Human CTL Epitopes Prostatic Acid Phosphatase-3 and Six-Transmembrane Epithelial Antigen of Prostate-3 as Candidates for Prostate Cancer Immunotherapy

Arthur Machlenkin,1 Adrian Paz,2 Erez Bar Haim,1 Ofir Goldberger,1 Eran Finkel,1 Boaz Tirosh,1 Ilan Volovitz,1 Ezra Vada1,1 Gilles Lugassy,3 Shmuel Cytron,2 Francois Lemonnier,1 Esther Tzehoval,4 and Lea Eisenbach1

1Department of Immunology, Weizmann Institute of Science, Rehovot, Israel; Departments of Urology and Hematology, Barzilai Medical Center, Ashkelon, Israel; and 4AIDS-Retrovirus Department, Antiviral Cellular Immunity Unit, Pasteur Institute, Paris, France

Abstract

Specific immunotherapy of prostate cancer may be an alternative or be complementary to other approaches for treatment of recurrent or metastasized disease. This study aims at identifying and characterizing prostate cancer–associated peptides capable of eliciting specific CTL responses in vivo. Evaluation of peptide-induced CTL activity in vitro was done following immunization of HLA-A2 transgenic (HHD) mice. An in vitro tumor rejection was tested by adoptive transfer of HHD immune lymphocytes to nude mice bearing human tumors. To confirm the existence of peptide-specific CTL precursors in human, lymphocytes from healthy and prostate cancer individuals were stimulated in vitro in the presence of these peptides and CTL activities were assayed. Two novel immunogenic peptides derived from overexpressed prostate antigens, prostatic acid phosphatase (PAP) and six-transmembrane epithelial antigen of prostate (STEAP), were identified; these peptides were designated PAP-3 and STEAP-3. Peptide-specific CTLs lysed HLA-A2.1+ LNCaP cells and inhibited tumor growth on adoptive immunotherapy. Furthermore, peptide-primed human lymphocytes derived from healthy and prostate cancer individuals lysed peptide-pulsed T2 cells and HLA-A2.1+ LNCaP cells. Based on the results presented herein, PAP-3 and STEAP-3 are naturally processed CTL epitopes possessing anti–prostate cancer reactivity in vivo and therefore may constitute vaccine candidates to be investigated in clinical trials. (Cancer Res 2005; 65(14): 6435-42)

Introduction

Prostate cancer is the most diagnosed noncutaneous cancer in the western male population and the second leading cause of cancer-related death in this population (1). Yet, treatment of hormone-refractory metastatic prostate cancer remains ineffective (1). Alternative approaches based on immunotherapy have used recent advances in identification of tumor-specific and tumor-associated antigens (TAA) and have shown therapeutic promise (2). Tumor-specific antigens come from mutated proteins and from viral proteins in virally associated tumors. TAA are embryonal reexpressed or overexpressed proteins and differentiation antigens (3). Clinical trials, which test peptide-based vaccination, have been conducted mainly in melanoma patients and resulted in partial clinical responses in 10% to 30% of patients (4). Moreover, metastatic melanoma regression was achieved by adoptive transfer of peptide-specific CTLs directed against differentiation antigens after a nonmyeloablative conditioning regimen (5).

Yet, relatively few prostate carcinoma–derived antigens have been identified and evaluated as potential reagents for immunotherapy. The earliest prostate-restricted antigens include prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), and prostatic acid phosphatase (PAP), all overexpressed in prostate cancer. PSA and PSMA peptides were tested in clinical trials (6). PSA-derived peptides generate in vitro T-cell responses when cultured in peripheral blood mononuclear cells (PBMC) of HLA-A2+ or HLA-A24+ donors (7, 8). In a phase II trial, dendritic cell vaccines pulsed with HLA-A2+-restricted PSMA peptides resulted in CTL induction of 2 of 33 patients not accompanied by clinical manifestations (9). In accordance, both peptides failed to induce effective CTL responses against prostate tumor cells expressing PSMA (10). There is evidence of CD8 T-cell response against PAP (11, 12). Yet, to the best of our knowledge, there are no reports demonstrating in vivo antitumor reactivity of PAP-derived peptide vaccines. Recent studies describe differentially expressed prostate-specific genes, which may be a potential source of candidates for peptide-based vaccination. These include six-transmembrane epithelial antigen of prostate (STEAP; ref. 13), prostate stem cell antigen (PSCA; ref. 14), prostate carcinoma tumor antigen-1 (PCTA-1; ref. 15), prostate tumor-inducing gene-1 (PTI-1; ref. 16), prostate-specific gene with homology to G protein–coupled receptor (17), and prostates, an androgen-regulated serine protease (18).

Direct and reverse strategies are used for identification of TAA epitopes. The direct approach depends on patient-derived established CTL lines and genetic or biochemical screening. However, it is difficult to establish human carcinoma lines as well as carcinoma-associated CTL lines. Moreover, conceptually CTL lines from cancer patients may partially represent the repertoire of the anergized immune system. Furthermore, the in vitro propagation of CTL lines may enhance emergence of sporadic clones rather than tumor-specific clones. Alternatively, the modified reverse approach involves prediction of putative TAA-derived epitopes with amino acid anchor motifs specific for the defined human HLA allele (often A2.1, a prevalent allele in the population). Next, the predicted peptides are screened in HLA transgenic mice to identify immunodominant epitopes. In the last few years, several studies have determined concordance between the CTL repertoires of HLA-A2.1-restricted peptides in human PBMC and the CTLs (19).
The dominant murine H-2-restricted responses that take place upon immunization with multipieptide proteins have been overcome in second-generation HLA transgenic mice [i.e., HHD mice (\(D^{b/-}\) × \(B_{2}m\)-null mice transgenic for a recombinant HLA-A21/D\(^{b}\)/\(B_{2}m\) single chain (20)]. Recently, novel breast tumor–associated MUC1 and BA46-derived peptides were characterized (21, 22). Noticeably, consistent with their immunogenicity in the HHD mice, BA46-derived peptides stimulated cytotoxic activity in PBMC from HLA-A2.1-positive breast carcinoma patients (22). Thus, the HHD model is an effective means for screening of candidate TAA peptides.

In the current study, we used the HHD mouse system to screen candidate prostate-associated TAA peptides for immunogenic HLA-A2.1-restricted CTL epitopes. The results show that CTLs against PAP-3 (ILLWQPPIPVI) and STEAP-3 (LLLGTIHAL) lyse HLA-A2.1 and antigen-negative tumor cells in vitro and inhibit tumor growth in vivo. Furthermore, PAP-3 and STEAP-3 prime antitumor CTLs in human PBMC from healthy and prostate cancer donors.

Materials and Methods

**Mice.** The derivation of HLA-A2.1/\(D^{b}\)/\(B_{2}m\) monochain, transgenic, H-2\(^{b}\)/\(B_{2}m\) double-knockout mice (HHD mice) was described previously (20). Mice (CD\(^{nu}\)/\(B_{2}m\) mice (8-12 weeks old) were bred in Weizmann Institute of Science (Rehovot, Israel). All experiments were conducted in accordance with Weizmann Institute of Science Animal Facility and NIH guidelines.

**Cell lines.** LNCaP-1740 (HLA-A2\(^{b}\))/, LNCaP-10995 (HLA-A2\(^{b}\))/, and PC-3 and DU 145 (both HLA-A2\(^{-}\)) are human prostate carcinoma cell lines obtained from the American Type Culture Collection (Manassas, VA). The PC-3/HHD clone is a HLA-A2.1/\(B_{2}m\) single-chain (HHD) transfectant of PC-3 cells. EL4-HHD and RMA-S-HHD clones are HHD transfectants of the murine T lymphoma EL4 and TAP-2-deficient RMA-S cells, respectively. The RMA-S-HHD-B7.1 clone is a HHD transfectant expressing the murine B7.1 costimulatory molecule. T2 is a TAP-2-deficient lymphoblastoid line of HLA-A2 genotype. PC-3, DU 145, and T2 cells were maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% FCS (Life Technologies, Rockville, MD) and combined antibiotics.

**Reverse transcription-PCR analysis.** Total cytoplasmic RNA was isolated from logarithmically growing cell cultures. Tissue samples were obtained from patients who underwent radical prostatectomy at Barzilai Medical Center (Ashkelon, Israel). All tissues were histologically confirmed as benign prostate hyperplasia (BPH) or carcinoma of the prostate. Tissue samples were frozen in liquid nitrogen, and RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Total RNA (4 \(\mu\)g) was reverse transcribed into cDNA with 200 units SuperScript II RNase H− reverse transcriptase (Life Technologies). The cDNA quality was assured by testing the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). cDNA corresponding to 500 ng total RNA was subjected to PCR amplification (PTC-100, MJ Research, Inc., Watertown, MA) for 40 cycles as follows: 1 minute at 92°C, 1 minute at 55°C (GAPDH, PAP, PSA, PSMA, PCTA, and PTI-1), 60°C (PSA), 62°C (STEAP), and 1 minute at 72°C. Amplification was followed by 10-minute incubation at 72°C. The following primer sequences (5′−3′) were used: PAP (forward) GAGCACTGCTCACCAGC and (reverse) AACATCTGCGCCTGCTTG; PSA (forward) TGCTGTCCCCTGTTGAGCAC and (reverse) CCAGAGCAGCCAGCCAGTGGCA; PSMA (forward) TACCTAAGTTCTACGGA and (reverse) CCTTGAACCGCCTCCAATTTGCT; PTI-1 (forward) ACAGGGGAGGATGGAAAA and (reverse) TGGCCGGCTCCCTTCATCGT; PCA (forward) AAGCTGAGCCTATTTGGA and (reverse) AACACAATGGATAACCGGGT; PSMA (forward) GGCTCTGAGTTCCTGTGACG and (reverse) TGGGCACTTTGAGACTTCCCT; STEAP (forward) ACTTTGTTGATGACCAGTGTGA and (reverse) CAGAATCTCAGCACACAGGAA; and prostatase (forward) GTCCAGAGATGCGCTGTCTC and (reverse) GAGGGTCCAGGAGTCCCTTCGG. Products were electrophoresed on 1% or 2% agarose gels and monitored under UV.

**Peptide synthesis.** Peptides were synthesized on an Abimed AMS 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany), employing the \(N\)-(9-fluorenyl)methoxycarbonyl strategy following the manufacturer's protocols. Crude peptides were purified to homogeneity by reverse-phase high-performance liquid chromatography. Composition of the products was determined by amino acid analysis and the molecular weight was ascertained by mass spectrometry.

**Peptide loading and measurement of peptide binding by stabilization of cell surface MHC.** Peptide binding to HHD monochain was measured by incubation of HHD on RMA-S-HHD cells using fluorescence-activated cell sorting (FACS) analysis. Cells (5 × 10\(^{5}\) per sample) were incubated with 0.1 to 100 \(\mu\)mol/L synthetic peptide for 2 hours at 26°C followed by 3-hour incubation at 37°C and incubated in the presence of B-9-12, W6/32 (anti-HLA-A\(^{-}\)-B\(^{-}\) and -C\(^{-}\)), 28-14-8 (anti-H2-Db α3 domain), and BB7.2 (anti-HLA-A2) and washed with PBS supplemented with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide. Goat anti-mouse FITC (The Jackson Laboratory, Bar Harbor, ME) was applied for 30 minutes at 4°C. Following washing, the samples were analyzed by FACScan and CellQuest software (Becton Dickinson, San Jose, CA).

**Generation of dendritic cells from murine bone marrow cells.** Generation of murine bone marrow–derived dendritic cells was described by Lutz et al. (23) and used with minor modifications. Briefly, bone marrow cells from femurs and tibiae of 4- to 6-week-old HHD male mice were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 \(\mu\)mol/L 2-mercaptoethanol, 2 \(\mu\)mol/L L-glutamine, combined antibi-otics, and 300 units/mL recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF; Prospec, Rehovot, Israel). On day 8, nonadherent cells were harvested and further cultured in fresh medium containing 100 units/mL GM-CSF for 24 hours. Dendritic cells were matured by addition of 1 \(\mu\)g/mL lipopolysaccharide (Sigma) for another 24 hours. Nonadherent cells were analyzed by FACS and found to express typical characteristics of mature dendritic cells (CD11c\(^{+}\), CD80\(^{+}\), CD86\(^{+}\), and MHC class II\(^{+}\)).

**Vaccination.** HHD mice were immunized i.p. thrice at 7-day intervals with 100 \(\mu\)g irradiated (5,000 rad) peptide-loaded RMA-S-HHD-B7.1 or 10\(^{6}\) peptide-pulsed (100 \(\mu\)mol/L for 2 hours at 37°C) dendritic cells. In mixed synthetic vaccines, RMA-S-HHD-B7.1 cells were loaded separately with each peptide and admixed before vaccination.

**In vitro cytotoxicity assay.** HHD mice were immunized as described above. In all vaccination modes, spleens were removed on day 10 after the last immunization and cell suspensions were prepared. One third of the splenocytes were pulsed with 100 \(\mu\)mol/L synthetic peptide for 2 hours at 37°C and then added to the rest of the splenocytes. Cultures were incubated for 5 days in lymphocyte medium [RPMI 1640 supplemented with 10% FCS, 2 \(\mu\)mol/L L-glutamine, 1 \(\mu\)mol/L sodium pyruvate, 1% nonessential amino acids 25 \(\mu\)mol/L HEPES (pH 7.4), 50 \(\mu\)mol/L \(\beta\)-mercaptoethanol, and combined antibiotics]. Viable cells (effector cells) were separated by Lymphocyte-M (Cedarlane Laboratories Ltd., Hornby, British Columbia, Canada) gradient centrifugation, washed, resuspended in lymphocyte medium, and admixed at different ratios with 5,000 \(^{35}S\)-methionine-labeled target cells. CTL assays were carried out as described previously (22) in four effector-to-target (E/T) ratios ranging from 100:1 to 12.5:1. Percentage of specific lysis was calculated as follows: % lysis = (cpm in experimental well − cpm spontaneous release) / (cpm maximal release − cpm spontaneous release) × 100.

**Adaptive immunotherapy of CD\(^{1}\)nu\(^{+/}\) mice bearing human prostate carcinoma.** HHD male mice were immunized ip, six times at 3-day intervals with 2 × 10\(^{6}\) peptide-loaded syngeneic dendritic cells. Spleens were removed 10 days after the last immunization and stimulated as above. Lymphocyte-M gradient purified cells were seeded in 24-well plates
(10^6 cells/mL) that were precoated with anti-CD3 monoclonal antibody (mAb; 145-2C11) for 72 hours at 37°C in 5% CO₂. The cells were harvested and resuspended to a concentration of 3 × 10^6 in lymphocyte medium supplemented with recombinant human interleukin (hiL)-2 (100 units/mL, Prospec). Following 3 days of incubation, the cells were diluted to 10^5/mL in lymphocyte medium with hiL-2 for an additional 4 days. Expression of CD4, CD8, and CD45R was detected by FACScan (all antibodies were purchased from BD Pharmingen, San Diego, CA). Male CD1<sup>imm</sup>/mice received 4 Gy total body irradiation from a 137Cs source followed by s.c. inoculation in the upper back of 5 × 10<sup>6</sup> PC-3 HHD cells admixed 1:1 with Matrigel (Becton Dickinson). Seven days later, the mice were injected around the tumors with indicated number of HHD-derived activated T cells followed by local s.c. administration of 1,000 units hiL-2 twice daily for 5 days. Tumor size was measured in two perpendicular dimensions thrice weekly. Mice were sacrificed 44 days from tumor inoculation.

**In vitro cytotoxicity assays of human peripheral blood mononuclear cells.** HLA-A2.1-positive healthy volunteers and patients (after informed consent) with localized prostate carcinoma, after surgery, were recruited for **in vitro** generation of human CTLs. Leukapheresed PBMCs (Barzilai Medical Center) were isolated by centrifugation on Ficoll-Plaque Plus gradients (Amer sham, Uppsala, Sweden). Mature autologous dendritic cells were prepared from adherent PBMC by incubation in the presence of IL-4 (1,000 units/mL, R&D, Minneapolis, MN) and GM-CSF (1,000 units/mL; R&D) followed by maturation with a cocktail of IL-1β, IL-6, tumor necrosis factor-α (each at 10 ng/mL, R&D), and prostaglandin E<sub>2</sub> (1 μg/mL, Alexis, Carlsbad, CA). Dendritic cells were pulsed with synthetic peptides and then overlaid with PBMC in the presence of IL-7 (20 ng/mL; R&D). Two days later, hiL-2 (12 units/mL, Prospec) was added and renewed every 2 days. Two additional restimulations were done every 7 days over peptide-pulsed monocytes. Seven days later, lymphocytes were harvested and cytolytic assays were done using peptide-pulsed T2 cells or human prostate carcinoma cells as targets.

**Results**

Expression patterns of candidate tumor-associated antigens in prostate cancer. Candidate targets for immunotherapy include prostate selective antigens overexpressed in prostate cancer. To evaluate the expression profile of such genes, we used reverse transcription-PCR (RT-PCR) analysis. We examined the expression patterns of PSA, PSMA, PAP, STEAP, PSCA, PCTA, PTI-1, and prostate in human prostate cancer cell lines LNCaP, DU 145, and PC-3, in five prostate cancer specimens and in six BPH samples. PAP, STEAP, PSA, and PSMA were overexpressed in most prostate cancer samples compared with BPH (Fig. 1; data not shown). In our hands, PTI-1, PCTA-1, and prostate were not overexpressed in most prostate cancer specimens (data not shown). Thus, PAP, STEAP, PSA, and PSMA are a possible source for immunogenic peptides.

**Screening for HLA-A2.1-restricted peptides.** The four selected antigens were screened for potential TAA peptides predicted to bind HLA-A2.1 molecules and scored according to their ability to stabilize MHC using "HLA-binding predictions" software (24). This software scores every possible peptide along the protein sequence for MHC binding and every amino acid in the evaluated peptide is scored according to its contribution to the binding (http://www.bimas.dcrt.nih.gov/molbio/hla_bind/index.html). For each candidate TAA, we synthesized five peptides with the highest binding score (Supplementary Table S1).

**Screening for immunogenic HLA-A2.1-restricted peptides: CTL responses in HHD mice.** To test the immunogenicity of the candidate peptides (i.e., their potential to induce T-cell responses), we established a two-step **in vitro** screening system using transgenic HHD mice. HHD mice were immunized with syngeneic dendritic cells pulsed with pools of two to four synthetic peptides derived from each examined protein followed by **in vitro** restimulation of splenocytes with the same peptides used for priming. We evaluated the lysis patterns of each of the individual peptides loaded on target cells. CTL results showed specific lysis of RMA-S-HHD-B7.1 target cells loaded either with PAP-derived peptide-3 (PAP-3, ILLWQIPPV) or with STEAP-derived peptide-3 (STEAP-3, LLLGTIHAL). Other peptides showed either background lysis (PAP-, PSA-, and PSMA-derived peptides) or less effective lysis (STEAP-derived peptides; Fig. 2A and B; data not shown). Subsequent immunization of HHD mice with dendritic cells pre pulsed with individual PAP-3 or STEAP-3 showed dose-dependent lysis of EL-4 HHD target cells loaded with either PAP-3 or STEAP-3 (Fig. 2C and D). CTL responses were peptide specific. Interestingly, immunization of HHD mice with homologous murine peptides (mPAP-3, RLLWQPMPV; mSTEAP-3, LLLGTIHAL) induced also peptide-specific CTLs comparable with human counterparts. Anti-mPAP-3 CTLs lysed 55 ± 3.2% and 38 ± 2.9% targets at 100:1 and 25:1 E:T ratio, respectively; anti-mSTEAP-3 CTLs lysed 48 ± 3.5% and 37 ± 2.4% targets at 100:1 and 25:1 E:T, respectively.

**Confirmation of predicted peptide binding by stabilization of cell surface MHC.** To examine the actual peptide binding to HLA-A2.1 molecules, we used TAP-2-deficient, HLA-A2.1-transfected RMA-S cells (RMA-S-HHD), which express low levels of peptide-free and therefore unstable class I MHC molecules. External loading of RMA-S-HHD cells with selected synthetic peptides at 0.1 to 100 μmol/L stabilized HLA-A2.1 molecules at a dose-dependent manner as determined by FACS analysis. MHC binding at 100 μmol/L gave the highest level of stabilization that was comparable with that of reference tyrosinase-derived, HLA-A2.1-restricted peptide (Fig. 3). A high correlation between predicted and actual binding has been confirmed (22).

**HLA-A2.1-restricted lysis of human prostate carcinoma cell lines by CTL induced against prostatic acid phosphatase-3 and six-transmembrane epithelial antigen of prostate-3.** Although PAP-3 and STEAP-3 induce an immune response, it is crucial to test whether these peptides are indeed presented on tumor cell surface...
by MHC class I molecules and therefore may serve as tumor rejection antigens. We assessed CTL responses against relevant prostate cancer cell lines as targets using the HLA-A2.1-positive LNCaP cells that were shown to express PAP and STEAP proteins as targets for CTLs raised against the peptides of interest (refs. 13, 25; Fig. 4C and D). To this end, HHD mice were immunized with RMA-S-HHD-B7.1 cells loaded with either PAP-3 or STEAP-3, and lysis of LNCaP cells was monitored (Fig. 4A). CTLs induced against PAP-3 or STEAP-3 showed similar and specific response profiles against target LNCaP cells. Furthermore, the lysis of HLA-A2.1-negative PC-3 cells by either PAP-3- or STEAP-3-specific CTLs was negligible, suggesting MHC-restricted lysis (Fig. 4A). Expression of PAP in PC-3 and PC-3-HHD transfectant was confirmed by immunoprecipitation (Fig. 4C). In contrast to previous reports describing PC-3 cells as negative for PAP (26, 27), we found that both lines express PAP (Fig. 4D). HHD-derived anti-PAP-3 CTLs lysed efficiently and in a dose-dependent manner PC-3-HHD but not parental PC-3 cells (Fig. 4B). Taken together, these data indicate that both PAP and STEAP proteins undergo intracellular processing in LNCaP cells.

Adoptive immunotherapy of CD1<sup>nu/nu</sup> mice bearing human prostate carcinoma. We next tested the in vivo antitumor reactivity of PAP-3- and STEAP-3-induced lymphocytes. Because neither LNCaP nor PC-3-HHD cells grow progressively in HHD mice, we established a model of adoptive transfer of HHD-derived lymphocytes into CD1<sup>nu/nu</sup> mice bearing prostate carcinoma. PC-3-HHD cells at 5 x 10<sup>6</sup> admixed with Matrigel but not LNCaP cells formed homogeneous s.c. tumors in 100% of the CD1<sup>nu/nu</sup> mice. To induce peptide-specific CTLs for adoptive transfer, we vaccinated HHD mice with PAP-3, STEAP-3, and HLA-A2.1-restricted, tyrosinase-derived peptide as an irrelevant control, all loaded on syngeneic dendritic cells. CD1<sup>nu/nu</sup> mice were inoculated s.c. with the PC-3-HHD, and 7 days later, after formation of visible tumors, CTLs were injected around the tumors as described. We observed that 11 x 10<sup>6</sup> per mouse of anti-PAP-3 CTLs completely prevented progressive growth of PC-3-HHD tumors in CD1<sup>nu/nu</sup> mice, whereas the same amount of irrelevant peptide-induced CTLs failed to inhibit tumor development (Fig. 5A). The CTLs induced by STEAP-3 (3 x 10<sup>6</sup> per mouse) were potent in decreasing the tumor volumes compared

Figure 2. Immunogenicity of PAP3 and STEAP3 peptides in HHD mice. Mice were immunized i.p. thrice weekly with 10<sup>6</sup> peptide-loaded syngeneic mature dendritic cells. DC were loaded separately with PAP-2, PAP-3, and PAP-5 (A) or STEAP-1, STEAP-2, STEAP-3, and STEAP-4 (B), washed, and pooled before immunization. DC were loaded with PAP-3 (C) or STEAP-3 (D) and injected individually. For all experiments, spleens were removed on day 10 after the last boost and splenocytes were stimulated in vitro by corresponding peptide as described. CTL assays were done on day 5 with individual peptides loaded on RMA-S-HHD (A and B) or EL4-HHD (C and D) as targets. A and B, irrelevant HIV-derived, peptide-loaded targets were used as negative controls. STEAP-3-loaded cells were used as nonspecific targets for PAP-3-induced lymphocytes (C) and vice versa (D). The percentages of specific lysis at E:T ratios of 100:1 to 12.5:1 are shown. Columns, mean of three replicates; bars, SD. Representative experiment of four. Specific lysis of PAP-3 and STEAP-3 is statistically significant (P < 0.001, unpaired Student’s t test) at all E:T ratios compared with background lysis.

Figure 3. Stabilization of cell surface MHC by PAP-3 and STEAP-3. PAP-3, STEAP-3, or a tyrosinase peptide, known to bind HLA-A2 molecules (positive control), was loaded at various concentrations (0.1-100 μmol/L) on TAP-2-deficient RMA-S-HHD cells as described. Indirect FACS analysis was done by incubating 5 x 10<sup>5</sup> loaded cells with anti-HLA-A2.1 mAb BB7.2 for 30 minutes at 4°C. Following washing with PBS containing 0.5% BSA and 0.1% sodium azide, the secondary antibody, goat anti-mouse FITC, was applied for 30 minutes at 4°C. Following another wash, the amounts of bound antibodies were detected by a FACScan. Mean fluorescence at 0.1 to 100 μmol/L peptide concentrations. Representative experiment of three.
with the control groups (Fig. 5B). Thus, using adoptive immunotherapy of human prostate carcinoma as an experimental model, we confirmed a strong in vivo antitumor reactivity of PAP-3- and STEAP-3-specific CTLs.

Activation of prostatic acid phosphatase-3–specific and six-transmembrane epithelial antigen of prostate-3–specific CTL precursors in peripheral blood mononuclear cells derived from healthy volunteers and prostate cancer patients. We then asked whether PAP-3- and STEAP-3-specific CTL precursors are found in PBMC of healthy volunteers and more importantly of prostate cancer patients. To this end, bulk lymphocytes from three healthy and two prostate cancer donors were in vitro primed over autologous dendritic cells pulsed with the synthetic peptides followed by two additional cycles of restimulation as described in Materials and Methods. Activated CTLs lysed the TAP-2-deficient HLA-A2.1+ T2 target cells loaded with PAP-3 or STEAP-3 and not unloaded cells (Fig. 6A). Moreover, peptide-specific CTLs lysed the HLA-A2.1-positive LNCaP cells (Figs. 4C and 6B and C). Based on these data, one may conclude that there are PAP-3- and STEAP-3-specific and potent CTL precursors in peripheral blood of healthy and prostate cancer donors.

Discussion

Two approaches govern active anticancer vaccination. The first uses vaccines directed against unidentified antigens as irradiated tumor cells, either intact or genetically modified, total tumor RNA, or cell lysates (28). An alternative strategy, allowing both monitoring of immune response and rational systematic improvement of vaccine design, is based on use of antigen-specific cancer vaccines that induce immune responses against defined antigens. Indeed, active vaccination with a single TAA epitope has been shown to protect from tumor challenge and to palliate established tumors in vivo (29). Relatively few prostate cancer–derived epitopes have been identified and examined in clinical trials thus far. Contrary to melanomas, prostate cancer–associated epitopes,
such as PSMA derived epitopes, were largely ineffective in specific CTL and clinical responses (9, 10).

In the current study, we identified two HLA-A2.1-restricted CTL epitopes, PAP-3 and STEAP-3, which induce antitumor immunity in vitro and in vivo. Identification of TAA peptides by the reverse approach is based on recognition of candidate TAAs, in silico prediction of HLA-binding epitopes, and testing the peptides for their ability to induce an immune response in human PBMC. This strategy is hard to reproduce and it is highly dependent on the individual heterogeneity of the population. To overcome these problems, we used the HHD mouse model for initial screening of immunogenic peptides derived from candidate TAAs. The HHD mouse combines classic HLA transgenesis (HLA-A2.1/D b-)

immunogenic peptides derived from candidate TAAs. The HHD mouse combines classic HLA transgenesis (HLA-A2.1/D b-) with selective destruction of murine H-2 (double knockout deletion of the murine H-2m single chain) with selective destruction of murine H-2 (double knockout deletion of the murine H-2m and D b- genes) that restricts the whole class I–derived T-cell repertoire to HLA-A2.1. Others and we have identified several HLA-A2.1-restricted epitopes from TAAs, such as MUC1 (21), BAAb (22), PTH-rP (30), HER-2/neu and hTERT (31), and MAGE-A8 (32). PAP, PSA, PSMA, and STEAP were found to be overexpressed in prostate cancer versus BPH samples (Fig. 1). Twenty HLA-A2.1-restricted peptides derived from the protein sequences were selected according to their calculated MHC-binding affinities (Supplementary Data). Positive correlation between calculated binding affinities and immunogenicity of CTL epitopes has been shown (24, 33). Using a two-step screening in HHD mice, we compared in the same experiment the immunogenic potential of each peptide with that of others. Two peptides, PAP-3 and STEAP-3, were highly immunogenic in HHD mice (Fig. 2). One possible explanation for the immunogenicity of PAP-3 and STEAP-3 could be that they represent sequences with minimal similarity to the murine homologues and therefore induce xenopCTLs. Sequence alignment between PAP or STEAP and their murine homologues displays 81% identity in protein sequences in both pairs. Moreover, the PAP-3 and STEAP-3 murine counterparts show conservation of eight/nine amino acids (I/L-LWQPPIP/Vc→RLLWQPPIP/V and LLLGT/HALc→LLLGT/HAL, respectively), keeping the anchor motifs in positions 2 and 9 (34). Finally, analysis of CTL responses against murine PAP-3 and STEAP-3 homologues showed comparable immunogenicity with the human peptides. Thus, the immune response to PAP-3 and STEAP-3 was not the result of differences between human and mouse sequences. Lymphocytes induced against PAP-3 or STEAP-3 preferentially lysed the prostate cancer cell line LNCaP and not HLA-A2–/cancer cells, indicating that PAP-3 and STEAP-3 are processed and presented by LNCaP cells (Fig. 4).

To test the immunotherapeutic potential of PAP-3 and STEAP-3, we established an adaptive transfer model in which PC-3-HHD tumor-bearing nude mice were treated by peptide-specific lymphocytes transferred to the tumor vicinity from immunized HHD mice. In immunoprotection experiments on TRAMP mice, memory lymphocytes specific for T antigen have been transferred from mice immunized with SV40-transformed fibroblasts (35). In our system, we achieved a strong therapeutic effect in palpable tumor-bearing mice using peptide-induced lymphocytes (Fig. 5). PC-3-HHD cells are lysed in vitro by anti-PAP-3 (Fig. 4B) but not by anti-STEAP-3 CTLs (data not shown). Two possible mechanisms could account for the lack of lysis of PC-3-HHD by anti-STEAP-3 CTLs: low expression of STEAP by PC-3-HHD cells or low affinity of STEAP-3 to HLA-A2.1 that decreases the effective concentration of the presented peptide. We observed, however, that on adoptive immunotherapy of PC-3-HHD bearing mice STEAP-3-induced CTL (3 × 10^6/mouse) impaired growth of the tumors (Fig. 5B). We have shown, we believe for the first time, that a CD8-mediated CTL response to STEAP can be induced in vitro.

Several studies have reported that higher frequencies of survivin (36), HER-2/neu, and carcinoembryonic antigen (37) specific CTL precursors exist in patients suffering from melanoma and colorectal cancer, respectively, than in healthy subjects. On the other hand, cancer patient-derived CTLs that were successfully activated against defined TAA epitope presented by antigen-presenting cells have shown inability to lyse cancer cells presenting the same epitope (38). In our study, PAP-3 and STEAP-3 induced human CTLs in blood lymphocytes from healthy and prostate cancer donors. Importantly, peptide-specific CTLs lysed not only peptide-loaded T2 cells but also MHC-positive LNCaP cells that naturally express the TAAs of interest (Fig. 6). Yet, HLA-A2–/cancer cells are not lysed. Thus, low MHC–expressing tumors might be resistant to peptide-based immunotherapy.

Figure 5. Adoptive immunotherapy of PC-3-HHD tumor-bearing CD1 nu/nu mice. CTLs specific to either PAP-3 (A, ▲, 11 × 10^6/mouse), STEAP-3 (B, ●, 3 × 10^6/mouse), or irrelevant peptide (A and B, ■, 11 × 10^6/mouse) were obtained on vaccination of HHD mice, restimulated in vitro, and injected around the 7-day-old PC-3-HHD tumors in CD1 nu/nu mice (n = 10/group) as described in Materials and Methods. All mice (n = 40) had palpable tumor at the time of adoptive transfer. Points, mean tumor size (in mm^3) of the treatment groups plotted against the days after tumor challenge; bars, SD. Tumor growth was followed for 40 days. CTLs from mice immunized with irrelevant tyrosinase-derived peptide or PBS-injected (No treatment) CD1 nu/nu tumor-bearing mice are the negative controls in the experiment. Adoptive transfer of specific CTLs significantly completely inhibited (A, PAP-3, P < 0.01, unpaired Student’s t test) or slowed down (B, STEAP-3, P < 0.01, unpaired Student’s t test) tumor growth.
In conclusion, this study presents a novel approach for in vivo two-step screening of candidate HLA-A2.1-restricted CTL epitopes for cancer immunotherapy. Using the HHD mouse as a model for in vivo evaluation of immunogenic and antigenic potential of putative TAA epitopes, PAP-3 and STEAP-3 have been identified as good vaccine candidates for immunotherapy of prostate cancer.

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References

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Arthur Machlenkin, Adrian Paz, Erez Bar Haim, et al.