant, HLA-DR-specific mAb (L243 at 10 μg/mL for 30 minutes, light gray columns). Specific recognition only of T2 cells pulsed with the E7-11-20 peptide was observed, as measured by IFN-γ ELISPOT activity, by HLA-A*0201-restricted, E7-11-20-specific CTL. B, avidity of the HLA-A*0201-E7-11-20–specific CTL line after serial dilutions of the HLA-A*0201-E7-11-20 tetramer. C, results of CTL recognition after E7-11-20 peptide titration using exogenously loaded T2 cells. A half-maximal recognition of ~50 nmol/L was observed.

Figure 4. E7-11-20 peptide–specific CTL recognition of HPV-16+ SCCHN cell line after IFN-γ. A, recognition of SCC90 cells by HLA-A*0201-restricted, E7-11-20–specific CTL following either incubation of target cells with exogenous E7-11-20 peptide (not shown) or with IFN-γ by ELISPOT IFN-γ assay. T2 cells pulsed with exogenous, irrelevant peptide (E7-86-93) were not recognized by these CTL. HLA-A*0201-restricted CTL activity was determined by incubating the target cells with no blocking mAb (dark gray columns), HLA-A2-specific mAb (BB7.2 at 10 μg/mL for 30 minutes, white columns), or irrelevant, HLA-DR-specific mAb (L243 at 10 μg/mL for 30 minutes, light gray columns). B, quantitative RT-PCR (qRT-PCR) indicates no significant change in E7 expression by SCC-90 cells over baseline expression (hashed column) after IFN-γ treatment (100 units/mL for 72 hours, white column). The relative expression was calculated according to that of the endogenous housekeeping gene, β-glucuronidase, using the 2^−ΔΔCt calculation as described in Materials and Methods. C, costimulatory molecules, CD80 or CD86, are not expressed (blue curve) or up-regulated (red curve) on SCC-90 cells after IFN-γ treatment (100 units/mL for 72 hours)
RT-PCR) after IFN-γ treatment, ruling out this possibility (Fig. 4B). We also tested expression of CD80 and CD86, which were unchanged after IFN-γ treatment (Fig. 4C). The induction of CTL recognition after incubation with exogenous incubation with exogenous E7 11-20 peptide (not shown) indicated a lack of processing and presentation of E7 peptide, prompting our analysis of antigen-processing machinery component expression in SCC90 cells.

**IFN-γ-induced human leukocyte antigen and antigen-processing machinery component up-regulation in SCC90 cells correlates with E7 peptide-specific CTL recognition.** To explain the lack of recognition of SCC90 cells by HPV-16 E7 11-20–specific CTL, we examined the expression of HLA class I antigens and components of the antigen-processing machinery before and after treatment with IFN-γ (100 units/mL for 72 hours). IFN-γ treatment enhanced expression of HLA and antigen-processing machinery components (see Fig. 5) and this correlated strongly with induction of T-cell recognition, not appreciably seen in SCC90 cells without prior IFN-γ treatment (Fig. 5A). Also, Fig. 5B shows the increase in expression of several antigen-processing machinery components, including the IFN-γ-inducible peptide transporters, TAP1 and TAP2, the immunoproteasome subunits LMP2 and LMP10, and the endoplasmic reticulum–resident chaperone tapasin. The antigen-processing machinery components found to be nearly absent were LMP2, TAP1, and tapasin, which were up-regulated by IFN-γ. This implicated a role for this pathway in resistance to CTL recognition at basal conditions in vitro.

Expression of antigen-processing machinery components that are not responsive to IFN-γ, namely the endoplasmic reticulum–resident chaperones calnexin and calreticulin, showed stable expression after IFN-γ treatment (Fig. 5C). Likewise, the proteasome subunits δ (Y) and MB1 (X) were expressed at stable or slightly decreased levels, likely reflecting up-regulation of the IFN-γ-inducible β-subunits, LMP2, LMP7, and LMP10, as described above. Thus, IFN-γ-dependent induction of CTL recognition was strongly associated with sufficient expression of IFN-γ-inducible antigen-processing machinery components.

To determine whether antigen-processing machinery component down-regulation in HPV-16+ SCCHN tumors was observed in a larger number of tumors in vivo, expression of HLA class I antigens and antigen-processing machinery components was measured by immunohistochemistry (Fig. 6). We found that the levels of expression of HLA and antigen-processing machinery components frequently decreased in HPV-16+ SCCHN tissues (n = 8) compared with surrounding normal squamous epithelium. Figure 6H shows the down-regulation of LMP2 in eight HPV-16+ SCCHN tissues, graphed according to the semiquantitative immunohistochemical staining (P < 0.05). These data suggest that low expression of antigen-processing machinery

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**Figure 5.** Expression of HLA and antigen-processing machinery components in HPV-16+ cells. A, expression of the total HLA class I antigens, β2m, and HLA-A*0201. B, up-regulation of antigen-processing machinery components, particularly LMP2, TAP1, and tapasin before (solid line) or after (dotted line) IFN-γ treatment. C, expression of constitutive antigen-processing machinery components, the endoplasmic reticulum–resident chaperones, before (blue) or after (red) IFN-γ treatment (100 units/mL for 72 hours).
components is generally found in vivo consistent with our in vitro findings.

Discussion

Our findings indicate that HPV+ SCCHN patients possess elevated frequencies of E7\textsubscript{11-20}-specific T cells that recognize HPV-16 E7+ SCCHN cells. Thus, we conclude that antiviral immunity exists against E7 oncogenic proteins despite unsuccessful tumor eradication. Whether direct (tumor) priming or cross-priming, through professional antigen-presenting cells (such as dendritic cells) occurs in vivo is not known. Although rapidly degraded proteins have been shown to be poor substrates for cross-priming of T cells, the E6 and E7 gene products, which target specific tumor suppressor proteins (p53 and Rb, respectively) for proteasomal degradation, may not undergo degradation themselves (25). If this is the case, cross-priming of E7-specific T cells by dendritic cell, presenting E7 peptides derived from HPV-16+ tumors, may account for the differences observed between our study populations. On the other hand, direct priming by tumor cells may lead to ineffective activation and maintenance of a memory T-cell compartment and has been shown to be less efficacious than dendritic cell priming (26, 27) in terms of phenotype and differentiation status (28). Direct priming by tumor cells may lead to a terminal differentiation phenotype that would be expected to be poorly capable of tumor elimination due to previously described T-cell signaling defects (5).

An important finding in our work was the ability of HPV-16 E7\textsubscript{11-20} or E7\textsubscript{86-93}-specific T cells to recognize SCC90 cells after incubation with exogenous E7 peptide or after IFN-γ treatment. Our findings that some antigen-processing machinery components were expressed in the absence of IFN-γ indicated that these proteins are unlikely to be responsible for processing and presentation of HLA-A*0201-E7\textsubscript{11-20} or HLA-A*0201-E7\textsubscript{86-93} peptide complexes, whereas up-regulation of low or absent expression of antigen-processing machinery components (LMP2, TAP1, and tapasin) are more likely to regulate expression of these HLA class I antigen-peptide complexes and thus CTL recognition. The lower expression of these components in HPV-16-infected tumors in vivo, compared with normal adjacent squamous epithelium, is consistent with this conclusion and suggests a general phenomenon of immune escape during viral carcinogenesis.

The ability of a naturally HPV-16 transformed SCCHN cell line to be recognized by HPV-16 E7-specific CTL is significant and provides support for future immunotherapeutic strategies targeting HPV antigens in patients with this disease. It also indicates that, although many authors have studied HPV-associated SCCHN

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**Figure 6.** Down-regulation of antigen-processing machinery component expression in HPV-16+ SCCHN tumor tissues compared with surrounding normal squamous epithelium. A to D, representative immunohistochemical staining for antigen-processing machinery components, LMP2 (A-B) or TAP1 (C-D) or for HLA class I antigens (mAb W6/32, anti-HLA-A, anti-HLA-B, anti-HLA-C; E-F) in HPV-16+ SCCHN lesions (n = 8). Consistently lower expression was observed by semiquantitative immunohistochemistry compared with surrounding normal squamous epithelium (P < 0.01) as summarized in the graph (G). Positive staining for these proteins was observed in surrounding normal squamous epithelium and in tumor-infiltrating stromal cells, confirming the efficacy of mAb staining in each tissue section.
using RNA-based analyses, virally infected SCCHN cells express HPV oncoproteins at the protein level, which can be substrates for processing and presentation to T cells. In addition, our data strongly implicate a role for LMP2, TAP1, and tapasin in generating sufficient HLA-A4*0201-E7 peptide complexes to permit CTL recognition. The conditions used (100 IU/mL of IFN-γ for 72 hours) did not significantly alter expression of costimulatory molecules or the tumor antigen, E7, in SCC90 cells.

Despite the ability of E711-20 specific CTL to recognize a naturally HPV-16-transformed SCCHN cell line in vitro, tumors expressing E7 clearly were present in SCCHN patients in our cohort and were found to have elevated levels of E711-20 tetramer–binding T cells in vivo. Our analysis of nearly absent LMP2, TAP1, and tapasin levels, both critical antigen-processing machinery components necessary for efficient peptide loading onto nascent HLA class I molecules, provides support for the notion of immune escape in SCC90 cells. Whether this is a general phenomenon, as has been reported in other tumor types without viral etiologies, or due to HPV-specific factors, as has been suggested in HPV-6 and HPV-11-associated laryngeal papillomas (29), remains to be clarified. Our preliminary immunohistochemical staining suggests that virally induced SCCHN may progress due to insufficient tumor cell antigen processing and presentation. Other mechanisms of immune dysfunction in cancer patients, such as defective effector responses and function, might also contribute to tumor progression, which we attempted to investigate using multicolor flow cytometry for surface memory and effector markers, including CD107a (Fig. 2B).

Despite some limitations imposed by the number of PBMC available for multicolor flow cytometric lymphocyte subset analyses, we studied phenotypic markers on E711-20-specific, tetramer+ T cells, which we hypothesized might indicate potential mechanisms of resistance of HPV-16+ tumor cells to CTL recognition and elimination (24). A disproportionate level of terminally differentiated/lytic phenotype (CD45RA+CCR7−) antigen-specific T cells (6, 24, 30) were retained in circulating HPV-16 E711-20 tetramer+ T cells (see Fig. 3). This population was further characterized by a high frequency of staining for CD107a in E7 tetramer+ T cells, consistent with their terminal differentiated lytic, degranulated status. These cells may account for the unsuccessful antiviral immune response (5) to these tumors, indicating that incomplete activation of tumor-specific T cells or suboptimal target recognition may enable tumor progression in vivo. On the other hand, the presence of a reservoir of E7-specific circulating memory T cells (CD45RA+CCR7+) may indicate the potential to respond favorably to immunotherapy (31), if appropriately activated and expanded through cancer vaccination. These findings in a pilot cohort must be confirmed in an expanded group of similar subjects to enable validation and further statistical analysis. Finally, further studies must characterize the failure of T-cell recognition and elimination of HPV-16+ tumors in vivo, through such mechanisms as HLA and antigen-processing machinery component defects, such as have been identified in cervical carcinomas (32, 33). Our preliminary data indicate that transfection of SCC90 cells with wild-type TAP1/2 cDNA leads to similar levels of TAPI and TAP2 expression as we show after IFN-γ treatment (100 units/mL for 72 hours). Transfection with TAP1/2 restored CTL recognition of tumor antigen–specific CTL (not shown), indicating the importance of these particular IFN-γ-inducible antigen-processing machinery components in regulating tumor antigen–specific CTL recognition. The constitutive proteasome subunits that we found to be expressed in SCC90 cells may be less crucial in this CTL reactivity. Strategies to enhance antigen-processing machinery component expression and function are likely to result in improved CTL recognition of HPV-16+ tumor cells and may need to be incorporated into T-cell-based immunotherapy against this disease.

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