Expression of CD56 by Human Papillomavirus E7-specific CD8+ Cytotoxic T Lymphocytes Correlates with Increased Intracellular Perforin Expression and Enhanced Cytotoxicity against HLA-A2-matched Cervical Tumor Cells

Alessandro D. Santin, Paul L. Hermonat, Antonella Ravaggi, Stefania Bellone, Juan J. Roman, Sudheer Jayaprabhu, Sergio Pecorelli, Groesbeck P. Parham, and Martin J. Cannon

Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, University of Arkansas for Medical Sciences, Little Rock, AR 72205-7199, and Division of Gynecologic Oncology, University of Brescia, Brescia, Italy [A. D. S., A. R., S. B., S. P.]

Abstract

Human papillomavirus (HPV) infection represents the most important risk factor for developing cervical cancer. In this study, we examine the potential of full-length E7-pulsed autologous dendritic cells (DCs) to induce antigen-specific CTL responses from the peripheral blood of healthy individuals against HLA-A2-matched HPV-16 and HPV-18-positive tumor target cells in vitro. We show that DCs pulsed with E7 oncoprotein can consistently stimulate antigen-specific CTL responses that recognize and lyse HPV-16 or HPV-18-positive naturally infected cervical cancer cell lines. HPV-negative, EBV-transformed lymphoblastoid cell lines (LCLs) sharing the HLA haplotype of the target tumor cells, as well as autologous donor LCLs, were not significantly killed by E7-specific CTLs. Cytotoxicity against HPV-A2-matched HPV-16 and HPV-18 tumor target cells could be significantly inhibited by anti-HLA class I and by anti-HLA-A2 monoclonal antibodies. CD8+ CTLs expressed variable levels of CD56 and showed a strongly polarized Type 1 cytokine profile. Sorting of the CD8+ T cells on the basis of CD56 expression demonstrated that the most highly cytotoxic CTLs were CD56+ and expressed higher levels of perforin and IFN-γ, compared with the CD8+CD56− population. Taken together, these data demonstrate that full-length, E7-pulsed DCs can consistently induce E7-specific CD8+ CTL responses in healthy individuals that are able to kill naturally HPV-16 and HPV-18-infected cancer cells, and that CD56 expression defines a subset of CD8+ CTLs with high cytolytic activity against tumor cells.

Introduction

The association between HPV infection and the development of cervical cancer is now well established (1). Although there are over 20 oncogenic HPV genotypes, HPV-16 and HPV-18 are the most strongly associated with invasive cancer (2). Because the E6- and E7-transforming oncoproteins are detected in the vast majority of HPV-positive cancer biopsies and almost all HPV-containing cell lines and play a crucial role in both transformation and maintenance of the malignant phenotype (2), these proteins might be ideal candidates as tumor rejection antigens for HPV-specific immunotherapy or vaccination.

Several lines of evidence suggest that cell-mediated immune responses are important in controlling both HPV infections and HPV-associated neoplasms: (a) there is an increased incidence of HPV-associated genital cancer in immunosuppressed patients, whereas only a minority of genital HPV infections result in the development of cancer in otherwise healthy individuals (3–5); (b) HPV-specific T-cell stimulation during the spontaneous regression of warts has been observed (6), whereas weak or negative responses were reported among individuals harboring longstanding lesions (7); (c) the generation of HPV E7-specific CTLs has been reported recently in cervical cancer patients (8–10).

Recently, we have reported that full-length, E7-pulsed autologous DCs can elicit a specific CD8+ CTL response against autologous tumor target cells in patients harboring HPV-16 and HPV-18 cervical cancer (11). In this report, using a similar in vitro immunostimulatory protocol, we analyzed HPV-16 and HPV-18 E7-specific CTL responses in healthy individuals. Here, we show DC-mediated induction of robust, HLA class I-restricted cytotoxicity against HPV-16 and HPV-18 naturally infected and partially HLA-matched tumor targets. In addition, we show that the most highly cytotoxic HPV-E7 oncoproteins by this in vitro DC-based CTL induction protocol. Finally, we demonstrate that the most highly cytotoxic HPV-

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2 To whom requests for reprints should be addressed, University of Arkansas for Medical Sciences Medical Center, Division of Gynecologic Oncology, University of Arkansas for Medical Sciences, Mail Slot 518, 4301 West Markham, Little Rock, AR 72205-7199. Phone: (501) 686-7162; Fax: (501) 686-8091.

The abbreviations used are: HPV, human papillomavirus; DC, dendritic cell; LCL, lymphoblastoid B-cell line; PBMC, peripheral blood mononuclear cell; IL, interleukin; MAb, monoclonal antibody; PMA, phorbol myristate acetate; NK, natural killer.
specific CD8+ T cells coexpressed CD56, and that CD56 expression correlated with increased perforin and IFN-γ expression, when compared with CD8+/CD56− T cells. These findings may have important implications for development of an HPV vaccine and the treatment of cervical cancer and HPV-related neoplasms with active or adoptive immunotherapy.

Materials and Methods

Tumor Cell Lines. Fresh primary tumor cells that shared with the healthy donors the HLA-A2 haplotype were obtained from patients harboring HPV-16 and HPV-18 invasive cervical cancers and have been described previously (11). Fresh tumor cell lines were maintained in serum-free keratinocyte medium (Life Technologies, Inc., Grand Island, NY) supplemented with 5 ng/ml epidermal growth factor and 35–50 μg/ml bovine pituitary extract (Life Technologies, Inc.) at 37°C, 5% CO2. EBV-transformed LCLs derived from the same HLA-A2-positive cancer patients that provided the HPV naturally infected primary tumor cell targets were established by coculture of PBMCs with EBV-containing supernatant from the B95.8 cell line in the presence of 1 μg/ml cyclosporin A (Sandoz, Cambridge, United Kingdom) and were maintained in RPMI 1640 supplemented with 10% FCS (Gemini Bioproducts, Calabasas, CA). LCLs derived from healthy donor PBMCs were established and maintained in similar culture conditions.

HLA Haplotypes. HLA class I typing of purified CD8+ T cells was performed by standard lymphocytopotoxicity assay in the Tissue Typing Laboratory of the Bone Marrow Transplantation and Blood Transfusion Service at the University of Arkansas for Medical Sciences. The healthy donors manifested the following haplotypes: donor 1-HLA A2, A32, B35, B51, CW4; and donor 2-HLA A2, A11, B35, B62, BW6, CW3, CW4. HPV-16- and HPV-18-positive tumor cells used as targets, as well as the matched LCL cell lines used as negative controls, presented the following haplotypes; HPV-16-infected squamous cell carcinoma line-HLA A1, A2, B7, B41, CW7; and HPV-18-infected adenocarcinoma cell line-HLA A1, A2, B7, B41, CW2, CW7 (11).

DC Cultures and Generation of HPV E7-specific T Cells. Heparinized PBMCs were collected from two healthy adult donors under protocols approved by the Institutional Review Board. The healthy volunteer studies had no prior history of clinical or cytological HPV infection and a normal routine cervical smear (donor 2) collected at the time of the study. The derivation of DCs from the donor PBMCs and their subsequent use for generation of HPV E7-specific T cells were carried out essentially as described (11). Briefly, between 40 and 50 ml of PBMCs were placed into six-well culture plates (Costar, Cambridge, MA) in AIM-V medium (Life Technologies, Inc.) at 0.5–1 × 10^7/ml/well. After 2 h at 37°C, nonadherent cells were washed, and the adherent cells were cultured at 37°C in a humidified 5% CO2/95% air incubator in AIM-V medium supplemented with recombinant human granulocyte/macrophage-colony-stimulating factor (800 units/ml; Immunex, Seattle, WA) and IL-4 (1000 units/ml; Genzyme, Cambridge, MA). Final maturation of monocyte-derived DCs was induced by exposure during the last 48 h of culture (i.e., days 6–8) to tumor necrosis factor-α (1000 units/ml), IL-1β (500 units/ml; R&D Systems, Minneapolis, MN), and prostaglandin E2 (0.5 μg/ml; Sigma Chemical Co., St. Louis, MO). After final maturation, DCs were generated by pulsing with E7 HPV-16 or HPV-18 oncoproteins generated using previously characterized plasmids encoding glutathione S-transferase E7 fusion proteins (11). The cationic lipid N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethyl sulfate (Boehringer Mannheim, Indianapolis, IN) was used to deliver the HPV-16 or HPV-18 E7 proteins into cells as described (11). Generation of HPV-E7-specific CTLs was achieved by culturing responder PBMCs (10–20 × 10^6 cells/well in six-well culture plates; Costar) in AIM-V with E7-pulsed autologous DCs (ratios from 20:1 to 30:1 responder PBMCs:DCs). The cultures were supplemented with recombinant human IL-2 (10 units/ml Aldesleukin; Chiron Therapeutics, Emeryville, CA) and restimulated once with E7-pulsed DCs after 10–14 days. At day 21–28, CD8+ T cells were separated from the bulk cultures by positive selection with CD8-Dynabeads (Dynal, Inc., Lake Success, NY) and further expanded in number for 5–7 days using autologous or allogeneic irradiated peripheral blood lymphocytes (5000 eGy; 1 × 10^6 cells/well) and anti-CD3 monoclonal antibody (Ortho Pharmaceutical Corp., Raritan, NJ; 0.2 μg/ml) plus 5% autologous plasma and 100 units/ml of IL-2 in 24-well plates (Costar), before being assayed for phenotype and CTL activity.

Flow Cytometric Analysis of Phenotype, Intracellular Perforin, and Cytokine Expression by HPV-specific CTLs. Flow cytometric analysis of cell surface antigens and intracellular cytokine expression was conducted essentially as described previously (11, 12). Briefly, flow cytometry for cell surface antigen expression was performed using directly conjugated MAb against CD8 (Leu-2a), CD56 (Leu-19), and isotype-matched controls (Becton Dickinson, San Jose, CA) and analyzed on a FACScan (Becton Dickinson). Expression of CD8α/β heterodimers was assessed with MAb specific for CD8 β-chain (MCA1722; Serotec, Oxford, United Kingdom), as described (12). For evaluation of intracellular perforin, harvested cells were washed and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. Cells were then washed and permeabilized by incubation in PBS plus 1% BSA and 0.5% saponin (S-7900; Sigma) for 10 min at room temperature. Both populations of cells were stained with FITC-anti-perforin MAb (Delta G9; PharMingen, San Diego, CA) and isotype-matched control MAb (FITC-anti-IgG2a; PharMingen). After staining, cells were washed twice with PBS plus 1% BSA and 0.5% saponin, once with PBS plus 0.5% BSA, and fixed a second time with 2% paraformaldehyde in PBS.

For intracellular staining for IFN-γ and IL-4 expression in unsorted CTLs, cells were activated with 50 ng/ml FMA and 500 ng/ml ionomycin for 6 h. Ten μg/ml Brefeldin A were added for the final 3 h of incubation. Controls (nonactivated cultures) were incubated in the presence of Brefeldin A only. Cells were then washed and permeabilized by incubation in PBS.
plus 1% BSA and 0.5% saponin for 10 min at room temperature. Activated and control cells were stained with FITC-anti-IFN-γ and phycoerythrin-anti-IL-4, or isotype-matched controls (FITC-anti-IgG2a and phycoerythrin-anti-IgG1) from Becton Dickinson. After staining, cells were washed twice with PBS plus 1% BSA and 0.5% saponin, once with PBS plus 0.5% BSA, and fixed a second time with 2% paraformaldehyde in PBS. Analysis was conducted with a FACScan, using Cell Quest software (Becton Dickinson).

For intracellular cytokine staining of CD8+/CD56+ and CD8+/CD56− cells, sorted cells were allowed to rest for 24–48 h in RPMI 1640 plus 5% autologous plasma and 100 units/ml of IL-2 in 24-well plates and thereafter activated with PMA and ionomycin as described above. Cells were then washed and permeabilized by incubation in PBS plus 1% BSA and 0.5% saponin for 10 min at room temperature. Activated and control cells were stained with FITC-anti-IL-2 or FITC-anti-IFN-γ or isotype-matched control MAb (FITC-anti-IgG2a) from Becton Dickinson. After staining, cells were washed twice with PBS plus 1% BSA and 0.5% saponin, once with PBS plus 0.5% BSA, and fixed a second time with 2% paraformaldehyde in PBS.

Cytotoxic Activity. A 6-h 51Cr release assay was performed as described previously (13) to measure the cytotoxic reactivity of DC-E7-stimulated CD8+ T lymphocytes. In some experiments, to directly assay and differentiate the cytolytic capacity of the two populations of CD8+ effector T cells obtained (i.e., CD8+/CD56+ and CD8+/CD56−), sorted fractions were cultured in RPMI 1640 plus 5% autologous plasma and 100 units/ml of IL-2 in 24-well plates for 24–48 h before being assayed for CTL activity. Targets included HPV-16+ or HPV-18+ HLA-A2-matched primary tumor cell lines, autologous LCLs from the healthy donors, and LCLs matched to the tumor cell lines. To determine the HLA restriction of the cytotoxic response against HLA-A2-matched tumor cell lines, HLA-specific MAbs were used to block cytotoxicity. Briefly, 51Cr-labeled tumor targets were preincubated with MAbs specific for monomorphic HLA class I (W6/32; 50 μg/ml) or anti-HLA-A2 (MAb BB7-2; 50 μg/ml; both hybridomas obtained from American Type Culture Collection, Rockville, MD). The effector cells and 51Cr-labeled targets were then incubated in a final volume of 200 μl for 6 h at 37°C with 5% CO2.

Results

Tumor-specific CD8+ CTL Responses. Cytotoxicity assays were conducted after a minimum of 5 weeks after stimulation of T lymphocyte cultures from both donors by HPV-16 or HPV-18 full-length E7-pulsed autologous DCs. Significant cytotoxicity against HLA-A2+ and HPV-16 or HPV-18 matched tumor-cell targets was demonstrated in the purified CD8+ T lymphocyte populations of both adult donors (Figs. 1 and 2). Cytotoxicity against the HPV-16, HLA-A2-matched tumor target ranged from 42 to 88% for donor 1 and from 14 to 37% for donor 2 at 20 effectors/target. Cytotoxicity against the HPV-18, HLA-A2-matched tumor target ranged from 45 to 89% in donor 1 and from 14 to 51% in donor 2 at 20 effectors/target (Figs. 1 and 2). The ability to kill HPV-16- and HPV-18-positive HLA-A2-matched target tumor cells but not HPV-negative targets sharing the HLA haplotype of the tumor cells was demonstrated repeatedly in several separate assays over a 6-month period. Autologous donor LCLs were also not lysed. Blocking studies demonstrated that the tumor-specific lytic activity could be significantly inhibited by pretreatment of tumor targets with MAb specific for HLA class I (W6/32) and/or anti-HLA A2 MAb (BB7-2; Figs. 1 and 2).

Because the phenotypic analysis of the E7-pulsed, DC-stimulated CD8+ CTLs in all primings revealed a significant CD8+/CD56− subpopulation (Fig. 3), experiments were initiated to isolate the CD8+/CD56+ and the CD8+/CD56− populations and to examine their cytolytic potential as well as to correlate CD56 expression with the level of lysis and the specificity thereof. The T cells in these experiments expressed CD8α/β heterodimers (not shown) as described (12), thus ruling out the presence of CD56− NK cells, which may also express CD8 but only as α/α homodimers. At 24 h from separation, CD8+/CD56− cells with 70% purity could be recovered and directly compared with a population of 90% CD8+/CD56− cells.
Fig. 2. HPV-16 and HPV-18 E7-specific CD8+ CTL responses induced by E7-pulsed DCs from donor 2 measured in a 6-h 51Cr-release assay. The percentage of lysis at a 20:1 E:T cell ratio is shown. Anti-HLA class I (W6/32) and anti-HLA-A2 (BB7-2) blocking antibodies were used at 50 μg/ml. A, HPV-16-primed T cells. Column 1, HPV-16+ tumor cells; column 2, HPV-16+ tumor cells + W6/32 anti-class I MAb; column 3, HPV-16+ tumor cells + BB7-2 anti-HLA-A2 MAb; column 4, tumor-matched LCLs; column 5, autologous donor LCLs. B, HPV-18-primed T cells. Column 1, HPV-18+ tumor cells; column 2, HPV-18+ tumor cells + W6/32 anti-class I MAb; column 3, HPV-18+ tumor cells + BB7-2 anti-HLA-A2 MAb; column 4, tumor-matched LCLs; column 5, autologous donor LCLs. A matched-pairs (two-tailed) t test was used for evaluation of statistical significance. Reduction of lysis against tumor cells in the presence of blocking antibodies compared with untreated controls was significant at P < 0.05.

Discussion

The E6 and E7 oncoproteins of HPV-16 and HPV-18 are endowed with transforming ability and have been implicated in the tumorigenicity of HPV. Both E6 and E7 can alter cellular...
growth regulation by inactivating the products of the tumor suppressor genes p53 and Rb, respectively, and the level of expression of the E6 and E7 genes has been linked directly to the proliferative capacity of HPV-infected cancer cells (2). Although the viral genome can exhibit significant rearrangements and deletions, the E6 and E7 genes are regularly preserved and transcribed in the tumorous tissue, as well as in cell lines derived therefrom (2). These viral products could be targets for the immune response against HPV and thus represent candidates for therapeutic vaccination. In agreement with this view, HLA-A2-restricted peptide epitopes of HPV-16 E6 and E7 have been identified (15), and these peptides have been shown to be able to stimulate antitumor T-cell immunity in vitro models (8). However, the use of peptides as antigens has a number of limitations. Because peptide vaccination protocols rely on knowledge of the HLA type of each patient as well as of the HLA restriction of each synthesized HPV-specific peptide, this approach may not be practical for large-scale vaccination protocols. In contrast, autologous DCs pulsed with full-length E7 oncoprotein of the HPV genotype involved in the disease offer the significant advantage of potentially presenting multiple immunogenic CTL epitopes, as well as allowing DCs to tailor those peptides fitting self HLA molecules without the necessity for previous knowledge of the individual HLA type. In addition, CD4+ T helper epitopes derived from full-length E6/E7 oncoproteins have also been reported (16). The requirement for both helper and CTL epitopes presented by the same antigen-presenting cells for efficient and long-lasting CD8+ T-cell activation (17, 18) might explain the efficacy and reproducibility of full-length, E7-pulsed DCs in generating potent CTL immune responses from cervical cancer patients against naturally HPV infected autologous tumor targets (11). Finally, because the selective loss of one HLA restriction element on cervical tumor cells is a much more frequent finding than the total loss of HLA expression (19, 20), it is likely that the use of full-length E7 oncoprotein-based vaccination, incorporating peptides able to be bound to multiple HLA class I restriction elements (10), might be a superior form of vaccination.

In this report, induction of high cytotoxic activity against HPV-16 and HPV-18-infected HLA-A2 matched tumor cell lines was demonstrated for CD8+ CTLs induced by full-length E7-pulsed autologous DCs from two healthy adult donors. Although previous studies have not detected HPV-specific CTLs in the PBMCs of healthy donors (10), the possibility that the responses observed in our adult healthy individuals might represent secondary in vitro restimulation of CTLs, rather than DC-induced priming in vitro, cannot be excluded. The killing of HPV naturally infected fresh tumor cell lines showed that CTLs generated by this method were able to recognize target cells expressing physiological levels of HPV antigens. This point is noteworthy in the light of previous observations showing that CTLs generated by in vitro primary stimulation with a high concentration of peptides often fail to lyse targets expressing endogenous antigens (21, 22).

The lack of significant cytotoxicity against HPV-negative LCLs sharing HLA-A2 with the HPV-positive tumor cell lines used as targets further supports the E7 specificity of the response. This negative control excludes the possibility that the reactivity against HPV-positive HLA partially matched tumor cell targets was attributable to cross-reactivity against allogeneic HLA molecules other than A2, as reported previously (23), rather than to E7 recognition. Also, a significant reduction in tumor cell killing was obtained using anti-HLA-A2 MAb, as well as with anti-class I MAb, demonstrating that HLA-A2-restricted cytotoxicity was an important component of the CD8+ T-cell response.

Phenotypic analysis of the E7-pulsed, DC-induced priming CD8+ CTLs in all donors revealed a significant CD8+/CD56- subpopulation. Because previous studies from us (11, 12) as well as of others (24) in cervical cancer patients have suggested that CD56- enriched CTL populations may exhibit significantly greater lysis of HPV-positive tumor targets, we isolated CD56+ and CD56- populations of CTLs to assess functional and phenotypic differences between the two subsets. In agreement with the result of Hilders et al. (24), we consistently found a higher cytotoxic activity of the CD8+/CD56- T cells against tumor target cells when compared with CD8+/CD56+ T cells.
Fig. 5 Two-color flow cytometric analysis of intracellular IFN-γ and IL-4 expression by HPV-16 and HPV-18 tumor-specific CD8+ T cells from donors 1 and 2. CD8+ T cells were tested at ~6 weeks after priming, after resting for 14 days after the last antigen stimulation prior to activation by PMA and ionomycin. HPV-16-specific (A) and HPV-18-specific (B) CTLs from donor 1 are shown. HPV-16-specific (C) and HPV-18-specific (D) CTLs from donor 2 are shown.

Fig. 6 Expression of intracellular perforin by HPV-18-specific CD8+/CD56- (light lines) and CD8+/CD56+ (heavy lines) CTLs, as analyzed by flow cytometry in donor 1. Dotted lines, histograms from cells stained with isotype control MAb.

over, a close correlation between increased cytotoxic activity and elevated expression of perforin and IFN-γ was demonstrated in the sorted CD8+/CD56+ subpopulation compared with CD8+/CD56-. Unlike the data of Hilders et al. (24), however, we were able to consistently demonstrate inhibition of tumor target cell lysis by anti-HLA class I MAb in both the CD56+ and the CD56- populations of CTLs. The reasons for these different results are not well understood. Previous reports have shown the possibility of a dual recognition of tumor antigens (i.e., HLA-restricted and HLA-unrestricted) in CD8+/CD56+ CTL clones (25, 26), suggesting that expression of CD56 may indicate the presence of a CD8+ cell population endowed with NK-like activity. However, as we have described previously (12), the cytotoxic cells in this study expressed CD3 and CD8α/β heterodimers (not shown), which are confined to the T-cell lineage, whereas CD8+ NK cells express CD8α/α homodimers and do not express CD3. Our observations are thus in accord with our earlier study, which showed that CD56 expression correlated with enhanced lytic function by CD3+, TCRα/β+, CD8α/β+ CTLs (12).

Fluorescence-activated cell sorting for CD56+ and CD56- CD8+ T cells failed to yield two phenotypically stable populations of CTLs (data not shown). Collectively, these results suggest that the coexpression of CD56 on CD8+ T cells may reflect a higher stage of CTL activation, resulting in an increased lytic activity against HPV-infected tumor cells that is at least in part correlated with increased perforin and IFN-γ expression. This stage of activation, however, seems to be transient. These observations further support the view that CD56 expression by CD8+ CTLs may be an activation antigen associated with higher cytotoxic function rather than a lineage-specific marker (11, 12, 24, 27).

In conclusion, we suggest that in vitro stimulation and selection of tumor-specific CD8+ CTLs for immunotherapy should not encompass depletion of CD56+ cells on the assumption that this marker is confined to the NK compartment. On the contrary, available evidence suggests that the CD56-CD8+ subset represents a population of HLA class I-restricted and tumor-specific CTLs with enhanced cytotoxic potential, rather than a nonspecific NK-like subset. Depletion of CD56- cells may markedly abrogate the cytotoxic capacity and immunother-
apeutic potential of tumor-specific CD8+ T cells, and thus we favor development of in vitro stimulation and selection strategies that enrich for CD56+CD8+ CTLs.

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References
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