Identification of a cytotoxic T-lymphocyte epitope in the human papillomavirus type 16 E2 protein

József Kónya, Carina Eklund, Veronika af Geijersstam, Fang Yuan, György Stuber and Joakim Dillner

Microbiology and Tumorbiology Centre and Department of Pathology, The Karolinska Institute, S-171 77 Stockholm, Sweden

Introduction

The human papillomaviruses (HPVs), a group of more than 70 different types, induce proliferation of infected epithelial cells. About one-third of the known HPV types infect the genital mucosa and are further classified as low-risk (HPV-6 and HPV-11) and high-risk types (HPV-16, HPV-18, etc.). The HPV types belonging to the former group may cause benign genital warts whereas the high-risk types are strongly associated with several common human cancers, notably carcinoma of the cervix uteri (zur Hausen & de Villiers, 1994). Anogenital HPV infections are transient in the majority of exposed people (Hildesheim et al., 1994; Evander et al., 1995). However, in some infected individuals the virus establishes a persistent infection that frequently results in development of squamous intraepithelial lesions (SILs) (Koutsky et al., 1992). The SILs can either regress spontaneously, persist or progress into invasive cancer in a process that may take several decades from the time of infection until the development of the invasive cancer (Ponten et al., 1995).

Little is known about the factors influencing the clinical outcome of papillomavirus infections. A protective role of the cellular immune responses is suggested by the increased incidence of papillomavirus-induced lesions and cervical neoplasias among iatrogenically immunosuppressed, organ transplant patients and among symptomatic, HIV-infected persons (Petry et al., 1994). Also, for women exposed to HPV-16, certain human leukocyte antigen (HLA) class II haplotypes (e.g. DR15, DQ6) confer an increased susceptibility to development of cervical neoplasias (Apple et al., 1994; Sanjeevi et al., 1996). Finally, a substantial proportion of cervical cancers exhibit complete or partial loss of HLA class I expression (Keating et al., 1995), implying a possible escape mechanism from immune surveillance.

Although antibodies may protect from challenge with infectious virus (Breitbart et al., 1995), the antibody responses do not appear to have any antiviral effect once HPV infection has been established. Also, some antibodies against early viral proteins may appear only in more advanced stages of HPV-related neoplasias (Dillner, 1995).

In mouse systems, both proliferative (Tindle et al., 1991) and cytotoxic (Felkamp et al., 1993) responses against defined epitopes in the E6 and E7 viral oncogenes have been found.

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Vaccination of mice with an E7 peptide conferred protection against challenge with a tumour cell line induced with HPV-16 (Feltkamp et al., 1993). In vitro stimulation of human lymphocytes with E6 and E7 peptides resulted in cytotoxic T-lymphocyte (CTL) lines capable of lysing the Caski cell line, which carries HPV-16 (Ressing et al., 1995). The E7 and E6 proteins have been the focus of most previous studies, since these proteins are uniformly present in cervical cancers (zur Hausen & de Villiers, 1994). Papillomavirus-containing tumours are also known to present class I-associated peptides of the E6 (Bartholomew et al., 1994) and the E7 (Ressing et al., 1995) oncogenes. However, during natural infection these proteins have restricted expression in infected tissue. Their abundant expression in cervical cancers rather suggests that they are not essential targets of the naturally evoked immune responses mediating virus clearance. Indeed, only a few cervical cancer patients with the relevant HLA haplotype (HLA-A*0201) showed a memory CTL response against an E7 epitope of HPV-16 (Ressing et al., 1996). In contrast, genes coding for the E1 and E2 proteins are frequently lost in cervical cancers (Stoler et al., 1992). Restoration of full-length E2 protein expression results in suppression of cellular proliferation driven by the E6 and E7 viral oncogenes (Dowhanick et al., 1995). The E1 and E2 proteins form a replication complex necessary for maintenance of the episomal viral genome (Turek, 1994) and should thus be uniformly expressed in cells containing episcopal HPV genomes. The possibility that cellular immunity against E1 and E2 proteins may determine the fate of HPV infection has not been thoroughly studied, although proliferative T-cell responses against these proteins have been described in patients with HPV-carrying lesions (Lehtinen et al., 1995).

We wished to investigate whether the HPV-16 E1 or E2 proteins contain epitopes that may be recognized by CTLs. Based on the HLA-A*0201 binding motifs, 9-mer and 10-mer peptides deduced from the nucleotide sequences of the E1 and the E2 open reading frames were screened for HLA-A*0201 binding and the ones with high binding-affinity were tested for their ability to stimulate virus-specific CTLs.

**Methods**

- **Peptide-binding assay.** Peptides with free carboxyl ends were synthesized by F-moc chemistry. Homogeneity of peptides was > 90% as determined by HPLC. HLA-A*0201 binding was measured as previously described (Stuber et al., 1995). Briefly, the peptide-processing-defective T2 cell line was seeded into 96-well plates (10⁵ cells per well) and incubated with the peptides overnight at 37 °C in RPMI 1640 medium buffered with 10 mM HEPES. The next day, the plate was put on ice and stained with the HLA-A2-specific monoclonal antibody BB7.2 (diluted in PBS, 1:1000), followed by a rabbit anti-mouse immunoglobulin–FITC conjugate (DAKO, 1:40 in PBS). After each step, the cells were washed three times in ice-cold PBS. Fluorescence intensity (FI) was measured in a flow cytometer (FACSort, Becton Dickinson). Samples with mean FI above the mean FI of cells incubated without peptide plus 2 × SD were regarded as positive.

- **HPV-16 capsid ELISA.** ELISA plates were coated overnight at 4 °C with HPV-16 recombinant virus-like particles (1 µg in 1 ml PBS) kindly provided by J. T. Schiller at NCI, Bethesda, Md., USA (Kirnbauer et al., 1994). Next morning, the plates were emptied, washed three times with PBS and blocked with 10% heat-inactivated horse serum in PBS (HS–PBS) for 1 h at 37 °C. Plasma samples diluted 1:30 in HS–PBS were added to both antigen-coated and uncoated wells and incubated for 1 h at 37 °C. After five washes in PBS–0.05% Tween 20, an anti-human IgG monoclonal antibody (Eurodiagnostica, 1:800 in HS–PBS) was added for 1 h at 37 °C. After a further five washes, goat anti-mouse immunoglobulin–horseradish peroxidase conjugates (Southern Biotechnology, 1:1000 in HS–PBS) were added for 1 h at 37 °C. For development, 20 mg/ml ABTS (2,2’-azino-di[3-ethyl-benzthiazolin-6-sulphanate] diammonium salt) diluted 1:50 in 0.1 M citrate buffer (pH 4.0) with 0.01% H₂O₂ was added and absorbances were read at 415 nm.

**In vitro CTL stimulation.** For stimulation, peripheral blood mononuclear cells (PBMCs) were separated on Lymphoprep (Nycomed) from buffy coat samples obtained from HLA-A2-positive, healthy blood donors. Presence of the HLA-A2 allele was determined by fluorescent staining with BB7.2; the staining protocol was identical to that used with the T2 cells. Aliquots of 10⁸ autologous PBMCs pulsed with high affinity binding peptides (10⁻¹⁰ M, 2 h, 37 °C) were used as antigen-presenting cells (APCs). After peptide loading, unbound peptide was washed off and the APCs were added back to the main cultures (2 × 10⁵ PBMCs for each start culture) in 10 ml complete medium (RPMI 1640, 12.5 mM HEPES, 8% pooled human serum from five healthy donors, 2 mM glutamine, 100 U/ml penicillin G and 100 µg/ml streptomycin). For weekly restimulation of the cultures, frozen aliquots of autologous PBMCs were thawed, regenerated in complete medium, irradiated (35 Gy), pulsed with peptides, washed and added to the lymphocyte cultures at a 1:5–1:2 responder:stimulator ratio. At each restimulation, the medium was replaced with fresh complete medium with 5 U/ml recombinant interleukin (IL)-2 (Amersham). The cell density of the restimulated cultures was adjusted to 2 × 10⁴ responders in 1 ml medium. After 4 weeks, further weekly restimulations were done with a mixture of irradiated (35 Gy), peptide-loaded PBMCs from two or three donors. Between the restimulations, half of the medium was replaced by fresh IL-2-containing (5 U/ml) complete medium from one to three times weekly depending on the growth of the individual cultures.

**Cytotoxicity assay.** The peptide-stimulated cultures were tested in a standard ³¹Cr-release assay. Effector cells were used 5–7 days after the last stimulation and were suspended in fresh complete medium. HLA-A2-matched, Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines (established in our department) were used as targets. Target cells were labelled with 100 µCi ³¹Cr (1 h, 37 °C) and washed twice. Aliquots were pulsed with 50 µM peptide for 30 min at 26 °C, then appropriately diluted and distributed into 96-well plates (5 × 10⁵ target cells in 20 µl medium into each well). After adding the effector cells (in 100 µl complete medium into each well), the final peptide concentration was approximately 1 µM. The cells were incubated together for 5 h at 37 °C in a CO₂ atmosphere. Specific lysis (%) was calculated as follows: 100 × (³¹Cr release into supernatant-spontaneous release)/total release in detergent-spontaneous release. Peptide-specific CTL activity was evaluated by comparing specific lysis of the target cells incubated with and without peptide.

**Establishment of an HPV-16-transfected cervical cancer cell line.** The cervical-cancer-derived epithelial cell line C33A (HLA-A2 positive as determined by BB7.2 immunofluorescent staining) does not contain any known papillomavirus sequences. The HPV-16 expression...
vector pMHPV16d provided by C. Woodworth at NIH, Bethesda, Md., USA (Woodworth et al., 1988) was used to transfect C33A cells. This recombinant plasmid contains a dimer of the whole HPV-16 genome cloned at the BamHI site of the vector and expression of HPV-16 DNA is initiated by an MMT promoter. The plasmid contains neomycin and ampicillin resistance genes. Before transfection, C33A cells were grown in RPMI 1640 medium supplemented with 10% foetal calf serum (FCS). Upon 60–70% confluency, cells were transfected with 5 µg of pMHPV16d plasmid DNA per 60 mm culture dish using the polybrene technique. After 6 h, the DNA/polybrene-containing medium was removed, cells were washed with serum-free medium, treated with 30% DMSO and washed again with serum-free medium. After addition of fresh medium, cells were incubated for an additional 48 h. Transfected cultures were trypsinized, resuspended in RPMI 1640 medium containing 10% FCS and 400 µg/ml G418 and transferred to a fresh culture dish. The stable G418-resistant transfectants were selected by culturing in selective medium for at least 3 weeks and single colonies from individual dishes were separated. Expression of HPV-16 DNA in the transfectants (C33A-HPV16) was verified by RT–PCR with HPV-16 E7 primers (nt 696–714 and nt 824–843).

Results

Identification of HLA-A*0201 binding peptides

Amino acid sequences of the E1 and E2 proteins were deduced from the nucleotide sequence of the HPV-16 genome (Seedorf et al., 1985) and were screened for the 9-mer and 10-mer stretches that contained the HLA-A*0201 binding motif (Kast et al., 1994). Altogether, 37 peptides from E1 and 24 from E2 were selected for synthesis. There exist alternatively spliced mRNAs that may code for putative, truncated forms of E1 and E2, designated E1C and E2C, respectively (Sherman & Alloul, 1992). Neither the splice site flanking region of E1C nor the unique N-terminal leading sequence of E2C contained additional HLA-A*0201 binding motifs.

A standard HLA-A2 upregulation assay on the antigen-processing-deficient T2 cell line was used to measure the HLA-A*0201 binding affinities of the synthesized peptides (Stuber et al., 1995). The peptides were tested initially at a 200 µM concentration, after which the binding peptides were further titrated in serial twofold dilution steps. The relative binding affinity of the peptides was expressed as the peptide concentration that induced half-maximal HLA-A*0201 up-regulation (C1/2max). As positive controls, we included the HLA-A*0201-restricted CTL epitopes of the HPV-16 E7 (12–20) (Ressing et al., 1995) and the influenza virus matrix (58–66, F62K) (Gotch et al., 1987). Seven E1 and three E2 peptides bound to HLA-A*0201 with affinities similar to those of the control epitopes (Table 1). An additional eight E1 and six E2 peptides that exhibited weak binding (C1/2max ≥ 200 µM) were not analysed further. Six of the seven E1 peptides with high binding-affinities cluster in the middle part of the protein; one is close to the C terminus. For the E2 protein, all three strongly binding peptides map to the transactivator region located at the 5’ part of the E2 ORF.

Cytotoxic lymphocytes against the E1 and E2 peptides

PBMCs were purified from buffy coat samples; the tested donors were found to be seronegative in HPV-16-capsid ELISA. The presentation of peptide epitopes was mimicked by loading the synthetic peptides onto aliquots of PBMCs known to contain professional APCs, of which the dendritic cells are the most potent, especially in activation of the naive, unprimed T-cell precursors (Croft, 1994). Autologous PBMCs were applied at the initiation of the lymphocyte cultures and at the three consecutive restimulation steps in order to avoid the outgrowth of T-cells with allogeneic specificity. Before the fourth restimulation, cultures were tested for peptide-specific cytolytic activity. HLA-A2-matched target cells (EBV-transformed lymphoblastoid cell lines) were 51Cr-labelled and sensitized to CTL killing with the cognate peptides. The same target cell line, untreated with peptide, was tested simultaneously as a negative control in each 51Cr-release assay. Among the E1 peptides, E1 253–262 (Fig. 1A) was able to elicit peptide-specific CTLs from two donors. In addition to this peptide, peptide-specific CTLs were also simultaneously generated against other peptides, i.e. against a peptide of the HIV protease from the first donor and against HPV-16 E7 12–20 from the second donor (Fig. 1C), a known CTL epitope of the E7 protein. This 10-mer peptide also contains a 9-mer fragment (E1 254–262) with similar binding affinity (Table 1) that was also recognized by CTLs stimulated with the 10-mer peptide (Fig. 1A). However, we failed to stimulate peptide-specific CTLs with the 9-mer peptide from both donors. For the E2 protein, peptide-specific CTLs could be raised against the E2

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>C1/2max (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 253–262†</td>
<td>TLLQYCYCLYL</td>
<td>6.0</td>
</tr>
<tr>
<td>E1 254–262</td>
<td>LLQQYCYLYL</td>
<td>12.0</td>
</tr>
<tr>
<td>E1 266–274</td>
<td>SLACSWGMV</td>
<td>75.0</td>
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<tr>
<td>E1 266–265</td>
<td>SLACSWGMVV</td>
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<tr>
<td>E1 292–300</td>
<td>KLSKLLCV</td>
<td>25.0</td>
</tr>
<tr>
<td>E1 304–312</td>
<td>CMMIEPPKL</td>
<td>25.0</td>
</tr>
<tr>
<td>E1 493–502</td>
<td>FLQGSLICFV</td>
<td>75.0</td>
</tr>
<tr>
<td>E2 69–77†</td>
<td>ALQAIEQOL</td>
<td>1.5</td>
</tr>
<tr>
<td>E2 93–101</td>
<td>TLQDVSSLV</td>
<td>6.0</td>
</tr>
<tr>
<td>E2 138–147</td>
<td>YICEASVT</td>
<td>12.0</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E7 12–20†</td>
<td>MLDLQPETT</td>
<td>18.5</td>
</tr>
<tr>
<td>Influenza virus matrix</td>
<td>GILGKVFTL</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* C1/2max is the peptide concentration (µM) inducing half-maximal HLA-A*0201 upregulation on T2 cells.
† Localization of peptides in the E1, E2 and E7 proteins of HPV-16.

Table 1. Peptides with high HLA-A*0201 binding affinity in the HPV-16 E1 and E2 proteins
69–77 peptide from the third donor (Fig. 1B). Among the E2-derived peptides, this peptide had the highest binding affinity. From this PBMC sample, we also obtained primary CTLs against an HIV protease peptide.

At the fourth and subsequent restimulations, a mixture of peptide-pulsed PBMCs from two or three donors was used as feeder–stimulator cells. By this time, the lymphocyte cultures were highly specific as assessed by the high cytolytic activity even at low effector:target ratios; the allogeneic feeder–stimulator cells did not affect the CTL specificity for the rest of the culture period. These further cytotoxicity tests were done 7–9 days after the last restimulation step and consumed one-third to one-half of the cultures. Under these conditions, CTLs could be maintained for 10–12 weeks. The peptide-specific CTLs were further tested for ability to lyse virus-carrying target cells. We transfected the whole HPV-16 genome with a neomycin resistance gene into one of the few HPV-negative cervical-cancer-derived cell lines, C33A, which is HLA-A2-positive. CTLs specific to the E2 and E7 peptides lysed the transfected cells (C33A-HPV16) but not the parental virus-negative cell line (Fig. 1E, F). The E1 peptide-specific CTLs did not recognize the HPV-16-carrying transfectant (Fig. 1D) indicating that E2 69–77, but not E1 253–262, can be presented by HPV-16-infected cells.

Discussion

The establishment of the MHC class I binding motifs (Kast et al., 1994) has enabled development of a straightforward method for identification of potential CTL epitopes. This study focused on HLA-A2, the most common allele in the Caucasian population where more than 95% of HLA-A2 positives carry the HLA-A*0201 allele. Although other alleles coding for HLA-A2 (notably A*0202–07) are more frequent in non-Caucasian populations, some of these alleles (e.g., A*0202, A*0205) share common antigen-presenting properties with A*0201 and define an HLA-A2-like supertype with regard to the presentation of microbial antigens (del Guercio et al., 1995). Thus, the results obtained with the HLA-A*0201 allele may be relevant for a considerable proportion of mankind. In line with other observations (Houbiers et al., 1993), the preselection of peptides according to the HLA-A*0201 binding motif was useful as several of the synthesized peptides (seven of the 37 E1 and three of the 24 E2 peptides) bound to HLA-A*0201 with high affinity and several peptides that did bind, albeit with weak binding-affinities, were also identified. Peptides that were able to evoke CTLs were those that had the highest binding affinity in the tested peptide set. In other studies also, high binding-affinity has been found to be a prerequisite for efficient CTL stimulation (Sette et al., 1994; Vanderburg et al., 1995). Our previous experience that CTLs were raised against an HIV peptide from regularly tested, seronegative, healthy blood donors indicates that the applied protocol is able to stimulate naive, unprimed CTL precursors (Kónya et al., 1997). In this study, the donors were seronegative for HPV-16 capsids and although not all HPV-16-exposed subjects seroconvert (Dillner, 1995), the elicited CTL responses may represent in vitro priming of naive T-cell precursors rather than recall of memory responses. Stimulation of primary T-cell responses provides a theoretical background for peptide-based prophylactic or therapeutic vaccines (Vanderburg et al., 1995; Vitiello et al., 1995).

Since more than 70 types of HPV have been identified, it is of interest to know whether the identified immunogenic peptides of the E1 and E2 proteins are type-16 specific or type-common epitopes. For both E1 253–262 and E2 69–77, the aligned sequences revealed several variations between HPV types belonging to groups other than the HPV-16 group (Myers et al., 1995). Since even a single residue change can be deleterious for CTL recognition, it is unlikely that peptides
Carcinogenesis appears to be the factors that determine... 1994), the most important limiting step in cervical... 12–20 is indeed a naturally... positive) (Ressing... 16-carrying cervical carcinoma line, Caski (also... AE1/IE1QL E2 69–77
31 . . . M E2 69–77
35 . . . . . M E2 70–78
52 . . . M E2 79–77
33 . . . F. V . . . M E2 69–77
58 . . . F. V . . . M E2 69–77

* Single-letter amino acid codes are used. In the aligned sequences of... HPV-31. In contrast, the E2 69–77 peptide is rather conserved in the HPV-16 group; the corresponding peptides of HPV-31 and HPV-35 revealed only... with many variations would cross-react. Even in the HPV-16-related group, the E1 peptide was variable with a similar sequence only found in HPV-31. In contrast, the E2 69–77 peptide is rather conserved in the HPV-16 group; the corresponding peptides of HPV-31 and HPV-35 revealed only... a naturally presented CTL epitope. Similar results were obtained with the E7 12–20 epitope. CTLs specific to this epitope have been reported to lyse the HPV-16-carrying cervical carcinoma line, Caski (also HLA-A2 positive) (Ressing et al., 1995). Since there is no HPV-negative, but otherwise identical, parental control cell line for Caski, our results using the C33A/HPV16 pair provide additional evidence that E7 12–20 is indeed a naturally presented CTL epitope. The E2 protein is likely to have a crucial role in cervical carcinogenesis. The frequent loss of E2 expression in cervical cancers could play a role in pathogenesis by causing deregulated expression of the viral oncogenes E6 and E7 (Dowhanick et al., 1995; Stoler et al., 1992) and might conceivably result in escape from immune surveillance. Since only a small proportion of HPV-16-exposed individuals will develop cervical dysplasia (Ponten et al., 1995; zur Hausen & de Villiers, 1994), the most important limiting step in cervical carcinogenesis appears to be the factors that determine persistence or clearance of the infection. Our identification of a CTL epitope in one of the likely target proteins of the cellular immune response, involved in the early steps of carcinogenesis, may be important in the elucidation of the natural history of HPV infection.

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References


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