we evaluated the efficacy of combined treatment with antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53* using the androgen-independent human prostate PC3 tumor model.

We initially evaluated the effect of Ad5CMV-*p53* treatment on clusterin expression level in PC3 cells, because clusterin expression has been shown to be highly up-regulated in various tissues undergoing apoptotic cell death (15–18, 26–28). As expected, clusterin expression in PC3 cells was markedly increased after Ad5CMV-*p53* treatment, suggesting that clusterin up-regulation is likely an adaptive response, which helps the cell survival against *p53*-dependent cell death signal. However, phosphorothioate antisense clusterin oligodeoxynucleotide inhibited clusterin mRNA in dose-dependent and sequence-specific manners, even after Ad5CMV-*p53* treatment, which resulted in a significant increase in clusterin expression. Furthermore, treatment of PC3 cells with antisense clusterin oligodeoxynucleotide reduced the IC_{50} of Ad5CMV-*p53* by 75% and enhanced Ad5CMV-*p53*-induced apoptosis, although no growth inhibitory effects were observed in PC3 cells treated with antisense clusterin oligodeoxynucleotide alone. These findings suggest that clusterin expression in androgen-independent prostate cancer cells may confer a phenotype resistant to apoptosis induced by Ad5CMV-*p53*; therefore, despite the lack of a significant effect on cell proliferation in the absence of other apoptotic stimuli or cell death signals, the inhibition of clusterin expression by antisense clusterin oligodeoxynucleotide may enhance the sensitivity to several therapies inducing *p53*-dependent apoptosis for androgen-independent prostate cancer.

Recently, several investigators have shown that overexpression of antiapoptotic genes in prostate cancer cells, such as mutant-type *p53*, *bcl-2*, and *clusterin*, helps mediate resistance to conventional treatment through the inhibition of apoptotic cell death (23, 43, 44). These findings suggest that the approach of enhancing sensitivity to stimuli, which induces apoptotic cell death, by decreasing the expression of the antiapoptotic gene seems to be a more rational strategy for patients with advanced androgen-independent prostate cancer than the conventional approach of combining several kinds of treatments. Furthermore, recent preclinical studies have provided proof of principle evidence that targeting antiapoptotic genes using antisense oligodeoxynucleotide enhances apoptosis induced by conventional therapeutic options (26–28, 30, 35). Accordingly, based on the findings of the present *in vitro* experiments, we then examined whether antisense clusterin oligodeoxynucleotide therapy enhances the cytotoxic effect of Ad5CMV-*p53* on the growth and metastasis of PC3 cells *in vivo*, and showed that a regimen combining antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53* markedly inhibited the growth of s.c. PC3 tumors *in vivo*. These findings suggest that it might be possible to achieve powerful cytotoxic effects of Ad5CMV-*p53* at tolerable doses by combining with antisense clusterin oligodeoxynucleotide. The combined regimen also significantly suppressed the incidence of metastasis after orthotopic injection of PC3 cells, resulting in a significant delay of tumor progression. This combined regimen may directly affect the metastatic process and also suppresses metastasis through inhibition of the growth of the orthotopically inoculated primary tumors. However, it may be potentially important for the clinical application of this combined regimen to develop the specific targeting delivery of viral vectors to the prostate as well as the stable oligodeoxynucleotide after *in vivo* administration.

We further evaluated the effect of combined treatment with antisense clusterin oligodeoxynucleotide, Ad5CMV-*p53*, and mitoxantrone on PC3 tumor growth *in vivo*, since mitoxantrone has been approved by the U.S. Food and Drug for use in hormone-refractory prostate cancer based on two randomized controlled trials demonstrating improved palliative response rates (45, 46). Surprisingly, the combined therapy with these three agents eradicated established PC3 tumor from 6 of 10 mice. There were no serious side effects were observed by the administration of antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53*, and the dose of mitoxantrone used in this experiment was tolerable; therefore, this combined treatment could be done safely and provide significant antitumor effects.

In conclusion, the present findings suggest that expression of clusterin inhibit the effect of Ad5CMV-*p53* treatment (i.e., *p53*-dependent apoptosis), resulting in androgen-independent prostate cancer progression, and that decreasing clusterin expression with the use of antisense oligodeoxynucleotide targeting clusterin gene may provide a feasible and safe strategy to enhance the therapeutic efficacy of Ad5CMV-*p53* in prostate cancer. The preclinical data shown here provides preliminary evidence supporting the design of clinical studies using a combination of antisense clusterin oligodeoxynucleotide plus Ad5CMV-*p53* therapy for patients with advanced androgen-independent prostate cancer.

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New Epitope Peptides Derived From Parathyroid Hormone-Related Protein Which Have the Capacity to Induce Prostate Cancer-Reactive Cytotoxic T Lymphocytes in HLA-A2$^+$ Prostate Cancer Patients

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BACKGROUND. Parathyroid hormone-related protein (PTHrP) is produced by cancer cells and has been suggested to be responsible for malignancy-associated hypercalcemia and osteolysis after bone metatsases. Therefore, PTHrP is a promising target in the treatment of metastatic prostate cancer.
METHODS. Seven PTHrP-derived peptides were prepared based on the HLA-A2 binding motif. These peptide candidates were screened by their ability to induce peptide-specific cytotoxic T lymphocytes (CTLs), and their ability to be recognized by immunoglobulin G (IgG).
RESULTS. Both the $PTHrP_{59-67}$ and $PTHrP_{42-51}$ peptides were found to efficiently induce peptide-specific CTLs from peripheral blood mononuclear cells of HLA-A2$^+$ prostate cancer patients with several HLA-A2 subtypes. These CTLs showed HLA-A2-restricted cytotoxicity toward prostate cancer cells. IgG reactive to the $PTHrP_{42-51}$ peptide was frequently detected in prostate cancer patients.
CONCLUSIONS. These results indicate that these two new PTHrP peptides will be useful in the peptide-based immunotherapy of HLA-A2$^+$ prostate cancer patients, especially those with bone metastases. *Prostate 62: 233–242, 2005.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; PTHrP; CTLs; peptide; antibody; HLA-A2**

INTRODUCTION

Prostate cancer is one of the most common cancers in elderly men [1]. Prostate cancer frequently metastasizes to the bone, and is treated with hormonal therapy. Although hormonal therapy can temporarily inhibit the progress of the disease, most patients subsequently develop hormone-refractory prostate cancer. Therefore, the development of new therapeutic modalities to treat distant metastatic prostate cancer is needed. One candidate is specific immunotherapy. In prostate cancer, tissue-specific antigens, which are expressed in normal prostate cells, can also be target molecules for

Abbreviations: CTLs, cytotoxic T lymphocytes; Flu, influenza; Ig, immunoglobulin; mAb, monoclonal antibody; OD, optical density; PBMCs, peripheral blood mononuclear cells; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related protein.

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specific immunotherapy for patients with this disease. Immunotherapy which targets prostate-specific antigens or prostate-specific membrane antigens has been carried out, but the antitumor effects have been unsatisfactory [2–6].

Parathyroid hormone-related protein (PTHrP) is a factor, which stimulates bone formation and reabsorption [7]. PTHrP is produced by several types of cancer cells, including prostate cancer cells [8], and has been reported to be expressed in most prostate carcinoma [9]. PTHrP is known to be a key agent in the development of bone metastasis in cases of prostate cancer [10], and is suggested to play an important role in the progression of prostate cancer [11]. Because PTHrP has limited homology with the parathyroid hormone (PTH) at its NH2 terminus, it can bind to the same receptor as PTH, resulting in similar biological activity [12]. Although a high extracellular calcium concentration inhibits PTH secretion and the proliferation of parathyroid cells as a result of negative feedback regulation, it allows the continuation of PTHrP secretion, and promotes worsening bone resorption [13]. Because a major barrier in treating prostate cancer patients is the development of bone metastases, PTHrP could be a promising target in the specific immunotherapy of prostate cancer patients with bone metastases. In this study, we identified two new PTHrP-derived peptides, which have the potential to induce prostate cancer-reactive cytotoxic T lymphocytes (CTLs).

MATERIALS AND METHODS

Patients

Informed consent was obtained from all HLA-A2$^+$ prostate cancer patients (three with bone metastases and seven with no bone metastases) and HLA-A2$^+$ healthy volunteers who were enrolled in this study. None of the participants were HIV-positive. Twenty milliliters of peripheral blood was obtained from all participants, and the peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll–Conray density gradient centrifugation. The expression of the HLA-A2 molecules on the PBMCs of the cancer patients and healthy donors was first determined by flow cytometry, and the HLA-A2 subtypes were determined using the sequence-specific oligonucleotide probe method.

Cell Lines

T2 is an HLA-A*0201-expressing lymphoma line. PC93 is an HLA-A2 negative prostate cancer cell line that was established by Dr. K. Ohnishi (Department of Urology, Kyoto University, Japan), and PC93-A2 is a subline that was stably transfected with the *HLA-A*0201* gene [14]. All cell lines were maintained in RPMI-1640 medium (Gibco-BRL, Grand Island, NY) supplemented with 10% FCS.

Peptides

Seven PTHrP-derived peptides (listed in Table I) were prepared based on the HLA-A2 binding motif [15,16]. All peptides were of >90% purity, and were purchased from Biologica Co., Nagoya, Japan. Influenza (Flu) virus-derived (GILGFVFTL), EBV-derived (GLCTLVAML), and HIV-derived peptides (SLYNTYATL) with the HLA-A2 binding motif were used as controls. All peptides were dissolved with dimethyl sulfoxide at a dose of 10 mg/ml.

Assay for Peptide-Specific CTLs in PBMCs

The assay for the detection of peptide-specific CTLs in PBMCs was performed according to a previously reported method [17]. In brief, PBMCs (1×10^5 cells/well) were incubated with 10 μg/ml of each peptide in a U-bottom-type 96-well microculture plate (Nunc, Roskilde, Denmark), at a volume of 200 μl of culture medium. The culture medium consisted of 45% RPMI-1640, 45% AIM-V medium (Gibco-BRL), 10% FCS, 100 U/ml of IL-2, and 0.1 mM MEM nonessential amino acid solution (Gibco-BRL). Every 3 days, half of the culture medium was removed and replaced with new medium containing a corresponding peptide (20 μg/ml). On the 15th day of culture, the cultured cells were separated into 4-wells. Two-wells were used for the PTHrP peptide-pulsed T2 cells, and the other 2-wells for the HIV peptide-pulsed T2 cells. After an 18-hr incubation period, the supernatants were collected, and the level of IFN-γ was determined by ELISA (limit of sensitivity: 10 pg/ml).

Cytotoxicity Assay

After in vitro stimulation with the PTHrP peptides, the peptide-stimulated PBMCs were additionally cultured with 100 U/ml IL-2 for approximately 10 days, in order to obtain a sufficient number of cells to carry out a cytotoxicity assay. These cells were then tested for cytotoxicity against both PC93 and PC93-A2 by a 6-hr ^{51}Cr-release assay. Two-thousand ^{51}Cr-labeled cells per well were cultured with effector cells in 96-round-well plates at the indicated effector/target ratios. In some experiments, either anti-HLA class I (W6/32: mouse IgG2a), anti-HLA-DR (L243: mouse IgG2a), anti-CD4 (NU-TH/I: mouse IgG1), anti-CD8 (NU-TS/C: mouse IgG2a), or anti-CD14 (H14: mouse IgG2a) monoclonal antibody (mAb) were added to the wells at a dose of 20 μg/ml at the initiation of the assay.

TABLE I. PTHrP Peptide Candidates and Comparison With Corresponding PTH Peptides

Peptides	Score[a]	Amino acid sequence[b]	Shared amino acids
PTHrP 103–111	320	YLTQETNKV	0
PTH		GEADKADVN	
PTHrP 59–67	110	FLHHLIAEI	1
PTH		W-RKKLQDV	
PTHrP 43–51	73	LLHDKGKSI	4
PTH		-M-NL- - -HL	
PTHrP 51–60	47	IQDLRRRFFL	2
PTH		LNSME-VEW-	
PTHrP 42–51	39	QLLHDKGKSI	5
PTH		- - -M-NL- - -HL	
PTHrP 165–173	<1	TSTTSLELD	0
PTH		(no corresponding peptide)	
PTHrP 59–68	<1	FLHHLIAEIH	2
PTH		W-RKKLQDV-	

PTHrP, parathyroid hormone-related protein; PTH, parathyroid hormone.
[a]The score represents the estimated half-time dissociation of the PTHrP peptides binding to HLA-A2 molecules.
[b]PTH-derived amino acids which share with the PTHrP peptides are shown as dash lines.

Cold Inhibition Assay

The specificity of the PTHrP peptide-stimulated CTLs was confirmed by a cold inhibition assay. In brief, ^{51}Cr-labeled target cells (2×10^3 cells/well) were cultured with the CTLs (4×10^4 cells/well) in 96-round-well plates with 2×10^4 cold target cells. T2 cells, which were pre-pulsed with either the HIV peptide or a corresponding PTHrP peptide, were used as cold targets.

Detection of Peptide Specific IgG

The peptide-specific immunoglobulin G (IgG) levels in the plasma were measured by ELISA, as previously reported [18,19]. In brief, peptide (20 μg/well)-immobilized plates were blocked with Block Ace (Yukijirushi, Tokyo, Japan) and washed with 0.05% Tween-20-PBS, after which 100 μl/well of plasma sample diluted with 0.05% Tween-20-Block Ace was added to the plate. After a 2-hr incubation at 37°C, the plates were washed and further incubated for 2-hr with a 1:1,000-diluted rabbit anti-human IgG (γ-chain-specific) (DAKO, Glostrup, Denmark). The plates were washed, and then 100 μl of 1:100-diluted goat anti-rabbit IgG-conjugated horseradish peroxidase (EnVision, DAKO) was added to each well, after which the plates were incubated at room temperature for 40 min. The plates were washed once, and 100 μl/well of tetramethyl benzidine substrate solution (KPL, Guildford, UK) was added. The reaction was then stopped by the addition of 1 M phosphoric acid. The values are shown as optical density (OD) U/ml. IgG reactive to a corresponding PTHrP peptide was judged to be positive when the difference of the OD in 1:100-diluted plasma exceeded 0.054. To confirm the specificity of IgG to the PTHrP peptide indicated, sample plasma was cultured with plates coated with either the corresponding PTHrP peptide, or an irrelevant PTHrP peptide. Thereafter, the levels of PTHrP peptide-specific IgG in the resulting supernatant were determined by ELISA.

Statistics

The statistical significance of the data was determined using a two-tailed Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Induction of PTHrP Peptide-Specific CTLs From HLA-A2$^+$ Healthy Donors and Prostate Cancer Patients

First, seven PTHrP-derived peptide candidates were prepared based on their binding affinity to HLA-A2 molecules (Table I). Because the $PTHrP_{1-36}$ peptide is a propeptide [20], peptides that include the first 36 amino acids were not included in these candidates. Two PTHrP peptides, the $PTHrP_{165-173}$ and $PTHrP_{59-68}$ peptides, which were previously reported to generate anti-cancer CTLs from HLA-A*0201$^+$ healthy donors [8], were included, whereas the predicted scores of

binding to the HLA-A*0201 molecules were less than 1. The difference in amino acids between PTHrP and PTH are also shown in Table I. All amino acids were different in the case of the $PTHrP_{103-111}$ peptide. PTH shared 1, 4, 2, and 5 amino acids with the $PTHrP_{59-67}$, $PTHrP_{43-51}$, $PTHrP_{51-60}$, and $PTHrP_{42-51}$ peptides, respectively. Next, the PBMCs of the 10 HLA-A2$^+$ healthy donors and 10 HLA-A2$^+$ prostate cancer patients were stimulated in vitro with each of the seven PTHrP peptides, and then examined for their IFN-γ production in response to T2 cells, which were pre-pulsed with either a corresponding PTHrP peptide, or the HIV peptide (Table II). Flu- and EBV-derived peptides were used as controls. The assay was carried out in quadruplicate. The cultured cells in 1-well were separated into 4-wells. Two-wells were used for the PTHrP peptide-pulsed T2 cells, and the other 2-wells were for the HIV peptide-pulsed T2 cells. The results which showed the best response are shown. The successful induction of peptide-specific CTLs was judged to be positive when significant values ($P < 0.05$ by two tailed Student's t-test) were observed. As a result, the $PTHrP_{59-67}$ and $PTHrP_{42-51}$ peptides induced peptide-specific CTLs in four and seven of ten HLA-A2$^+$ healthy donors, respectively. Although only one amino acid is different between the $PTHrP_{59-67}$ and $PTHrP_{59-68}$ peptides, no peptide-specific CTLs were induced when PBMCs from the HLA-A2$^+$ healthy donors were stimulated with the $PTHrP_{59-68}$ peptide. The reported $PTHrP_{165-173}$ induced peptide-specific CTLs in four of ten HLA-A2$^+$ healthy donors. However, the $PTHrP_{59-67}$ and

TABLE II. Reactivity of PTHrP Peptide-Stimulated PBMCs From HLA-A2$^+$ Healthy Donors and Prostate Cancer Patients

	Peptides								
	PTHrP 103–111	PTHrP 59–67	PTHrP 43–51	PTHrP 51–60	PTHrP 42–51	PTHrP 165–173	PTHrP 59–68	Flu	EB
	IFN-γ production (pg/ml)[a]								
Healthy donors									
#1: A0201	34	140	17	0	215	0	23	N.D.	N.D.
#2: A0201	217	100	21	79	127	222	51	408	302
#3: A0201	9	0	23	0	0	0	0	172	163
#4: A0201	0	6	27	314	122	294	0	135	0
#5: A0206	0	112	0	0	138	0	0	N.D.	N.D.
#6: A0206	0	296	0	46	143	0	46	498	0
#7: A0206	0	38	0	0	0	27	0	114	340
#8: A0206	0	22	0	16	380	325	0	0	82
#9: A0206	0	23	0	0	346	395	0	0	0
#10: A0207	0	0	0	14	17	46	0	399	0
Total	1/10	4/10	0/10	2/10	7/10	4/10	0/10	6/8	4/8
Cancer patients									
#1: A0206	144	118	40	55	155	202	31	0	24
#2: A0210	41	183	56	187	172	26	0	0	141
#3: A0210	57	129	185	34	179	60	48	166	0
#4: A0206	86	221	0	33	22	151	138	0	0
#5: A0206	0	0	39	0	153	103	154	0	0
#6: A0207	68	37	51	0	116	0	0	N.D.	N.D.
#7: A0206	49	0	0	0	0	0	222	N.D.	N.D.
#8: A0206	29	59	23	2	30	80	146	173	77
#9: A0201	56	0	14	67	0	0	43	172	163
#10: A0207	0	49	0	21	0	24	112	27	0
Total	1/10	4/10	1/10	2/10	5/10	3/10	5/10	3/8	2/8

[a]The PBMCs of HLA-A2$^+$ healthy donors and prostate cancer patients were stimulated in vitro with the PTHrP peptide indicated, as described in Materials and Methods.
On day 15th, the cultured PBMCs were tested for their reactivity to T2 cells, which were pre-pulsed with a corresponding peptide or an HIV peptide.
The values represent the mean of 2-wells, and the background IFN-γ production in response to the HIV peptide was subtracted.
Significant values ($P < 0.05$ by two-failed Student's t-test) are underlined.
N.D., not done.

$PTHrP_{42-51}$ peptides induced peptide-specific CTLs in four and five of ten HLA-A2$^+$ cancer patients, respectively. The reported $PTHrP_{165-173}$ and $PTHrP_{59-68}$ peptides induced peptide-specific CTLs in three and five of ten HLA-A2$^+$ cancer patients, respectively. The most cases of successful induction of peptide-specific CTLs were different when stimulated with either the $PTHrP_{59-67}$ or $PTHrP_{59-68}$ peptide. Because the $PTHrP_{42-51}$ and PTH corresponding peptides share 5 amino acids, we determined whether or not $PTHrP_{42-51}$ peptide-stimulated PBMCs could show reactivity against the corresponding PTH peptide. As a result, $PTHrP_{42-51}$ peptide-stimulated PBMCs from patients #1 and #2 produced IFN-γ in response to $PTHrP_{42-51}$ peptide-pulsed T2 cells, but not to corresponding PTH peptide-pulsed T2 cells (data not shown). In total, these findings indicate that both the $PTHrP_{59-67}$ and $PTHrP_{42-51}$ peptides are new candidates for generating peptide-specific CTLs from HLA-A2$^+$ prostate cancer patients.

Induction of Prostate Cancer-Reactive CTLs Using the $PTHrP_{59-67}$ and $PTHrP_{42-51}$ Peptides

Next, we determined whether CTLs that were induced by either the $PTHrP_{59-67}$ or $PTHrP_{42-51}$ peptide could show cytotoxicity against HLA-A2-expressing prostate cancer cells. The expression of the HLA-A*0201 molecules on PC93-A2 was previously reported [14]. In addition, the PC93 cells produced PTHrP at a level of 2.2 pmol/L (1 × 10^6 cell/ml for 24 hr). Next, we determined whether PBMCs stimulated by either the $PTHrP_{59-67}$ or the $PTHrP_{42-51}$ peptide could induce prostate cancer-reactive CTLs from HLA-A2$^+$ healthy donors and prostate cancer patients (Fig. 1). PBMCs from two HLA-A2$^+$ healthy donors (HD #1 and HD #6) and two HLA-A2$^+$ cancer patients (Pt #1 and Pt #2) were repeatedly stimulated with the PTHrP peptide indicated, according to the culture protocol described in Materials and Methods. These PTHrP peptide-stimulated PBMCs could produce IFN-γ in response

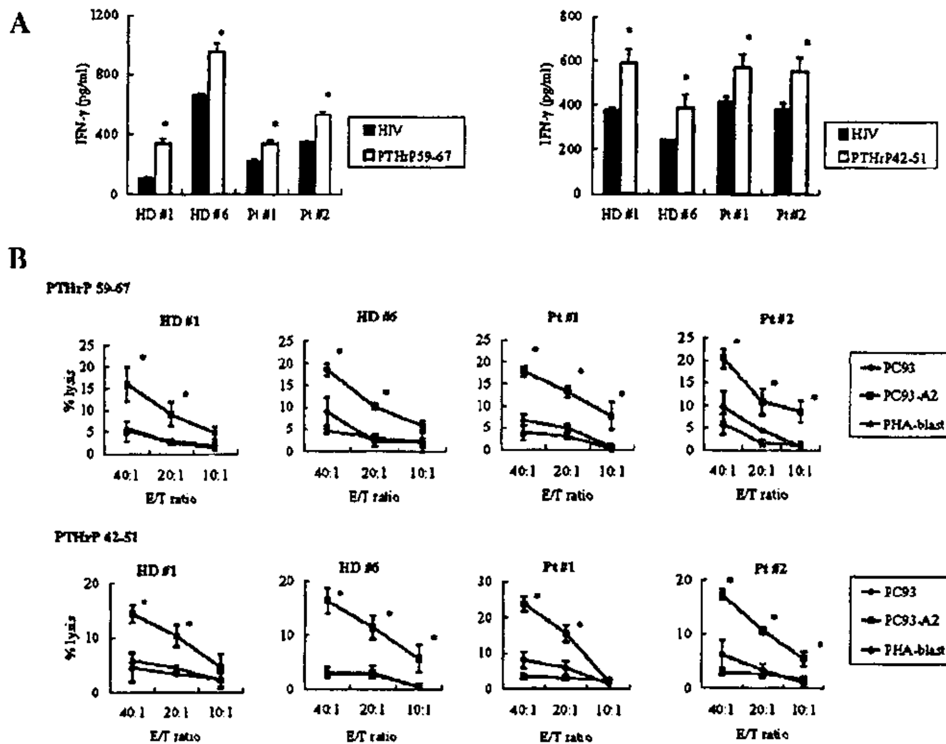

Fig. 1. Induction of HLA-A2-restricted and prostate cancer-reactive cytotoxic T lymphocytes (CTLs) from the peripheral blood mononuclear cells (PBMCs) of healthy donors and cancer patients. A: PBMCs from two HLA-A2$^+$ healthy donors (HD #1 and HD #6) and from two HLA-A2$^+$ prostate cancer patients (Pt #1 and Pt #2) were stimulated in vitro with the PTHrP peptides indicated, as described in Materials and Methods. On day 15, half of the cultured cells were harvested, pooled from 4-wells, and cultured with T2 cells, which were pre-pulsed with an HIV peptide (closed symbol) and the indicated PTHrP peptide (open symbol) for 18-hr. The levels of IFN-γ in the supernatants were then determined by ELISA. * $P < 0.05$ was considered statistically significant. B: Thereafter, these cells were examined for their cytotoxicity against PC93 cells (HLA-A2$^-$), PC93-A2 cells (HLA-A2$^+$), and PHA-blastoid T cells (HLA-A2$^+$). A 6-hr ^{51}Cr-release assay was performed. Values represent the mean of triplicate assays. *, $P < 0.05$ was considered statistically significant.

to the corresponding PTHrP peptide-pulsed T2 cells (Fig. 1A). After an additional 10 days culture with IL-2 100 U/ml, these peptide-stimulated PBMCs were examined for their cytotoxicity against three targets, PC93, PC93-A2, and PHA-stimulated HLA-A2$^+$ T cell blasts. As a result, these PTHrP peptide-stimulated PBMCs showed higher levels of cytotoxicity against the PC93-A2 than against the PC93 or HLA-A2$^+$ T cell blasts (Fig. 1B).

The cytotoxicity of the PTHrP peptide-stimulated PBMCs against the PC93-A2 cells was further examined by blocking antibodies and the cold inhibition assay. As shown in Figure 2A, their cytotoxicity toward the PC93-A2 cells was significantly inhibited by the addition of anti-HLA-class I or anti-CD8 mAbs, but not by the addition of anti-HLA-class II, anti-CD4, or anti-CD14 mAbs. Furthermore, their cytotoxicity against the PC93-A2 cell line was significantly suppressed by the addition of corresponding PTHrP peptide-pulsed T2 cells, as a cold target, whereas this suppression was not observed by the addition of HIV peptide-pulsed T2 cells (Fig. 2B). In total, these results indicate that both the PTHrP$_{59-67}$ and PTHrP$_{42-51}$ peptides have the potential to generate HLA-A2-restricted and prostate cancer-reactive CTLs from HLA-A2$^+$ prostate cancer patients, and that their cytotoxicity against prostate cancer is dependent on PTHrP peptide-specific CD8$^+$ T cells.

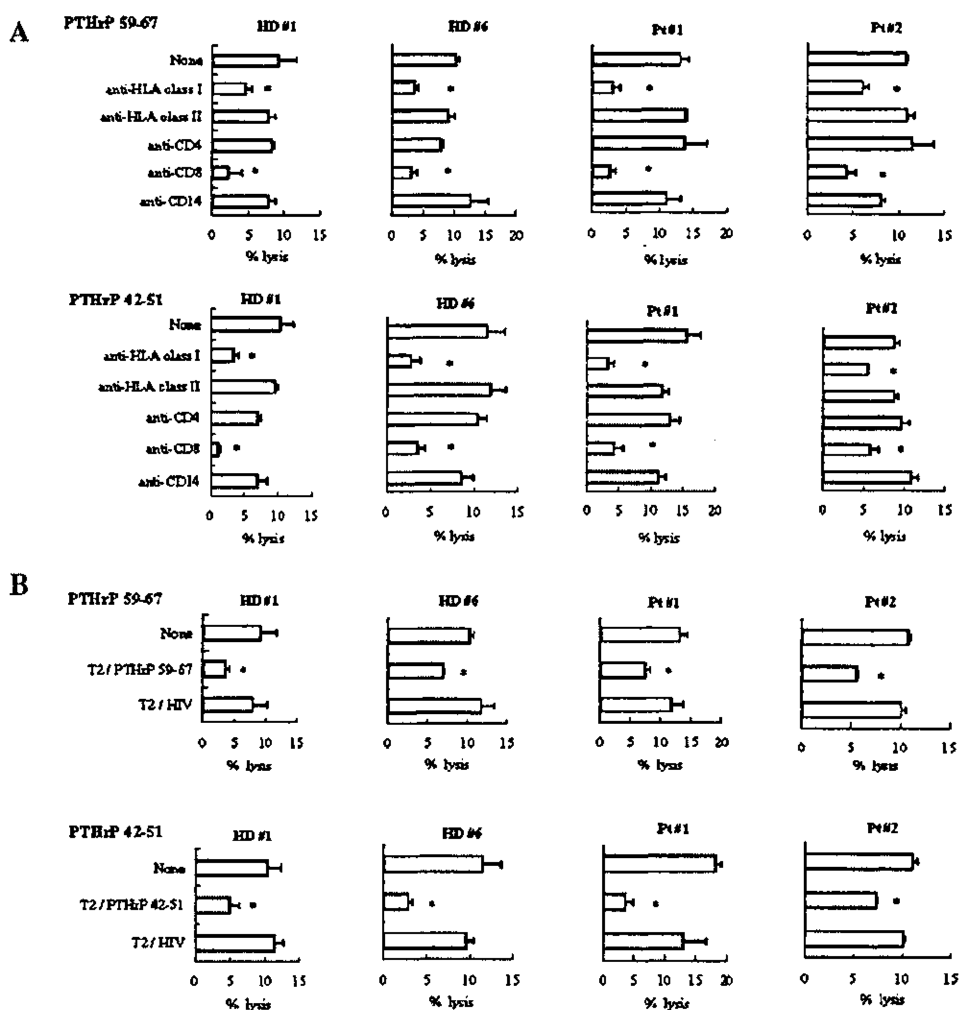

Fig. 2. Cytotoxicity of CD8$^+$ T cell-dependent and PTHrP peptide-specific CTLs. **A**: The PTHrP peptide-stimulated PBMCs were examined for their cytotoxicity against the PC93-A2 cell line, with or without anti-HLA class I, anti-HLA class II, anti-CD4, anti-CD8, or anti-CD14 mAb at a dose of 10 µg/ml. The values represent the mean of triplicate assays. *, $P < 0.05$ was considered statistically significant. **B**: The cytotoxicity against the PC93-A2 cell line (2×10^3 cells/well) was also examined in the presence of unlabeled T2 cells (2×10^4 cells/well), which were prepulsed with the HIV peptide or a corresponding PTHrP peptide. The values represent the mean of triplicate assays. * $P < 0.05$ was considered statistically significant.

TABLE III. IgG Reactive to the PTHrP Peptides in Plasma of HLA-A2$^+$ Healthy Donors and Prostate Cancer Patients

	Peptides						
	PTHrP 103–111	PTHrP 59–67	PTHrP 43–51	PTHrP 51–60	PTHrP 42–51	PTHrP 165–173	PTHrP 59–68
Healthy donors							
#1	0.0745	—	—	0.0945	—	—	0.0855
#2	0.092	—	0.102	0.13385	0.055	—	—
#3	—	—	—	—	—	—	—
#4	0.128	—	0.155	—	0.1115	—	—
#5	0.0575	—	—	—	0.0615	—	—
#6	—	0.078	—	—	0.0845	0.0865	—
#7	—	—	—	0.0675	0.0874	0.089	0.119
#8	0.0585	—	0.121	—	0.1275	0.193	0.167
#9	—	0.112	—	—	0.169	0.0925	0.065
#10	—	—	—	—	0.091	0.099	0.112
Total	5/10	2/10	3/10	5/10	8/10	5/10	5/10
Cancer patients							
#1	—	—	—	—	0.0685	—	—
#2	—	0.1225	0.078	—	0.055	—	—
#3	—	—	—	0.0675	0.0715	0.078	0.0795
#4	—	—	—	—	—	—	—
#5	—	—	—	—	0.106	—	—
#6	—	—	0.0895	—	0.0885	—	—
#7	0.0655	0.0655	0.0795	—	0.099	—	—
#8	—	—	—	—	—	—	—
#9	—	—	—	—	—	—	—
#10	0.112	—	—	0.0995	0.0995	0.1515	0.1505
Total	2/10	2/10	3/10	2/10	7/10	2/10	2/10

IgG reactive to a corresponding peptide was judged to be positive when a difference of CD in a 1:100-diluted plasma was more than 0.054, which was determined as described in Materials and Methods.

Detection of IgGs Reactive to the PTHrP Peptides

We previously reported that IgGs reactive to CTL epitope peptides were detected in healthy donors and patients with various types of epithelial cancer [18,19]. IgGs reactive to prostate-related antigens, including prostate-specific membrane antigen, prostate-specific antigen, and prostate stem cell antigen, were also detected in healthy donors and prostate cancer patients [14,20–22]. Therefore, we attempted to determine whether IgGs reactive to any of seven PTHrP-derived peptides could be detected in the plasma of ten healthy donors and ten cancer patients (Table III). The mean ± 2 SD value (OD: 0.054), which was determined as the level of IgG reactive to the HIV peptide in plasma from HIV-negative healthy donors, was used as a cut-off value at a 1:100-diluted plasma in the ELISA. The result was that IgGs reactive to the $PTHrP_{42-51}$ peptide were detected in eight of ten healthy donors, and in seven of ten prostate cancers. IgGs reactive to the $PTHrP_{59-67}$ peptide were detected in two of ten healthy donors, and in two of ten cancer patients. IgGs reactive to the reported two PTHrP peptides, the $THPrP_{165-173}$ and $PTHrP_{59-68}$ peptides, were detected in five of ten healthy donors, and in two of ten cancer patients. The representative results of two healthy donors (HD #1 and HD #6) and two cancer patients (Pt #1 and Pt #2) are shown in Figure 3A. As shown in Figure 3B, the levels of PTHrP peptide-specific IgG from either HD #6 and Pt #2 were significantly diminished by culturing the plasma in the corresponding PTHrP peptide-coated wells. This peptide-specific absorption demonstrated the validity of the ELISA system. In addition, because the $PTHrP_{42-51}$ and corresponding PTH peptides share five amino acids, we determined whether or not IgG reactive to the $PTHrP_{42-51}$ peptide could be absorbed by culturing the plasma in the corresponding PTH peptide-coated plates. However, no definite absorption was observed in seven patients whose plasma contained IgG reactive to the $PTHrP_{42-51}$ peptide, as shown in Table III (data not shown).

DISCUSSION

Prostate cancer appears to be a good target for the development of specific immunotherapies [23]. In recent years, our group has attempted to identify CTL epitope peptides derived from prostate-related

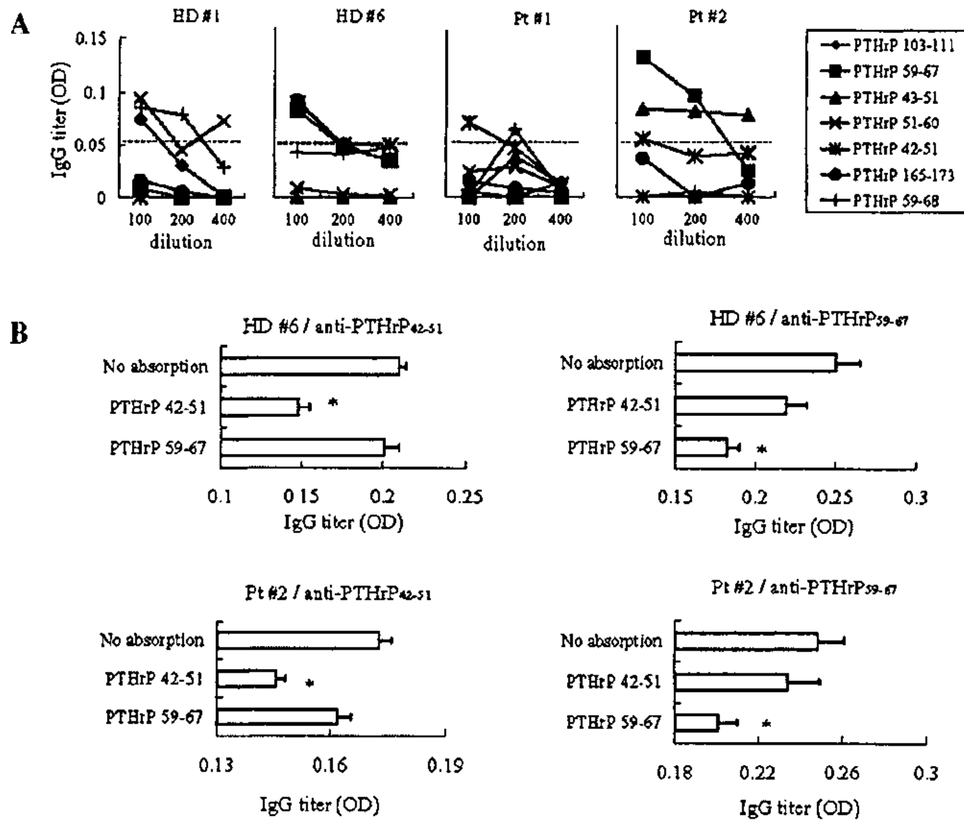

Fig. 3. IgG reactive to the PTHrP peptides in plasma from healthy donors and prostate cancer patients. A: Representative results from two healthy donors and two prostate cancer patients are shown. These values are shown as optical density (OD), and responses to the HIV peptide were subtracted. IgG reactive to a corresponding PTHrP peptide was judged to be positive when the difference of the OD in 1:100-diluted plasma exceeded 0.054. The cut-off value, 0.054, was determined as the level of IgG reactive to the HIV peptide in plasma from HIV-negative healthy donors. B: To confirm the specificity of IgG to the PTHrP peptides indicated, 100 μl of sample plasma from either HD #6 or Pt #2 was cultured in a plate pre-coated with either a corresponding PTHrP peptide or an irrelevant PTHrP peptide. Thereafter, the levels of IgG reactive to either the $PTHrP_{42-51}$ peptide or $PTHrP_{59-67}$ peptide in the resultant samples were determined by ELISA. *, $P < 0.05$ was considered statistically significant.

antigens that would be able to generate prostate cancer-reactive CTLs from prostate cancer patients [14,20–22,24]. We undertook the present study to extend the possibility of developing a peptide-based vaccine against metastatic prostate cancer. Up to the present, the $PTHrP_{59-68}$ and $PTHrP_{165-173}$ peptides had been reported to generate CTLs from HLA-A201^{+} healthy donors [8], but their immunogenicity in prostate cancer patients had not been determined. In this study, we identified two new epitope peptides which have the potential to induce prostate cancer-reactive CTLs in HLA-A2^{+} prostate cancer patients. PBMCs from HLA-A2^{+} prostate cancer patients showed peptide-specific IFN-γ production in four or five of ten patients when stimulated with the $PTHrP_{59-67}$ peptide or $PTHrP_{42-51}$ peptide, respectively. More importantly, PBMCs that were stimulated with these PTHrP peptides showed cytotoxicity against prostate cancer cells in an HLA-A2-restricted manner. These results indicate that these two PTHrP peptides are immunogenic, and therefore, potentially useful for specific immunotherapeutic treatment of HLA-A2^{+} prostate cancer patients with metastases.

The $PTHrP_{59-67}$ and $PTHrP_{42-51}$ peptides also induced peptide-specific and tumor-reactive CTLs from the PBMCs of HLA-A2^{+} healthy donors. This result is consistent with that of a previous report demonstrating the induction of PTHrP peptide-specific CTLs from the PBMCs of HLA-A2^{+} healthy donors [8]. Because the nonamer $PTHrP_{59-67}$ and the octamer $PTHrP_{42-51}$ peptides share only one and five amino acids with PTH, respectively, cross-reactivity between the PTHrP peptides and PTH could be excluded. However, low levels of PTHrP have been sporadically detected in keratinocytes, uterus, and mammary glands during lactation [25]. Recent advances in tumor immunology have revealed that self-antigens on human cancer cells are the most prevalent antigens

recognized by the immune system [26,27]. CTL precursors reactive to non-mutated self-antigens might circulate in the peripheral blood of both certain healthy donors and cancer patients.

We determined whether or not IgG against PTHrP peptides would be detectable in the plasma from HLA-A2$^+$ prostate cancer patients, because antibodies against CTL epitope peptides had already been observed in certain cancer patients and healthy donors [18,19]. We previously reported that IgG reactive to peptides derived from prostate-related antigens was frequently detectable in healthy donors and prostate cancer patients [14,20–22]. In this study, IgG reactive to the PTHrP$_{42-51}$ peptide was frequently detected in prostate cancer patients, as well as in healthy donors. This means that the PTHrP$_{42-51}$ peptide was effectively recognized by both the cellular and humoral immune systems. Our clinical trials revealed that a peptide vaccination frequently resulted in the induction of IgG reactive to the administered peptides [28,29]. In addition, the induction of IgG reactive to the vaccinated peptides was positively correlated with a longer survival rate of advanced lung cancer patients [30]. With regards to the use of a peptide vaccination in patients with gastric cancer, prolonged survival has been observed in patients showing not only cellular, but also humoral immune responses to vaccinated peptides [31]. Furthermore, the induction of IgG reactive to the administered peptides was correlated with a clinical response among patients with recurrent gynecologic cancer [32]. Vaccination with the PTHrP$_{42-51}$ peptide into such patients could elicit the induction of peptide-specific IgG, and might lead to positive clinical responses. We recently reported that HLA-DR-restricted CD4$^+$ T cells recognizing an administered CTL epitope peptide were induced in a lung cancer patient who showed a drastic increase in peptide-specific IgG after peptide vaccination [33]. This finding may hint at the involvement of the CD4$^+$ T cells. Further study could reveal the role of peptide-specific IgG in the anti-tumor immune response.

Most Caucasians are HLA-A*0201 positive, but HLA-A2 subtypes vary considerably in Japanese, as shown in Table II. PTHrP-derived peptides were prepared based on the binding motif to HLA-A*0201 molecules, and both the T2 cells and PC93-A2 cells express HLA-A*0201 molecules. CTLs reactive to PTHrP-derived peptides were induced not only from the HLA-A*0201 participants, but also from participants with the HLA-A2 subtypes, including HLA-A*0206, -A*0207, -A*0210 (Table II). We have previously reported that epithelial tumor antigen-derived peptides, which were prepared based on the binding motif to HLA-A*0201 molecules, are also immunogenic in patients with several HLA-A2 subtypes [34–36]. Therefore, the PTHrP$_{59-67}$ and PTHrP$_{42-51}$ peptides can be promising target molecules for specific immunotherapy of HLA-A2$^+$ prostate cancer patients with bone metastases. The frequency of the HLA-A2 allele is relatively high throughout the world [37]. The information provided here might increase the possibility of treating HLA-A2$^+$ prostate cancer patients with metastases using peptide-based immunotherapy.

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The Prostate 61:318 –323 (2004)

Protection of Androgen-Dependent Human Prostate Cancer Cells From Oxidative Stress-Induced DNA Damage by Overexpression of Clusterin and its Modulation by Androgen

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BACKGROUND. Recent studies reported that oxidative stress is one of the major factors associated with the progression of prostate cancer through the accumulation of DNA damage. In the present study, we investigated the effect of oxidative stress on cell injury using androgen-dependent human prostate cancer LNCaP cells overexpressing clusterin, which has been shown to play crucial roles in the acquisition of resistance to several apoptotic stimuli.
METHODS. We introduced clusterin cDNA into LNCaP cells which do not express a detectable level of clusterin expression, and generated a clusterin-overexpressing cell line (LNCaP/Cl) and a control vector only-transfected cell line (LNCaP/Co). The effects of hydrogen peroxide (H_2O_2) treatment on the LNCaP sublines with and without the addition of dihydrotestosterone (DHT) were analyzed using the in vitro mitogenic assay and lipid peroxidation assay, and morphological changes in the LNCaP sublines after H_2O_2 treatment were examined by staining with Hoechst 33258. The degrees of DNA damage induced by H_2O_2 into the LNCaP sublines were evaluated by the measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG) level.
RESULTS. H_2O_2-induced apoptosis in LNCaP/Cl was significantly suppressed compared with that in LNCaP/Co through the inhibition of membrane damage; however, the measurement of 8-OHdG level demonstrated that DNA damage was more intensively accumulated in LNCaP/Cl cells than LNCaP/Co cells. Furthermore, DHT suppressed the incidence of apoptotic cell death and enhanced the formation of 8-OHdG in both LNCaP/Cl and LNCaP/Co cells after H_2O_2 treatment in a dose-dependent manner.
CONCLUSIONS. These findings suggest that clusterin may contribute to conferring resistance to oxidative stress-mediated cellular injury on prostate cancer cells, especially in the presence of androgen. *Prostate 61: 318–323, 2004.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **clusterin; oxidative stress; DNA damage; apoptosis**

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer mortality in men in Western industrialized countries, and the risk of prostate cancer has been shown to increase markedly after age 50 years [1]. Aging is closely associated with a shift in the prooxidant-antioxidant balance of many tissues, including the prostate,

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toward an oxidative state under the damage of reactive oxygen species [2]. However, data are still limited regarding the effects of oxidative stress on the initiation and progression of prostate cancer [2–4].

Clusterin, also known as testosterone-repressed prostate message-2 or sulfated glycoprotein-2, was first isolated from ram rete testes fluid, and has been shown to be implicated in various biological processes, including tissue remodeling, reproduction, lipid transport, and apoptotic cell death [5]. Clusterin was initially regarded as a marker for cell death, because its expression is highly up-regulated in various normal and malignant tissues undergoing apoptosis [6,7]. Recent studies, however, revealed conflicting findings on the association between enhanced clusterin expression and apoptotic activity [8,9]. We also demonstrated the protective role of clusterin against therapeutic cell death signals using several kinds of animal models for human malignancies, including prostate cancer [10–14]. Furthermore, Schwochau et al. reported that clusterin protects against oxidative stress in porcine renal epithelial cells [8]. However, the functional significance of clusterin expression in the resistance to oxidative stress-induced apoptosis has not been well characterized.

In the present study, we investigated the effects of clutserin overexpression on the oxidative stress-induced apoptotic cell death and its modulation by androgen treatment using androgen-dependent human prostate cancer LNCaP cells.

MATERIALS AND METHODS

Cell Culture

LNCaP cells were generously provided by Dr. L. W. K. Chung (University of Virginia, Charlottesville, VA) and maintained in RPMI 1640 (Life Technologies Inc., Gaithersburg, MD) supplemented with 5% heat-inactivated fetal calf serum. In our previous study, we generated clusterin-overexpressing LNCaP cell lines (LNCaP/Cl#1 and LNCaP/Cl#2) by the transfection of pRC-CMV expression vector containing the 1.6-kb cDNA fragment encoding human clusterin, and confirmed that abundant levels of clusterin mRNA were detected in LNCaP/Cl#1 and LNCaP/Cl#2, whereas the parental LNCaP (LNCaP/P) and the control vector-transfected cell line (LNCaP/Co) did not express detectable clusterin mRNA levels [10]. Steroid hormones-depleted charcoal-stripped media (CSM) was prepared as described previously [15].

In Vitro Mitogenic Assay

In vitro mitogenic assay was performed as described previously [11]. Briefly, 3×10^3 cells were seeded in each well of 96-well microtiter plates and allowed to attach overnight. After exchanging the normal media for CSM with and without dihydrotestosterone (DHT) (Sigma Chemical Co., St. Louis, MO), the LNCaP sublines were treated with hydrogen peroxide (H_2O_2). Following a 48 hr incubation period, cells were fixed with 1% glutaraldehyde (Sigma Chemical Co.), and stained with 0.5% crystal violet (Sigma Chemical Co.). The optical density was determined with a microculture plate reader (Becton Dickinson Labware, Lincoln Park, NJ) at 540 nm. Absorbance values were normalized to the values obtained for the vehicle-treated cells in order to determine the percent of surviving cells. Each assay was performed in triplicate.

Quantification of Apoptosis

To evaluate apoptotic morphological features in the LNCaP sublines after the treatments described above, tumor cells were stained with Hoechst 33258 (Sigma Chemical Co.) as described previously [14]. Briefly, harvested tumor cells were suspended in 200 μl of 3% paraformaldehyde and incubated for 30 min at room temperature. The fixative was then removed, the cells were washed twice with PBS, resuspended in 20 μl of PBS containing 8 μg/ml of Hoechst 33258, and incubated for 15 min at room temperature. Aliquots of 10 μl were then placed on glass slides, and 500 cells were counted and scored for the incidence of apoptotic morphological changes including nuclear condensation and fragmentation observed by fluorescence microscopy. Each assay was performed in triplicate.

Lipid Peroxidation Assay

The peroxidation assays were performed using the lipid peroxidation kit (Calbiochem, La Jolla, CA) as described previously [16]. This assay is based on the formation of malondialdehyde, an end product of peroxidation of polyunsaturated fatty acids in the plasma membrane.

Determination of 8-Hydroxy-2′-Deoxyguanosine (8-OHdG) Level

8-OHdG levels were measured as previously reported [17–20]. Briefly, culture supernatants after the treatment as described above were centrifuged at 10,000*g* for 10 min, and the supernatants were used for the determination of 8-OHdG level using a quantitative sandwich ELISA kit (NOF Cooperation, Tokyo, Japan) with the determination range between 0.64 and 2,000 ng/ml. All analyses and calibrations were performed in duplicate. Optical density was determined with a microculture plate reader (Becton Dickinson Labware) at 450 nm. The blank value was

subtracted from the duplicate readings for each standard and sample. A standard curve was created using Stat View 4.02 (Abacus Concepts Inc., Berkeley, CA) by plotting the logarithm of the mean absorbance of each sample versus the sample concentration.

RESULTS

Effects of Clusterin Overexpression on H_2O_2-Induced DNA Damage in LNCaP Cells

We initially compared the cell growth rates among LNCaP/P, LNCaP/Co, LNCaP/CL#1, and LNCaP/CL#2, when cultured in normal media; however, there were no significant differences among these LNCaP sublines (data not shown). The LNCaP sublines were subsequently examined for their response to H_2O_2. LNCaP/CL#1 and LNCaP/CL#2 had acquired resistance to H_2O_2 in comparison with LNCaP/P and LNCaP/Co (Fig. 1A); that is, overexpression of clusterin in LNCaP cells increased the IC50 of H_2O_2 approximately threefold. To evaluate the apoptotic features induced by H_2O_2, the LNCaP sublines treated with various concentrations of H_2O_2 for 24 hr were analyzed by staining with Hoechst 33258. After the exposure to H_2O_2, the incidence of apoptotic morphological changes observed in LNCaP/CL#1 and LNCaP/CL#2 were significantly decreased compared with LNCaP/P and LNCaP/Co (Fig. 1B). To assess the mode of H_2O_2 action in apoptotic cell death, its cellular effects were examined by the measurement of cellular lipid oxidation levels. The degree of apoptotic cell death induced by H_2O_2 into the LNCaP sublines was closely associated with the cellular lipid oxidation levels (Fig. 1C), suggesting that H_2O_2 had caused considerable membrane damage in these cells. Moreover, treatment of LNCaP/CL#1 and LNCaP/CL#2 with H_2O_2 resulted in a significant increase in the formation of 8-OHdG compared with LNCaP/P and LNCaP/Co (Fig. 1D).

Moderated Effects of Androgen on H_2O_2-Induced DNA Damage in the LNCaP Sublines

We then evaluated the effects of DHT on H_2O_2-induced cell death in LNCaP sublines maintained in steroid hormones-depleted CSM. As previously reported [15], DHT treatment resulted in the enhanced growth of LNCaP sublines against H_2O_2 treatment irrespective of the introduction of the clusterin gene (Fig. 2A). Furthermore, DHT reduced the incidence of apoptosis as well as the cellular lipid oxidation level in the LNCaP sublines treated with H_2O_2 in a dose-dependent manner (Fig. 2B,C). The formation of 8-OHdG in the LNCaP sublines after treatment with H_2O_2 was significantly enhanced by DHT, and its formation in LNCaP/CL#1 and LNCaP/CL#2 was significantly greater than that in LNCaP/P and LNCaP/Co (Fig. 2D).

DISCUSSION

Oxidative stress induced by reactive oxygen species have been shown to be involved in several pathophysiological processes, such as cell proliferation, differentiation, apoptosis, and carcinogenesis [21]. Low levels of reactive oxygen species mainly play important roles in physiologic functions, while several studies have reported that at high concentrations, reactive oxygen species are regarded as cytotoxic and implicated in carcinogenesis of various types of malignancies, including prostate cancer [2–4,22]. However, it has not been well documented whether antiapoptotic genes diminish the cytotoxicity accumulated by reactive oxygen species, and consequently inhibit apoptotic cell death. Recent studies have clearly demonstrated the powerful antiapoptotic activity of clusterin against various kinds of stimuli [8–14]; however, to our knowledge, the functional significance of clusterin in DNA damage by oxidative stress and its induced apoptotic cell death has not been well characterized. In this study, therefore, we analyzed the effects of clusterin expression on H_2O_2-induced apoptosis into the androgen-dependent human prostate cancer LNCaP cells.

We initially showed the inhibitory effect of clusterin overexpression on H_2O_2-induced cellular injury, and the observation of morphological changes in the LNCaP sublines after H_2O_2 treatment demonstrated that clusterin confers resistance to cellular injury-induced by H_2O_2 through the inhibition of apoptotic cell death. Furthermore, H_2O_2 was shown to cause considerable membrane damage in the LNCaP sublines by measurement of cellular lipid oxidation levels, and this membrane damage was associated with the degree of apoptosis in each cell line. Furthermore, judging from the measurement of 8-OHdG in culture medium, higher levels of DNA damage tended to accumulate in clusterin-transfected LNCaP cells compared with control LNCaP cells. These findings suggest that overexpression of clutserin may help accelerate prostate cancer development, at least in part, through the inhibition of membrane damage induced by reactive oxygen species, resulting in the accumulated DNA damage in clusterin-overexpressing cells, which possibly causes enhancing malignant potential of prostate cancer cells.

The exact mechanism of prostate cancer development is not well characterized; however, the importance of androgen in prostate carcinogenesis is suggested by the finding that prostate cancer seldom

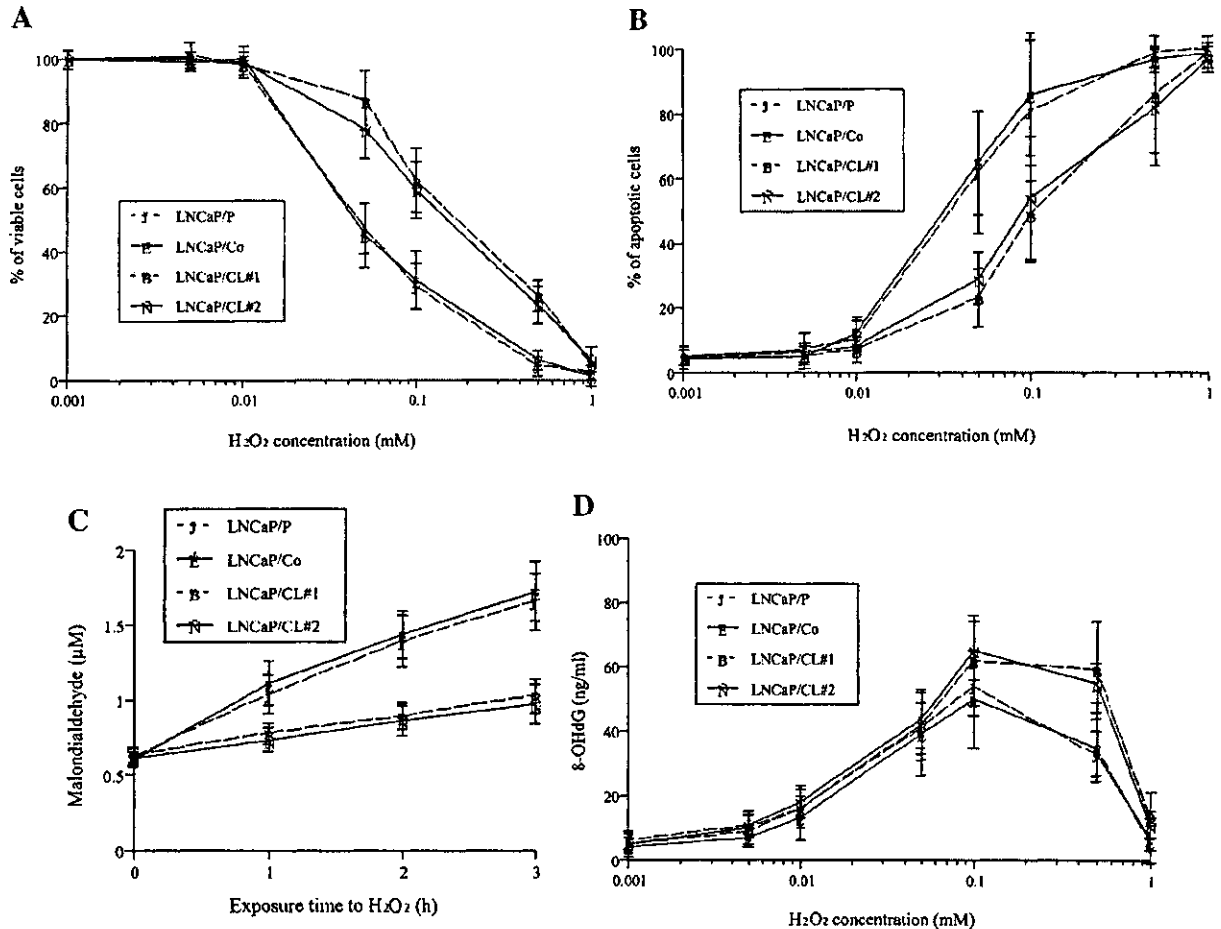

Fig. 1. **A**: Three thousand cells of each cell line (LNCaP/P, LNCaP/Co, LNCaP/Cl#1, and LNCaP/Cl#2) were seeded in 96-well microtiter plates. Cells were then treated with various concentrations of H_2O_2 for 24 hr. Cell viability was determined by in vitro mitogenic assay. Values are the mean of three independent experiments. Bars indicate standard deviations. The differences between clusterin-transfected LNCaP cells and control cells were significantly different by repeated-measure analysis of variance (ANOVA) model ($P < 0.01$). **B**: The percentage of cells in LNCaP sublines exhibiting morphological changes of apoptosis was assessed. Cells of each cell line were treated with various concentrations of H_2O_2 for 24 hr, then stained with Hoechst 33258. A total of 500 cells was counted for each data point. Values are the mean of three independent experiments. Bars indicate standard deviations. The differences between clusterin-transfected LNCaP cells and control cells were significantly different by repeated-measure analysis of variance (ANOVA) model ($P < 0.05$). **C**: The formation of malondialdehyde in each of the LNCaP sublines was measured after exposure to 0.1 mM H_2O_2 for 0 to 3 hr to assess the severity of lipid peroxidation. Values are the mean of three independent experiments. Bars indicate standard deviations. The differences between clusterin-transfected LNCaP cells and control cells were significantly different by repeated-measure analysis of variance (ANOVA) model ($P < 0.01$). **D**: 8-hydroxy-2′-deoxyguanosine level in each of the LNCaP sublines was measured after treatment with various concentrations of H_2O_2 for 24 hr. Values are the mean of three independent experiments. Bars indicate standard deviations. *The differences between clusterin-transfected LNCaP cells and control cells were significantly different by repeated-measure analysis of variance (ANOVA) model ($P < 0.05$).

occurs in eunuchs or men lacking 5α-reductase, which is the enzyme responsible for converting testosterone to its more active form, 5α-DHT [23]. Furthermore, development of the normal prostate and its functional maintenance depend on androgen, and more than 75% of tumors in men with extraprostatic disease are androgen-dependent at initial diagnosis [24]. Recently, Ripple et al. reported that physiological levels of androgen induce a shift in the prooxidant–antioxidant balance toward the prooxidant state in LNCaP cells [2]. We also clearly showed that DHT suppressed the incidence of apoptotic cell death and enhanced the formation of 8-OHdG after treatment of LNCaP sublines with H_2O_2 in a dose-dependent manner. Considering these findings, androgen may be capable of increasing the effect of oxidative stress on the LNCaP sublines resulting in the accumulation of DNA damage, irrespective of clusterin expression; however,

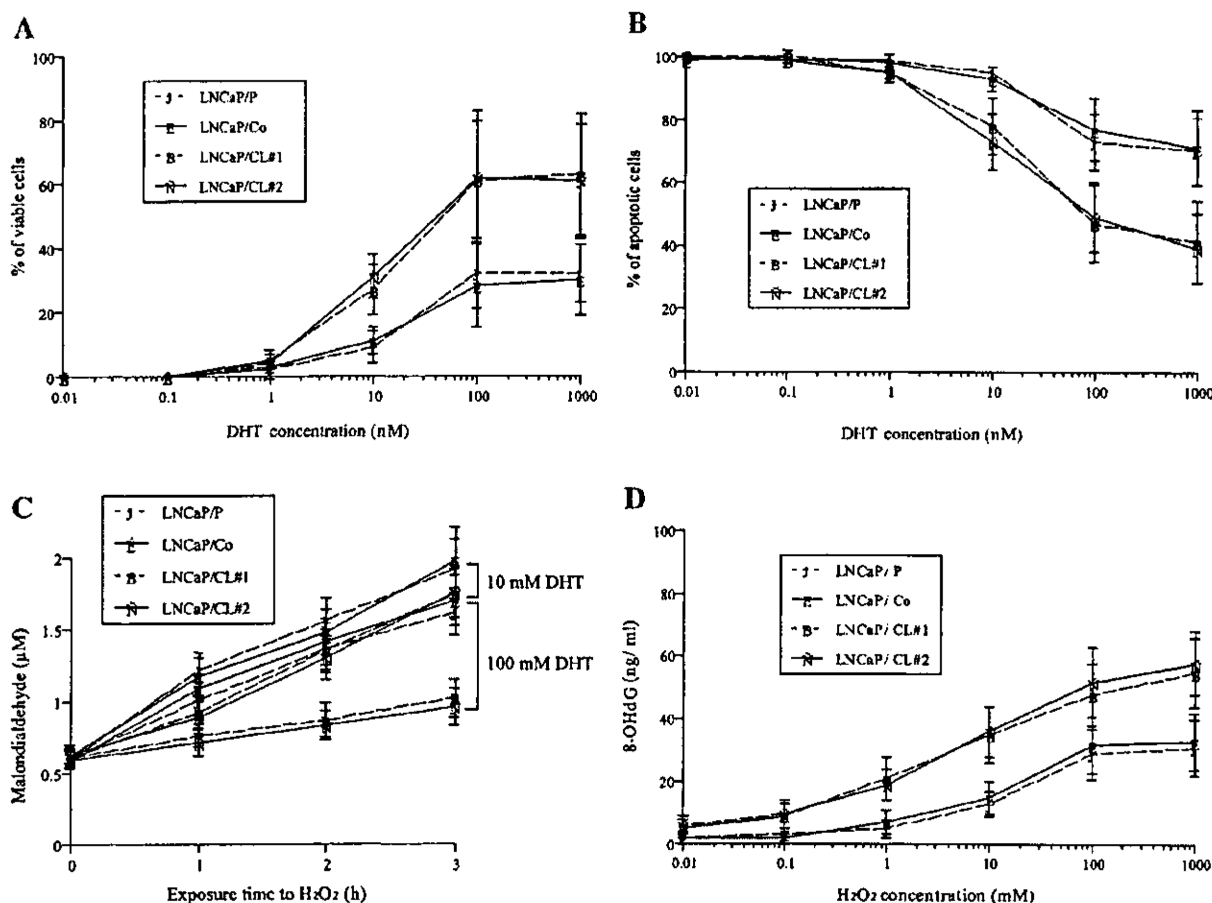

Fig. 2. **A**: Three thousand cells of each LNCaP subline were seeded in 96-well microtiter plates. After exchanging the normal media for steroid hormones-depleted CSM containing various concentrations of dihydrotestosterone (DHT), cells were treated with 0.1 mM H_2O_2 for 24 hr. Cell viability was determined by the in vitro mitogenic assay. Values are the mean of three independent experiments. Bars indicate standard deviations. The differences between clusterin-transfected LNCaP cells and control cells were significantly different by repeated-measure analysis of variance (ANOVA) model ($P < 0.05$). **B**: The percentage of cells in LNCaP sublines exhibiting the morphological changes of apoptosis was assessed. Cells of each cell line cultured in CSM containing various concentrations of DHT were treated with 0.1 mM H_2O_2 for 24 hr, then stained with Hoechst 33258. A total of 500 cells was counted for each data point. Values are the mean of three independent experiments. Bars indicate standard deviations. The differences between clusterin-transfected LNCaP cells and control cells were significantly different by repeated-measure analysis of variance (ANOVA) model ($P < 0.01$). **C**: The formation of malondialdehyde in each of the LNCaP sublines cultured in CSM containing 10 or 100 nM DHT was measured after the exposure to 0.1 mM H_2O_2 for 0–3 hr to assess the severity of lipid peroxidation. Values are the mean of three independent experiments. Bars indicate standard deviations. The differences between clusterin-transfected LNCaP cells and control cells cultured in CSM containing 100 nM DHT were significantly different by repeated-measure analysis of variance (ANOVA) model ($P < 0.01$), while there were no significant differences when cultured in CSM containing 10 nM DHT. **D**: 8-hydroxy-2′-deoxyguanosine level in each of the LNCaP sublines cultured in CSM containing various concentrations of DHT was measured after treatment with 0.1 mM H_2O_2 for 24 hr. Values are the mean of three independent experiments. Bars indicate standard deviations. The differences between clusterin-transfected LNCaP cells and control cells were significantly different by repeated-measure analysis of variance (ANOVA) model ($P < 0.01$).

these effects became remarkable in clusterin-transfected LNCaP cells compared with control LNCaP cells, when treated with lower concentrations of DHT, suggesting the cooperative effects of clusterin and DHT.

The significance of oxidative stress in association with clusterin expression in the development of a novel strategy for preventing prostate cancer development is of interest. We have recently reported the usefulness of antisense oligodeoxynucleotide therapy targeting clusterin using preclinical animal models for prostate cancer [10–12]. Furthermore, several experimental as well as epidemiological studies demonstrated the efficacies of antioxidants in inhibiting prostate cancer progression [25,26]. For example, Fleshner et al. showed that the mechanism of dietary fat-induced

growth of LNCaP tumor is mediated by oxidative stress, and that vitamin E, a potent intracellular antioxidant, inhibits the growth of LNCaP tumors [25]. These findings, therefore, suggest that targeting the clusterin gene and reducing oxidative stress might be efficacious as a therapeutic strategy for prostate cancer.

In conclusion, clusterin may contribute to conferring a resistant phenotype to oxidative stress-mediated DNA injury on androgen-dependent prostate cancer cells, especially in the presence of androgen; therefore, it raises the possibility of a therapeutic benefit of antioxidant administration.

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Experience With Conformal Proton Therapy for Early Prostate Cancer

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Abstract: A study was conducted to evaluate the use of proton beam therapy for the treatment of organ-confined prostate cancer. This is a preliminary assessment of treatment-related morbidity and tumor response. Sixteen patients with T1-T2b prostate cancer underwent proton beam therapy. Acute and late toxicity was scored according to the National Cancer Institute Common Toxicity Criteria Grading System (version 2.0, April 1999) and to the Radiation Therapy Oncology Group grading system, respectively. Local control was assessed using magnetic resonance imaging (MRI) and prostate-specific antigen (PSA) values. Although skin toxicity and bladder irritability were commonly observed, none of the patients developed grade III or IV toxicity. Of 9 patients in whom the primary lesion was detected by MRI, partial response and no change (NC) was observed in 6 (66.7%) and 3 (33.3%) patients, respectively. Four patients presented normal PSA value before treatment due to the previous endocrine therapy. However, the other 12 patients with elevated PSA value before treatment showed complete response. No patients showed PSA failure within the median follow-up period of 11.9 months. Although longer follow-up is necessary, minimum toxicity and good short-term clinical responses were observed following proton beam therapy in T1-T2 prostate cancer patients.

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The discovery of prostate-specific antigen (PSA) had a great impact on the diagnosis and treatment of prostate cancer.[1] Early detection of prostate cancer was very difficult prior to this discovery. However, the number of individuals being diagnosed with early prostate cancer is rapidly increasing as the PSA measurement is now widely included in health examinations.[2] Before the discovery of PSA, hormonal therapy was the standard treatment for prostate cancer because so few prostate cancer patients were diagnosed in the early stages. As early prostate cancer has been diagnosed more frequently, several treatment modalities have become available. Anatomical radical prostatectomy, established by Walsh,[3] was a gold standard treatment for organ-confined prostate cancer. Furthermore, excellent clinical outcomes for laparoscopic prostatectomy have recently been reported.[4–6] Radiation therapy is also a widely used treatment for early prostate cancer. However, increases in irradiated dose may cause serious complications, particularly in adjacent organs such as the rectum. Due to the characteristics of proton beams, delivery of higher doses of radiation is possible, without influencing adjacent organs. Proton therapy has therefore been attracting considerable attention. Although we have only recently begun using proton therapy for the treatment of organ-confined prostate cancer, we report herein our experiences to date.

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PATIENTS AND METHODS

Patients Characteristics

Patient characteristics are summarized in Table 1. Sixteen patients with biopsy-proven stage T1-T2b prostate cancer underwent proton therapy between May and December 2001. Magnetic resonance imaging (MRI), digital rectal examination, results of prostate biopsy, and PSA value were considered to determine clinical staging. Patient age ranged from 54 to 82 years old. Nine of them elected proton therapy rather than surgery. The remaining 7 patients were not able to undergo radical prostatectomy due to age or complications. The Gleason scores of the patients were 6 (two patients), seven (seven patients), 8 (four patients), and 9 (three patients). Four patients had undergone hormonal therapy prior to proton therapy. The mean PSA values of patients who underwent hormonal therapy and of naive patients were 0.45 ng/ml (0.2–0.8) and 11.0 ng/ml (5.9–23.0), respectively.

Treatment Planning and Delivery

Patients were immobilized for treatment planning using custom-shaped foam material. This device minimized motion

TABLE 1. Patient Characteristics

No.	Age	PS	Op	Reason For Not Having Operation	TNM	Gleason	Pretreatment	PSA
1	62	PS 0	+		T2aN0M0	3 + 4 = 7	–	12.0
2	71	PS 0	+		T1cN0M0	4 + 4 = 8	Hormonal therapy	0.7
3	67	PS 1	–	Complication (angina pectoris)	T1cN0M0	3 + 3 = 6	–	22.0
4	70	PS 0	–	Complication (angina pectoris)	T1cN0M0	4 + 3 = 7	–	12.0
5	67	PS 1	+		T2aN0M0	4 + 5 = 9	Hormonal therapy	0.8
6	60	PS 0	+		T2aN0M0	4 + 3 = 7	–	8.7
7	69	PS 1	–	Complication (scoliosis)	T2aN0M0	5 + 4 = 9	–	23.0
8	82	PS 0	–	Age	T2aN0M0	4 + 4 = 8	–	13.0
9	79	PS 1	–	Age	T2aN0M0	4 + 3 = 7	–	5.9
10	66	PS 0	+		T1cN0M0	4 + 4 = 8	Hormonal therapy	0.2
11	66	PS 0	+		T2aN0M0	4 + 3 = 7	–	8.4
12	66	PS 0	+		T1cN0M0	3 + 3 = 6	–	10.0
13	54	PS 0	+		T2aN0M0	3 + 4 = 7	–	17.0
14	77	PS 0	–	Age	T2aN0M0	4 + 4 = 8	–	10.0
15	67	PS 2	–	Complication (angina pectoris)	T2aN0M0	3 + 4 = 7	–	8.2
16	64	PS 0	+		T2aN0M0	4 + 5 = 9	Hormonal therapy	0.2

Op, operation; PS, performance status; PSA, prostate-specific antigen; TNM, tumor, nodes, metastasis.

and maintained a constant distance from the skin surface to the distal edge of the target volume. Rectal balloon was inserted before each treatment to distend the posterior rectal wall to remove it from the planned treatment field. Then computed tomography (CT) scanning was performed to decide on a treatment plan. The radiation oncologist outlined the target volume and critical normal tissues at each appropriate interval. The total dose delivered to the prostate was 74 gray equivalent in 37 fractions. Relative biologic effectiveness was 1.1.[7]

Follow-up End Points

Follow-up ranged from 7.9 to 14.5 months after radiation therapy. All patients underwent follow-up examinations every month during the first year posttreatment. PSA was measured as part of each examination. MRI was also assessed to evaluate clinical response. Routine CT scans and bone scintigraphy were not conducted unless PSA value increased or clinical symptoms were observed. Biochemical failure was defined as three consecutive elevations of PSA value.

Acute treatment-related toxicity was graded using the NCI Common Toxicity Criteria Grading System (version 2.0, April 1999), and late treatment-related toxicity was graded using the Radiation Therapy Oncology Group late morbidity criteria.[8,9]

RESULTS

Response Assessed by Tumor Volume

Tumors were detected in 9 of 16 patients by MRI. Although complete response (CR) was not observed in any patients, partial response was seen in 6 patients (66.7%). No tumor growth was observed in any of the patients (Table 2).

Response assessed by PSA

Since PSA response was not evaluated in the 4 patients who had undergone hormonal therapy, pre- and posttreatment PSA values in 12 patients were compared (Table 3). Elevated PSA value in all these patients returned to normal range. In other words, 100% CR was observed based on PSA. Moreover, none of these patients showed PSA failure in the mean follow-up period of 11.9 months (Fig. 1).

The four patients who had undergone hormonal therapy ceased it after the proton radiation. No PSA failure has been observed in these patients so far (Fig. 2).

Toxicity

None of the patients developed grade 3 or 4 toxicity (Fig. 3). All patients showed grade I skin toxicity. The next most common side effect was bladder irritability (grade I: 69%, grade II: 6%). Only 2 patients (12%) showed rectal bleeding and none developed anal pain or diarrhea.

DISCUSSION

Radical prostatectomy is still the standard treatment for early prostate cancer. However, radiation therapy is becoming a more widely used treatment modality.[10] Which treatment—radical prostatectomy or radiation therapy—may be better applied to organ-confined prostate cancer is still a subject of controversy. The disease-specific 10-year survival

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