Prostate Stem Cell Antigen Is a Promising Candidate for Immunotherapy of Advanced Prostate Cancer


ABSTRACT

Immunotherapy of prostate cancer (CaP) may be a promising novel treatment option for the management of advanced CaP. However, the lack of suitable tumor antigens remains a major obstacle for the rational design of vaccines. To characterize potential CaP antigens, we determined the mRNA expression of the prostate-specific genes C1, C2, C5, PAGE-1, and prostate stem cell antigen (PSCA) in hormone-refractory CaP, benign prostatic hyperplasia, CaP cell lines, and CaP specimens. Among these gene products, only expression of PSCA appears to be retained in the majority of advanced CaP samples, as shown by reverse transcription-PCR analyses. Peptide fragments of PSCA presented in the context of major histocompatibility molecules could serve as recognition targets for CD8 T cells, provided these lymphocytes were not clonally deleted or peripherally tolerant. Our goal was to determine whether the human T-cell repertoire could recognize PSCA-derived peptide epitopes in the context of a common class I allele, HLA-A0201. Of nine peptides that, according to HLA-A0201 binding motifs, were candidate ligands of A0201 class I molecules, three peptides were able to stabilize HLA-A0201 molecules on the cell surface. One of the latter peptides, encompassing amino acid residues 14–22, was capable of generating a PSCA-specific T-cell response in a human lymphocyte culture from a patient with metastatic CaP. PSCA-specific CTLs recognized peptide-pulsed targets as well as three prostate carcinoma lines in cytotoxicity assays, indicating that this peptide could be endogenously processed. In conclusion, our findings establish PSCA as a potential target for antigen-specific, T cell-based immunotherapy of prostate carcinoma.

INTRODUCTION

CaP is the most common cancer diagnosis and the second leading cause of cancer-related deaths in men. Despite recent advances in detection of CaP and treatment of localized disease, significant challenges unique to CaP remain to be overcome. In particular, there is no effective treatment for patients who develop recurrent disease after surgery or radiation therapy or those who have metastatic disease at the time of diagnosis. Although hormone ablation therapy may palliate patients with advanced disease temporarily, the progression to incurable hormone-refractory CaP is almost inevitable (1). Therefore, the development of novel therapeutic modalities for the treatment of hormone-refractory CaP is of paramount importance. Several new CaP treatment approaches aim to eradicate CaP cells by inducing systemic immunity to antigens expressed by CaP cells as well as normal prostate (2–6). However, the identification of target tumor antigens that are capable of overcoming immune tolerance against proteins expressed in normal prostate remains a major obstacle for developing rational strategies in CaP immunotherapy. Over the past years, several prostate-specific gene products have been reported. These include PSA (7), PSMA (8), PAP (9), prostate carcinoma tumor antigen 1 (10), PAGE-4 (11), PSP 94 (12), six-transmembrane epithelial antigen of the prostate (13), differential display 3 (14), and prostate androgen-regulated transcript 1 (15). The rationale of using prostate-specific genes as target antigens for immunotherapy is based on a decade of intensive research in the melanoma field, leading to the insight that prominent antigens of melanoma-specific CTLs were expressed in melanocytes in a tissue-specific manner (16). Apparently, the presumed tolerance of peripheral T cells against these self antigens can be overcome if aberrant expression in tumors occurs. Moreover, this antitumor response could be successfully enhanced by several vaccination procedures using melanocyte antigens (17–19). The consequence of this type of antitumor response was on the one hand regression of melanoma lesions but on the other hand vitiligo as a result of CTL-mediated melanocyte destruction.

Because many of the so-called “cancer testis” antigens, which are expressed in testis and in several different malignancies, are not prevalent in the majority of CaP specimens, organ-specific gene products were considered as target antigens in CaP. This approach seems reasonable because in organ-confined CaP, the prostate is surgically removed and because the life of vaccinated patients would not be endangered if healthy prostate tissue was damaged by CTLs. Unfortunately, the majority of defined prostate-specific gene products display properties that limit their utilization as antigens in specific immunotherapy of CaP. PSA, PSMA, PAP, PSP 94, and prostate carcinoma tumor antigen 1 are secretory proteins that are found in considerable concentrations in the serum and hence are likely to induce peripheral tolerance. The expression of PSA and PAP in tissue is reduced in neoplastic cells of poorly differentiated tumors compared with normal prostate tissue and well-differentiated adenocarcinomas (20). In addition, PSA has a high degree of homology with members of the kallikrein family, and PSMA has been found to be expressed in various human tissues (21), thus bearing the risk of generating autoimmune disease upon protein-based vaccination. PSP 94 (22) and PAGE-4 (11) seem to be down-regulated in tumor tissue, and expression of six-transmembrane epithelial antigen of the prostate appears to be expressed at low levels in several other tissues (13). Prostate androgen-regulated transcript 1 expression is regulated by androgens (15), which is a drawback for strategies aiming to eliminate hormone-refractory tumors. Lastly, the mRNA of differential display 3 does not contain extensive open reading frames and has, therefore, been suggested to function as a noncoding RNA (14).

However, there are new and partially characterized prostate-specific genes that warrant further investigation regarding their potential as CaP antigens. Little is known about the CaP expression status of C1, C2, and C5, which are prostate-specific mRNAs identified from expressed sequence tag libraries (23). On the other hand, PAGE-1 (24) and PSCA (25–27) have been identified as gene prod-
ucts specifically overexpressed in hormone-independent CaP cell lines or tumor tissue, respectively. PAGE-1 was identified by differential display PCR as an mRNA that is up-regulated in androgen-insensitive metastatic sublines of the CaP cell line LNCaP. The PAGE-1 protein shares 45% homology with antigens of the “cancer-testis” family, and its expression was found to be restricted to LNCaP sublines, testes, and placenta. PSCA was isolated by representational difference analysis in the LAPC-4 xenograft model and was found to be up-regulated in tumor xenografts when compared with normal prostate. The PSCA gene codes for a 123-amino acid glycoprotein that is not homologous to other genes except for a 30% identity to stem cell antigen 2. Topologically, PSCA was characterized as a glycosylphosphatidylinositol-anchored cell surface antigen. Interestingly, PSCA was not differentially expressed between androgen-dependent and -independent LAPC-4 tumors, which would make it an attractive target for vaccination against hormone-refractory CaP.

In this study, we asked whether PAGE-1, PSCA, C1, C2, and C5 are suitable as target antigens for immunotherapy of hormone-refractory CaP in terms of expression in tumor specimens and, furthermore, that antigen-specific cytotoxic T cells recognizing these antigens can also be generated in vivo. Although C1, C2, C5, and PAGE-1 were not expressed in the majority of CaP specimens, PSCA was found in all samples analyzed. Using a “reverse immunology” approach, we identified an HLA-A0201-restricted T-cell epitope from PSCA. CTLs against this epitope could be generated from peripheral blood of a CaP patient that recognized and killed CaP cell lines, suggesting that PSCA protein as well as this peptide epitope may be valuable antigens against this epitope could be generated from peripheral blood of a CaP patient that recognized and killed CaP cell lines, suggesting that PSCA protein as well as this peptide epitope may be valuable antigens for immunotherapy of hormone-refractory CaP.

Materials and Methods

Cell Lines and Antibodies. For HLA-A201 typing, donors and cell lines were prescreened with the HLA-A2-specific antibody BB7.2 (American Type Culture Collection, Manassas, VA) by flow cytometry. HLA-A2-positive cell lines were subtyped by PCR as described previously (28). The cell lines K562 (chronic myelogenous leukemia), HepG2 (hepatocellular carcinoma), Hep-2 (cervix carcinoma), T89G (glioblastoma), MCF-7 (breast carcinoma), Daudi cells (Burkitt’s lymphoma), and LNCaP10990, DU-145, and PC-3 (all CaP) cell lines were obtained from the American Type Culture Collection. Lymphokine-activated killer-sensitive T2 cells were kindly provided by Maries van den Berge (Erasmus University Rotterdam, the Netherlands). Cell lines were maintained in complete RPMI 1640 (RPMI 1640, 10% heat-inactivated FCS, 2 mM glutamine, and 100 units/ml penicillin/streptomycin). The androgen-insensitive metastatic sublines of the CaP cell line LNCaP. The PAGE-1 was identified by differential display PCR. Oligodeoxynucleotide primers for PCR reactions were purchased from the Microsynth Company (Balgarh, Switzerland). PAGE-1 was found to be up-regulated in tumor xenografts when compared with normal prostate.

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T2 Binding Assays. Each peptide was tested for concentration-dependent binding to T2 cells in HLA-A201 stabilization assays. T2 (TAP-deficient) cells were incubated at room temperature overnight with the indicated PSCA peptides over a range of peptide concentrations from 0.5 to 10 μM in the presence of 1 μg/ml B2-microglobulin (Sigma). Stability of HLA-A201 was assayed by flow cytometry (FACSscan; Becton Dickinson) after staining the cells with antibody BB7.2 (5 μg/ml) and goat antimouse-FITC (AMRAD, Melbourne, Australia). The peptide GilGFVFTL of influenza matrix protein, residues 58–66, was used as a positive control. Alternatively, in “off-assays,” T2 cells were incubated overnight at room temperature in the presence of 10 μM peptide, followed by an incubation at 37°C in the presence of 10 μM emetine (Sigma). The loss of HLA-A201 molecules from the cell surface was monitored by flow cytometry after 1, 2, 3, 4, and 6 h, respectively.

Cytolytic Assay. Target cells were harvested, washed, and counted, and labeled with 100 μCi of Na2-131I (EGT Chemie, Tageri, Switzerland) in 0.5 ml complete RPMI 1640 at 37°C for 1.5 h. Exogenous loading of cells with 10 μM synthetic peptide was performed simultaneously with labeling reactions. Infection of target cells with recombinant vaccinia viruses was performed 10 h before labeling at an multiplicity of infection of 5. CTLs were washed, counted, and diluted to the desired density in complete RPMI 1640 and plated in duplicate wells in a round-bottomed 96-well plate. Target cells were washed three times, diluted to 103 cells/ml, and plated with CTLs. To control for nonspecific lysis by natural killer cells or lymphokine-activated killer cells, respectively, K562 and Daudi cells were included as targets in our assays. The plates were spun briefly at 800 × g and incubated for 4–5 h at 37°C. Supernatants were harvested and counted in a gamma counter. Duplicate wells were averaged, and the percentage of specific lysis was calculated as:

\[
\%\text{ specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100
\]

CTL Generation from Peptide-pulsed PBMCs. Peptide-specific, short-term CTL cultures were generated to the PSCA peptides. HLA-A201-positive PBMCs from a patient with metastatic prostate cancer were pulsed with 10 μM peptide for 2 h at 37°C in serum-free media (X-VIVO 15; BioWhittaker, Verviers, Belgium), washed, and cultured in the presence of 10 units/ml IL-2 in complete RPMI 1640 at 103 cells/200 μl per well. IL-2 (Sigma) was added twice weekly at 10 units/ml. Cells were restimulated every 2 weeks by addition of autologous, peptide-pulsed, washed, and irradiated (80 Gy) PBMCs at 1:1 ratio. After 8 weeks in culture, cells were tested for cytotoxicity.

Patients. All clinical material was obtained by prostatectomy, transurethral resection of prostate, or autopsy, after informed consent from patients according to an Institutional Review Board–approved protocol. Surgically harvested...
specimens were examined by a pathologist and frozen at −70°C within 30 min. The material was classified as BPH, locally confined CaP, locally advanced CaP, or metastatic disease as detailed in Table 1. Autopsies were performed within 2 h after death.

RESULTS

RT-PCR Analysis of CaP Cell Lines, BPH, and CaP Tissue. The qualification of a prostate-specific protein as a target antigen for vaccination of CaP relies on a maintained expression in the majority of CaP specimens. To determine the mRNA expression levels of the candidate genes C1, C2, C5, PAGE-1, and PSCA, we performed RT-PCR analysis on a panel of CaP cell lines, one BPH sample, and seven tumor specimens (two hormone-refractory metastases, three hormone-refractory primary tumors, and two hormone-dependent primary tumors; Fig. 1). As positive controls for the quality of our cDNA, we performed amplifications of β-actin (data not shown) and PSA (Fig. 1, top panel) mRNA. As expected, PSA mRNA can be detected in LNCaP 1740 (Lane 1), LNCaP 10990 (Lane 3), BPH tissue (Lane 8), and all CaP specimens, although to varying degrees. Consistently, PSA expression is absent in androgen receptor-negative cell lines DU-145, PC-3, and Tsu-pr (Lanes 5, 6, and 7). An androgen-independent (ai) subline of LNCaP 1740 (Lane 2), which we have generated by androgen deprivation of the parental line, reveals a similar PSA signal as the parental line, whereas expression of PSA is down-regulated in LNCaP 10990-ai (Lane 4). PSCA mRNA can be found in all CaP cell lines at similar levels, except for lower expression in LNCaP 1740-ai and Tsu-pr (Lanes 2 and 7). Furthermore, PSCA mRNA could be detected in BPH and at different levels, but consistently, in most specimens examined (see also Fig. 4). PAGE-1 mRNA could not be detected in any of the samples analyzed. However, in agreement with published data (24), PAGE-1 expression could be found in testis, which demonstrates that the former result is not attributable to inappropriate amplification conditions in our PCR reactions (data not shown). Chen et al. (24) could detect expression of PAGE-1 only in androgen-independent, metastatic sublines of LNCaP that had been isolated from castrated nude mice but not in parental LNCaP cells, PC-3 cells, DU-145 cells, or even normal prostate.

Additionally, a recent publication suggests that PAGE-1 is most likely an unknown member of the GAGE family, i.e., a shared cancer-testis antigen, rather than a prostate-specific gene product (31).

C1 mRNA could only be detected in one CaP specimen as well as in BPH at a very low level, C2 expression was evident only in BPH as well as in one CaP specimen, and expression of C5 could be found in PC-3 cells as well as in LNCaP cell lines, at low levels. These data clearly demonstrate that among the gene products analyzed, only PSCA exhibited consistent expression in both androgen-dependent and androgen-independent samples, although at varying levels. Therefore, we decided to further characterize PSCA as a candidate gene for CaP vaccination.

Tissue Specificity of PSCA Expression. To determine the tissue specificity of PSCA expression, we harvested nonprostatic tissues during the autopsy of a patient who died from metastatic prostate cancer and subjected isolated RNA to RT-PCR analyses. This patient had metastatic lesions in os sacrum, os ileum, lumbar spine, and liver. Bone marrow was harvested from the femur. Among the tissues analyzed, PSCA expression was only found in bone marrow (Fig. 2, Lane 6). This result is unexpected, and we asked if the PSCA signal was attributable to the presence of CaP cells in the bone marrow or if our PCR picked up a gene product expressed in hematopoietic tissue. Therefore, we harvested three additional bone marrow samples, one from a healthy donor, one from a leukemia patient, and one during autopsy of a female patient who died from nonmalignant disease. As can be seen in Fig. 3, PSCA could only be detected in the bone marrow derived from the patient with metastatic CaP (Fig. 3, Lane 1). Amplification of β-actin RNA (Fig. 3, bottom) demonstrates that equal amounts of cDNA have been included in our RT-PCR. These results suggest that PSCA expression in the bone marrow of the patient with metastatic CaP was attributable to the presence of CaP.
cells in his bone marrow. It is interesting to note that the PSCA signal in this bone marrow sample is exceptionally high, because PSCA expression in primary tumor and a liver metastasis (>90% CaP tissue) derived from the same patient is significantly lower (Fig. 1, Lane 15).

**Semiquantitative RT-PCR of CaP Specimens and BPH Samples.** Given that PSCA appeared to be a promising candidate for CaP tumor vaccination, we decided to investigate the PSCA expression at the mRNA level by semiquantitative RT-PCR for a larger panel of 10 hormone-dependent primary CaP samples, 10 hormone-independent primary CaP samples, 6 hormone-refractory CaP metastases, and 2 samples of BPH. As shown in Fig. 4A, PSCA was expressed in all samples examined, and no evidence for complete down-regulation of PSCA expression, either in hormone-dependent or -refractory primary CaP or in CaP metastases, could be obtained. Most of the hormone-dependent CaP samples displayed a higher level of PSCA expression compared with BPH specimens, whereas some of the metastases and hormone-refractory primary CaP samples appeared to express slightly reduced levels of PSCA mRNA. Although we used an amplification of β-actin mRNA to control for the integrity and amount of mRNA in the preparations (Fig. 4B), the determination of PSCA mRNA levels by RT-PCR is at best semiquantitative. Taking these limitations into consideration, we conclude from our analysis that, compared with BPH, there is no evidence for either a selective loss or for a general up-regulation of PSCA expression in metastases or hormone-refractory CaP samples.

**Identification of HLA-A0201-binding PSCA Peptides.** We next sought to identify potential HLA-A0201-restricted PSCA peptide epitopes that may be recognized by PSCA-specific T cells. Computer-based analysis (32, 33) of the published human PSCA sequence (25) was performed to identify 9-mer peptides whose sequences conformed to the well-characterized binding motif for HLA-A0201. Table 2 presents the HLA-A0201 binding properties of PSCA peptide epitopes and those of the immunodominant HLA-A0201-restricted influenza matrix peptide M1$_{48-66}$, and of the PSMA peptide LL-HETDSAV, which is currently being used for dendritic cell-based vaccination of patients with metastatic CaP (30). All peptides analyzed bound strongly to HLA-A0201 and stabilized MHC molecules at peptide concentrations $<$0.5 μM, except for PSCA$_{20-28}$ (4 μM) and PSCA$_{43-51}$ (1 μM), which contain only one HLA-anchor (leucine).
residue at position 2. However, significant differences between peptide epitopes could be detected when analyzing the duration of HLA-A0201 stabilization on the cell surface of peptide-pulsed cells. Only peptides PSCA\textsubscript{7–16}, PSCA\textsubscript{14–22}, and PSCA\textsubscript{115–123} were capable of stabilizing MHC molecules for up to 6 h. For these peptides, we determined whether they were capable of stimulating human PSCA-specific T cells in 	extit{vivo}.

**Generation and Characterization of CTLs Specific for an HLA-A0201-restricted PSCA Epitope.** The peptides PSCA\textsubscript{7–16}, PSCA\textsubscript{14–22}, and PSCA\textsubscript{115–123} were used to stimulate human T cells in 	extit{vitro}. Bulk T-cell cultures were generated from PBMCs obtained from a patient with hormone-refractory metastatic CaP by pulsing the cells with the respective synthetic peptides in the presence of IL-2. After 8 weeks of expansion, the T-cell culture was assayed for the ability to lyse HLA-A0201\textsuperscript{+} T2 lymphoblastoid cells, which were pulsed with the respective peptides in a chromium release assay. Only CTL cultures that were restimulated with the PSCA\textsubscript{14–22} peptide were able to specifically lyse target cells presenting the respective epitope (Fig. 5), and it was also this T-cell culture that proliferated most vigorously upon restimulation. At the time of specificity analysis, 70% of the CTL culture consisted of CD3\textsuperscript{+} cells, whereas there were still some 30% CD3\textsuperscript{−} cells present, as determined by flow cytometry (data not shown).

To test whether the PSCA\textsubscript{14–22} epitope could be intracellularly processed in CaP cells that endogenously express the PSCA protein, we tested in a multistep PCR whether the HLA-A0201 allele would be present in the CaP cell lines LNCAP1740, DU-145, PC-3, and Tsu-pr1. Only LNCAP 1740 was positive for HLA-A0201 and expressed HLA-A0201 on the cell surface at low levels, which did not significantly increase when the cells were treated with IFN-\(\gamma\) for 2 days (data not shown). When LNCAP cells were used as targets in a cytolytic assay, they were lysed by PSCA\textsubscript{14–22}-specific CTLs in the presence but not in the absence of IFN-\(\gamma\), indicating that the PSCA\textsubscript{14–22} epitope could be endogenously processed and presented by IFN-\(\gamma\)-stimulated CaP cells (Fig. 5). To test whether the lysis by the PSCA\textsubscript{14–22}-reactive CTLs was specific for PSCA, other tumor cell lines, which we determined to be HLA-A0201\textsuperscript{+}, were used as targets.

**Table 2. HLA-A0201 binding characteristics of PSCA peptide epitopes**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Anchor residues to stabilize MHC ((\mu)M)</th>
<th>Off-kinetics (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSCA\textsubscript{5–13}</td>
<td>LLALLMAGL</td>
<td>2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PSCA\textsubscript{7–16}</td>
<td>ALLMAGLAL</td>
<td>2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PSCA\textsubscript{14–22}</td>
<td>ALQPGTALL</td>
<td>2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PSCA\textsubscript{20–28}</td>
<td>ALLCYSCKA</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>PSCA\textsubscript{43–51}</td>
<td>QLGEQCDWA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PSCA\textsubscript{105–113}</td>
<td>ALLPALGLL</td>
<td>2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PSCA\textsubscript{108–116}</td>
<td>ALLPALGLL</td>
<td>2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PSCA\textsubscript{115–123}</td>
<td>LLLWGPGQL</td>
<td>2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PSCA\textsubscript{109–117}</td>
<td>LLPALGLLL</td>
<td>2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>PSCA\textsubscript{110–118}</td>
<td>LLPALGLL</td>
<td>2</td>
<td>&lt;0.5</td>
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<tr>
<td>Flu M1\textsubscript{45–53}</td>
<td>GILGFVFTL</td>
<td>2</td>
<td>&lt;0.5</td>
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Fig. 6. Cytolytic assay for recognition of PSCA\textsubscript{14–22}/HLA-A0201 in prostate cancer and control cell lines. A, IFN-\(\gamma\)-treated LNCAP cells and PSCA\textsubscript{14–22} peptide-loaded T2 cells were lysed at E:T ratios ranging from 12.5 to 100; HLA-A0201\textsuperscript{+} control cell lines were lysed at E:T = 100, as indicated. T2 cells (lymphoblastoid, Lane 1), T2 cells pulsed with PSCA\textsubscript{14–22}, and lysed at E:T ratios of 12.5, 25, 50, and 100 (Lanes 2–5). IFN-\(\gamma\)-stimulated HLA-A0201\textsuperscript{+} control cell lines HepG2 (hepatoma, Lane 6), MCF-7 (breast carcinoma, Lane 7), SW620 (colon carcinoma, Lane 8), and T98G (glioblastoma, Lane 9), K562 (chronic myelogenous leukemia, Lane 10), Daudi (Burkitt’s lymphoma, Lane 11), and IFN-\(\gamma\)-stimulated LNCAP cells lysed at E:T = 12.5 (Lane 12), E:T = 25 (Lane 13), E:T = 50 (Lane 14), and E:T = 100 (Lane 15). B, comparison of HLA-A0201\textsubscript{+}/PSCA\textsubscript{14–22} specific lysis of T2 cells, PSCA\textsubscript{14–22}-loaded T2 cells, IFN-\(\gamma\)-treated LNCAP, PSCA\textsubscript{14–22}-loaded LNCAP cells, HLA-A0201-deficient prostate carcinoma lines DU145 and PC3 infected with recombinant vaccinia virus-HLA-A0201 (rVV-HLA-A0201) and recombinant vaccinia virus control (rVVcontrol) as indicated. The cytolytic assays were performed at an E:T ratio of 50. Values represent means of triplicates with SE <10%.

Fig. 5. PSCA specificity of human lymphocyte culture pulsed with PSCA\textsubscript{14–22}. After 8 weeks of expansion, PBMCs were assayed for cytotoxicity in a chromium release assay at an E:T ratio of 50:1. Cytotoxicity was assayed against T2 cells, PSCA\textsubscript{14–22}-loaded T2 cells, natural killer-sensitive K562 cells, lymphokine-activated killer-sensitive Daudi cells, LNCAP cells, and LNCAP cells that had been stimulated with IFN-\(\gamma\). Values represent means of triplicates with SE <5%.
simultaneously with IFN-γ-treated LNCAP cells at the highest E:T ratio (Fig. 6A). In contrast to LNCAP cells that were lysed at E:T target ratios ranging from 12.5 to 100 (Fig. 6, Lanes 12–15), the tumor cell lines HepG2 (hepatocarcinoma, Lane 6), MCF-7 (mammary carcinoma, Lane 7), SW620 (colon carcinoma, Lane 8), and T98G (glioblastoma, Lane 9) were not lysed to a significant extent at an E:T ratio of 100. Moreover, the cell lines K562 (chronic myelogenous leukemia, Lane 10) and Daudi (Burkitt’s lymphoma, Lane 11), which are sensitive to lysis by natural killer cells and lymphokine-activated killer cells, respectively, were not lysed by the PSCA14–22-reactive CTLs. The relatively low lysis of LNCAP cells was not attributable to a poor ability of these cells to present peptides on HLA-A0201 in general because the specific lysis could be enhanced by exogenously pulsing LNCAP cells with the PSCA14–22 peptide (Fig. 6B). Most likely, the moderate specific lysis of LNCAP cells is attributable to a relatively low level of endogenous PSCA expression, as we have determined by RT-PCR (Fig. 1) and a quantitative real time PCR analysis (data not shown). The presentation of the PSCA14–22 peptide was not confined to LNCAP cells but occurred also in the PSCA-expressing but HLA-A0201-deficient prostate carcinoma lines PC-3 and DU-145 when these were infected with a recombinant vaccinia virus expressing the HLA-A0201 molecule (34) but not when they were infected with a control recombinant vaccinia virus expressing the influenza matrix protein (Ref. 35; Fig. 6B). Taken together, our data indicate that the generated CTLs are specific for the peptide ALQPGTALL encompassing residues 14–22 of PSCA. Because CTLs reactive to this epitope lyse three independent PSCA-positive prostate carcinoma cell lines, PSCA and this epitope should be promising target antigens for vaccination against CaP.

DISCUSSION

In the present study, we analyzed a number of prostate-specific genes for their suitability as antigens for specific vaccination against CaP. Although PAGE-1 and the expressed sequence tags C1, C2, and C5 were not expressed in the majority of CaP samples, PSCA fulfilled the criteria for serving as an antigen in CaP tumor vaccination: (a) PSCA was expressed in the majority of CaP samples and appeared not to be down-regulated in advanced stage disease; (b) according to RT-PCR analysis, PSCA appeared not to be expressed in human tissues other than the prostate and CaP; and (c) CTLs could be raised from the blood of a CaP patient that specifically recognized a PSCA peptide and lysed a PSCA-expressing CaP cell line, indicating that tolerization or exhaustion of PSCA-reactive CTLs in patients with progressing metastatic CaP does not prevail.

The CTLs that we could raise against PSCA were directed against the HLA-A0201-restricted peptide ALQPGTALL covering residues 14–22 of the PSCA sequence. This peptide could stabilize HLA-A0201–b2-microglobulin complexes on the cell surface of TAP-deficient lymphoblastoid T2 cells. The fact that two additional peptides (PSCA7–16 and PSCA115–123), which stabilized HLA-A0201 cell surface expression with similar off-rates as PSCA14–22, did not give rise to peptide-specific T cells in parallel cultures may indicate that the T-cell precursor frequency of PSCA14–22-specific peptides was enhanced in this patient, or alternatively, that there were no T cells in the repertoire reacting to the other two epitopes. More importantly, our PSCA14–22-specific CTLs were able to lyse three independent CaP cell lines, suggesting that this epitope can be processed in CaP cells from endogenously expressed PSCA proteins. The finding that IFN-γ stimulation of LNCAP target cells was required for recognition by CTLs may be attributable to an insufficient expression of HLA-A0201 molecules in unstimulated LNCAP cells, although our flow cytometric analyses revealed only a minor up-regulation of HLA-A0201 cell surface expression in some of the experiments. A more likely explanation is that the intracellular production and transport of the PSCA14–22 epitope may be limiting and that factors that are induced by IFN-γ, e.g., the two subunits of the TAP or the proteasome subunits LMP2, LMP7, and MECL-1 as well as PA28α/β, are required to achieve T-cell recognition (36). We have not yet determined whether the intracellular production of the PSCA14–22 epitope can be inhibited by specific inhibitors of proteasome activity. Nevertheless, it is important that IFN-γ stimulation does not abrogate PSCA presentation because it has been shown recently for a number of tumor epitopes that they could be processed by constitutively expressed proteasomes of unstimulated tumor cells but not by immunoproteasomes in mature dendritic cells or after induction of tumor cells with IFN-γ (37).

To avoid autoimmune destruction of tissues upon vaccination, it would be ideal if the expression of a target antigen for CaP immunotherapy was strictly confined to CaP and/or the prostate. We analyzed PSCA expression by RT-PCR and found no expression in testis, spleen, liver, bone marrow, kidney, lymph node, lung, bladder, cerebellum, or colon. Principally, this finding is consistent with the original Northern analyses by Reiter et al. (25), who found PSCA to be predominantly expressed in prostate and in placenta. However, a minor PSCA expression was reported in this study for kidney and small intestine, which was ~100-fold lower compared with expression levels in the prostate. Recently, an immunohistochemical analysis by Gu et al. (27) revealed that within these organs the PSCA protein expression is confined to the renal collecting ducts and neuroendocrine cells of the stomach and the colon. Because these cells constitute only a small fraction of the respective organs, this may explain why we did not find PSCA expression in colon and kidney by RT-PCR. Although the expression level of PSCA in these cells appeared to be lower than in CaP and epithelial cells of the prostate, it will be necessary to quantify the amounts of PSCA in isolated neuroendocrine cells and in prostate epithelial cells by real time PCR or Western analysis for a direct comparison. Moreover, it would be important to test whether the PSCA expression in cell lines of colonic or gastric neuroendocrine origin is high enough to be recognized and lysed by our PSCA-specific CTL line.

Our quantification of PSCA mRNA levels in BPH tissue and CaP specimens revealed that PSCA was consistently expressed in BPH, primary CaP, and metastases, but at least according to semiquantitative RT-PCR analyses, we obtained no evidence for a correlation between up-regulation of PSCA and tumor grade. Furthermore, we did not detect higher levels of PSCA mRNA in tumor tissue as compared with BPH specimens. At first glance, these data appear to contradict recent observations by Gu et al. (27), who found that the level of PSCA expression increased with higher Gleason score, higher tumor stage, and progression to androgen independence. However, Reiter and colleagues used different techniques in their analyses, such as in situ hybridizations (26) and immunohistochemical analyses using PSCA-specific monoclonal antibodies (27). Both methods can distinguish CaP tissue from surrounding nonneoplastic prostatic tissue, which is not possible when isolating total RNA for RT-PCR or Western hybridizations (26) and immunohistochemical analyses using PSCA-specific monoclonal antibodies (27). Both methods can distinguish CaP tissue from surrounding nonneoplastic prostatic tissue, which is not possible when isolating total RNA for RT-PCR or Western analysis for a direct comparison. Moreover, it would be important to test whether the PSCA expression in cell lines of colonic or gastric neuroendocrine origin is high enough to be recognized and lysed by our PSCA-specific CTL line.
stages of CaP in the vast majority of cases, which would make it a promising target for immunotherapy of CaP.

Interestingly, we observed in a single CaP patient that PSCA expression in a bone metastasis was considerably higher than in the primary tumor and in a liver metastasis, suggesting that PSCA expression might be up-regulated in bone metastases. A very similar finding was reported by Gu et al. (27) in three cases where it was possible to compare PSCA expression in metastases from bone and primary tumors. A potential explanation for these observations may be that factors are produced in bone marrow that either lead to an up-regulation of PSCA expression or to a selective expansion of PSCA-expressing CaP cells. Because CaP preferentially metastasizes to the bone, the up-regulation of PSCA in bone tissue would be a strong argument for using this protein as an antigen for CaP immunotherapy.

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