Table 2
Frequency of $CD8^+$TL activities induced by antigenic peptides.

Peptide	Frequency (%)	Patient																			
		1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
SART-1_{690}	9/35 cases (25.7%)	271[b]	783	279	185	238	<0	<0	51	11	107	19	<0	29	<0	<0	17	<0	713	751	385	<0
SART-2_{93}	7/35 cases (20.0%)	624	<0	146	195	<0	<0	<0	13	2	27	86	<0	124	56	56	664	256	844	<0	<0	116
SART-2_{161}	1/35 cases (2.9%)	166	<0	77	105	<0	<0	<0	47	<0	<0	ND	<0	62	<0	<0	227	<0	948	<0	<0	<0
SART-2_{899}	5/35 cases (14.3%)	<0	801	93	197	<0	124	<0	85	24	81	ND	<0	<0	<0	<0	29	253	252	158	280	76
SART-3_{109}	6/35 cases (17.1%)	<0	<0	28	167	<0	<0	<0	119	84	10	336	<0	<0	<0	2	689	199	206	241	417	<0
SART-3_{315}	4/35 cases (11.4%)	<0	682	34	84	<0	<0	2	47	<0	105	ND	1119	<0	<0	<0	29	180	<0	365	<0	ND
CyB_{84}	5/35 cases (14.3%)	<0	914	303	146	37	<0	60	4	9	134	115	76	64	119	<0	89	<0	<0	<0	<0	16
CyB_{91}	0/35 cases (0%)	<0	<0	38	<0	15	<0	5	79	48	<0	ND	<0	<0	21	<0	<0	<0	<0	<0	<0	<0
Lck_{208}	4/35 cases (11.4%)	471	<0	41	<0	<0	<0	<0	119	<0	123	5	<0	26	48	94	<0	<0	656	<0	<0	147
Lck_{486}	6/35 cases (17.1%)	1013	<0	48	72	<0	<0	<0	200	<0	110	ND	139	20	<0	228	267	<0	396	<0	383	ND
Lck_{488}	2/35 cases (5.7%)	494	<0	106	176	<0	<0	<0	41	16	<0	ND	<0	<0	<0	56	<0	<0	<0	<0	<0	<0
$ART4_{13}$	2/35 cases (5.7%)	159	<0	100	<0	<0	<0	<0	190	29	112	<0	26	<0	<0	82	<0	<0	458	<0	311	<0
$ART4_{75}$	7/35 cases (20.0%)	<0	<0	301	162	34	<0	95	446	<0	224	ND	<0	23	204	274	111	<0	<0	<0	<0	<0
EBV-derived	35/35 cases (100.0%)	611	528	200	169	192	81	49	176	84	137	190	101	90	171	188	528	283	624	321	407	135

ND: not detected.
[a] Patient number is indicated.
[b] The induced $CD8^+$TL activity was measured as described in Fig. 1. Detectable levels of $CD8^+$TLs were adjudged as positive (the score is underlined in the Table) if the mean value of IFN-γ production by the peptide-stimulated PBMCs in response to the corresponding peptide was significantly ($P = 0.035$; two-tailed Student's *t*-test) higher than that in response to no peptide. The background IFN-γ production in response to no peptide was subtracted from the values. Mean values are shown at E/T ratio of 10:1 in quadruplicate assays.

able for the HLA-$A24^+$ patients. Apart from these peptides, there are many other vaccine candidates with the ability to induce HLA class I-restricted $CD8^+$TLs reacting for cancer cells [25–27]. Therefore, an increased number of peptides for assaying would be associated with an increased percentage of patients with positive peptides, as well as with an increased number of positive peptides per patient. Regardless of these limitations, this study shows that peptide-specific $CD8^+$TL activities were detectable in the majority (60.0%) of HLA-$A24^+$ patients with oral SCC. The HLA-A24 allele is found in more than 60% of the Japanese population (in 95% of these individuals the genotype is A2402), 20% of Caucasians, and 12% of Africans [28]. Therefore, the method employed in this study might be available for detecting peptide-specific $CD8^+$TLs in a large number of patients with oral SCC.

Moreover, in patients with detectable SART-1_{690}-specific $CD8^+$TLs, specific $CD8^+$TL activities were induced for more peptides than in patients without the SART-1_{690}-specific $CD8^+$TLs. In these patients, the *in vivo* immunogenicity of the tumor cells should be high due to their expression of HLA class I. Furthermore, the PBMCs from 14 patients showed no detectable levels of $CD8^+$TLs to any of the tested peptides. The cellular immunity of these patients may have been suppressed. Therefore, additional supportive immunotherapies may be needed for these patients in order to boost immunity prior to peptide-based specific immunotherapy.

The present study also shows that the profile of positive peptides varied greatly among the patients, suggesting that the peptides that can be used in CTL-oriented vaccination differ from patient to patient. These variations are probably due to the heterogeneity of tumor cells and the immunologic diversity of T cells among individual patients. In the future, this new immunotherapeutic approach may be developed into a tailor-made tumor immunotherapy. The same approach may be applicable to the treatment of malaria, HIV and other infectious diseases for which no effective vaccine protocols have been established [29–31].

This *in vitro* induction shows HLA-$A24^+$-restricted $CD8^+$TLs could be cytotoxic against C1R-A2402 cells. As the medium that contained 10 mM of each peptide was replaced every 3 days, higher concentrations of the peptides could be maintained throughout the culture period, which, in turn, should facilitate the generation of HLA class I-restricted $CD8^+$TLs. This assumption is based on the notion that high concentrations of peptides in the culture result in a high density of peptides in the groove of the HLA class I molecule of the antigen-presenting cells, which would tend to stimulate $CD8^+$TLs.

A recently developed HLA-tetramer assay has been shown to be sensitive and accurate [32–34]. However, it requires labeled tetramer and a relatively high number of cells per assay and, furthermore, it cannot measure CTL activity. Although the method employed in the present study has several disadvantages in terms of sensitivity and accuracy with regard to monitoring the frequency of peptide-specific $CD8^+$TLs, it has the advantages of simplicity and the ability to handle relatively large samples, and it can measure $CD8^+$TL activity. The $CD8^+$TLs induced by these peptides could be cytotoxic for cancer cells but not for normal proliferating cells (PHA-blastoid T cells), as demonstrated in previous studies [17–20,23,35,36]. Therefore, vaccination with these peptides may not be associated with adverse effects on normal cells and normal tissues. Indeed, no severe adverse effects were observed in the phase I clinical studies carried out at Kurume University Hospital, in which peptide vaccines were analyzed using 13 different peptides [37,38]. The same peptides were used in the *in vitro* experiments that involved HLA-$A24^+$ patients in the present study.

The use of tumor resection, chemotherapy, radiotherapy and combinations of them as treatments for oral SCC has maintained the overall 5-year survival rate at about 80% [10]. The present

Table 3
Correlation between $CD8^+$TL activity induced by SART-1_{690} peptide and cellular response.

Patient	$CD8^+$TL activity by SART-1_{690}[a]	Cellular response[b]		Infiltration of $CD3^+$ T cell[c]		Differentiation[d]	
	IFN-γ (pg/ml)						
1	271	1		+++		well	
2	783	1		+++		well	
3	279	1		+++		well	
4	185	1		+++		well	
5	238	3		+		moderate	
18	713	2		++		well	
19	751	1		+++		well	
6	<0	2		++		well	
7	<0	2		++		well	
8	51	2		++		well	
9	11	2		++		well	
11	19	2	$P = 0.027$[e]	++	$P = 0.041$[e]	well	NS[e]
12	<0	2		++		well	
13	29	2		++		well	
14	<0	1		+++		well	
15	<0	2		++		well	
16	17	2		++		well	
17	<0	3		+		moderate	
21	<0	2		++		moderate	
22	13	2		++		moderate	
23	29	2		++		well	
24	<0	2		++		well	
25	8	2		++		well	

NS: not significant.
+++: >75% reactive cells (strong).
++: 74–50% reactive cells (moderate).
+: 49–25% reactive cells (weak).
−: <25% reactive cells (negative).
[a] $CD8^+$TL activity was measured as described in Fig. 1.
[b] The histologic extents of the cellular responses were determined according to the criteria of Willen et al. [22].
[c] Infiltration of $CD3^+$ T cells was estimated with three cut-off points, as described in Section 2.
[d] The histologic grades of differentiation were determined according to the criteria of the WHO [21].
[e] The statistical significance of the association was determined by Fisher's exact probability test.

study shows that the frequency of $CD8^+$TL induction by each peptide is not so high. Therefore, it seems reasonable to use a combination of 3 or 4 peptides as a peptide vaccination for oral SCC or precancerous conditions. As surgery, radiotherapy, chemotherapy, or the combination therapy would be chosen as a first choice of SCC treatment, peptide-based specific immunotherapy for oral SCC should be considered as a fourth modality, possibly as a sequential therapy. For patients who have a poor prognosis after tumor resection or other treatments, peptide immunotherapy might be used to prolong their life. Also, peptide immunotherapy should be considered as a treatment for precancerous conditions in the oral cavity such as leukoplakia and erythroplakia, as in patients at a very early stage of disease this treatment might help to prevent precancerous conditions from developing into oral SCC. Therefore, peptide-based specific immunotherapies may have applications as adjuvant modalities for oral SCC patients who have undergone surgery or who have precancerous lesions.

In conclusion, we have shown that peptide-specific $CD8^+$TLs in the PBMCs of patients with oral SCC respond to SART-1_{690}, SART-2_{93}, and $ART4_{75}$. Therefore, these peptides could be applicable as peptide-based specific immunotherapies for the majority of patients with oral SCC. However, our finding, that some of the patients mounted no significant $CD8^+$TL response to any of the peptides, presents an obstacle to the use of this type of immunotherapy.

Acknowledgment

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (Grant numbers 12470441, 13557179, and 15390618).

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Fukui et al. Virology Journal 2012, 9:199
http://www.virologyj.com/content/9/1/199

VIROLOGY JOURNAL

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Identification of B cell epitopes reactive to human papillomavirus type-16L1- derived peptides

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Abstract

Background: Persistent infection of human papillomavirus (HPV) types 16 and 18 causes cervical cancer. To better understand immune responses to the prophylactic vaccine, HPV 16/18 L1 virus-like particles (HPV-VLPs), we investigated B cell epitopes of HPV16 L1-derived peptides.

Methods: Sera from mice immunized with HPV-16/18 L1 VLPs were analyzed for their IgG titers against 10 different HPV16 L1-derived peptides (20-mer) that contain human leukocyte antigen (HLA)-class I A-2, A-24 and class II DR.

Results: One 20-mer peptide at positions 300 to 319 was identified as a common B cell epitope in both Balb/c (H-2^d) and C57BL/6 (H-2^b) mice. Mapping analysis showed that the 10-amino-acid sequence at positions 304to 313 was an immunogenic portion. It is of note that the binding capability of this 10-mer peptide to the HLA-A2 and HLA-A24 molecules was confirmed by the HLA class I stabilization assay. In addition, one unique 20-mer was determined as a B cell epitope in each strain.

Conclusions: These results might provide new information for better understanding of immune responses to HPV 16 L1.

Keywords: Human papillomavirus, Prophylactic vaccine, Anti-peptide antibody, Virus-like particles

Background

Cervical cancer is the second most prevalent cancer in women worldwide. HPV 16 is the most common type associated with cervical cancer [1]. HPV-16/18L1 virus-like particles (HPV-VLPs), which induce neutralizing antibody responses, have been used as a prophylactic vaccine with great success [2,3]. Although the preventive effect of the HPV-VLPs vaccine has been reported to last up to 7.3 years [4], the durability is unclear at the present time, either for the entire vaccinated population or for individuals. This hurdle could be in part overcome if predictable biomarkers were identified. One of the biomarkers could be based on the measurement of specific humoral immune responses to the vaccine. However, little information is presently available with regard to humoral responses against the HPV-VLPs, primarily because of the limited availability of the assay reagents [5,6]. The main aim of this study was to better understand humoral immune responses to HPV16 L1-derived peptides in an animal model.

Results

Detection of IgG antibodies in serum of Balb/c mice

Serum of the Balb/c mice was obtained before immunization, and 3, 5, 8, 11, 14 weeks after the first immunization. Each group consisted of 6 mice, and serum from each mouse was independently measured for IgG level. A 100-fold dilution of samples was used to determine the levels of IgG reactive to each of 10 different HPV16 L1-derived peptides (20-mer), and the results were given in fluorescent intensity units (FIU) (Figure 1). Representative results of a kinetic study showed that IgG against peptide 4 and peptide 6, but not any others, became detectable at 3 weeks and reached a maximum at 5 weeks ($P<0.05$) followed by decline thereafter until 11 weeks. IgG levels were somewhat increased again at 14 weeks, which might be in part a reflection of the

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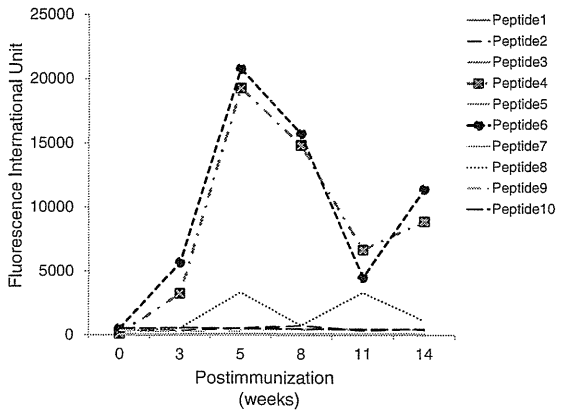

Figure 1 The kinetics of antibody production in Balb/c mice. Serum (100-fold dilution) of each of 6 Balb/c mice was obtained before immunization, and 3, 5, 8, 11, 14 weeks after the first immunization followed by the measurement. The representative results are given in fluorescent intensity units (FIU). IgG levels against peptide 4 and peptide 6, but not any others, became detectable at 3 weeks and reached a maximum at 5 weeks, followed by a decline thereafter until 11 weeks ($^{*}P<0.05$; 0 vs. 5 weeks). This analysis was done using Friedman's test.

second immunization at 3 weeks after the first injection. A dim level of IgG against peptide 8 was detectable at 8 and 11 weeks, but the levels were not significant.

Detection of IgG antibodies in serum of C57BL/6 N (H-2^b) mice

We then measured IgG levels in serum from the vaccinated C57BL/6N (H-2^b) mice before and 5 weeks after the immunization to address any difference between Balb/c and C57BL/6N mice. As a result, IgG against both peptide 6 and peptide 8, but not any others, were detected at 5 weeks ($P < 0.05$) (Figure 2).

Epitope mapping of peptide

These results indicated that peptide 6 is the major common B cell epitope shared by the two strains. Subsequently, epitope mapping of peptide 6 was conducted with eight different 10-mers (Figure 3). Five amino acids sequences from the HPV16 L1-derived sequence at position 295 to 299 were added to the N-terminal of the first 10-mer, and those at position 320 to 324 were also added to the C-terminal of the eighth 10-mer. Each of these eight peptides shared seven amino acids sequences with each other. As a result, one 10-mer at amino acid positions of 304–313 of HPV16 L1, aqifnkpywl, was determined to be an immunogenic portion using the antibodies (Figure 3). Then, we addressed the reactivity of the 9-mer peptide (qifnkpywl) at position 305 to 313, which had binding motifs to HLA-A2 and HLA-A24, to immunized sera. A modest level of reactivity to this 9-mer was observed (Figure 3).

Binding capability of the immunogenic B cell epitope to HLA class I molecules

The identified immunogenic B cell epitope contains binding motifs to the HLA- A2 and HLA-A24 molecules. Therefore, we examined whether they actually bind to the HLA-A2- and HLA-A24 molecules by the HLA class I stabilization assay with TAP-deficient cell lines RMA-S/A2 and RMA-S/A24. As illustrated in Table 1, both of the 9-mer (qifnkpywl) and 10-mer (aqifnkpywl) peptides showed substantial binding capability to HLA-A2 and HLA-A24.

Discussion

We analyzed immune responses to the 10 different HPV-VLP L1-derived peptides (20-mers) that had binding motifs to both HLA-class I (A2 or A24) and HLA-class II (DR) in animal model. We used BALB/c and C57BL/6N mice that have been regarded as Th2- and Th1-skewed strains, respectively, and widely known to express different immune responses in normal and pathological states [7]. When we examined humoral immune responses to the 10 different HPV-VLP L1-derived peptides in the Th2-skewed BALB/c mice, the levels of IgG to the peptide 4 and peptide 6 were clearly elevated in the sera after immunization with HPV-VLPs. We also used the Th1-skewed C57BL/6N mice to examine whether the selection of mouse strains tested has considerable and variable impacts on humoral immune responses to HPV-VLPs. In the C57BL/6N mice, one of the identified peptides , peptide 6 , but not another one (peptide 4), was also immunogenic, suggesting that the peptide 6 is a major common B cell epitope peptide in mice. Notably, our preliminary study has shown that the peptide 6 is also immunogenic in humans who are vaccinated with HPV-VLPs (data not shown), suggesting that the peptide 6 is a common immunogenic B cell epitope shared between mice and humans. A dim level of IgG against peptide 8 was detected in Balb/c mice; thus, peptide 8 might also contain a common epitopic portion. This issue remains to be studied further.

Mapping analysis of peptide 6 showed 10-mer (aqifnkpywl) at positions of 304 to 313 of HPV16 L1 as an immunogenic portion. In addition, the 9-mer peptide (qifnkpywl) at position 305 to 313 with binding motifs to HLA-A2 and -A24 molecules was also recognized by immunized sera. It is of note that both these 9-mer and 10-mer peptides showed substantial binding capability to HLA-A2 and HLA-A24 molecules, the two dominant HLA-class I A types among Japanese and other ethnics [8]. The peptide 6 at positions 300 to 319 of HPV16 L1 also contains binding motifs to HLA-class-II DR 1, 3, and 4 molecules, the dominant molecules expressed in Japanese and other ethnics [9]. Therefore, the peptide 6 and its fragment might be one of the appropriate

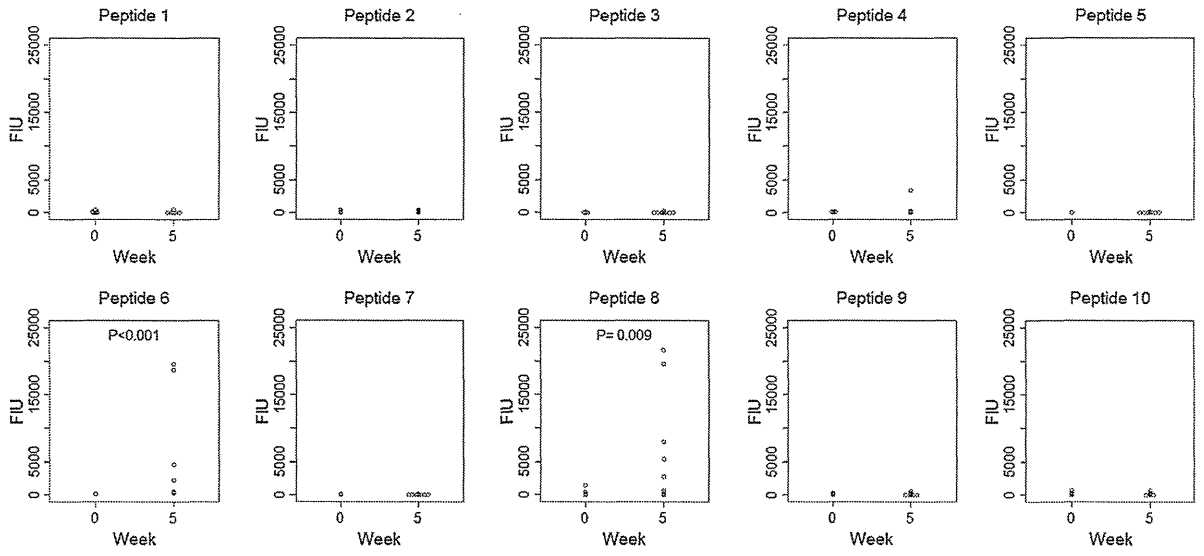

Figure 2 Antibody production in C57BL/6N mice. Serum from C57BL/6N mice was obtained before (n = 9)and 5 weeks after the first immunization (n = 9), followed by the measurement. Each of fluorescent intensity units (FIU) from the 9 individual mice was shown. IgG against both peptide 6 and peptide 8, but not any others, was detected at 5 weeks after the immunization. Only the *P* values that were statistically significant($P < 0.05$)are shown. Wilcoxon rank sum test was used for this analysis.

candidate antigenic sequences for monitoring immune responses to HPV-VLP after vaccination both in animal model and humans. This hypothesis is now under investigation in humans.

Whether the vaccine-induced IgG to the peptide 6 or 8 possesses biological activity to either neutralize HPV infection or facilitate the prophylactic effect of the HPV 16/18L1 vaccine needs to be studied in near future. In addition, since we have known that the same epitopes are often recognized by both B cells and T cells [10], T cell responses to the peptide 6 and its fragment may also be of great interest.

Conclusions

In summary, one 20-mer peptide at positions 300 to 319 was identified as a common B cell epitope in both Balb/c (H-2^d) and C57BL/6N (H-2^b) mice. These results

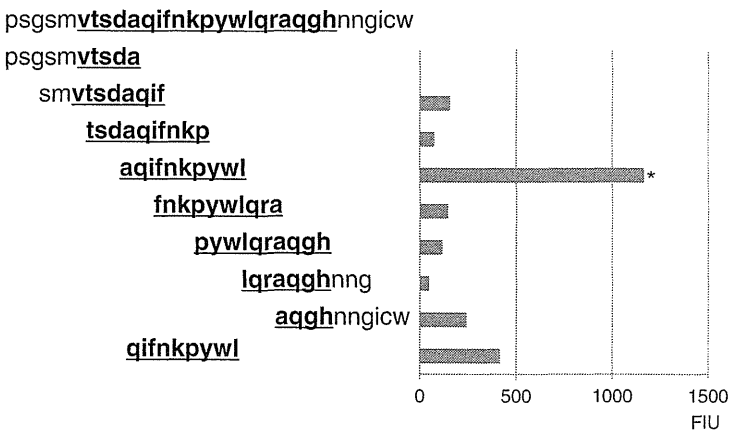

Figure 3 Epitope mapping of peptide 6 with eight different 10-mer peptides. Five amino acids sequences from the HPV16 L1-derived sequence at position 295 to 299 were added to the N-terminal of the first 10-mer, and those at position 320 to 324 were added to the C-terminal of the eighth 10-mer (left side of the figure). Each of these eight peptides shares seven amino acids sequences with each other. We also addressed the reactivity to immunized sera of the 9-mer peptide (qifnkpywl) at positions 305 to 313, which had binding motifs to HLA-A2 and HLA-A24. Representative results are given in the right side of the figure. The highest reactivity to one 10-mer (aqifnkpywl at position 304 to 313) is shown ($^*P < 0.05$ to any others tested). Wilcoxon rank sum test was used for this analysis.

Table 1 HLA-binding capability of the B cell epitopse derived from HPV16 L1

Peptide	Sequence	Binding capability (%)*	
		HLA-A2	HLA-A24
9-mer	QIFNKPYWL	66.8	40.3
10-mer	AQIFNKPYWL	74.9	51.7
Flu M1	GILGFVFTL	116.7	ND
EBV	TYGPVFMCL	ND	170.6
K-Ras	KLVVVG AGGV	3.3	3.8

*HLA-A2 or HLA-A24-binding capability was estimated by increase in mean fluorescence intensity (MFI), determined by flowcytometry after staining of RMA-S/A2 or RMA-S/A24 cells with anti-HLA-A2 or anti-HLA-A24 mAb, as follows; MFI increase (%) = (MFI with a given peptide - MFI without peptide)/(MFI without peptide) X 100. As positive controls, an HLA-A2-binding peptide derived from influenza virus M1 (Flu M1) or an HLA-A24-binding peptide derived from Epstein-Barr virus LMP2 (EBV) was used. As a negative control, a peptide derived from oncogene K-ras was used. ND, not determined.

might provide new information for better understanding of immune responses to HPV 16L1.

Materials and methods

Immunization of mice

Female Balb/c (H-2^d) and C57BL/6N (H-2^b) mice used in this study were 6 weeks of age and were maintained in a pathogen-free environment. Balb/c mice (H-2^d) were mainly provided for the study because they have been regarded as a Th2- skewed strain [7] and the binding motifs of the peptides to the H-2^d class I-A molecules were similar to those of human leukocyte antigen (HLA) class I-A2402, which are expressed in 60% of Japanese [11]. We administered the bivalent HPV-16/18 virus-like particle AS04 vaccine, containing 2 μg each of HPV-16 and HPV-18L1 VLPs with AS04 adjuvant that contained 50 μg aluminum hydroxide and 5 μg 3-deacylated monophosphoryl lipid A. Mice were immunized twice (day 1 and day 21) into the muscle at the thigh with HPV16/18 VLP AS04 vaccine based on the report by Didierlaurent et al. [12].

All experiments with live animals were performed under the institutional guidelines of the animal experiment in Kurume University after the approval by the Committee of Animal Experiment in Kurume University (Approval Number: 2010-107-1).

Collection of serum samples

The blood of each mouse was collected from the orbital sinus. Blood collection was performed before vaccination, and 3, 5, 8, 11, and 14 weeks after the first immunization in Balb/C (n = 6 in each group). In C57BL/6 mice, blood was collected before (n = 9) and 5 weeks after the first immunization (n = 9). Collected blood was allowed to coagulate by keeping it at room temperature for 1 h followed by centrifugation and collection. Serum from each mouse was sealed and stored at −80°C until use. Measurement of IgG titers was conducted simultaneously to avoid possible *in vitro* biases.

Peptides

Ten different HPV16 L1-derived peptides (20-mer) with binding motifs to both HLA-class I (A2 or A24) and HLA-class II (DR) were selected by the web software (MULTIPRED) (Table 2). This choice was based on consideration of future applications to the human immune system. For epitope mapping, 8 different 10-mer and one 9-mer peptides were selected from the 20-mer peptide 6. These peptides were purchased from Greiner Bio-One (Thermo Fisher Scientific, Ulm, Germany). Each peptide was dissolved in dimethyl sulfoxide (DMSO), stored at −80°C.

Preparation of xMAP beads

The xMAP carboxylate beads and Luminex system platform were obtained from Luminex Corp. (Austin, TX) as

Table 2 HPV16 L1-derived peptides used in this study and their binding motifs to HLA-A2 and -A24

	DR		A2			4A2		
	position	Amino acid sequence	position	Amino acid sequence	Score	position	Amino acid sequence	Score
Peptide 1	54-73	KPNNNKILVPKVSGLQYRVF	60-68	ILVPKVSGL	30	59-68	KILVPKVSGL	14
Peptide 2	392-422	HSMNSTILEDWNFGLQPPPGG	398-406	ILEDWNFGL	23	397-406	TILEDWNFGL	16
Peptide 3	62-81	VPKVSGLQYRVFRIHLPDPN	67-75	GLQYRVFRI	22	66-75	SGLQYRVFRI	24
Peptide 4	112-131	PLGVGISGHPLLNKLDDTEN	118-126	SGHPLLNKL	22	117-126	ISGHPLLNKL	12
Peptide 5	243-262	GDSLFFYLRREQMFVRHLFN	249-257	YLRREQMFV	22	248-257	FYLRREQMFV	12
Peptide 6	300-319	VTSDAQIFNKPYWLQRAQGH	305-313	QIFNKPYWL	21	305-313	QIFNKPYWL	12
Peptide 7	144-162	RECISMDYKQTQLCLIGCK	148-156	SMDYKQTQL	20	148-156	SMDYKQTQL	11
Peptide 8	293-312	PTPSGSMVTSDAQIFNKPYW	298-306	SMVTSDAQI	20	298-306	SMVTSDAQI	10
Peptide 9	384-403	TADVMTYIHSMNSTILEDWN	390-399	YIHSMNSTIL	20	389-398	TYIHSMNSTI	23
Peptide 10	152-171	KQTQLCLIGCKPPIGEHWG	157-165	CLIGCKPPI	23	156-165	LCLIGCKPPI	12

Anchor residues for HLA class I are shown in boldface.

reported previously [13]. The 96-well filter plates (MABVN12) and vacuum manifold apparatus (MAVM 09601) were from Millipore Corp. (Bedford, MA). Biotinylated goat anti mouse IgG (gamma chain-specific) (SouthernBiotech, AL) was purchased from Vector Laboratories Inc. (Burlingame, CA). Streptavidin-PE (S-866) was purchased from Molecular Probes (Eugene, OR). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, 22980) was obtained from PIERCE (Rockford, IL). Peptides were coupled to xMAP beads according to the modified manufacturer's instructions as reported previously [13]. In brief, 100 μ of xMAP beads were washed with 0.1 M MES buffer, pH 7.0, followed by mixing with 100 μl of peptide (1 mg/ml in 0.1 M MES buffer, pH 7.0). The peptide-loaded beads were incubated with EDC (1 mg/ml) at room temperature for 30 min in darkness, and then incubated twice more under the same conditions, after which the beads were washed with 0.05% Tween 20-PBS. Finally, the beads were treated with 2-aminoethanol for 15 min at room temperature in darkness, then washed twice and re-suspended with 1 ml of 0.05% NaN_3 in Block-Ace.

Anti-peptide antibody measurement by multiplexed bead-based Luminex assay

Blood samples were obtained from each of the mice at each scheduled point. Peptide-specific IgG levels in serum were measured by flowmetry assay using the Luminex system as reported previously [13]. In brief, serum was incubated with 100 μl of the peptide-coded beads for 1.5 hours at room temperature in a 96-well filter plate on a plate shaker. After incubation, the plate was washed using a vacuum manifold apparatus and incubated with 100 μl of biotinylated goat anti mouse IgG (gamma chain-specific) for 1 hour at room temperature on a plate shaker. The plate was then washed, 100 μl of streptavidin-PE was added to the wells, and the plate was incubated for 40 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μl of Tween 20-PBS into each well, and the plate was placed for 3 min on a plate shaker.

HLA class I stabilization assay

The actual binding of the peptides to HLA-A2 or HLA-A24 molecules was evaluated by MHC class I stabilization assay with the TAP2-deficient RMA-S cells stably transfected with the HLA-A*0201 gene (RMA-S/A2) or with the HLA-A*2402/K^b gene (RMA-S/A24), according to a previously reported method with several modifications [14]. Briefly, RMA-S/A2 or RMA-S/A24 cells ($5x10^5$ cells per well in a 24-well plate) were cultured for 18 hours at 26°C in 1 ml of RPMI 1640 medium (Invitrogen Inc, Carlsbad, CA) containing 10% FBS (MP Biologicals, Solon, OH) in the presence of synthetic peptides (25 μg/ml) and β2-microglobulin (2 μg/ml; Fitzgerald Industries International, Acton, MA). After washing, the cells were cultured for 3 hours at 37°C, and then stained with anti-HLA-A2 mAb (BB7.2; BD Bioscience, San Jose, CA) or anti-HLA-A24 mAb (One Lambda, Inc. Canoga Park, CA), followed by incubation with PE-conjugated rabbit anti-mouse IgG Ab (MP Biomedicals, Solon, OH). After washing, the cells were suspended with 1 ml of PBS containing 1% formaldehyde, and analyzed with FACSCanto (BD Bioscience). The binding capability of each peptide to HLA-A2 or HLA-A24 molecules was evaluated by the increase in mean fluorescence intensity (MFI) assessed by flow cytometry, as follows: MFI increase (%) = (MFI with a given peptide – MFI without peptide)/(MFI without peptide) X 100. As positive controls, an HLA-A2-binding peptide derived from influenza virus M1 (Flu M1, GILGFVFTL) or an HLA-A24-binding peptide derived from Epstein-Barr virus LMP2 (EBV, TYGPVFMCL) was used. As a negative control, a peptide derived from oncogene K-ras (KLVVVG AGGV) was used.

Statistics

The statistical significance of the data was determined using Friedman`s test and the Wilcoxon rank sum test. P-values less than 0.05 were considered statistically significant.

Abbreviations

HPV: Human papillomavirus; VLPs: Virus-like particles; HLA: Human leukocyte antigen; FIU: Fluorescent intensity units.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

A.F. performed most of the experiments and involved in manuscript preparation. T.S. and N.K. coordinated laboratory manipulation. KK proposed the hypothesis of this study edited the manuscript. K.K., N.T., K.U., K.I. and T.K. designed this study. S.S. and A.F. designed the peptides analyzed. A.F., S.M. gave vaccination to mice and obtained blood sample, A.F., S.M., N.K. and T.S. measured the antibody. A.F., T.S., S. H., and K.I. analyzed the data. KI and TK are the project leaders and were involved in project design, manipulation, data analysis and finalization of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported in part by the Supporting Fund of Obstetrics and Gynecology, Kurume University, by the grant from Regional Innovation Cluster Program of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the the grant from Sendai-Kousei Hospital.

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Received: 22 February 2012 Accepted: 11 September 2012
Published: 14 September 2012

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doi:10.1186/1743-422X-9-199
Cite this article as: Fukui *et al.*: Identification of B cell epitopes reactive to human papillomavirus type-16L1- derived peptides. *Virology Journal* 2012 9:199.

Cancer Science
The official journal of the Japanese Cancer Association

Prostate Cancer Chemoprevention Study: An investigative randomized control study using purified isoflavones in men with rising prostate-specific antigen

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(Received August 7, 2011/Revised September 25, 2011/Accepted September 28, 2011/Accepted manuscript online October 11, 2011/Article first published online November 10, 2011)

Our previous case–control study suggested that equol, a metabolite of isoflavone, has a preventive effect on prostate cancer. To examine the prostate cancer risk based on isoflavone intake and equol production, we carried out a phase II, randomized, double-blind, placebo-controlled trial of oral isoflavone (60 mg/day) for 12 months. The inclusion criteria were Japanese men between 50 and 75 years of age, a serum prostate-specific antigen level of 2.5–10.0 ng/mL, and a single, negative prostate biopsy within 12 months prior to enrollment. The study included 158 men in eight Japanese centers. Their median age was 66.0 years, and the numbers of equol producers and non-producers were 76 (48%) and 82 (52%), respectively. The majority of adverse events were mild or moderate in severity, and the scheduled intake of tablets was completed by 153 patients (96.8%). The prostate-specific antigen value showed no significant difference before and after treatment. Of the 89 patients evaluated by central pathological review, the incidence of biopsy-detectable prostate cancer in the isoflavone and placebo groups showed no significant difference (21.4% *vs* 34.0%, $P = 0.140$). However, for the 53 patients aged 65 years or more, the incidence of cancer in the isoflavone group was significantly lower than that in the placebo group (28.0% *vs* 57.1%, $P = 0.031$). These results support the value of isoflavone for prostate cancer risk reduction. A large-scale phase III randomized study of isoflavone tablets in men with different hereditary factors and living environments is warranted. Registered with the UMIN Clinical Trials Registry (UMIN-CTR) for clinical trials in Japan (C000000446). (*Cancer Sci* 2012; 103: 125–130)

Prostate cancer is the second most common cancer in men and the third most common cause of male cancer death worldwide.[1] The incidence of clinical cancer in Japan is low, however, the incidence of total clinical and latent prostate cancer is the same between Japanese and American populations.[2] Diet is thought to play an important role in the progression from microscopic to clinical cancer.[1,3] Fat and calcium have been reported to be risk factors for prostate cancer. Conversely, lycopene, selenium, soy isoflavone, and vitamin E were reported to be preventive factors.[4–6] However, the SELECT (Selenium and Vitamin E Cancer Prevention Trial) study,[7] a recent large-scale, double-blind study, was unable to show a preventive effect for selenium or vitamin E on prostate cancer.

Basic research, including epidemiologic studies, suggested that soy isoflavone exerts an anticarcinogenic effect on prostate cancer.[8,9] Our case–control study of the serum isoflavone levels in patients with prostate cancer and healthy volunteers[8] found that some individuals were able to degrade daidzein into equol (equol producers) whereas others were not (non-producers).

In another case–control study involving residents in Japan, Korea, and the USA,[9] we found that the percentage of equol producers in patients with prostate cancer was significantly lower than in the healthy controls (30.3% *vs* 49.5%; $P = 0.013$). The percentage of equol producers in patients and controls was 29% and 46% in Japan ($P = 0.004$) and 30% and 59% in Korea ($P = 0.001$), respectively. The serum isoflavone level was markedly lower and the percentage of equol producers was also lower (17% for patients and 14% for controls) for Americans as compared to the Japanese and Koreans.

In another study,[10] we carried out an age-stratified dietary survey of soybean food consumption and measured the serum isoflavone levels in healthy Japanese and Korean men. The daily intake of genistein and daidzein in the teenage group was significantly lower than in the age group ≥30 years ($P < 0.05$). In the Japanese cohort, the proportion of equol producers in the teenage group was only 10%, which was significantly the lowest among all age-strata. Those results suggest that equol or equol-producing ability may be deeply involved in prostate cancer risk, and decreased intake of isoflavones in the young generation may lead to an increase in the prostate cancer incidence in Japan and Korea.

Recently, we clarified the mechanism of biodegradation from daidzein into equol by two kinds of intestinal bacteria.[11] As a strategy for chemoprevention of prostate cancer, clinical intervention by changing equol non-producers to producers, as well as by ingesting equol-containing supplements, are anticipated.

Considering this background, we carried out a phase II, randomized, double-blind, placebo-controlled trial of oral isoflavone (60 mg/day) for 12 months.

Patients and Methods

Patients. This was a phase II, randomized, double-blind, placebo-controlled trial of isoflavone (60 mg/day), given orally for

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doi: 10.1111/j.1349-7006.2011.02120.x

12 months. The inclusion criteria were Japanese men between 50 and 75 years of age, a serum PSA level of 2.5–10.0 ng/mL (50–60 years) or 3.0–10.0 ng/mL (>60 years), and a single, negative prostate biopsy (6–12 cores) within 12 months prior to enrollment. Men with HG-PIN or ASAP in the baseline biopsy, or a history of prostate cancer, were excluded from the study. None of the patients were using a steroidal or non-steroidal antiandrogen. The protocol was approved by the Institutional Review Board of each study site. The study was carried out in accordance with the Helsinki Declaration, and all participants signed informed consent forms.

Study design. The ingredients in the soy isoflavone tablet are shown in Table 1. Eligible patients were randomized to receive 10 isoflavone tablets (isoflavone 6 mg/tablet, totally 60 mg/day) or 10 placebo tablets for 12 months. Six-core transrectal ultrasound-guided biopsies were planned to be carried out at 12 months. The investigator was allowed to carry out "protocol-independent" (for-cause) biopsies whenever deemed clinically necessary. Protocol-mandated biopsies and for-cause biopsies were to be submitted for confirmation by central pathological review (University of Kyushu, Fukuoka, Japan). However, central pathological review was not the essential requirement for registration. No biopsy samples were collected at baseline. The negative biopsy prior to enrolment was confirmed by local pathological review only.

Because this study was planned as a pilot phase II study of the large-scale clinical trial, the primary endpoints were the tolerability of the soy isoflavone tablet and the changes in PSA and sex hormones including testosterone, DHT, SHBG, and estradiol. The secondary endpoints were the incidences of biopsy-detectable prostate cancer, HG-PIN, and ASAP at 12 months.

Measurement of serum isoflavone levels. Blood samples were drawn at 0, 3, and 12 months (serum isoflavones and sex hormones) or 0, 3, 6, 9, and 12 months (PSA). They were drawn before breakfast, and the sera were separated and stored at −10°C or less. The frozen samples were transported to the laboratory of SRL (Tokyo, Japan). The details of the measurement of serum isoflavone levels have been described elsewhere.[8] The assayed isoflavones were genistein, daidzein, and equol. Equol producers were defined as having a baseline serum equol concentration above the lower limit of detection of the present assay system, that is, 0.5 ng/mL.[8]

Statistical analyses. The efficacy analysis population consisted of patients who ingested the study medication for 12 months, in accordance with the study protocol. The safety analysis population included all randomized patients.

Table 1. Ingredients of soy isoflavone tablet given to study participants for 12 months

Component	mg/10 tablets (%)
Daidzin	19.1 (31.9)
Genistin	3.5 (5.8)
Glycitin	10.4 (17.3)
Malonyl daidzin	8.1 (13.5)
Malonyl genistin	2.2 (3.7)
Malonyl glycitin	3.4 (5.7)
Acetyl daidzin	7.3 (12.2)
Acetyl genistin	1.9 (3.2)
Acetyl glycitin	3.6 (6.0)
Daidzein	0.2 (0.3)
Genistein	0.1 (0.1)
Glycitein	0.2 (0.3)
Total	60.0 (100.0)

Ten isoflavone tablets (6 mg/tablet, totally 60 mg/day) are divided twice.

Statistical analyses were carried out using Wilcoxon's test (non-parametric), the chi-square-test and Fisher's exact test. For the individual changes of laboratory tests we used the paired *t*-test. A *P*-value of <0.05 was defined as representing a statistically significant difference. Data were analyzed using SAS version 8 software (SAS Institute, Cary, NC, USA).

Results

Baseline patient characteristics. The study enrolled 158 men in eight Japanese centers. Their median age at the first blood collection was 66.0 years (range, 50–75 years). The number of equol producers and non-producers was 76 (48%) and 82 (52%), respectively. These patients were randomized into an isoflavone group (n = 78) and a placebo group (n = 80).

Table 2 shows data on the baseline patient characteristics. These baseline characteristics, age, family history of prostate cancer, total PSA, prostate volume, and equol production, were generally similar in each treatment group to those of the overall study population.

Safety and tolerability. The planned intake of tablets was completed in 153 of 158 patients (96.8%). Of the five patients who did not complete the treatment course, three decided for themselves to quit taking the tablets. The other two patients had grade 3 adverse events: one in the isoflavone group suffered iliac artery stenosis, and the other in the placebo group suffered ileus. However, the majority of adverse events were mild in severity. The completion rates in the isoflavone and placebo group were 96.2% (75/78) and 97.5% (78/80), respectively. No significant changes in laboratory data were observed during the study (data not shown).

Serum isoflavone levels. Figures 1 and 2 show the median serum levels of daidzein and equol, stratified by treatment and baseline equol production. Daidzein was significantly increased in the isoflavone groups, and its level was lower in the isoflavone/producer group compared with the isoflavone/non-producer group (Fig. 1).

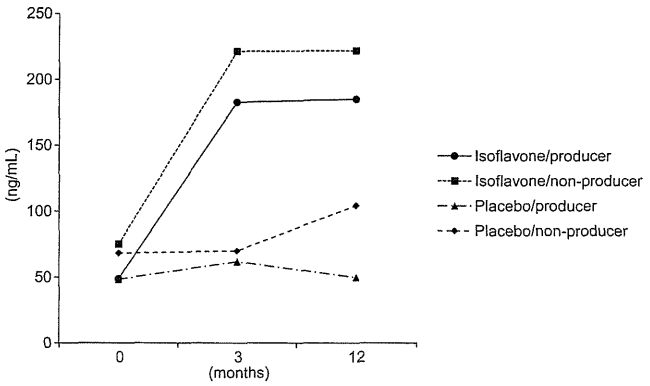
In Figure 2, equol producers who received isoflavone greatly increased equol production and showed the highest equol level. Equol producers given the placebo showed no change in the serum equol level from before treatment. The two groups of non-producers, given either isoflavone or placebo, showed the lowest equol levels.

Table 2. Baseline patient characteristics

	Total (n = 158)	Isoflavone (n = 78)	Placebo (n = 80)	*P*-value
Age (years)				
Median	66.0	66.5	65.0	0.974†
Range	50.0–75.0	52.0–75.0	50.0–75.0	
Family history, no. (%)	3 (2)	1 (1)	2 (3)	0.873‡
Total PSA (ng/mL)				
Median	5.75	5.83	5.73	0.784†
Range	2.76–10.20	2.76–9.77	3.00–10.20	
Prostate volume (mL)				
Median	37.7	37.6	37.6	0.349†
Range	0.5–93.5	12.3–93.5	0.5–84.0	
Equol production, no. (%)				
Producer	76 (48)	38 (49)	38 (48)	0.502‡
Non-producer	82 (52)	40 (51)	42 (53)	

Non-producers, individuals unable to degrade daidzein into equol; producers, individuals able to degrade daidzein into equol.
†Wilcoxon's test; ‡Fisher's exact test.

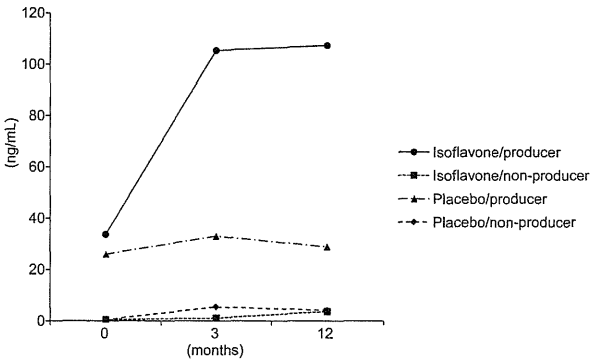
doi: 10.1111/j.1349-7006.2011.02120.x



	0 month	3 months	12 months	*P*-value†
Isoflavone/producer	48.69	182.68	185.04	<0.0001*
Isoflavone/non-producer	75.08	221.27	221.72	0.0005*
Placebo/producer	48.37	61.66	49.84	0.4140
Placebo/non-producer	68.27	69.78	104.20	0.1240

† Paired *t* test (0 month *vs.* 12 months)
* $P < 0.05$

Fig. 1. Median serum levels of daidzein in Japanese men given oral isoflavone (60 mg/day) or placebo for 12 months (n = 158). Daidzein was significantly increased in the isoflavone groups, and its level was lower in individuals who could also degrade daidzein into equol (isoflavone/producer group) compared with those who could not (isoflavone/non-producer group).



	0 month	3 months	12 months	*P*-value†
Isoflavone/producer	33.68	105.35	107.34	0.0007*
Isoflavone/non-producer	0.50	1.12	3.70	0.0830
Placebo/producer	25.97	32.99	28.88	0.6080
Placebo/non-producer	0.50	5.39	4.13	0.1270

† Paired *t* test (0 month *vs.* 12 months)
* $P < 0.05$

Fig. 2. Median serum levels of equol in Japanese men given oral isoflavone (60 mg/day) or placebo for 12 months (n = 158). Individuals who could degrade daidzein into equol (equol producers), who received isoflavone, greatly increased equol production and showed the highest equol level. Equol producers given the placebo showed no change in the serum equol level from before treatment. The two groups of non-producers (those unable to degrade daidzein into equol), treated with either isoflavone or placebo, showed the lowest equol levels.

Prostate-specific antigen and sex hormones. The serum PSA value showed no significant change during the study (Fig. 3). There were also no differences among the treatment groups.

The interval changes in testosterone, DHT, and SHBG were not significant. In the non-producer groups, estradiol decreased significantly, independent of treatment (Table 3).

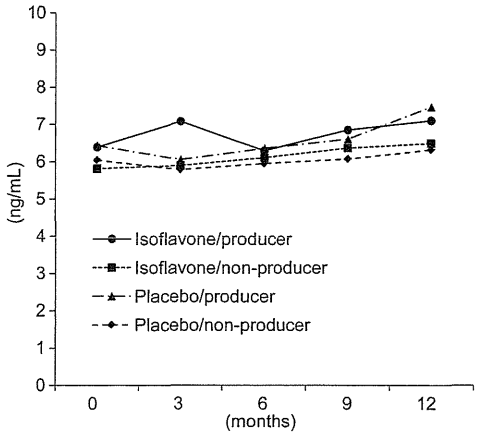
Pathologic endpoints. Of the 153 patients who completed the planned intake of tablets, 121 underwent needle biopsy of the prostate at 12 months. The remaining 32 patients did not agree to undergo the needle biopsy.

Of the 121 patients, 89, consisting of 42 in the isoflavone group and 47 in the placebo group, were evaluated by central pathological review, and 112 patients, consisting of 55 in the isoflavone group and 57 in the placebo group, were evaluated by local pathological review (Table 4). The specimens for 23 of the 112 patients were not approved to be sent to the central pathology laboratory by the Institutional Review Board. Thus, 89 were evaluated by both central and local pathological reviews. There were no significant differences between the results of central pathology and local pathology (data not shown). The following discussion focuses on the central pathology results.

The incidence of biopsy-detectable prostate cancer in the isoflavone and placebo groups was 21.4% (9/42) and 34.0% (16/47), respectively. These incidences of cancer detection were not statistically significantly different. However, for the patient stratum aged 65 years or more, the incidence of cancer in the isoflavone group was significantly lower than that in the placebo group (28.0% [7/25] *vs* 57.1% [16/28], P = 0.031).

The incidence of a Gleason score of 6 or less and HG-PIN were numerically lower in the isoflavone group compared with the placebo group, but the difference was not statistically significant.

Table 5 presents the central pathology results for prostate cancer incidence based on isoflavone intake and equol production. The incidence of prostate cancer in the isoflavone group was significantly lower than that in the placebo group for the



	0 month	3 months	6 months	9 months	12 months
Isoflavone/producer	6.39	7.08	6.29	6.85	7.10
Isoflavone/non-producer	5.81	5.89	6.10	6.37	6.49
Placebo/producer	6.43	6.05	6.36	6.60	7.46
Placebo/non-producer	6.04	5.79	5.95	6.07	6.32

Fig. 3. Serum prostate-specific antigen (PSA) levels in Japanese men given oral isoflavone (60 mg/day) or placebo for 12 months (n = 158). The PSA value showed no significant change during the study. There were also no differences among the treatment groups. Non-producers, individuals unable to degrade daidzein into equol; producers, individuals able to degrade daidzein into equol.

Table 3. Changes in hormone levels in Japanese men given oral isoflavone (60 mg/day) or placebo for 12 months (n = 158)

	0 month (pre)	3 months	12 months	P-value†
Testosterone				
Isoflavone	5.45	5.12	5.31	0.131
Producer	5.33	4.92	5.28	0.405
Non-producer	5.57	5.33	5.34	0.200
Placebo	5.12	5.36	5.07	0.286
Producer	5.25	5.22	5.19	0.508
Non-producer	5.01	5.48	4.99	0.411
DHT				
Isoflavone	0.94	0.95	1.01	0.533
Producer	0.92	0.89	1.00	0.708
Non-producer	0.96	1.01	1.02	0.619
Placebo	0.91	0.99	0.98	0.337
Producer	1.02	1.09	1.04	0.869
Non-producer	0.83	0.91	0.94	0.207
Estradiol				
Isoflavone	28.56	27.41	26.53	0.027*
Producer	27.44	26.39	26.29	0.416
Non-producer	29.59	28.46	26.76	0.023*
Placebo	27.20	26.65	25.27	0.0002*
Producer	26.71	26.97	25.70	0.122
Non-producer	27.61	26.36	24.94	0.007*
SHBG				
Isoflavone	52.00	55.43	55.97	0.064
Producer	52.76	52.70	57.00	0.261
Non-producer	51.31	58.17	54.95	0.138
Placebo	50.11	48.82	51.71	0.321
Producer	53.98	50.11	53.48	0.511
Non-producer	46.89	47.64	50.32	0.468

DHT, dihydrotestosterone; non-producers, individuals unable to degrade daidzein into equol; producers, individuals able to degrade daidzein into equol. SHBG, sex hormone binding protein. †Paired t-test (0 *vs* 12 M); *P < 0.05.

patient stratum aged 65 years or more and equol non-production.

Discussion

Because of the high incidence of microscopic prostate cancer and the long latency period from microscopic lesions to clinical disease, development of strategies for reducing the risk of prostate cancer is a reasonable and promising approach. Clinical research has been carried out regarding the preventive effect of isoflavones on prostate cancer, including dietary supplement intervention trials for prostate cancer patients.[12,13] In those studies, patients were given isoflavone-containing drugs and the serum PSA was examined as a surrogate marker. Kumar *et al.*[12] gave a soy isoflavone beverage for 12 weeks to men with prostate cancer on watchful waiting, randomizing 59 patients to a soy group and a placebo group. Serum total PSA decreased or was unchanged in 69% of the subjects in the isoflavone-treated group compared to 55% in the placebo group. Schröder *et al.*[13] gave an isoflavone-containing drug to men with prostate cancer with increasing PSA after primary treatment. Forty-two patients were examined in a placebo-controlled, double-blind, crossover study that involved 10 weeks of intervention, followed by a 4-week washout period prior to crossover. Although no statistically significant difference was found in either total (P = 0.076) or free (P = 0.988) PSA between the two groups, the free PSA doubling-time was significantly increased in the supplement group compared with the control group: 1150 *vs* 445 days (2.6-fold, P = 0.041). These studies establish the need to further explore the effects of prolonged and consistent soy consumption.

In the present study, pure isoflavone was administered to 158 patients over a comparatively long period of 12 months. In an interventional study, if some patients more aggressively consume foods containing isoflavones, such as tofu, miso, and natto, the influence on intervention was supposed. In the present study, however, the plasma isoflavone concentrations in the isoflavone groups were clearly higher than in the placebo control groups. Therefore, such influence to the results is able to be excluded.

The incidence of prostate cancer was lower in the isoflavone group, but not significantly. However, for the patient stratum aged 65 years or more, the incidence of cancer in the isoflavone group was significantly lower than that in the placebo group (28.0% *vs* 57.1%, P = 0.031). The reason why a significant difference was not shown in the total patient cohort might be related to the fact that the incidence of prostate cancer was small.

One of the primary endpoints, the effect on PSA level, was not proven. However, one of the secondary endpoints, the incidence of biopsy-detectable prostate cancer, was confirmed to have been significantly reduced in the group aged ≥65 years. Serum PSA is well-established as a biomarker of prostate cancer, but it is not specific to neoplasia, and the data do not suggest that the level is related directly to the extent of neoplastic progression.

The incidence of Gleason scores of 6 or less and HG-PIN were numerically lower in the isoflavone group compared with the placebo group, but not significantly. Based on this result, isoflavone may suppress small and low-grade cancers. This may be one of the reasons why an effect of isoflavone on the serum PSA level was unable to be proven.

We are also interested in prostate cancer risk reduction based on isoflavone administration and equol production. Unfortunately, because of the insufficient number of enrolled men, we were unable to analyze for a relationship between isoflavone intake and equol production.

Although intake of isoflavone suppressed the incidence of prostate cancer, the hormonal data did not show significant changes.

doi: 10.1111/j.1349-7006.2011.02120.x

Table 4. Numbers and proportions of men with prostate cancer and high-grade prostatic intraepithelial neoplasia (HG-PIN) who participated in this study (n = 158)

	Central pathology			Local pathology		
	Isoflavone (n = 42)	Placebo (n = 47)	P-value†	Isoflavone (n = 55)	Placebo (n = 57)	P-value†
No. of patients with tumors	9/42 (21.4%)	16/47 (34.0%)	0.140	8/55 (14.6%)	14/57 (24.6%)	0.137
Age (years)						
<64	2/17 (11.8%)	0/19 (0.0%)	0.220	2/27 (7.4%)	0/26 (0.0%)	0.255
≥65	7/25 (28.0%)	16/28 (57.1%)	0.031*	7/31 (22.6%)	14/31 (47.1%)	0.035*
Equol production						
Producer	5/22 (22.7%)	8/22 (36.4%)	0.255	5/29 (17.2%)	6/19 (24.0%)	0.390
Non-producer	4/20 (20.0%)	8/25 (32.0%)	0.288	3/26 (11.5%)	8/32 (25.0%)	0.168
No. of positive cores						
1	6	11	0.713	5	10	0.510
2–4	3	5	(1 *vs* 2–4)	3	4	(1 *vs* 2–4)
Gleason score						
5	0	0		0	1	
6	5	12		4	8	
7	3	4		3	2	
8	0	0		1	3	
9	1	0		0	0	
5–6	5/9 (55.6%)	12/16 (75.0%)	0.287	4/8 (50.0%)	9/14 (64.3%)	0.416
7–9	4/9 (44.4%)	4/16 (25.0%)	(G5–6 *vs* 7–9)	4/8 (50.0%)	5/14 (35.7%)	(G5–6 *vs* 7–9)
HG-PIN	2/42 (4.8%)	8/47 (17.0%)	0.660	NE	NE	NE

The Gleason score is the sum of the two most common histological patterns. NE, not evaluated; non-producers, individuals unable to degrade daidzein into equol; producers, individuals able to degrade daidzein into equol. †Fisher's exact test; *P < 0.05.

Table 5. Numbers and proportions of men with prostate cancer and high-grade prostatic intraepithelial neoplasia (HG-PIN), based on an isoflavone intake administration and equol production (central pathology)

	Equal producer			Equal non-producer		
	Isoflavone (n = 22)	Placebo (n = 22)	P-value†	Isoflavone (n = 20)	Placebo (n = 25)	P-value†
No. of patients with tumors	5/22 (22.7%)	8/22 (36.4%)	0.255	4/20 (20.0%)	8/25 (32.0%)	0.288
Age (years)						
<64	1/9 (11.1%)	0/6 (0.0%)	0.600	1/8 (12.5%)	0/13 (0.0%)	0.381
≥65	4/13 (30.8%)	8/16 (50.0%)	0.293	3/12 (25.0%)	8/12 (66.7%)	0.049*
No. of positive cores						
1	4	7	0.641	2	2	0.727
2–4	1	1	(1 *vs* 2–4)	4	4	(1 *vs* 2–4)
Gleason score						
6	2	6		3	6	
7	2	2		1	2	
8	0	0		0	0	
9	1	1		0	0	
5–6	2/5 (40.0%)	6/8 (75.0%)	0.250	3/4 (75.0%)	6/8 (75.0%)	0.764
7–9	3/5 (60.0%)	2/8 (25.0%)	(G6 *vs* 7–9)	1/4 (25.0%)	2/8 (25.0%)	(G6 *vs* 7–9)
HG-PIN	1/22 (4.6%)	4/22 (18.2%)	0.172	1/20 (5.0%)	4/25 (16.0%)	0.251

The Gleason score is the sum of the two most common histological patterns. †Fisher's exact test; *P < 0.05.

These results suggest that isoflavone exerts a cancer chemoprevention effect through an action other than hormonal. In published reports, effects such as apoptosis induction, tyrosine kinase inhibition, anti-angiogenic action, anti-oxygenation, and antipromotion have been reported for isoflavones based on *in vitro* and *in vivo* studies.[14,15] In the non-producer groups, estradiol decreased significantly, independent of treatment. We are not able to definitely interpret the phenomenon. Estrogen may decrease if the body weight tends to increase in non-producer groups. In this study, the change in body weight was not measured.

This study was carried out in Japanese patients. Isoflavone intake showed an effect on prostate cancer, even though Japanese ordinarily ingest considerable isoflavone in daily life. It can be expected that this effect would become even greater in Europe and America, who ordinarily ingest little isoflavone. A global study including Europe and America is recommended.

In the PCPT[16] and REDUCE[17] studies, which used 5AR inhibitors, the incidence of prostate cancer was reduced by 25%.[18] However, this treatment strategy is high in cost and leads to complications such as sexual dysfunction.[18] Therefore, its suitability can be considered to be limited to men at very high risk of prostate cancer. However, foods and supplements including isoflavones would be suitable for men in general, because of

their safety, low cost, and high feasibility. In addition, isoflavones are effective not only against prostate cancer but also cardiovascular diseases, osteoporosis, and hyperlipidemia.

The isoflavone tablets used in the present study showed no specific safety problems and were well tolerated, with 96.2% (75/78) of the patients in the isoflavone group completing the treatment regimen. Equol binds specifically with 5a-DHT and sequesters it from the androgen receptor.[19] The end result is similar to that achieved with 5AR inhibitors. We are able to obtain a similar effect to 5AR inhibitors, and safely, using isoflavone.

In conclusion, the incidence of prostate cancer in the isoflavone group was lower than that in the placebo group. In addition, the safety and tolerability of isoflavone intake were proven. These results support the value of isoflavone treatment for prostate cancer risk reduction. A large-scale phase III randomized study, preferably international, that takes into account different hereditary factors and living environments is warranted.

Acknowledgments

The authors wish to acknowledge the contribution of Dr Kentaro Kuroiwa (Department of Urology, Kyushu University, Fukuoka, Japan) for central pathology. This study was supported by the a grant-in-Aid for Scientific Research on Priority Area; Cancer A04, Ministry of Education, Science, Sports and Culture, Japan (Chief investigator; Hideyuki Akaza). The authors thank the cooperative researchers, Wataru Obara, Akira Yokomizo, Atsushi Mizokami, Daisaku Hirano, Kiyohide Fujimoto, Hisamitsu Ide, and Yuichiro Kurimura. We express our thanks to Fuji Oil Company (Osaka, Japan) for kindly providing the isoflavone tablets. [Correction added after online publication 2 December 2011: Acknowledgement added for Dr Kentaro Kuroiwa.]

Disclosure Statement

The authors have no conflicts of interest.

Abbreviations

5AR	5-α-reductase
ASAP	atypical small acinar proliferation
DHT	dihydrotestosterone
HG-PIN	high-grade prostatic intraepithelial neoplasia
PSA	prostate-specific antigen
SHBG	sex hormone binding protein

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doi: 10.1111/j.1349-7006.2011.02120.x

The Prostate 72:741–751 (2012)

Downregulation of Phosphodiesterase 4B (PDE4B) Activates Protein Kinase A and Contributes to the Progression of Prostate Cancer

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BACKGROUND. Prostate cancer is the most commonly diagnosed non-cutaneous cancer in American men. Unfortunately, few successful therapies for castration-resistant prostate cancer (CRPC) exist. The protein kinase A (PKA) pathway is a critical mediator of cellular proliferation and differentiation in various normal and cancerous cells. However, the PKA activity and the mechanism of regulation in CRPC remain unclear. Then, in this study, we intended to reveal the PKA activity and the mechanism of regulation in CRPC.

METHODS. Western blotting, quantitative real-time polymerase chain reaction, cytotoxicity analysis, and cell proliferation assay were used to resolve the regulatory role of PKA in prostate cancer cell line, LNCaP and their derivatives.

RESULTS. cAMP-specific phosphodiesterase 4B (PDE4B) was downregulated and the PKA pathway was activated in castration-resistant LNCaP derivatives (CxR cells). Rolipram activated the PKA pathway via inhibition of PDE4B, resulting in AR transactivation while the PKA inhibitor, H89 reduced AR transactivation. In response to hydrogen peroxide and in hydrogen peroxide-resistant LNCaP derivatives (HPR50 cells) PDE4B was decreased and as a result PKA activity was increased. Moreover, PDE4B expression was reduced in advanced prostate cancer and PDE4B knockdown promoted castration-resistant growth of LNCaP cells.

CONCLUSIONS. Oxidative stress may suppress PDE4B expression and activate the PKA pathway. The PDE4B/PKA pathway contributed to progression of androgen-dependent prostate cancer to CRPC. This pathway may represent an attractive therapeutic molecular target. *Prostate 72:741–751, 2012.* © 2011 Wiley Periodicals, Inc.

KEY WORDS: **PDE4B; PKA; CRPC; prostate cancer; oxidative stress**

INTRODUCTION

In its early stages, prostate cancer cell growth is androgen dependent. At this stage, androgen-deprivation therapies cause repression of prostate tumors. Unfortunately, the majority of prostate cancers eventually progress to the castration-resistant phenotype. In the treatment of castration-resistant prostate cancer (CRPC), docetaxel-based chemotherapy is often administered, but not curative [1]. Furthermore, the prognosis of patients after relapse of CRPC

Grant sponsor: The Ministry of Health, Labour and Welfare, Japan; Grant sponsor: The Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT), Japan; Grant number: KAKENHI 22591769; Grant sponsor: The Japanese Foundation for Prostate Research, Japan; Grant sponsor: The Fukuoka Foundation for Sound Health, Japan.

No potential conflicts of interest were disclosed.

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Received 16 May 2011; Accepted 29 July 2011
DOI 10.1002/pros.21478
Published online 30 August 2011 in Wiley Online Library (wileyonlinelibrary.com).

is extremely poor. Thus, novel treatment strategies for CRPC are gravely needed.

Androgen receptors (AR) play a vital role in both androgen-dependent and CRPC. In CRPC, AR are inappropriately activated under the condition of castration levels of androgens [2]. AR signaling is influenced by a web of complex signal cascades, such as signal transducers and activators of transcription (STAT), mitogen-activated protein kinase (MAPK), and Janus-activated protein kinase (JAK) [3–5]. One of the mechanisms of progression to CRPC is suspected to involve the protein kinase A (PKA) signaling pathway [6]. The PKA pathway is a critical mediator of cellular proliferation and differentiation in various normal and cancerous cells [7]. PKA overexpression has been reported to be associated with poor prognosis in several tumor types including colorectal, breast, and prostate cancers [8–10]. Inhibition of PKA prevents lung and prostate tumor cell proliferation and progression [11]. The relationship between PKA and AR is becoming elucidated by a number of recent investigations. For instance, G protein coupled receptors (GPCRs) activate stimulatory G proteins (Gs), which then stimulate PKA and activate AR [12]. Vasoactive intestinal peptide (VIP) can also transactivate AR via PKA [13]. Moreover, PKA signaling phosphorylates and activates cAMP responsible element-binding protein (CREB). Subsequently, CREB binds to a cAMP responsive element (CRE) site in the prostate-specific antigen (PSA) promoter, thereby increasing its transcriptional potential [3,14], and PKA modulates AR function by phoshorylation. Nevertheless, the actual activity of PKA and the mechanism modulating its activity in CRPC remain unclear.

By specifically hydrolyzing cAMP, a secondary messenger that activates the PKA pathway, cAMP-specific phosphodiesterase 4B (PDE4B) promotes prostate cancer proliferation [15]. The expression of PDE4B is abundantly observed in the fibromuscular stroma as well as in glandular structures of the transition zone [16]. Thus, we investigated PDE4B as a candidate of PKA modulation in prostate cancer.

In this report, we measured actual PKA activity in CRPC cells and revealed the mechanism of PKA activation. We also uncovered that oxidative stress is a key mediator in the activation of PKA pathways via the downregulation of PDE4B. We previously revealed that castration-induced oxidative stress caused castration resistance of prostate cancer through Twist1 and AR overexpression [17]. On the basis of the connection between oxidative stress and PKA activity, it was suggested that oxidative stress may have another critical influence on prostate cancer progression independent of Twist1.

MATERIALS AND METHODS

Cell Cultures

Human prostate cancer LNCaP cells were purchased from the American Type Cell Collection, cultured in RPMI1640 purchased from Invitrogen, and supplemented with 10% fetal bovine serum. LNCaP cells that propagated between 10 and 30 times were used. Castration-resistant derivatives of LNCaP cells (LNCaP-CxR cells, referred to as CxR cells) and hydrogen peroxide-resistant derivatives of LNCaP cells (LNCaP-HPR50 cells, referred to as HPR50 cells) were established and maintained, as previously described [17]. All cell lines were maintained in a 5% CO^2 atmosphere at 37°C.

Antibodies and Drugs

Antibody against AR (sc-815) was purchased from Santa Cruz Biotechnology. Anti-CREB (#9197) and anti-phosphoCREB (ser133, #9198) antibodies were purchased from Cell Signaling Technology. Anti β-actin and anti-PSA antibodies were purchased from Sigma and Epitomics, Inc., respectively. H89 was purchased from LC Laboratories, while rolipram was purchased from Sigma.

Western Blot Analysis

Western blot analysis was performed as described previously [18]. To prepare whole cell lysates, cells were sonicated for 20 sec with buffer-Y. Whole-cell lysates (30 μg) were separated by SDS–PAGE and transferred onto PVDF membranes. Western blot analysis was carried out using appropriately diluted antibodies, while the membrane was developed using a chemiluminescence protocol. Images were obtained using an image analyzer (LAS-3000 mini; Fujifilm).

Knockdown Analysis Using siRNAs

Knockdown analysis using siRNAs was performed as described previously [18]. siGENOME's human PDE4B set of four MQ-007648-01-0002 was purchased from Dharmacon. D-007648-02 and D007648-03 are referred to 2 and #3, respectively. LNCaP cells were transfected with various amounts of the siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Cytotoxicity Analysis

Cytotoxicity analysis was performed as previously described [18]. LNCaP, CxR, and HPR50 cells (2×10^3) were seeded into 96-well plates. The following day, various concentrations of H89 were applied

in medium. After 48 hr, the surviving cells were stained with the Alamar Blue assay (TREK Diagnostic Systems) for 180 min at 37°C. The absorbances of the wells were measured using a plate reader (ARVO MX; Perkin Elmer, Inc.).

RNA Isolation and ReverseTranscriptase PCR

Total RNA was prepared from cultured cells using an RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized from 1.0 μg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's protocol.

Quantitative Real-Time PCR

The synthesized cDNA was diluted to a 1:2 ratio, and 2.0 μl of the diluted sample was used. TissueScan Prostate Cancer Tissue qPCR array III (HPRT303) was purchased from OriGene. The product contains first-strand cDNAs prepared from 48 human prostate tissues including both malignant and healthy controls. These 48 cDNAs have been normalized against β-actin by RT-PCR, and arrayed onto PCR plates. Quantitative real-time PCR was done by the Perfect Real-time Support System (Takara Bio) specific for PDE4B (HA113673). Assays were performed on QuantiTectSYBR Green PCR Kits (Qiagen) and ABI PRISM7900HT (Applied Biosystems), and GAPDH values were used for normalization. The results are representative of at least three independent experiments.

Cell Proliferation Assay

A cell proliferation assay was performed as described previously [19,20]. Briefly, LNCaP cells (2.5×10^4) were seeded into 12-well plates and transfected with the indicated siRNA under an androgen deprived condition. After 96 hr, the cells were harvested with trypsin and counted using a cell counter (Beckman Coulter). The results were normalized by the cell transfected with control siRNA at 96 hr, and are representative of at least three independent experiments.

PKA Activity Assay

LNCaP, CxR, and HPR50 cells were cultured in an androgen deprived condition for 48 hr. PKA activity was determined using a commercially available kit (Enzo Life Sciences) according to the manufacturer's instructions. Briefly, the wells of the PKA microtiter plate were soaked with 50 μl kinase assay dilution buffer for 10 min at room temperature. The samples were added to the wells of a PKA substrate microtiter plate. Reaction was initiated by the addition of 10 μl of diluted ATP to each well, which was then incubated for up to 90 min at 30°C. Subsequently, 40 μl of phospho-specific substrate antibody was added to each well and incubated at room temperature for 60 min. After washing, 40 μl of diluted anti-rabbit IgG:HRP conjugate was added to each well for 30 min, followed by exposure to the tetramethylbenzidine substrate for 30–60 min at room temperature. The reaction was stopped by adding stop solution. Absorbance at 450 nm was determined using a plate reader (ARVO MX).

Statistical Analysis

The Mann–Whitney's *U*-test was used for statistical analysis, and significance was set at the 5% level.

RESULTS

PDE4B Expression Is Downregulated While PKA Activity Is Further Activated in Castration-Resistant LNCaP Derivatives (CxR Cells) Compared With LNCaP Cells

First, we investigated mRNA levels of PDE4B and PKA activity in LNCaP and CxR cells. PDE4B mRNA was significantly decreased in CxR cells compared with that in parental cells (Fig. 1A). By hydrolyzing cAMP, which normally activates the PKA pathway, PDE4B causes an overall inhibition of PKA activity. Next, we investigated PKA activity in both LNCaP and CxR cells. Expectedly, PKA activity in CxR cells was increased in comparison to parental cells (Fig. 1B).

CREB is a transcription factor that is activated after phosphorylation by PKA [21]. Hence, we used CREB phosphorylation as another measure to confirm the activation of PKA. Consistent with the in vitro PKA kinase results (Fig. 1B), the level of phosphorylated CREB (serine133) was also increased in CxR cells (Fig. 1C).

Downregulation of PDE4B by siRNA Activates PKA Pathways and Increases CREB Phosphorylation

These above observations led us to investigate the potential ability of PDE4B suppression to control PKA activity. To determine if downregulation of PDE4B can activate PKA and phosphorylate CREB, we suppressed PDE4B by using PDE4B-specific siRNAs. PDE4B-specific siRNAs successfully suppressed PDE4B mRNA (Fig. 2A), downregulated PDE4B-activated PKA pathways (Fig. 2B), and increased phosphorylation of CREB (Fig. 2C). Because PKA controls

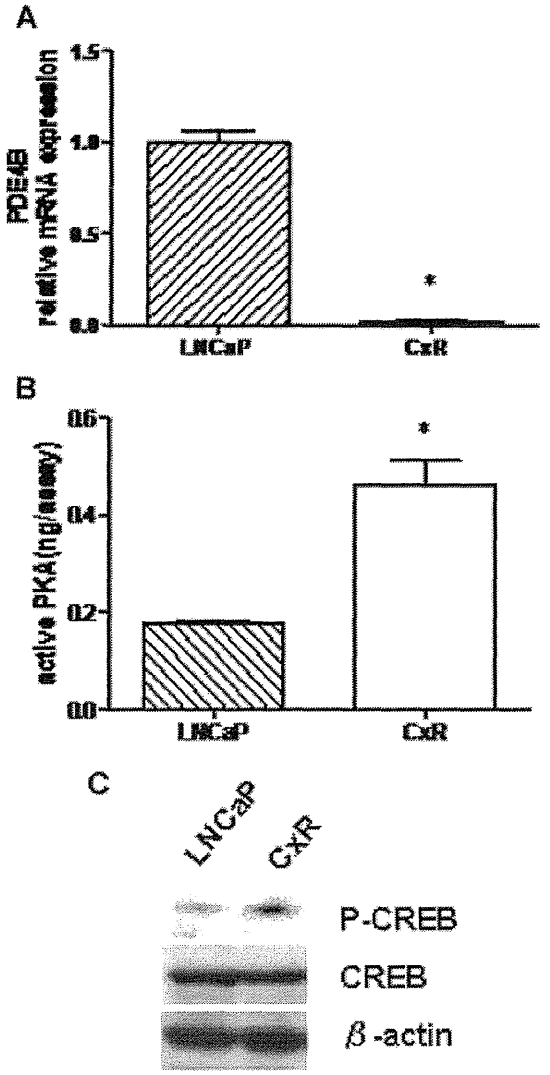

Fig. 1. mRNA level of PDE4B and PKA activation in CxR cells. **A**: mRNA level of PDE4B was analyzed by quantitative real-time PCR using the primers for PDE4B and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The target transcript levels were corrected by the corresponding GAPDH transcript levels. Boxes, mean; bars, ±s.d. $^*P < 0.05$ (compared with LNCaP cells). **B**: Both LNCaP and CxR cells were cultured in charcoal-stripped medium for 48 hr, and PKA kinase assay was performed as described in detail elsewhere. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±s.d. $^*P < 0.05$ (compared with LNCaP cells). **C**: Whole-cell extracts (30 μg) from LNCaP and CxR cells were analyzed for phosphoCREB and CREB by SDS–PAGE and Western blot analysis using the anti-phosphoCREB, anti-CREB, and anti-β-actin antibodies. The anti-β-actin antibody was used as a loading control.

transcription of PSA, we also examined PSA expression. As expected, PSA was upregulated by PDE4B suppression, while AR was not changed (Fig. 2D).

A PDE4B Inhibitor (Rolipram) Also Activates PKA Pathways and Increases CREB Phosphorylation

Because PDE4B suppression by siRNA activated PKA pathways, we also sought to confirm the relationship between PDE4B and the PKA pathway by pharmacological manipulation. PKA was activated in LNCaP cells by adding a PDE4B inhibitor (rolipram) in a dose-dependent manner (Fig. 3A). Rolipram also enhanced the phosphorylated form of CREB (Fig. 3B) and increased the expression of PSA, but not AR (Fig. 3C). Thus, we confirmed that PDE4B can negatively regulate PKA pathways as well as expressions of its downstream protein.

A PKA Inhibitor (H89) Decreases CREB Phosphorylation and PSA, But Not AR

We showed that PDE4B knockdown and rolipram addition activated PKA pathways and increased CREB phosphorylation. The above findings prompted us to investigate the effect of H89, a selective PKA inhibitor, on PKA pathways. LNCaP cells were treated with escalating concentration of H89. As expected, H89 induced the concentration-dependent abrogation of CREB phosphorylation (Fig. 4A). Additionally, PSA was downregulated in a dose-dependent manner but AR was not (Fig. 4B).

Oxidative Stress Decreases PDE4B Transcript Expression Level and Activates the PKA Pathway in LNCaP Cells

As we previously revealed that castration-induced oxidative stress causes castration resistance of prostate cancer [17], we hypothesized that oxidative stress may suppress PDE4B expression. When LNCaP cells were exposed to 50 μM of hydrogen peroxide for 2 hr in serum-free medium, PDE4B expression was reduced (Fig. 5A).

Next, we investigated whether oxidative stress regulates PKA activity. As shown in Figure 5B,C, PKA was activated and CREB was phosphorylated by hydrogen peroxide. Furthermore, hydrogen peroxide induced AR and PSA expression (Fig. 5D). As previously reported, upregulation of AR by oxidative stress was due to Twist1 expression [17]. On the other hand, PKA activation did not upregulate AR expression.

PDE4B Expression Is Downregulated and PKA Activity Is Enhanced in Hydrogen Peroxide-Resistant LNCaP Derivatives (HPR50 Cells) Compared With LNCaP Cells

The fact that oxidative stress promoted PKA activation through PDE4B downregulation in LNCaP cells led us to investigate PKA activity in hydrogen

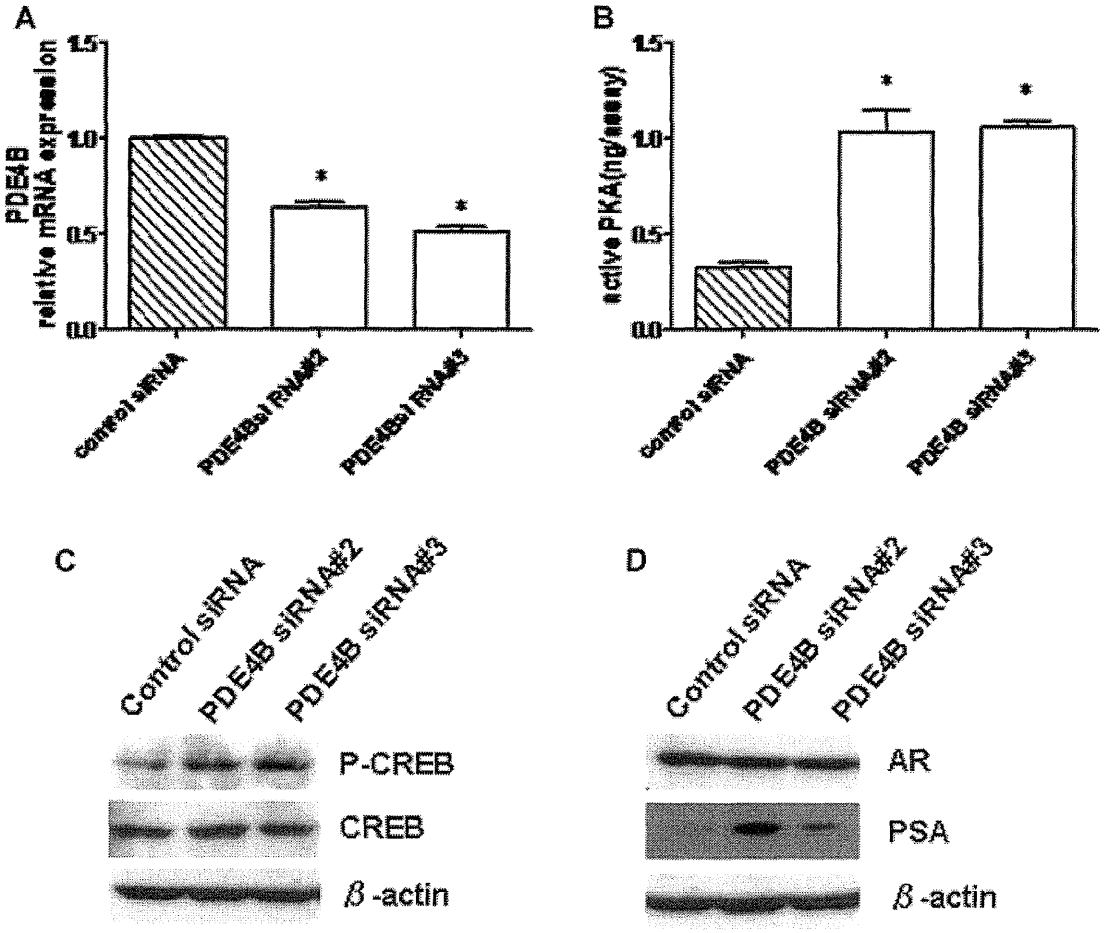

Fig. 2. Downregulation of PDE4B by siRNA activates the PKA pathway and increases CREB phosphorylation. **A**: LNCaP cells were transfected with 40 nM of control siRNA, PDE4B siRNA#2, or PDE4B siRNA#3. At 48 hr after transfection, quantitative real-time PCR was performed as described previously and the mRNA level of PDE4B was analyzed. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±s.d. $^*P < 0.05$ (compared with LNCaP cells transfected with control siRNA). **B**: LNCaP cells were transfected with 40 nM of control siRNA, PDE4B siRNA#2, or PDE4B siRNA#3. At 48 hr after transfection, PKA kinase assay was performed as described previously. All values represent the mean of at least three independent experiments. Boxes, mean; bars, ±s.d. $^*P < 0.05$ (compared with that of LNCaP cells transfected with control siRNA). **C,D**: LNCaP cells were transfected with 40 nM of control siRNA, PDE4B siRNA#2, or PDE4B siRNA#3. At 72 hr after transfection, whole-cell extracts (30 μg) were analyzed for phosphoCREB (C), CREB (C), AR (D), and PSA (D) by SDS–PAGE and Western blotting with specific antibodies. β-actin was used as a loading control.

peroxide-resistant LNCaP derivatives (HPR50 cells) which exhibit a castration-resistant phenotype [17]. As expected, PDE4B mRNA was decreased (Fig. 6A) and PKA activity was increased in HPR50 cells (Fig. 6B). Moreover, CREB phosphorylation (another measurement of PKA activity) was also increased (Fig. 6C). The results showed that oxidative stress is a robust activator of PDE4B/PKA pathways.

PDE4B Knockdown Induces Cell Growth in Androgen-Derived Medium by PKA Activation

We hypothesized that castration-resistant LNCaP-cell growth is influenced, at least in part, by PDE4B. To confirm this hypothesis, LNCaP cells transfected with PDE4B-specific siRNAs were cultured in an androgen-deprivation medium and subjected to a cell proliferation assay. PDE4B knockdown in LNCaP cells accelerated cell growth in androgen-deprivation medium after 96 hr (Fig. 7A). These data indicate that the PDE4B/PKA pathway accelerates castration-resistant cell growth.

H89 Suppresses the Cell Growth of LNCaP As Well As CxR and HPR50 Cells

PKA is a critical mediator of cellular proliferation, and our results further revealed that PKA was