Recognition of Prostate Tumor Cells by Cytotoxic T Lymphocytes Specific for Prostate-specific Membrane Antigen

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ABSTRACT

The development of immunotherapy for cancer, such as synthetic peptide-based vaccines, relies heavily on the identification of appropriate epitopes capable of eliciting antitumor T-cell responses. We have used a combination of computer-based algorithms to predict peptide sequences from prostate-specific membrane antigen (PSMA) capable of stimulating in vitro CTLs restricted by the HLA-A2 MHC molecule. Four of the five peptides that were predicted by these algorithms were capable of inducing antigen-specific CTLs that killed target cells that were pulsed exogenously with the corresponding peptides. However, only one of the four peptides, PSMA27, induced CTLs that were effective at recognizing prostate tumor cells expressing the HLA-A2 and PSMA molecules. These results underline the importance of demonstrating antitumor reactivity of peptide-induced CTLs for the selection of epitopes destined to become immunotherapeutic for prostate cancer.

INTRODUCTION

Prostate adenocarcinoma continues to be the most common malignancy affecting American men and the second leading cause of cancer death in the United States (Cancer Facts and Figures 2002, American Cancer Society, Inc., and Ref. 1). Although surgery and radiation remain the treatments of choice for the early (localized) stages of prostate cancer, there is no clear effective treatment for patients who develop recurrences or those who have metastatic disease at the time of diagnosis. T-cell-based immunotherapy has been seriously considered as a promising novel noninvasive treatment option for prostate cancer, which could be used to prevent metastatic spread or delay recurrences. However, the uncertainty of the existence of appropriate prostate tumor antigens that are capable of triggering effective antitumor T-cell responses remains one of the major obstacles for developing effective immunotherapies against prostate cancer. For many tumor types such as melanoma and ovarian/breast and colorectal carcinomas, there is clear evidence that peptide epitopes derived from conventional tumor markers such as gp100, NY-ESO-1, MAGE, CEA, and HER2/neu can be effectively recognized by tumor-reactive CTLs in the context of MHC class I molecules (2). There are numerous examples of CTLs that are stimulated with synthetic peptides (derived from the sequence of the above-mentioned tumor markers) that are able to effectively recognize and kill tumor cells. The epitopes identified in these studies are usually selected by one of numerous algorithms that predict the capacity of these peptides to bind to specific MHC alleles (3–5). A similar approach has been tried with several “prostate markers” such as PSA, prostate specific phosphatase (PAP), and prostate stem cell antigen (PSCA), which are all preferentially expressed by prostatic epithelial cells and continue to be produced after malignant transformation (6–12). However, in most cases, the results obtained regarding the effectiveness of the peptide-stimulated CTLs to kill prostate tumor cells have been suboptimal as compared with what we have observed in other tumor systems, in which high levels of cytolysis against tumor cells (60–80% specific lysis) are readily observed (13–16).

Another potential prostatic tumor marker that has been considered for immunotherapy is PSMA, which is a Mr 100,000 type-II membrane glycoprotein bearing some homology with the transferring receptor (17, 18). PSMA is expressed on normal prostatic epithelial cells and found elevated in most prostate cancers, especially in poorly differentiated, metastatic, and hormone-refractory carcinomas. Two synthetic peptides from PSMA containing HLA-A2.1-binding motifs (PSMA4 LLHETDSAV and PSMA711 ALFDIESKV) were selected for clinical studies using peptide-pulsed DCs for vaccination of prostate cancer patients (19–21). Preliminary results indicate that some of the treated patients responded immunologically to this vaccine and that positive effects in surrogate markers (drop or stabilization of serum PSA levels) were observed in some patients. However, clear evidence that the peptides selected for these clinical studies represent true CTL epitopes expressed on prostatic tumor cells has yet to be provided. Moreover, to our knowledge, there are no reports of PSMA-reactive CTLs that are capable of killing prostate tumor cells that express PSMA.

The purpose of the present study was to assess whether antitumor CTL responses to PSMA-derived epitopes could be generated using an in vitro immunization protocol that we developed (16, 22). The results presented herein show that four of the five peptide epitopes that were selected for this study based on predictive algorithms, were able to elicit strong CTL responses that were evident against peptide-pulsed target cells. However, only one peptide (PSMA27, VLAGGF-FLL) proved to be effective in triggering antitumor CTL responses as demonstrated by the capacity of these CTLs to lyse LNCaP prostate tumor cells. The results are discussed in terms of the significance of these findings for the development of peptide-based vaccines for prostate cancer.

MATERIALS AND METHODS

Epitope Selection and Peptide Synthesis. We used the combination of two computer algorithms available in the public domain and are accessible through the Internet. The predictive algorithm from the BIMAS (Biomolecular Analysis Section of the NIH) that was developed by Parker et al. (3), ranks potential MHC binders according to the predictive one-half-time dissociation of peptide/MHC complexes. The second algorithm, “SYFPEITHI,” was developed by Rammensee et al. (4) and ranks the peptides according to a score that takes into account the presence of primary and secondary MHC-binding anchor residues. The selected peptides from the PSMA sequence were synthesized according to standard solid-phase synthesis methods using Applied Biosystems apparatus and were purified by high-performance liquid chromatography. The purity (>95%) and identity of peptides were determined by analytical high-performance liquid chromatography and mass spectrometry analysis. Peptides were dissolved at 10 mg/ml in DMSO containing 0.1% trifluoroacetic acid and were aliquoted in small volumes to be maintained frozen at −20°C until further use.

Cell Lines. The T2 cells, a transplantable cell line (23), was used as target for CTL assays to demonstrate recognition of peptide and was kept in tissue culture using RPMI

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3 The abbreviations used are: PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; DC, dendritic cell; IL, interleukin; rIL, recombinant IL; DAPI, 4',6-diamidino-2-phenylindole.

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1640 supplemented with 10% fetal bovine serum (v/v), L-glutamine, nonessential amino acids, sodium pyruvate, and gentamicin (complete RPMI medium). The prostate cancer cell line LNCaP (24) was purchased from the American Type Culture Collection (Manassas, VA) and was maintained in tissue culture as recommended by the supplier. The melanoma cell line 624mel was kindly provided by Dr. S. Rosenberg (National Cancer Institute, NIH, Bethesda, MD) and was grown in complete RPMI medium. All of the culture materials were purchased from Life Technologies, Inc. (Rockville, MD). To increase the level of MHC class I expression, tumor cell lines (except for T2) were treated with 1000 units/ml IFN-γ for 72 h before the CTL cytotoxicity assays.

**In Vitro Generation of Tumor-Reactive CTLs.** DCs that were generated in vitro from adherent monocytes were used as antigen-presenting cells to stimulate CTL precursors with the candidate synthetic peptides, as described previously (16, 22). Briefly, purified monocytes were cultured for 7 days in the presence of 50 ng/ml granulocyte macrophage colony-stimulating factor and 1000 units/ml rIL-4 in complete RPMI medium. The tissue culture-generated DCs were pulsed with 4 μg/ml synthetic peptides together with 3 μg/ml β2-microglobulin in PBS containing 1% BSA for 4 h at room temperature. The peptide-pulsed DCs were washed twice, irradiated (4200 rads), and mixed with autologous CD8+ T cells (puriﬁed with Miltenyi antibody-coated magnetic beads by positive selection) at 1:20 (DC:T-cell) ratio. The CTL immunization cultures were done in 48-well plates, for which each well contained 0.25 × 10^5 DCs and 5 × 10^4 CD8+ T cells in 0.5 ml of complete RPMI medium containing 5% human AB serum instead of fetal bovine serum. This medium was supplemented with 10 ng/ml rIL-7. One day later, 10 ng/ml rIL-2 was added to the cultures to increase the efficiency of CTL induction. On days 7 and 14, the T-cell cultures were individually restimulated with irradiated peptide-pulsed autologous antigen-presenting cell (adherent monocytes) as described (16, 22), adding IL-10 on the following day. Starting on day 9, the T-cell cultures were fed with fresh medium containing 10 units/ml IL-2 every 2–3 days. The first screening cytotoxicity assay was performed after three rounds of peptide stimulation. And those cultures that exhibited cytotoxic activity toward peptide-pulsed T2 cells (>20% lysis as compared with the unpulsed target cells) were selected and expanded in tissue culture for further analysis. The Institutional Review Board on Human Subjects (Mayo Foundation) approved this research, and informed consent for blood donation was obtained from all of the volunteers.

**CTL Cytotoxicity Assays and Antibody Blocking Assays.** Cytotoxic activity of CTLs was determined in a 51Cr-release assay as described previously (16, 22). Peptide-pulsed targets were prepared by incubating T2 cells with 10 μg/ml peptides at 37°C overnight. Adherent tumor cells were removed from culture flasks with trypsin-EDTA. Target cells were labeled with 300 μCi sodium 51CrChromate (Amersham Pharmacia Biotech, Piscataway, NJ) for 1–2 h at 37°C in a water bath. Various numbers of effector cells were mixed with 2 × 10^4 labeled targets at different E:T ratios in 96-round-bottomed-well plates at a final volume of 0.2 ml of complete RPMI medium. After a 4-h (for peptide-pulsed targets) or a 12-h (for tumor targets) incubation period at 37°C, 30 μl of supernatant were collected from each well, and the percentage of specific lysis was determined according to the formula: [(cpm of the test sample − cpm of spontaneous release)/(cpm of the maximal release − cpm of spontaneous release)] × 100. For the first screening assay, the labeled target cells were mixed with cold (unlabeled) K562 cells at 1:20 labeled-target: cold-target ratio to decrease the nonspecific killing attributable to natural killer cells. Results show average specific lysis ± SE of triplicate determinations.

For MHC restriction assays, we tested the capacity of anti-MHC class I (W6/32; Ref. 25) or anti-MHC class II (9.3F10; Ref. 26) monoclonal antibodies to inhibit the lysis of tumor lines. Target cells were preincubated in 10 μg/ml mAb W6/32 or 10 μg/ml 9.3F10 for 1 h at 37°C before the cytotoxicity assay. Antibodies were prepared from culture supernatants of hybridoma cells obtained from the American Type Culture Collection.

**Cytokine Release Assay.** The cytokine production of PSMA27-specific CTLs was tested using standard ELISA tests. T2 cells pulsed with 10 μg/ml PSMA27, T2 alone, LNCaP, or 624mel were first irradiated at 8000 rads and then were placed in 96-well culture plates at 3 × 10^5 cells/well. PSMA27-specific CTLs were added at a 1:1 E:T ratio; and after 40 h of incubation, at 37°C, the concentration IFN-γ secreted by the CTLs was measured using a human IFN-γ ELISA kit (BD Pharmingen, San Diego, CA).

**Flow Cytometry and Immunofluorescence Microscopy.** The expression of HLA-A2 on tumor cells was evaluated by flow cytometry analysis. LNCaP or control melanoma cells 624mel were washed twice and resuspended in “FACS” buffer (PBS supplemented with 2% FCS and 0.2% sodium azide), then stained with anti-HLA-A2 monoclonal antibody BB7.2 (3 μg/sample), followed by FITC-labeled goat antimouse IgG (BD Pharmingen) for flow analysis in a FACScan analyzer (BD Immunocytometry Systems). The effect of IFN-γ was tested by pretreating LNCaP cells with 1000 U IFN-γ for 72 h before staining. The PSMA expression on LNCaP cells was evaluated by immunofluorescence microscopy. Briefly, LNCaP cells or 624mel cells were cultured overnight on glass slides and then fixed with cold acetone for 3 min. The fixed-cell monolayers were labeled with anti-PSMA monoclonal antibody 7E115C for 1 h in a humidified chamber, followed by staining with FITC-conjugated goat antimouse IgG (Vector Laboratories, Burlingame, CA). After washing, the slides were mounted and counterstained for DNA with Vectashield mounting medium containing DAPI (Vector). The stained cells were examined and photographed with a Nikon Eclipse E400 fluorescence microscope. The BB7.2 (27) and 7E115C (17) monoclonal antibodies were prepared from culture supernatants of hybridoma cells obtained from the American Type Culture Collection.

**RESULTS**

Selection of HLA-A2-binding Peptides from PSMA Using Predictive Algorithms. We recently reported the identification of an HLA-B7-restricted CTL epitope for CEA by the combined use of two algorithms that are accessible through the Internet and that help predict MHC-binding peptides (16). For the present studies, the amino acid sequence of PSMA was analyzed on both of the computer-based algorithms (3, 4) for the existence of 9-amino-acid peptides predicted to bind to HLA-A*0201, the most common human MHC class I allele. The number of candidate peptides was narrowed down according to two criteria. First, those peptides that did not contain canonical HLA-A*0201-binding anchor residues (L or M at position 2 and L or V at position 9; Ref. 28) were eliminated from the list of potential CTL epitopes. Second, we selected the three highest-ranking epitopes remaining from the results of each algorithm. As shown in Table 1, a total of five peptide sequences were selected using these criteria because one of the peptides (PSMA2711) scored in the top three places on both algorithms.

**Identification of HLA-A2-restricted CTL Epitopes Using PSMA-derived Peptides.** The five selected PSMA-derived peptides shown in Table 1 were synthesized and tested for their capacity to elicit primary in vitro CTL responses using peripheral blood mononuclear cells from HLA-A2+ normal donors. Each peptide was tested in at least four separate HLA-A*0201-typed donors (two males and two females). For these experiments, purified CD8+ CTL precursors were first stimulated with autologous peptide-pulsed DCs; and, after subsequent expansion, by two rounds of peptide restimulation, the CTL cultures (48 separate microcultures for each donor) were tested for their cytolytic activity against peptide-pulsed T2 target cells. Positive microcultures were considered to be those that displayed an.
least 20% specific lysis of the peptide-pulsed targets above the control unpulsed targets. In the original screening assays, four of the peptides, PSMA27, PSMA469, PSMA663 and PSMA711 were able to induce CTL responses against peptide-pulsed T2 target cells in at least one of the four donors that were tested (data not shown). On the other hand, we were unable to elicit CTLs using peptide PSMA4, in any of the four blood donors (data not presented). In those cases in which CTL responses to the peptides were observed, the microcultures were expanded for further analysis. The efficacy and specificity of representative CTL lines to recognize the four immunogenic PSMA peptides was evaluated by performing peptide-dose titration cytotoxicity assays. The results, presented in Fig. 1, indicate that the CTL-recognizing peptides PSMA27 and PSMA711 had a relatively high avidity for their ligands because 50% of the maximal responses (Fig. 1, dotted lines) could be achieved at concentrations below 1 µM peptide. On the other hand, the CTL responses to peptides PSMA469 and PSMA663 appeared to be of lower avidity because >1 µM peptide was required to attain 50% of the maximal response. In all cases, the CTLs were antigen specific because they did not kill targets pulsed with an irrelevant HLA-A*0201-binding peptide (Fig. 1, open symbols).

Antitumor Reactivity of PSMA-specific CTLs. The PSMA peptide-reactive CTL clones were then tested for their ability to recognize tumor cells that express PSMA and HLA-A2. The expression levels of both cell surface HLA-A2 molecules and PSMA will dictate whether the CTLs will be able to interact with the tumor cells in an antigen-dependent manner. Thus, we first assessed the expression levels of HLA-A2 and PSMA in two tumor cell lines to be used as targets for cytotoxicity assays, the LNCaP prostate tumor line and the 624mel melanoma line (to be used as a negative control target). Immunofluorescence microscopy using an anti-PSMA monoclonal antibody revealed, as expected, that LNCaP expressed high levels of PSMA (Fig. 2). Furthermore, all of the cells expressed similar levels of PSMA, which indicated a rather homogeneous cell population. On the other hand, the 624mel cells stained poorly with the anti-PSMA antibody, indicating that this protein is absent in these tumor cells (Fig. 2). The levels of cell-surface HLA-A2 molecules on these tumor cell lines were evaluated by flow cytometric analysis using an anti-HLA-A2-specific monoclonal antibody. As clearly shown in Fig. 3, significantly lower levels of HLA-A2 were present on LNCaP as compared with the 624mel cells. Moreover, pretreatment of LNCaP with IFN-γ (5000 IU/ml for 72 h) did not significantly increase the expression of PSMA.
HLA-A2 on these cells. These results suggest that LNCaP could be used as targets for cytotoxicity assays using PSMA peptide-specific CTLs. However, because LNCaP expresses low levels of surface HLA-A2, it is likely that only those CTLs expressing T-cell receptors of very high affinity for its corresponding MHC/peptide complexes will be able to interact with these target cells. The data also indicates that 624mel should function as an appropriate negative control for these assays because these HLA-A2-positive tumor cells do not express significant amounts of PSMA.

As shown in Fig. 4, the PSMA27-specific CTLs were quite effective in killing the LNCaP tumor cells in a dose-dependent manner. The cytotoxicity was specific because the 624mel cells were not recognized by these CTLs. On the other hand, the CTLs specific to PSMA469, PSMA663, and PSMA711 failed to kill the LNCaP and 624mel targets. It should be pointed out that these high levels of cytotoxicity were obtained using a 12-h 51Cr-release assay and that only approximately one half of this response (30% specific 51Cr release at E:T of 100:1) was observed in a conventional 4-h cytotoxicity assay (data not shown).

Another effector function of CTLs is their ability to secrete lymphokines on antigen stimulation. The results in Fig. 5 indicate that the PSMA27-specific CTLs secreted significant amounts of IFN-γ when challenged with peptide-pulsed T2 cells or with LNCaP tumor cells. On the other hand, the production of IFN-γ by the CTLs was negligible when unpulsed T2 cells or 624mel cells were used as stimulators. In similar experiments, CTL specific for peptides PSMA469, PSMA663, and PSMA711 did not produce significant amounts of IFN-γ when stimulated with LNCaP cells (data not shown).

Antibody blocking experiments were used to confirm that the CTL recognition of the naturally processed epitope (represented by peptide PSMA27), on the LNCaP cells was MHC class I restricted. Thus, we tested the capacity of anti-MHC class I and class II monoclonal antibodies to inhibit the cytolytic activity of CTL cells against PSMA-expressing tumor cells. As shown in Fig. 6, cytotoxicity of LNCaP by PSMA27-specific CTLs was significantly decreased by the anti-MHC class I but not by the anti-MHC class II antibodies. These results demonstrate that the CTL epitope represented by peptide PSMA27 is processed and presented by MHC class I molecules on prostate tumor cells.
DISCUSSION

The identification of peptide epitopes capable of triggering antitumor T-cell responses is important for the development of immunotherapy against cancer (29, 30). Although significant advances have been made in this area for tumor types such as melanoma and colon, breast/ovarian, and cervical carcinomas, progress for prostate cancer has been more modest. Although numerous potential tumor antigens exist for prostate cancer, one of the limiting factors that has hampered the identification of T-cell epitopes is the lack of suitable tumor cell lines expressing sufficient amounts of surface MHC molecules to be used as targets in cytoxicity/cytokine-release assays. The LNCaP cell line has been extensively used for prostate cancer-related studies including immunological assays because these cells express typical products of prostatic epithelial cells such as PSA, prostatic acid phosphatase, and PSMA. However, as shown here, LNCaP expresses very low levels of surface MHC class I molecules, which cannot be significantly up-regulated by IFN-γ (Fig. 3) or tumor necrosis factor-α (data not shown) treatments. Moreover, we have tried on numerous occasions to transduce these tumor cells with the genes encoding HLA-A2.1 and β2-microglobulin, but the selected drug-resistant cell lines did not express higher levels of MHC (data not presented). Concurrent with our results, it has been reported that LNCaP cells have a molecular defect in the formation of MHC class I chains that cannot be restored with IFN-γ treatment (31).

For the present studies we selected PSMA as a potential source of T-cell epitopes for HLA-A2-restricted CTLs. Following the same strategy that has allowed us to identify T-cell epitopes for other tumor markers, we selected five potential CTL epitopes from the PSMA sequence as the result of a combined algorithm analysis that predicts MHC-binding peptides (Table 1). Interestingly, two of the five peptides, PSMA4 and PSMA1111, that were selected on the basis of their high algorithm scores, correspond to the peptides “PSM–P1 and PSM–P2,” which were chosen for clinical studies (19–21) apparently solely based on the presence of HLA-A2 canonical primary binding anchors. Although there is some evidence of antitumor responses in prostate cancer patients treated with a DC-based vaccine incorporating PSMA4 and PSMA1111, to our best knowledge, no evidence has been reported demonstrating that these peptides correspond to CTL epitopes presented on tumor cells. In fact, in our hands we were unable to elicit any kind of CTL response with peptide PSMA4, which suggests either that this peptide fails to bind with sufficient affinity to HLA-A2 or, alternatively, that CTLs to this epitope have been eliminated through central or peripheral tolerance. Because HLA-A2-binding assays suggest that this peptide may bind to some extent to HLA-A2, it is more likely that the lack of immunogenicity to PSMA4 is caused by the absence (or low numbers) of precursor T cells, which would be the result of immunological tolerance. Our results indicate that the other peptide used in clinical studies, PSMA1111, was capable of inducing peptide-reactive CTLs (Fig. 1). However, these CTLs were unable to kill the prostate LNCaP cell line in a 12-h cytotoxicity assay (Fig. 4).

Similar results were obtained in a 2-day IFN-γ-release assay (data not presented). The lack of recognition of naturally processed epitopes on tumor cells by PSMA1111-reactive CTLs may be explained by one or two possibilities: (a) low affinity of the T-cell receptor for epitope; and/or (b) insufficient peptide/MHC complexes for this epitope expressed on the surface of LNCaP cells. The peptide titration curves presented in Fig. 1, which indirectly reflect the affinity of the CTLs for their epitope, illustrate that the PSMA1111-reactive CTLs probably exhibit the highest affinity of all 4 CTL lines because less peptide was required to achieve 50% of the maximal lysis. These results suggest that the reason for the lack of tumor-killing activity of the PSMA1111-specific CTLs is most likely attributable to insufficient specific MHC/peptide complexes on the surface of the LNCaP cells.

The present results cannot rule out that PSMA469, PSMA663, and PSMA1111 will not function as epitopes for CTLs on other HLA-A2+ prostate tumor cells besides LNCaP. It is possible that PSMA-positive prostate tumor cells expressing higher HLA-A2 levels than LNCaP could be able to serve as targets for CTLs reacting with any of these 3 peptides. Nevertheless, our results provide strong evidence that PSMA2 is naturally processed by the LNCaP cells and is presented in the context of HLA-A2 in sufficient amounts to allow recognition by CTLs. It would be expected that PSMA2-reactive CTL would also recognize PSMA-producing tumors expressing higher levels of HLA-A2 than does LNCaP. Unfortunately, at present, it is difficult to test this prediction because of the lack of additional prostate tumor cell lines. Nevertheless, the present results indicate that the CTL epitope represented by peptide PSMA2 should currently be the choice candidate among other PSMA peptides for developing epitope-based T-cell-based immunotherapy for HLA-A2 prostate cancer patients.

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