Screening of HLA-A24-restricted Epitope Peptides from Prostate-specific Membrane Antigen That Induce Specific Antitumor Cytotoxic T Lymphocytes

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ABSTRACT

Purpose: Prostate-specific membrane antigen (PSMA), which is a transmembrane glycoprotein predominantly expressed in prostate cancer, is an attractive target for tumor-specific immunotherapy. To identify human leukocyte antigen (HLA)-A24-restricted epitope peptides from PSMA for further application of the dendritic cell (DC)-based immunotherapy targeting prostate cancer, we have screened several PSMA-encoded HLA-A24-binding peptides for their capabilities to elicit specific antitumor CTL response in vitro.

Experimental Design: The amino acid sequence of PSMA was screened for peptides consisting of 9 or 10 amino acids, which possess the known HLA-A24-binding motif. Nine candidate peptides were screened for binding to HLA-A24 molecules. Then, each of these nine peptides was studied to determine whether CTL responses could be induced by primary in vitro immunization of CD8+ T cells using peptide-pulsed autologous DCs derived from peripheral blood mononuclear cells of HLA-A24+ healthy donor as antigen-presenting cells. The antigen specificity of the CTL lines was confirmed using several tumor cell lines as target cells, which were genetically modified to express both HLA-A24 and PSMA.

Results: Two peptides, LYSDPADYF and NYART-EDFF, were demonstrated to elicit CTL lines that lyse peptide-pulsed, HLA-A24+ B-lymphoblastoid cells. Each of the CTL lines recognized their specific PSMA-expressing target cells in a HLA-A24-restricted manner. The capability to release IFN-γ by the CTL lines was specifically inhibited by anti-MHC class I and anti-CD8 monoclonal antibodies but not by anti-MHC class II and anti-CD4 monoclonal antibodies.

Conclusion: Two novel HLA-A24-restricted PSMA-derived epitopes were identified in this study. These epitopes can be used to further evaluate the clinical utility of DC-based immunotherapeutic strategies for treatment of hormone-refractory prostate cancers.

INTRODUCTION

Prostate cancer is a significant and growing health problem in the Japanese population, and hormone-refractory disease is known to be highly resistant to the conventional radiotherapy and/or chemotherapy. Therefore, it is of great interest to develop an alternative strategy to treat these hormone-refractory prostate cancer patients. Immunotherapy targeting PSMA, a 100,000 type II transmembrane glycoprotein abundantly expressed on the surface of prostatic epithelial cells (1, 2), is one such promising approach. PSMA was initially defined by the mAb 7E11-C5 and has been shown to be highly specific for prostate cancer cells (3). Clinically, the mAb 7E11-C5 has been used in radiolabeled imaging studies to detect prostate cancer metastases (4, 5). More importantly, several PSMA mAb-based therapies against prostate cancers have been proposed and tested for their clinical feasibility (6–8).

A second utility of PSMA for targeting prostate cancer is for active cellular immunotherapy using antigen-presenting DCs loaded with epitope peptides derived from PSMA to activate prostate cancer-specific CTLs (9, 10). Murphy et al. (11) and Tjoa et al. (12) have conducted Phase I/I clinical trials using autologous DC pulsed with HLA-A2.1-restricted epitope peptides derived from PSMA, and ~30% of study participants showed treatment benefit, defined as a steady decrease in serum levels of PSA and/or stabilization or decrease in measurable metastatic tumor burden. Because HLA-A24 is the most com-
mon MHC class I allele among Japanese population and is also frequently present in other Asians and Caucasians (13), it is of great interest to identify HLA-A24-restricted epitope peptides from PSMA for further application of the DC-based immunotherapy targeting of prostate cancer.

In the present report, a selected series of PSMA-derived peptides harboring a HLA-A24-binding motif were evaluated as candidate CTL epitopes. The HLA-A24-binding affinity of each peptide was determined using a competitive inhibition assay. Each candidate peptides was then tested for the ability to induce peptide-specific CTLs from PBMCs obtained from HLA-A24+ healthy volunteers. Finally, three CTL lines that exhibited peptide-specific activity were tested for recognition of endogenously processed antigen using PSMA-expressing HLA-A24+ target cells that were generated using an adenovirus-mediated gene-modification technique. From these studies, we have identified two HLA-A24-restricted PSMA epitopes that should be useful for DC-based immunotherapy in the HLA-A24+ prostate cancer patients.

MATERIALS AND METHODS

Cell Lines. The prostate cancer cell line, LNCaP (HLA-A24+) and the colon carcinoma SW480 cell line (HLA-A24+) were obtained from the American Type Culture Collection (Manassas, VA). The TISI, a HLA-A24+ B-lymphoblastoid cell line, was supplied by Takara Shuzo Co., Ltd. (Shiga, Japan). The gastric cancer cell lines, MKN28 (HLA-A24+) and MKN45 (HLA-A24+), the esophageal carcinoma cell line T.T. (HLA-A24+), and the chronic myeloid leukemia K562 cell line were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The melanoma cell lines, 526mel (HLA-A24+) and 88mel (HLA-A24+), were kindly provided by Dr. Y. Kawakami (Keio University, Tokyo, Japan). EPV-transformed B-lymphoblastoid cell line, EHM cells (HLA-A3/3), was obtained from the American Society for Histo compatibility and Immunogenetics Repository Collection. All of these cell lines were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum and appropriate antibiotics. The HLA-A genotypes of the cells used in our study were determined by the PCR-sequence-specific oligonucleotide probe method as described previously (13).

Generation of HLA-A24+ and PSMA+ Cells. Adenovirus Expression Vector kit (Takara Shuzo, Co., Ltd.) was used for transient transfection of either HLA-A24 or PSMA cDNA into the HLA-A24+/PSMA+ LNCaP cells or HLA-A24+/PSMA+ various cancer cells, respectively. HLA-A24 and PSMA cDNA were obtained by PCR using pRSV5-PSMA-HLA-A24 given by Epimmune Inc. (San Diego, CA; Ref. 14) and pSPORT1-PSMA, generously given by Dr. W. W. W. Heston (The Cleveland Clinic Foundation, Cleveland, OH; Ref. 3), respectively. Each cDNA fragments of HLA-A24 and PSMA, which contain 10 bp upstream from the ATG start codon through the stop codon, were inserted into the SmaI site in the expression Cosmid vector, pAxCawt. After each rAd was cloned, the DNA sequences of each inserted fragment were verified by automated DNA sequencer (ABI 377 automated sequencer; Applied Biosystems, Tokyo, Japan). Final virus titers were 5.8 × 10⁸ PFU/ml and 2.9 × 10⁸ PFU/ml for rAd (HLA-A24) and rAd (PSMA), respectively. Subsequently, the rAd (HLA-A24) was used to transduce LNCaP cells at 20 MOI, and various PSMA cell lines were transduced with the rAd (PSMA) at 30 MOI. Forty-eight h later, transduced cells were evaluated by flow cytometry (Ortho Diagnostic Systems Inc., Raritan, NJ) for the expression of HLA-A24 and PSMA.

Antibodies. The expression of MHC class I and HLA-A24 molecules was evaluated by flow cytometric analysis using mAbs, W6/32 (Immunotec, Marseille, France) and anti-HLA-A24/24 (One Lambda, Canoga Park, CA), respectively, as first antibody. The membrane expression of PSMA was measured by flow cytometric analysis using a polyclonal antibody prepared by immunization of BALB/c mice (7-week-old, female) with PSMA expression plasmid pPSMA1056 (7672 bp). This expression vector was obtained by Sall digestion to remove the extra fragments of the PSMA Cosmid vector described above, and then by recircularization. The plasmid was purified using Qiagen kit (Mid prep kit; Qiagen, Tokyo, Japan), and then animals were immunized by i.m. injections of the plasmid (100 μl of 1 mg/ml solution) into the thighs on days 0 and 21, 4 days after injection of cardioxigen (Sigma Chemical Co., St. Louis, MO; 100 μl of 10 μm solution) into the thighs. Antisera obtained on day 51, which recognized PSMA protein contained in LNCaP cell lysate by immunoblotting, were used as a polyclonal antibody. Antisera used as a control were prepared from mice immunized with PBS on the same schedule as the immunization with pPSMA1056. We also used PSMA mAb, Y-PSMA-1 (clone 4G5; Yes Biotech Lab., Ontario, Canada; Ref. 15), for the detection of PSMA protein and expression of PSMA on the cell surface of the transfected cells.

Synthetic Peptides. Peptides were synthesized according to the standard solid-phase synthesis method and purified by reversed-phase HPLC. The purity (<90%) and identity of peptides were determined by analytical HPLC and mass spectrometric analysis, respectively. Peptides were dissolved in DMSO (Sigma Chemical Co.) at 20 mg/ml and stored at −30°C.

MHC-binding Assay. The binding capacity of peptides to HLA-A24 molecules was measured based on the inhibition of binding of a radiolabeled standard peptide to purified MHC class I molecules as described previously (16). Briefly, various concentrations of the test peptides were incubated with 125I-labeled standard peptide and with purified and detergent-solubilized HLA-A24 molecules in the presence of a mixture of protease inhibitors and β₂-microglobulin (Scripps Laboratories, San Diego, CA). The amino acid sequence of standard peptide used for the MHC-binding assay for HLA-A24 was AYIDNYK. The percentage of HLA-A24-bound radioactivity was determined by gel filtration, and the concentration of the test peptides that inhibited 50% of the binding of the labeled standard peptide (IC₅₀) was calculated. In addition, NIH-binding score, which is available through the Internet web site, was evaluated as HLA peptide-binding predictions.

Generation of DCs. PBMCs were isolated from apheresis of HLA-A24+ healthy volunteers by Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient centrifugation at 580 × g for 20
min, followed by washing with PBS three times. The adherent monocytes were cultured with 1000 units/ml recombinant IL-4 (R&D Systems, Inc., Minneapolis, MN) and 1000 units/ml recombinant granulocyte-macrophage colony stimulating factor (R&D Systems, Inc.) in RPMI 1640, supplemented with 5% human AB serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 20 μg/ml gentamicin (SH-RPMI) for 7 days at 37°C. At the time of CTL induction in vitro, the DCs were pulsed with 40 μg/ml PSMA epitope peptide in the presence of 2 μg/ml β2-microglobulin for 4 h at room temperature in 1% BSA/PBS, washed extensively, and then used for antigen presentation.

**Primary CTL Induction Cultures.** The CTL induction was performed according to the procedures as previously described (17). Briefly, peptide-pulsed DCs were irradiated (55 Gy) and mixed at a 1:20 ratio with autologous CD8+ T cells, obtained by positive selection with Dynabeads M-450 CD8+ (Dynal, Lake Success, NY) and Detachabead (Dynal). Then, these cocultures were set up in 48-well plates, each well containing 2.5 × 10^5 peptide-pulsed DCs, 5 × 10^3 CD8+ T cells, and 10 ng/ml recombinant IL-7 (Genzyme, Boston, MA) in 0.5 ml of SH-RPMI. One day later, the CTL cultures were supplemented with recombinant IL-10 (R&D Systems, Inc.) to a final concentration of 10 ng/ml. On days 7 and 14, the T-cell cultures were restimulated with autologous peptide-pulsed APC. At 2 and 5 days after every restimulation, the cultures were fed with a fresh medium containing 30 IU/ml IL-2 (Shionogi, Osaka, Japan). The CTL activity was assessed after two rounds of peptide restimulation on day 21 using peptide-pulsed T2i cells as target. Responder cells in the positive wells were further expanded as described in the following section and tested for specific recognition of various cancer cell lines.

**CTL Cytotoxic Assay.** Target cells were labeled with 51Cr by incubating with 100 μCi of Na51CrO4 (Tai-ichi Kagaku Yakuhin, Tokyo, Japan) for 1 h at 37°C and were washed twice. Effector cells were plated onto round-bottomed 96-well microtiter plates at various concentrations. The radiolabeled target cells were then added at a concentration of 1 × 10^3 cells/0.1 ml/well (maximum E:T ratios in these assays were 10:1). To eliminate nonspecific lysis, the cytotoxic activity was tested in the presence of a 30-fold excess of unlabeled K562 cells. After incubation for 4 h, the release of 51Cr in the supernatant was measured by an automated gamma counter. The percentage specific 51Cr release was calculated by the following formula:

\[
\text{Percentage specific } 51\text{Cr release} = \left(\frac{\text{Release by CTL} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}}\right) \times 100
\]

Spontaneous release generally 15–20% of the maximum release in our experiments.

**Cytokine Release.** The supernatants of PSMA-specific CTL lines were collected after 24-h coculture with the various target cells and stored at −80°C until the later IFN-γ measurement. To determine the IFN-γ activity, a commercially available immunoenzymometric assay kit was used (Genzyme Technne, Minneapolis, MN).

**CTL Expansion Procedure.** For more detailed characterization, CTL lines were expanded in tissue culture following a method similar to the one described previously (18). A total of 5 × 10^4 CTLs were resuspended in 25 ml of 10% HyClone-RPMI (Logan, UT) with irradiated (33 Gy) PBMC and irradiated (80 Gy) EHM cells in the presence of 30 ng/ml anti-CD3 mAbs (Ortho Diagnostic Systems, Tokyo, Japan). One day later, 120 IU/ml IL-2 were added to the cultures. The cultures were fed with fresh 10% HyClone-RPMI containing 30 IU/ml IL-2 on days 5, 8, and 11, and were split if the T-cell concentration reached numbers greater than 1.5 × 10^6/ml.

**Inhibition of IFN-γ Productivity by Blocking mAbs.** To determine the mechanism of immunological response in the recognition of antigens by the PSMA-reactive CTL lines, PSMA-transfected 888mel cells were pretreated with anti-HLA class I (W6/32) or II (L243; Becton Dickinson, San Jose, CA) mAbs for 1 h at room temperature. Also, the CTL lines were pretreated with anti-CD4 (MT310; DAKO Japan, Kyoto, Japan) or CD8 (DK25; DAKO Japan) mAbs. These pretreated target and effector cells were tested for their capacity to inhibit the IFN-γ productivity by the PSMA-specific CTL lines in response to the target cells.

**RESULTS**

**Generation of HLA-A24+/PSMA+ Target Cells.** LNCaP cells were transiently transfected with rAd (HLA-A24), and after 48-h cultivation, flow cytometric analysis was carried out for evaluating the transfection efficiency. Approximately 48% cells were positively expressing HLA-A24 molecules on the surface of transfected LNCaP cells (Fig. 1A). MKN45 gastric cancer cells, which were transfected with rAd (PSMA), were evaluated, and ~81% of the cells were positive for PSMA expression (Fig. 1B). Thus, 73–96% of the other rAd (PSMA)-transduced HLA-A24+ cell lines transiently expressed PSMA in each of the later experiments.

**Screening of HLA-A24-binding Epitope Peptides from PSMA.** The amino acid sequence of PSMA (GenBank accession no. AAC83972) was screened for peptides consisting of 9 or 10 amino acids, which possess the known HLA-A24-binding motif (Y or F at position 2 and F, I, W, or L at the COOH terminus; Ref. 19). A total of nine motif-containing peptides...
were selected for evaluation as candidate of HLA-A24-restricted PSMA epitopes and designated as PSMA24-1 through -9 (Table 1). NIH binding scores used for predicting the half-life of peptide-MHC dissociation ranged from 100 to 300 for the nine peptides analyzed. These values did not correlate with the actual HLA-A24-binding affinities, for which the IC50 ranged from 14 to 10,000 nM, as determined by 50% inhibition of binding of standard peptide.

**Table 1** List of HLA-A24-binding peptides from PSMA

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Position<strong>a</strong></th>
<th>Size<strong>b</strong></th>
<th>Sequence</th>
<th>Binding score<strong>c</strong></th>
<th>IC50 (nM)</th>
<th>No. of positive wells/Total no. of wells<strong>e</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>PSMA24-1</td>
<td>298</td>
<td>9</td>
<td>GYYDAQKLL</td>
<td>240</td>
<td>&gt;10,000</td>
<td>0/40 (1) 0/48 (2)</td>
</tr>
<tr>
<td>PSMA24-2</td>
<td>624</td>
<td>9</td>
<td>TYSVSFDSDL</td>
<td>240</td>
<td>197</td>
<td>0/40 (1) 0/48 (2)</td>
</tr>
<tr>
<td>PSMA24-3</td>
<td>227</td>
<td>9</td>
<td>LYSDPADYF</td>
<td>120</td>
<td>406</td>
<td>1/48 (1) 0/48 (3)</td>
</tr>
<tr>
<td>PSMA24-4</td>
<td>606</td>
<td>9</td>
<td>KYADKIYSI</td>
<td>120</td>
<td>15</td>
<td>0/48 (1) 0/48 (3)</td>
</tr>
<tr>
<td>PSMA24-5</td>
<td>178</td>
<td>9</td>
<td>NYARTEDFF</td>
<td>100</td>
<td>67</td>
<td>2/48 (1) 0/48 (4)</td>
</tr>
<tr>
<td>PSMA24-6</td>
<td>74</td>
<td>10</td>
<td>LYNFTQIYHL</td>
<td>300</td>
<td>1,268</td>
<td>0/48 (1) 0/48 (5)</td>
</tr>
<tr>
<td>PSMA24-7</td>
<td>565</td>
<td>10</td>
<td>FYDFMFKYHL</td>
<td>240</td>
<td>153</td>
<td>0/48 (1) 0/48 (5)</td>
</tr>
<tr>
<td>PSMA24-8</td>
<td>699</td>
<td>10</td>
<td>KYAGESFPQGI</td>
<td>120</td>
<td>838</td>
<td>0/40 (1) 0/40 (6)</td>
</tr>
<tr>
<td>PSMA24-9</td>
<td>624</td>
<td>10</td>
<td>TYSVSFDNL</td>
<td>100</td>
<td>14</td>
<td>0/48 (1) 0/48 (6)</td>
</tr>
</tbody>
</table>

**a** The relative positions in the protein sequence of PSMA.  
**b** The numbers represent peptide size.  
**c** NIH binding score, which is available through the Internet website, was evaluated as HLA peptide binding predictions.  
**d** Measured as concentration of test peptide required for 50% inhibition of binding of standard peptide as described in “Materials and Methods.”  
**e** The numbers of wells that showed positive CTL response are presented. The numbers with parentheses indicate each of the individuals whose PBMCs were used for the study.

Induction of Antigen-specific CTL Responses by PSMA Epitope Peptides. Each of the nine peptides was studied for the capacity to elicit CTL responses by primary in vitro immunization of CD8+ T cells using peptide-pulsed autologous APCs derived from PBMCs of a HLA-A24+ healthy donor. Two peptides, LYSDPADYF (PSMA24-3) and NYARTEDFF (PSMA24-5), stimulated the induction of peptide-specific CTL, generating 1 and 2 positive wells of 48 wells, respectively. To obtain a sufficient number of effector cells for more comprehensive studies on the cytotoxicity and specificity of the CTL, these three CTL lines, referred to as PSMA24-3 no. 46, PSMA24-5 no. 8, and PSMA24-5 no. 17, were expanded using anti-CD3 mAbs as a mitogen and a mixture of feeder cells. The three CTL lines were composed mostly (<90%) of CD8-positive T cells (data not shown) and showed a significantly high cytotoxicity against peptide-pulsed TISI cells when compared with the non-peptide-pulsed TISI cells (Fig. 2). The PSMA-specific CTL lines PSMA24-3 no. 46 and PSMA24-5 no. 8 were also evaluated in terms of their antigen-dose responsiveness. Peptide concentrations as low as 0.1 ng/ml could sensitize the target TISI cells with significant levels of specific lysis in both of the CTL lines, which indicates that these CTL lines were capable of recognizing the PSMA epitope peptides with considerably high affinity (Fig. 3). In a second experiment, none of the other peptides were able to induce specific CTL responses, even after another induction with these antigens using PBMCs from another HLA-A24+ healthy donor (Table 1).

Antigen Specificity of the PSMA-specific CTL Lines. The PSMA24-3 no. 46 CTL line produced high levels of IFN-γ when cultured with various HLA-A24+ cancer cell lines, which transfected with rAd (PSMA) at MOI of 30 for 48 h before the experiment. On the other hand, IFN-γ production was negligible when the same CTL line was cultured with PSMA-expressing HLA-A24+ MKN28 gastric cancer and 526mel melanoma cell lines (Fig. 4A). Similarly, the PSMA24-5 no. 8 CTL line produced high levels of IFN-γ, which is one order higher scale of 200–900 pg/ml, when cultured with HLA-A24+ PSMA-
expressing cell lines (Fig. 4B). This CTL activity was so strong that it could be observed even at a low E:T ratio of 1.

We also monitored IFN-γ production when the PSMA24-5 no. 8 CTL line was cultured with LNCaP cells (PSMA A24 +, HLA-A24 +) transfected with rAd (HLA-A24) at MOI of 20 for 48 h. As expected, a significant amount of IFN-γ release to the media was observed with HLA-A24-transfected LNCaP cells, whereas minimal IFN-γ release was seen with control LNCaP cells transfected with a mock rAd (Fig. 5B). As for the PSMA24-3 no. 46 CTL line, however, in this particular experiment, we could just observe trace levels of IFN-γ even with HLA-A24-transfected LNCaP cells (Fig. 5A). As it has been previously documented (20), the levels of expression of HLA class I molecules on the surface of HLA-A24-transfected LNCaP cells were up-regulated, when they were pretreated with TNF-α for 48 h (Fig. 6). The levels of IFN-γ release were significantly increased when each CTL line was cultured with these TNF-α-pretreated, HLA-A24-transfected LNCaP cells, indicating that the capabilities of antigen recognition by these CTL lines were sufficient when the target cells expressed sufficient levels of PSMA and HLA class I molecule (Fig. 5).

HLA Class I and CD8 Restriction of the PSMA-specific CTL Lines. To determine the mechanism of immunological response in the recognition of antigen by the PSMA-reactive CTL lines, anti-HLA class I and II mAbs, as well as anti-CD4 and CD8 mAbs, were tested for their capacity to inhibit the IFN-γ productivity by the PSMA-specific CTL lines when they were cultured with PSMA-transfected 888mel cells. The levels of IFN-γ released by both PSMA24-3 no. 46 and PSMA24-5 no. 8 CTL lines were significantly inhibited by anti-HLA class I mAbs and anti-CD8 mAbs but not by anti-HLA class II mAbs or anti-CD4 mAbs, indicating that the CTL lines recognize the PSMA-derived epitopes in an HLA-class I-restricted manner using their CD8 T-cell receptors (Fig. 7). The levels of IFN-γ released by CTL lines were negligible when 888mel cells were transfected with control adenoviral vector, pAxCAwt (data not shown).

DISCUSSION

PSMAs are well known to be abundantly expressed as a type-II transmembrane protein on the surface of prostate cancer cells. Interestingly, PSMA expression in the tumor-associated neovascularature of several solid tumors has recently been reported (21, 22). It is also noteworthy that the PSMA gene expression is up-regulated inversely rather than down-regulated as in PSA in prostate cancer patients with hormone-refractory status (23–25). These unique features of PSMA, in contrast to PSA, make PSMA a very attractive target for various prostate-specific targeting strategies in real clinical settings.

There have been a series of reports of PSMA epitope peptides for prostate cancer-specific CTLs that are restricted to the HLA-A2.1 (A*0201) subtype that is found in more than 90% of HLA-A2 + Caucasians (9, 11, 26). However, HLA-A2 is highly heterogeneous, and only 45% of HLA-A2 + Japanese are of the HLA-A2.1 subtype (13, 27). In contrast, the HLA-A24 allele is much less heterogeneous and more than 90% of HLA-A24 is HLA-A*2402 subtype, which is highly expressed in Asians and in ~60% of the Japanese population (13). HLA-A24 is also found in significant numbers of individuals belonging to other ethnic groups (33% in Chinese, 27% in Hispanics, 17% in Caucasians, and 9% in African Americans; Ref. 13).

The data reported herein demonstrate that two HLA-A24-binding epitope peptides derived from the amino acid sequence of PSMA were able to elicit HLA-class I-restricted CTLs that would kill tumor cells expressing both PSMAs and the corresponding HLA molecules. Because, as discussed previously, it will be fundamental to identify HLA-specific epitopes restricted to the most common allele for developing peptide-based immunotherapy in the general population, the identification of two novel epitopes restricted by HLA-A24, the most common MHC class I allele in the Japanese population, represents an important addition to the peptide epitopes available for the development of a safe and effective DC-based vaccine treatment strategy against prostate cancer in Japan and worldwide.

The possibility that TAAs such as PSA and PSMA would behave as “self” antigens and exhibit an insurmountable level of immunological tolerance in the host immune system is a potential concern relating to the development of cancer immunotherapies (28, 29). However, a number of previous reports using various TAAs as immunomodulators revealed that the tolerance could be overcome (30–33), yielding CTLs of sufficient avidity to recognize target cells expressing endogenous antigen. Furthermore, we have previously reported several HLA-restricted CTL epitopes from various TAAs in vitro in normal individuals as well as in cancer patients using peptide-pulsed autologous DC (17, 34–37). In the present report, we provide further evidence that immunological tolerance to PSMA may not prevent the development of CTL-based immunotherapies for prostate cancers, because two additional epitope peptides capable of eliciting tumor-reactive CTLs were found in the PSMA amino acid sequence. The two highest-affinity HLA-A24-binding peptides, PSMA24-4 and PSMA24-9, did not elicit any anti-PSMA CTL responses (Table 1), which might suggest that the tolerance of CTLs to these particular epitopes may be actually present. However, because they were tested in only two experiments and a negative result must be considered inconclusive, further testing of these epitopes would be required to address this possibility. Because this study addressed only a subset of the HLA-A24 motif-bearing PSMA peptides, future studies will likely allow the identification of additional HLA-
A24-binding epitopes, and characterization of these PSMA-derived epitopes should provide further insight into this question.

The CTL line PSMA24-5 no. 8 showed a scale order higher IFN-γ/H9253 production levels compared with the PSMA24-3 no. 46 CTL line, when cultured with various HLA-A24/H11001/PSMA-transfected tumor cell lines. This result may suggest that the PSMA24-5 epitope peptide is more potent than PSMA24-3 as an immunogen. Although based on a limited study, this interpretation is consistent with the observation that PSMA24-5 has higher binding affinity than does PSMA24-3 (IC50 of 67 nM versus 406 nM), and the demonstration that HLA binding correlates with immunogenicity (38). Despite the potential difference in in vitro potency for the two reported epitopes, both can be considered appropriate for testing in epitope-based immunotherapy clinical trials.

Because there are no established cancer cell lines that express both HLA-A24 molecules and PSMA, in our current study, we used an adenovirus-based system to generate HLA-A24-restricted LNCaP cells and various PSMA-transduced HLA-A24+ cancer cells as target cells for characterization of CTL responses. In general, adenoviral vector is itself immunogenic and may perturb HLA expression and/or antigen processing. However, we think that this is not the case in our experiments because the control cells transfected with “mock” adenoviral vector, defined as “AxCAwt” in Fig. 5, induced a minimal level of IFN-γ release. Also, in Fig. 4, rAd (PSMA)-transduced MKN28 and 526mel cells (HLA-A24+) did not induce IFN-γ production by PSMA-specific CTL lines, which

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Because there are no established cancer cell lines that express both HLA-A24 molecules and PSMA, in our current study, we used an adenovirus-based system to generate HLA-A24-restricted LNCaP cells and various PSMA-transduced HLA-A24+ cancer cells as target cells for characterization of CTL responses. In general, adenoviral vector is itself immunogenic and may perturb HLA expression and/or antigen processing. However, we think that this is not the case in our experiments because the control cells transfected with “mock” adenoviral vector, defined as “AxCAwt” in Fig. 5, induced a minimal level of IFN-γ release. Also, in Fig. 4, rAd (PSMA)-transduced MKN28 and 526mel cells (HLA-A24+) did not induce IFN-γ production by PSMA-specific CTL lines, which

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would convince us that immunogenicity of the adenovirus-transduced cells was not attributable to the adenoviral vector itself. This system should have general utility for creating target cells with TAA-MHC combinations not currently available in existing cell lines.

Herein we have demonstrated that using an in vitro priming protocol based on peptide-pulsed DCs as APCs enables us to identify new prostate cancer-relevant, HLA-A24-restricted PSMA peptides that are capable of inducing an epitope-specific CTL response. CTL epitopes identified in this study offer the opportunity to design novel epitope-based immunotherapies for the treatment of patients with advanced hormone-refractory prostate cancers. Peptide-based vaccines using these epitopes may be composed of epitope peptides administered in either adjuvant or peptide-pulsed DCs, and we are currently investigating the utility of these PSMA epitopes in clinical trials performed in Kelo University and Tokyo Medical University.

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REFERENCES


Fig. 7 Inhibition of specific recognition of PSMA-reactive CTL lines by mAbs. PSMA-transfected 888mel cells were preincubated with anti-HLA class I or II mAbs, or the PSMA24-3 no. 46. PSMA24-5 no. 8 CTL lines were preincubated with anti-CD4 or CD8 mAbs, for 1 h at room temperature. Then, the pretreated PSMA24-3 no. 46 (A) or PSMA24-5 no. 8 (B) CTL lines and the pretreated target 888mel were cocultured at an E:T ratio of 1. IFN-γ concentrations in each supernatant 24 h later were measured by an immunoenzymometric assay.
Screening of PSMA/HLA-A24 Peptide


Screening of HLA-A24-restricted Epitope Peptides from Prostate-specific Membrane Antigen That Induce Specific Antitumor Cytotoxic T Lymphocytes

Yutaka Horiguchi, Ikuei Nukaya, Kazuhide Okazawa, et al.


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