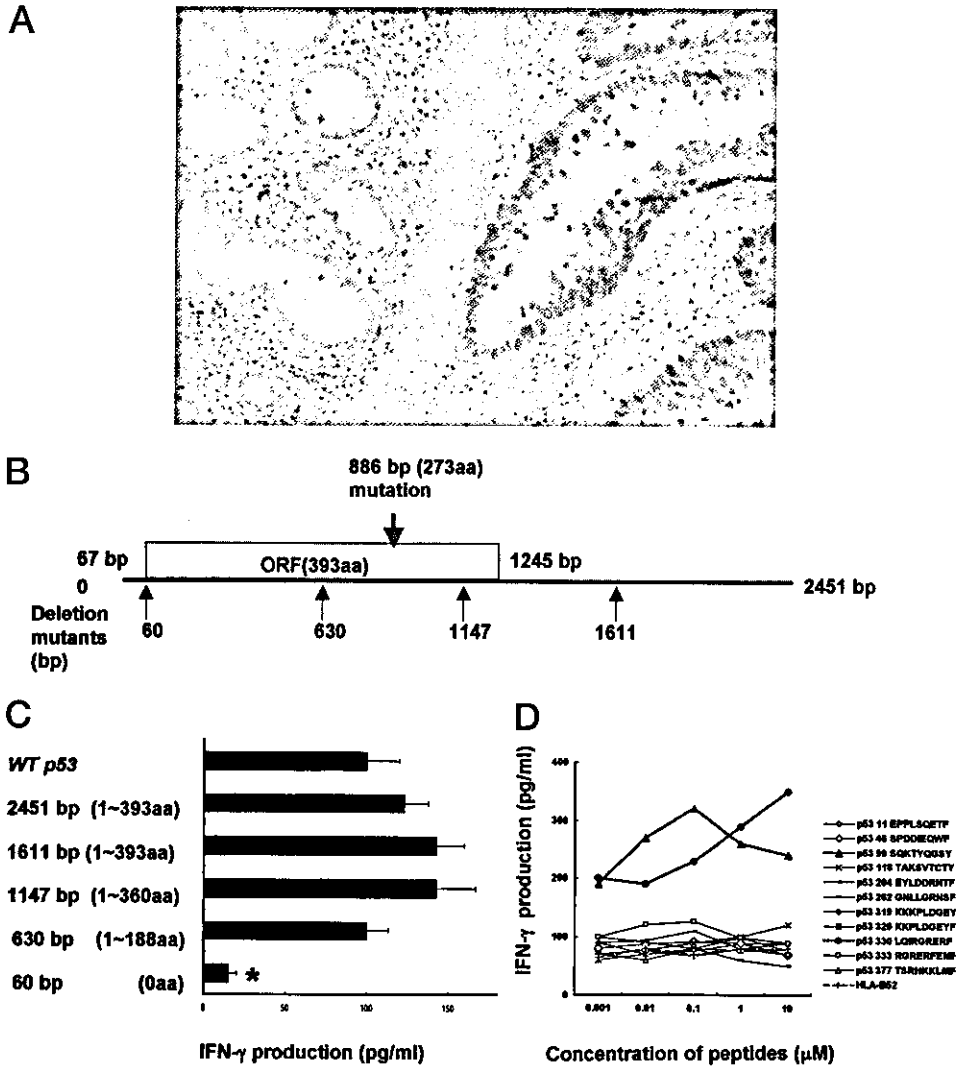

Fig. 2. *A*, expression of the p53 protein in cancer tissue. Immunostaining of p53 protein was performed on colon cancer tissue from which the OKB-CTL line was generated, and p53 was subjected to immunostaining by anti-p53 mAb (1:50 dilution, DO-7). Photomicrograph of colon cancer displays immunohistochemical localization of p53 confined to the nuclei of the cancer cells (*right side*; original magnification, ×400). However, p53 accumulation was not seen in the normal epithelial cells (*left side*). *B*, scheme of deletion mutants of *clone 7F*. *Clone 7F* had a missense point mutation at position 273 (R to H). *C*, the capability of wild-type *p53* and also the deletion mutants to stimulate IFN-γ production by the OKB-CTL cells. cDNA of wild-type *p53*, *clone 7F*, or one of the four deletion mutants corresponding to $p53_{1-1611}$, $p53_{1-1147}$, $p53_{1-630}$, or $p53_{1-60}$ was cotransfected with the *HLA-B*4601* gene into COS-7 cells, and IFN-γ production by the OKB-CTL cells was assessed. Values represent the mean of the triplicate assays. *, $P < 0.05$ by Student's *t* test. *D*, recognition of a p53-derived peptide by the OKB-CTL cells. Various doses of peptide were loaded onto *HLA-B*4601*-transfected COS-7 cells for 2 h, followed by the addition of OKB-CTL cells at an E:T ratio of 10:1. After an 18-h incubation, the culture supernatants were collected for measurement of IFN-γ. The values represent the means of duplicate assays.

*B*4601* gene and wild-type *p53*, the *HLA-B*4601* gene, and one of the deletion mutants corresponding to $p53_{1-2451}$ (full length), $p53_{1-1611}$, or $p53_{1-1147}$ were transfected to the COS-7 cells (Fig. 2, *B* and *C*). In contrast, the OKB-CTL cells failed to recognize COS-7 cells cotransfected with the *HLA-B*4601* gene and $p53_{1-60}$. The results suggest that a region coding for a CTL-directed epitope may be located within the range of 60–630 bp (1–188 aa) of the p53 protein, which had no mutation, as mentioned above. Eleven kinds of p53-derived nonapeptides with HLA-B46 molecule-binding motifs were loaded on COS-7 cells transfected with the *HLA-B*4601* or *HLA-B*5201* gene as a negative control and then tested for their ability to stimulate IFN-γ production by the OKB-CTL cells. Two peptides, $p53_{99-107}$ and $p53_{330-338}$, were recognized by the OKB-CTL cells in a dose-dependent fashion (Fig. 2*D*). The highest IFN-γ production was observed in 0.1 or 10 μM $p53_{99-107}$ or $p53_{330-338}$ peptide, respectively.

Immunogenic Epitopes Capable of Inducing CTLs. $p53_{99-107}$ and $p53_{330-338}$ peptides were tested for their ability to induce HLA-B46-restricted and tumor cell-reactive CTLs from the PBMCs of six HLA-B46$^+$ cancer patients and three healthy donors. $p53_{99-107}$ and $p53_{330-338}$ peptide-stimulated PBMCs from three and four of six cancer patients produced significant levels of IFN-γ by recognition of the corresponding peptide-loaded COS-7 cells transfected with the *HLA-B*4601* gene, but not by recognition of unloaded COS-7 cells transfected with the *HLA-B*4601* gene, respectively (Table 1). Among them, $p53_{99-107}$-stimulated PBMCs from patients 1 and 2 produced significant levels of IFN-γ in response to OSC20 cells (HLA-B46$^+$, *p53* mutation positive) but not in response to Kuma-1 cells (HLA-B46$^-$, *p53* mutation positive; Table 1) or COLO320 cells (HLA-B46$^-$, *p53* mutation positive; data not shown).

The $p53_{99-107}$ peptide-induced CTL activity against tumor cells in the PBMCs from patients 1 and 2 was confirmed by a 6-h ^{51}Cr release assay, and representative results are shown in Fig. 3*A*. The PBMCs stimulated by the $p53_{99-107}$ peptide showed significant levels of cytotoxicity against the OSC20 cells (HLA-B46$^+$, *p53* mutation positive) but failed to lyse Kuma-1 cells (HLA-B46$^-$, *p53* mutation positive), MKN45 cells (HLA-B46$^+$, *p53* mutation negative), and PHA-blastoid T cells (HLA-B46$^+$; Fig. 3*A*). Unstimulated but IL-2-activated PBMCs did not show such cytotoxicity (data not shown). This CTL activity was inhibited by anti-CD8 and anti-HLA-B/C mAbs, but not by any other mAbs tested (Fig. 3*B*). This CTL activity increased when the OSC20 cells were preincubated with a corresponding peptide ($p53_{99-107}$), but not with a control peptide ($p53_{204-212}$); Fig. 3*C*). Furthermore, this CTL activity against OSC20 cells was neutralized by the addition of unlabeled PHA-blastoid T cells preloaded with the corresponding peptide, but not by those preloaded with a control peptide (Fig. 3*C*). Taken together, these results support the hypothesis that the $p53_{99-107}$ peptide on the groove

Table 1 *Induction of HLA-B46-restricted CTL activity in HLA-B46$^+$ PBMCs by p53 peptides*[a]

Donors	Cancer and staging	Peptide	IFN-γ production (pg/ml) in response to[b] HLA-B46-transfected COS-7 pulsed with: Corresponding peptide	HLA-B46-transfected COS-7 pulsed with: Irrevant peptide	OSC20 HLA-B46	Kuma-1 HLA-B62/44
Patient 1	Prostate cancer Stage IV	None	0	0	0	0
		$p53_{99-107}$	624	0	277	0
		$p53_{330-338}$	205	0	218	133
Patient 2	Lung adenocarcinoma Stage II	None	0	0	0	0
		$p53_{99-107}$	402	0	196	0
		$p53_{330-338}$	109	0	229	340
Patient 3	Vulvur cancer Stage IV	None	0	0	0	0
		$p53_{99-107}$	0	0	0	0
		$p53_{330-338}$	0	0	0	0
Patient 4	Colon cancer Stage IV	None	0	0	0	0
		$p53_{99-107}$	149	0	0	0
		$p53_{330-338}$	25	0	0	0
Patient 5	Colon cancer Stage IV	None	0	0	0	0
		$p53_{99-107}$	0	0	0	0
		$p53_{330-338}$	126	0	171	143
Patient 6	Prostate cancer Stage IV	None	0	0	0	0
		$p53_{99-107}$	0	0	0	0
		$p53_{330-338}$	264	0	0	0
Healthy donor 1		None	0	0	0	0
		$p53_{99-107}$	0	0	0	0
		$p53_{330-338}$	0	0	0	0
Healthy donor 2		None	0	0	0	0
		$p53_{99-107}$	0	0	0	0
		$p53_{330-338}$	0	0	0	0
Healthy donor 3		None	0	0	0	0
		$p53_{99-107}$	0	0	0	0
		$p53_{330-338}$	0	0	0	0

[a] PBMCs from six HLA-B46$^+$ cancer patients and three HLA-B46$^+$ healthy donors were stimulated in vitro with 10 μM peptide and tested for their ability to produce IFN-γ by recognition of various target cells at an E:T ratio of 5.

[b] Values represent the means of duplicate determinations. The background IFN-γ production in response to HLA-B46-transfected COS-7 cells (50–100 pg/ml) has been substracted from the experimental values shown in the table.

of HLA-B46 molecules of OSC20 tumor cells is recognized by the PBMCs stimulated with the corresponding peptide. In contrast to the $p53_{99-107}$ peptide, the $p53_{330-338}$ peptide failed to induce HLA-B46-restricted and tumor cell-reactive CTLs in PBMCs from any of the cancer patients tested (data not shown).

DISCUSSION

We have newly established a HLA-B*4601-restricted and tumor cell-reactive CTL line (OKB-CTL) from the TILs of a colon cancer patient. The OKB-CTL cells produced significant levels of IFN-γ in response to HLA-B46$^+$ cancer cells (QG56 and Ca9-22) but failed to produce IFN-γ in response to HLA-B46$^-$ cancer cells (KWS and COLO320). The OKB-CTL cells also showed significant levels of cytotoxicity against HLA-B46$^+$ cancer cells (QG56, CA9–22, and OSC20) with *p53* mutation, but they failed to lyse either HLA-B46$^-$ SW620, Kuma-1, and COLO320 cells with a *p53* mutation or HLA-B46$^+$ MKN45 cells without a *p53* mutation. Furthermore, OKB-CTL activity was inhibited by anti-HLA-B/C mAb. All of these results, when taken together, indicate that the OKB-CTL cells recognized HLA-B46$^+$ tumor cells with a mutated *p53* gene.

The p53 protein has been divided into five domains (19, 20). The immunogenic epitope identified in this study at aa positions 99–107 was located between a proline-rich domain and a sequence-specific DNA-binding domain, and its biological activity has not yet been reported. Although the sites of *p53* mutations vary greatly, most of them are single-base mutations, and they are mostly restricted to the regions within the sequence-specific DNA-binding domain (1, 2). Subsequently, nonmutated CTL epitopes other than those with single-base mutations could be maintained in many types of tumor cells. If this is the case, nonmutated epitopes in conserved regions may become common antigens recognized by the host CTLs in many cancer patients whose tumors display the *p53* mutation. Because the immunogenic epitope identified in this study, $p53_{99-107}$, is encoded in a conserved region, this peptide could be an appropriate molecule for use as a part of a peptide-based cancer vaccine for HLA-B46$^+$ cancer patients with *p53* mutation.

We constructed deletion mutants of the *p53* gene to determine the antigenic peptide recognized by HLA-B46-restricted CTLs. The results of the experiments using these genes suggest that antigenic epitopes may be located within 60–630 bp (1–188 aa) of p53. The peptide $p53_{99-107}$ was identified as a CTL-directed epitope using synthesized peptides. These results from two separate experiments suggest that peptide $p53_{99-107}$ is an immunogenic peptide recognized by OKB-CTL cells. In addition, $p53_{330-338}$ was also recognized by the OKB-CTL cells when it was loaded on COS-7 cells transfected with *HLA-B*4601*. Subsequently, the two peptides ($p53_{99-107}$ and $p53_{330-338}$) were tested for their ability to induce HLA-B46-restricted CTL activity in the PBMCs of cancer patients and healthy donors. Both peptides stimulated IFN-γ production in the PBMCs of cancer patients, but not in those of healthy donors, in response to HLA-B46-transfected COS-7 cells loaded with the corresponding peptide. However, only the $p53_{99-107}$ peptide, but not the $p53_{330-338}$ peptide, induced HLA-B46-restricted and tumor cell-reactive CTLs in the PBMCs of cancer patients. One explanation for this discrepancy may be that higher or lower activity with regard to the binding to HLA-B46 molecules may be observed in the $p53_{99-107}$ and $p53_{330-338}$ peptides, respectively. This assumption is supported by the result that the highest IFN-γ production was observed when the HLA-B*4601-transfected COS-7 cells were loaded with 0.1 or 10 μM $p53_{99-107}$ or $p53_{330-338}$ peptide, respectively. Additional studies are needed to clarify this issue.

Mutations of the *p53* gene occur in approximately 50% of human cancers, and our study demonstrated that HLA-B46-restricted and p53-specific CTLs reactive to tumor cells with the *p53* mutation were

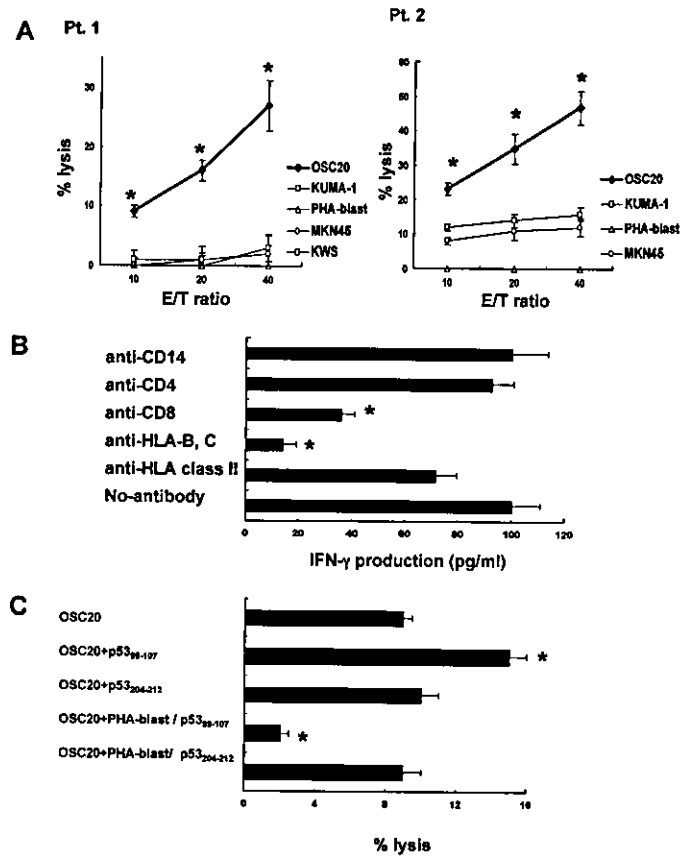

Fig. 3. Cytotoxicity of $p53_{99-107}$-induced CTL cells. *A*, $p53_{99-107}$-stimulated PBMCs from patients 1 and 2 were tested for their cytotoxicity against OSC20 (HLA-B46$^+$, *p53* mutation positive), Kuma-1 and KWS (HLA-B46$^-$, *p53* mutation positive), and MKN45 (HLA-B46$^+$, *p53* mutation negative) tumor cells. PHA-blastoid T cells (HLA-B46$^+$, *p53* mutation negative) were also used as a negative control. Six-h ^{51}Cr release assay was performed at three E:T ratios. *, $P < 0.05$ by the Student's *t* test. *B*, IFN-γ production by the $p53_{99-107}$-stimulated PBMCs in response to OSC20 tumor cells was tested in the presence of 20 μg/ml anti-HLA class II, anti-CD4, anti-CD8, anti-CD14, or anti-HLA-B/C mAb. Values represent the mean of the triplicate determinations. *, $P < 0.05$ by Student's *t* test. *C*, an excess amount (20 μg/ml) of a corresponding peptide ($p53_{99-107}$) or a control peptide ($p53_{204-212}$) was preloaded onto ^{51}Cr-labeled OSC20 tumor cells, which were used as target cells in a 6-h ^{51}Cr release assay. Unlabeled PHA-blastoid T cells preloaded with either a corresponding peptide ($p53_{99-107}$) or a control peptide ($p53_{204-212}$) were added to wells containing the ^{51}Cr-labeled OSC20 tumor cells at a cold:hot cell ratio of 10:1. Values represent the mean of the triplicate determinations. *, $P < 0.05$ by Student's *t* test.

induced by *in vitro* stimulation with the $p53_{99-107}$ peptide of PBMCs from cancer patients with various types of tumors. Because the mutation of *p53* is generally associated with tumor cells resistant to chemotherapy and radiotherapy, specific immunotherapy using the p53 peptides may provide an attractive new strategy for the treatment of HLA-B46$^+$ cancer patients with *p53* mutation in various histological types of cancers.

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Phase 1 Clinical Study of Cyclophilin B Peptide Vaccine for Patients With Lung Cancer

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Summary: Cyclophilin B (CypB) possesses two antigenic epitopes ($CypB_{84-92}$ and $CypB_{91-99}$) recognized by HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes (CTLs). To determine the safety of CypB-derived peptides and its ability to generate antitumor immune responses, patients with advanced lung cancer received subcutaneous vaccinations of these peptides or their modified peptides. All 16 patients were vaccinated with $CypB_{91-99}$ or its modified peptide, whereas only two patients were vaccinated with the modified $CypB_{84-92}$, as immediate-type hypersensitivity to $CypB_{84-92}$ or its modified peptide was observed in the remaining patients. No severe adverse events were associated with the vaccination. No significant increase in cellular responses to either peptides or tumor cells was observed in the postvaccination PBMCs by the conventional CTL assays in any patients tested. These results suggest that the vaccination of $CypB_{91-99}$ peptide was safe, but failed to induce objective immune responses at this regimen. **Key Words:** Cytotoxic T lymphocyte-peptides—Immunotherapy—Lung cancer—Phase 1 study.

Recent advances in molecular biology and cellular immunology in the field of tumor immunology have resulted in identification of a large number of antigens and epitopes recognized by HLA class I restricted cytotoxic T lymphocytes (CTLs) from melanomas and epithelial cancers (1–6), thereby opening the door to new peptide-based specific immunotherapy of cancers. Several subsequent clinical studies of patients with melanoma have shown increased immune responses to vaccinated peptides and tumor cells in PBMCs during the postvaccination period (7–11). To our knowledge, however, there have been no reports of peptide-based immunotherapy for patients with lung cancer. Lung cancer is among the most commonly occurring malignancies in the world and is one of the few that continues to show an increasing incidence (12). The prognosis for advanced lung cancer is extremely poor, despite recent clinical trials using gene therapy and numerous chemotherapeutic agents (13,14). Therefore, the development of new treatment modalities is needed, one of which should be specific immunotherapy. We have previously reported that the two cyclophilinB (CypB)-derived peptides and their modified peptides, which are recognized by the HLA-A24-restricted CTLs established from T cells infiltrating into lung adenocarcinoma, can induce CTLs from patients with cancer (15). In this report, we investigated the safety and immune responses in patients with advanced lung cancer vaccinated with CypB peptides combined with IFA.

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MATERIALS AND METHODS

Peptide Selection

The peptides used in the current study were prepared under Good Manufacturing Practice conditions by the

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Multiple Peptide System (San Diego, CA, U.S.A.): CypB-derived peptide at sequence positions 84–92 ($CypB_{84-92}$, KFHRVIKDF), its modified peptide in which tyrosine replaced phenylalanine at peptide position 2 ($CypB_{84-92}$2F-Y, KYHRVIKDF), at positions 91–99 ($CypB_{91-99}$, DFMIQGGDF), and its modified peptide in which tyrosine replaced phenylalanine at position 2 ($CypB_{91-99}$2F-Y, DYMIQGGDF). Peptides were > 95.5% pure with endotoxin levels of less than 0.1 endotoxin units per mL. The peptides were supplied as a white powder soluble in dimethylsulfoxide (DMSO), except for $CypB_{84-92}$2F-Y, which was soluble in saline. Each peptide was dissolved in DMSO or saline at 20 mg/mL, aseptically aliquoted, and stored at −80°C. Stock solutions were diluted with saline for $CypB_{84-92}$ and $CypB_{84-92}$2F-Y, or with 0.004 N NaOH-saline for $CypB_{91-99}$ and $CypB_{91-99}$2F-Y just before use. The other peptides used for in vitro assays as controls were an HIV-derived peptide with an HLA-A24-binding motif (RYLRQQLLGI), and an Lck-derived peptide at position 488 to 497 ($Lck_{488-497}$, DYLRSVLEDF), which was shown to induce HLA-restricted and tumor-specific CTLs (16). An EBV-derived peptide with an HLA-A24-binding motif (TYGPVFMCL) was also used as a control peptide.

Eligibility Criteria and Clinical Protocol

All patients had histologically confirmed lung cancers and underwent a complete clinical evaluation including x-rays and CT scans of all tumor sites. All patients were confirmed to be HLA-A24$^+$ by a conventional serologic method and by staining of PBMCs with anti-A24 mAb, as reported previously (4–6). Patient characteristics are listed in Table 1. No patients had received any treatments, steroids, or any other immunosuppressive drugs in the 4 weeks before the study. For the skin test, 10 μg of $CypB_{84-92}$ and $CypB_{91-99}$ peptides for the six patients, or 10 μg of their modified peptides for the other six patients in 50 μL of saline was independently injected intradermally using a tuberculin syringe with a 26-gauge needle. Ten μg was chosen since this was 1/100 of 1 mg, and 1/100 dose was generally used for the skin test of the other drugs. Immediate- and delayed-type hypersensitivity reactions were determined at 20 minutes and 24 hours after the skin test, respectively. If immediate-type hypersensitivity was negative, the peptide was injected as follows: group 1 patients (n = 3) received 1 mg each of the native peptides; group 2 patients (n = 5) received 3 mg each of the native peptides; group 3 patients (n = 3) received 1 mg each of the modified peptides; and group 4 patients (n = 5) received 3 mg each of the modified peptides. For groups 1 and 3, 1.5 mL of the peptide solution at 1 mg/mL was mixed with an equal volume of the IFA (Montanide ISA-51, Seppic, France) and emulsified in 5-mL sterilize-glass syringes, and 2 mL was injected into the subcutaneous tissue of the anterior thigh. For groups 2 and 4, 2 mL of the peptide solution at 2 mg/mL was mixed with an equal volume of IFA and

TABLE 1. *Demographics, adverse events, and clinical evaluation of CypB vaccination*

Group (patient no.)	Therapy before vaccination*	Peptide (dose)	Number of vaccination	Age	Sex	PS	Stage†	Adverse events	Clinical response at 5 weeks	Clinical response at 11 months	Time to progression (weeks)	Overall survival (weeks)
1 (001)	OP/Chemo	$CypB_{91-99}$ (1 mg)	18	63	Female	0	IIIb	Local reaction	SD	PD	53	92+
1 (002)	Chemo	$CypB_{91-99}$ (1 mg)	14	63	Female	1	IV	Local reaction	SD	PD	27	89+
1 (003)	Chemo/Rad	$CypB_{91-99}$ (1 mg)	5	41	Male	1	IV	Local reaction	SD	PD	8	33
2 (004)	OP/Chemo	$CypB_{91-99}$ (3 mg)	2	47	Female	2	IV	None	NE§	NE	NE	3
2 (005)	OP	$CypB_{91-99}$ (3 mg)	4	74	Female	0	IV	Local reaction	SD	SD	—	77+
2 (006)	OP/Chemo	$CypB_{91-99}$ (3 mg)	1	65	Female	1	IV	None	NE	NE	NE	43
2 (007)	Chemo/Rad	$CypB_{91-99}$ (3 mg)	8	55	Male	2	IV	None	SD	PD	11	38
2 (008)	OP	$CypB_{91-99}$ (3 mg)	10	78	Female	0	IV	Local reaction	SD	SD	—	75+
3 (011)	OP	$CypB_{91-99}$ 2F-Y (1 mg)	5	66	Male	0	IV	Local reaction	SD	PD	8	53+
3 (012)	OP/Chemo/Rad	$CypB_{84-92}$ 2F-Y and $CypB_{91-99}$ 2F-Y (1 mg)	4 6	50	Male	1	IV	Local reaction	SD	PD	5	40+
3 (013)	Chemo	$CypB_{91-99}$ 2F-Y (1 mg)	6	50	Male	2	IV	Local reaction	SD	PD	8	21
4 (014)	Chemo	$CypB_{84-92}$ 2F-Y (3 mg)	2	64	Male	2	IIIa	Local reaction	NE	NE	NE	7
4 (015)	Chemo/Rad	$CypB_{84-92}$ 2F-Y (3 mg)	5	70	Male	1	IV	Local reaction	SD	PD	20	38+
4 (016)	OP	$CypB_{84-92}$ 2F-Y and $CypB_{91-99}$ 2F-Y (3 mg)	3 6	63	Male	1	IV	Local reaction	SD	PD	5	16
4 (017)	Chemo	$CypB_{91-99}$ 2F-Y (3 mg)	4	77	Female	1	IV	Local reaction	SD	PD	3	17
4 (018)	Chemo	$CypB_{91-99}$ 2F-Y (3 mg)	6	45	Male	1	IV	Local reaction	SD	PD	4	15

* Therapy before vaccination was noted. OP, Surgery, Chemo, Chemotherapy; Rad, radiotherapy.
† UICC-TNM classification of malignant tumors was used for determination of clinical stage.
‡ Toxicity-grading criteria of the Japanese Clinical Oncology Group were used for grading of adverse events.
§ Not eligible for evaluation.

emulsified in the 5-mL glass syringes, and 3 mL was injected into the subcutaneous tissue. It was intended that all patients would receive at least three vaccinations at 2-week intervals. This protocol was reviewed and approved by the Kurume University Review Board and the Independent Ethical Committee (Protocol numbers 9905 and 9910). All patients were required to comprehend and sign an informed consent form before the clinical trial.

Response Evaluation

All known sites of disease were evaluated 7 days after every third vaccination, and these findings were compared with those obtained before the vaccinations. Cellular immune responses in the postvaccination PBMCs were evaluated 7 days after every third vaccination. Patients who received less than three vaccinations were excluded from clinical and immunologic evaluations. Patients were assigned a response category according to the following response criteria: (i) a complete response was considered to be a complete disappearance of all clinical evidence of the indicated lesion for a minimum of 4 weeks; (ii) a partial response was defined as a decrease of $\geq$ 50% in the product of the perpendicular diameters of the indicator lesion for a minimum of 4 weeks; (iii) progressive disease was defined as an increase of $\geq$ 25% in the product of the perpendicular diameters of the indicator lesion or as an appearance of tumors in a new region; and (iiii) stable disease (SD) was defined as any variation of the indicator lesion not meeting the above three criteria.

Cellular Immune Responses

PBMCs were cultured in vitro for 7 days with or without the corresponding peptide (10 μg/mL) used for in vivo vaccination in wells of a 24-well culture plate (2 mL/well). These cells were harvested, then tested for their ability to produce IFN-γ in response to HLA-A24$^+$11–18 lung adenocarcinoma cells or HLA-A24$^-$ QG56 lung squamous cell carcinoma cells, or to produce IFN-γ in the absence of tumor cells as a negative control in triplicate assays at three different E:T ratios by ELISA (limit of sensitivity: 10 pg/mL). The effector cells were also tested for their ability to produce IFN-γ in response to C1R-A2402 cells alone as a negative control, or to those pulsed with either a corresponding peptide or an irrelevant HIV peptide in triplicate assays at three different E:T ratios. The effector cells were further expanded in the complete medium for 21–25 days, and were tested for their surface phenotypes and their cytotoxic activity by a 6-hour 51Cr-release assay at different E:T ratios in triplicate assays. The target cells were 11–18, QG56 cancer cells, VA13 fibroblast cells, and HLA-A24$^+$ PHA-activated normal T cells.

RESULTS

Demographics, Skin Tests, Adverse Events, and Clinical Evaluation

Sixteen patients with advanced lung cancer (inoperable stages IIIa, IIIb, and IV) were treated in this regimen, and their demographics are given in Table 1. The histologic diagnosis was adenocarcinoma (nos. 001–008, 011, 012, and 016–018), squamous cell carcinoma (no. 013), large-cell carcinoma (015), or small-cell carcinoma (014). Immediate-type hypersensitivity to $CypB_{84-92}$ and its modified peptide was observed in skin tests before vaccination in all but two (nos. 012 and 016) patients. A representative skin reaction is shown in Figure 1. Subsequently, all 16 patients received vaccinations with $CypB_{91-99}$ (n = 8) or its modified peptide (n = 8), while two patients (nos. 012 and 014) received the $CypB_{84-92}$2F-Y peptide. The numbers of injections varied among patients from 1 to 18, with this number being primarily dependent on patients' own requests and also on clinical conditions as evaluated by the corresponding physicians. No delayed-type hypersensitivity was observed in any of the patients before or after vaccination.

All 16 patients were evaluated for all common toxicities; the overall toxicities are given in Table 1. The CypB peptide vaccine was well tolerated, but 13 of 16 patients had grade I local reactions at the injection sites. No medication was needed for these local immune reactions. No other adverse events were observed in this clinical trial. The 13 patients receiving more than three vaccinations were eligible for evaluation of clinical responses at two time points, 5 weeks after the first vaccination and at 11 months, which was the median observation time for all 16 patients (Table 1). All 13 patients were evaluated as having SD at 5 weeks. At 11 months after the first vaccination, two patients (nos. 005 and 008) vaccinated with 3-mg injections of $CypB_{91-99}$ peptide were clinically SD, whereas the other 11 patients showed progressive disease (PD). The median time to progression of the four patients vaccinated with the $CypB_{91-99}$ peptide or of the seven patients vaccinated with the modified peptides was 25 or 8 weeks, respectively. The overall survival of the six patients vaccinated with the $CypB_{91-99}$ peptide or the seven patients vaccinated with the modified peptides was 67+ and 29+ weeks, respectively.

assay. Further, the increased cellular responses were not observed in any patients other than patient no.008 by the standard ^{51}Cr-release assay. These results indicate that the CypB vaccination failed to induce objective immune responses to either peptide or tumor cells under the conventional CTL assays at the used regimen. An assay using the labeled HLA class I-A tetramer was recently carried out to monitor immune responses (24,25). Therefore, we prepared a labeled HLA-A24 tetramer loaded with $CypB_{91-99}$ peptide and used it to monitor the post-vaccination PBMCs of two patients (nos. 001 and 005) in this study, but the percentage of positive cells was < 0.5% of the gated $CD8^+$ cells. This tetramer assay was not conducted for the other ten cases because only a limited number of PBMCs was available for in vitro analyses.

The present results indicated that the vaccination of $CypB_{91-99}$ peptide was safe, but failed to induce immune responses at the used regimen. Further clinical studies with different regimen shall be taken to determine whether CypB peptide is suitable in use as cancer vaccine for the treatment of patients with lung cancer.

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Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method

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Among a number of human tumor antigens identified using the serological analysis of recombinant cDNA expression libraries (SEREX), only MAGE-1, tyrosinase, and NY-ESO-1 have been reported to be immunogenic tumor antigens that have the potential to elicit both humoral and cellular immunity. In this study, we determined whether our SEREX-defined pancreatic cancer antigens could be recognized by CTL, and report that one SEREX-defined antigen, coactosin-like protein (CLP), encoded cellular epitopes recognized by HLA-A2-restricted and tumor-reactive CTL. Three CLP peptides at positions 15–24, 57–65, and 104–113 possessed the ability to induce HLA-A2-restricted and tumor-reactive CTL from the PBMC of cancer patients. Subsequently, humoral responses to these peptides were investigated. IgG antibodies specific to the CLP 15–24, 57–65, and 104–113 peptides were detected in sera from 12, 0, and 12 of 12 cancer patients tested, and were also found in 5, 0, and 0 of 9 healthy donors, respectively. IgE antibodies specific to these peptides were also detected in sera from certain cancer patients and healthy donors. Since peptide-specific IgE was detected, type-I allergy to these peptides was tested. Unexpectedly the CLP 57–65 peptide, to which IgE was found in only 2 healthy donors, but not the other two peptides, was found to elicit an immediate-type hypersensitivity in all 10 healthy volunteers tested. These results indicate that identical antigenic peptides can be recognized by both cellular and humoral immune systems to a tumor-associated antigen. The CLP 15–24 and 104–113 peptides might be appropriate vaccine candidates for peptide-based immunotherapy of HLA-A2$^+$ cancer patients.

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1 Introduction

The recent development of molecular technology for analyzing cellular and humoral immune reactivity to cancer cells at the gene level has led to the identification and characterization of a large number of human tumor antigens recognized by CD8$^+$ T cells and antibodies. Many genes encoding tumor antigens and peptides that are recognized by CTL have been identified by cDNA expression cloning methods [1–6], thereby introducing the possibility of a peptide-based cancer immunotherapy. Post-vaccination PBMC became to show CTL activity against tumor cells in the clinical studies of peptide-based cancer immunotherapy, but these immunotherapies have rarely resulted in tumor regression [7, 8]. The failure to obtain tumor regression could be in part due to that immunogenicity of these tumor antigenic peptides was not strong enough to induce adaptive immunity against tumor cells. From this point of view, peptides with the ability to induce both CTL responses and humoral immunity could be better than those with the ability to induce either one. Over 1,500 types of tumor antigens have been identified using the serological analysis of recombinant cDNA expression libraries (SEREX) method [9–11]. However, only three (MAGE-1, tyrosinase, and NY-ESO-1) have been reported to have the ability to elicit both cellular and humoral immune responses to tumor cells [12–15]; CTL responses to the

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The last two authors contributed equally to this work.

Abbreviations: SEREX: Serological analysis of recombinant cDNA expression libraries **TIL:** Tumor-infiltrating lymphocyte **CLP:** Coactosin-like protein

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majority of SEREX-defined tumor antigens remain to be elucidated. Furthermore, there is no report on antigenic epitopes recognized by both cellular and humoral immune system to tumor-associated antigens.

We previously reported 18 SEREX-defined pancreatic cancer antigens [16]. This study has extended these studies and investigated whether five SEREX-defined pancreatic cancer antigens could be recognized by tumor-reactive CTL. We also provide evidence that one of the antigens is able to encode the identical epitopes recognized by both the cellular and the humoral immune system.

2 Results

2.1 Recognition of coactosin-like protein-derived peptides by an HLA-A2-restricted and tumor-reactive CTL line

Five genes coded for SEREX-defined pancreatic cancer antigens were considered from the perspective of whether or not their products could be recognized by an HLA-A2-restricted and tumor-reactive OK-CTL line by co-transfection of these cDNA and *HLA-A*0207* cDNA into COS-7 cells. This cell line was established from the tumor-infiltrating lymphocytes (TIL) of a patient (OK) with colon cancer, and responds to tumor cell lines in an HLA-A2-restricted manner, as reported previously [4, 6]. The OK-CTL line produced a significant level of IFN-γ in response to the COS-7 cells transfected with both *KM-PA-4* and *HLA-A*0207* cDNA (Fig. 1A). Maximum production of IFN-γ was observed when 100 ng *KM-PA-4* cDNA was transfected. In contrast, the OK-CTL line failed to produce significant levels of IFN-γ in response to COS-7 cells transfected with both *KM-PA-4* cDNA and an irrelevant *HLA-A*2402* cDNA, or with one of four kinds of cDNA (*KM-PA-5*, *KM-PA-14*, *KM-PA-15*, and *KM-PA-18*) together with *HLA-A*0207* cDNA. In our previous study [16], the KM-PA-4 was found to encode coactosin-like protein (CLP) consisting of 142 amino acids, as shown. These results indicate that KM-PA-4/CLP-derived antigens could be recognized by HLA-A2-restricted and tumor-reactive CTL. To determine the antigenic peptides recognized by the OK-CTL, eight different CLP-derived peptides with HLA-A2 binding motifs were prepared, and these peptides were then loaded onto T2 cells at a concentration of 10 μM; samples were tested for the ability to induce IFN-γ production by the OK-CTL (Fig. 1B). Three peptides (CLP 15–24, CLP 57–65, and CLP 104–113) had the ability to induce IFN-γ production by the OK-CTL.

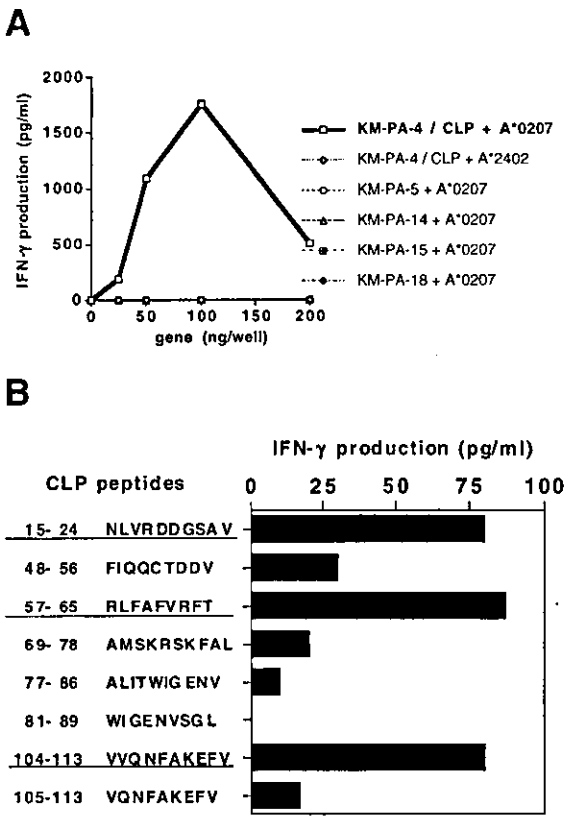

Fig. 1. Recognition of CLP-derived peptides by HLA-A2-restricted and tumor-reactive CTL. One of the highly reproducible results in a triplicate assay is indicated. (A) Different amounts of five SEREX-defined cDNA clones and 100 ng *HLA-A*0207* or *-A*2402* cDNA were co-transfected into COS-7 cells, followed by a test of the capacity to stimulate IFN-γ production by the HLA-A2-restricted and tumor-reactive OK-CTL. The experimental values, with the background production of IFN-γ release by the CTL in response to untransfected COS-7 cells (less than 100 pg/ml) subtracted, are shown. (B) Eight CLP-derived peptides with HLA-A2 binding motifs were loaded onto T2 cells at a concentration of 10 μM, and were tested for their ability to induce IFN-γ production by the OK-CTL. The experimental values, with the background production of IFN-γ (less than 30 pg/ml) by the CTL in response to T2 cells pre-loaded with the irrelevant HIV peptide subtracted, are shown. Three peptides, which were judged to be positive for this assay, are underlined.

2.2 Induction of tumor-reactive CTL by CLP peptides

We next attempted to generate tumor-reactive CTL from the PBMC of HLA-A2$^+$ patients with pancreatic or colon cancer, or of HLA-A2$^+$ healthy donors. The PBMC were stimulated with either the CLP 15–24, CLP 57–65, or CLP 104–113 peptide. These *in vitro*-sensitized PBMC

were tested for their cytotoxicity against various kinds of tumor cell lines in a 6-h ^{51}Cr-release assay (Fig. 2). The mRNA expression of the *CLP* gene in these cell lines used as target cells was confirmed by both reverse transcription (RT)-PCR and Northern blot analysis (data not shown). The CLP 15–24 peptide-sensitized PBMC from three of five cancer patients and two of six healthy donors showed significant levels of CTL activity against HLA-A2$^+$ Panc-1 and YPK-3 cells, but not against HLA-A2$^-$ PaCa-2 cells, HLA-A2$^+$ PHA-blasts or an HLA-A2$^+$ EBV-B cell line (Fig. 2). This was also the case with the CLP 57–65 peptide-sensitized PBMC in three of eight cancer patients, and in two of nine healthy donors (Fig. 2). Similarly, the CLP 104–113 peptide-sensitized PBMC from three of five cancer patients and one of seven healthy donors showed HLA-A2 restricted and tumor-reactive CTL activity (Fig. 2). The summary is shown in Table 1. In all cases, the percentage of CD8$^+$ T cells in peptide-stimulated PBMC was more than 80% (data not shown). These PBMC produced IFN-γ in response to T2 cells pre-loaded with a corresponding peptide in a dose-dependent manner (data not shown), and their IFN-γ production in response to HLA-A2$^+$ Panc-1 was inhibited by the addition of anti-HLA-class I, anti-CD8 or anti-HLA-A2 mAb (data not shown). These results indicate that these cytotoxicities were mediated by the peptide-specific and HLA-A2-restricted CD8$^+$ CTL.

2.3 Detection and quantification of serum IgG and IgE antibodies reactive to the CLP-derived peptides

We determined whether IgG specific to the whole CLP antigen could be detected in sera from pancreatic cancer patients and healthy donors using the SEREX method. IgG antibodies against the CLP antigen were found in the sera from 9 of 10 pancreatic cancer patients, and from 3 of 10 healthy donors (data not shown). We then examined if the three CLP peptides identified above could be recognized by the serum antibodies from cancer patients and healthy donors. ELISA was used to quantify levels of serum IgG and IgE specific to the CLP-derived peptides. Serum samples were judged as positive for peptide-specific antibody when the absorbance (unit) values changed in relation to the dilution of the serum samples; representative results are shown in

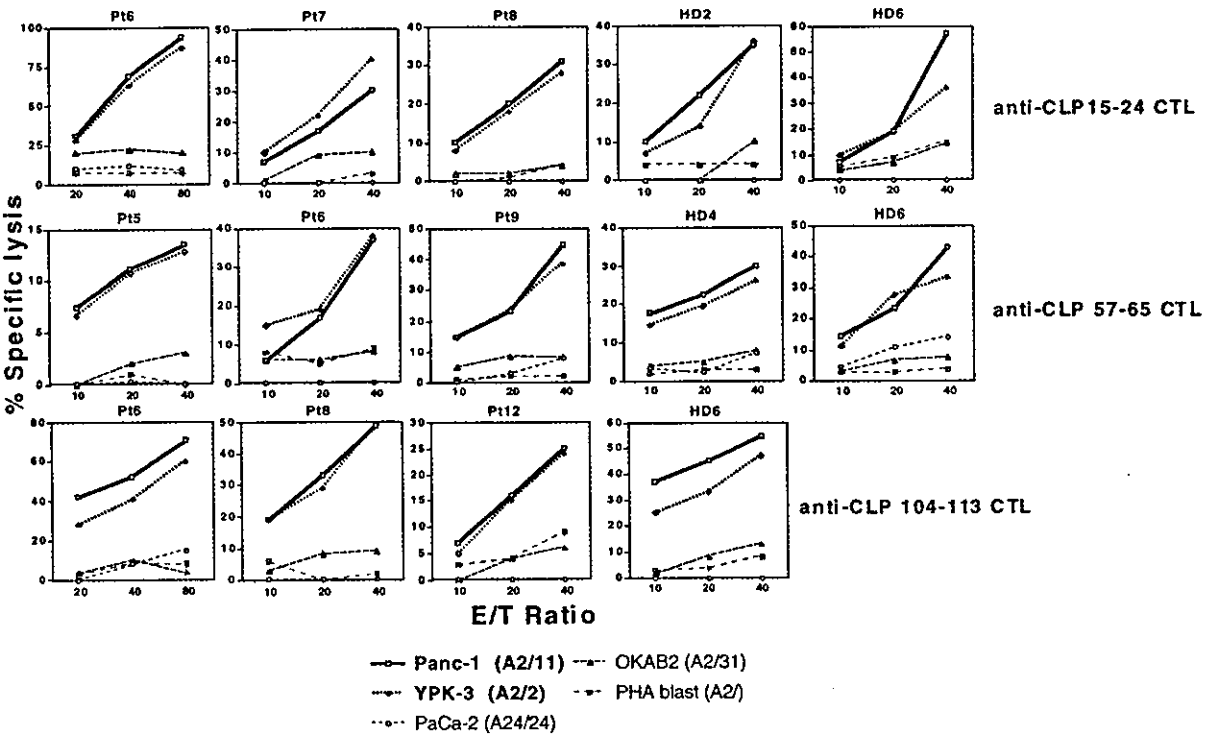

Fig. 2. Cytotoxic activity of PBMC stimulated with the CLP peptides. PBMC from patients with cancer and from healthy donors, listed in Table 1, were stimulated with the indicated peptides, followed by a test of the cytotoxicity against HLA-A2$^+$ Panc-1 and YPK-3, HLA-A2$^-$ PaCa-2 pancreatic cancer cell lines, HLA-A2$^+$ PHA-blasts, and the HLA-A2$^+$ EBV-B cell line, OKAB2, by a 6-h ^{51}Cr-release assay. Values represent the mean of triplicate assays. One of two highly reproducible results is indicated.

Table 1. Serum levels of IgG and IgE specific to the CLP peptides and induction of peptide-specific CTL from PBMC of HLA-A2$^+$donors

	CLP 15-24			CLP 57-65			CLP 104-113		
HLA-A2$^+$ Donor	CTL induction	IgG	IgE	CTL induction	IgG	IgE	CTL induction	IgG	IgE
Pancreatic Cancer									
Pt1	NT[c]	**3.7**[d]	**0.1**	-	<0.1	<0.1	NT	**1.0**	<0.1
Pt2[a]	NT	**4.5**	<0.1	NT	<0.1	<0.1	NT	**2.3**	<0.1
Pt3	NT	**6.0**	**0.4**	-	<0.1	<0.1	NT	**1.7**	**0.1**
Pt4	NT	**2.3**	<0.1	-	<0.1	<0.1	NT	**1.6**	<0.1
Pt5	NT	**3.8**	**1.9**	+	<0.1	<0.1	NT	**1.5**	**1.5**
Pt6	+[e]	**5.2**	**0.3**	+	<0.1	<0.1	+	**2.6**	<0.1
Pt7	+	**3.7**	**0.4**	NT	<0.1	<0.1	-	**1.0**	**1.0**
Pt8	+	**4.5**	<0.1	NT	<0.1	<0.1	+	**2.0**	<0.1
Colon Cancer									
Pt9 (OK)[b]	-	**4.5**	**0.1**	+	<0.1	<0.1	-	**2.0**	<0.1
Pt10	NT	**4.4**	**0.5**	-	<0.1	<0.1	NT	**1.9**	**0.4**
Pt11	NT	**5.2**	<0.1	NT	<0.1	<0.1	NT	**2.1**	<0.1
Pt12	-	**4.3**	**0.5**	-	<0.1	<0.1	+	**1.8**	**1.4**
Healthy Donor									
HD1	-	**0.3**	**0.1**	-	<0.1	<0.1	-	<0.1	<0.1
HD2	+	**1.9**	**0.4**	-	<0.1	**0.7**	-	<0.1	**0.1**
HD3	NT	**1.8**	<0.1	-	<0.1	<0.1	-	<0.1	<0.1
HD4	NT	**2.0**	**0.2**	+	<0.1	<0.1	NT	<0.1	**1.1**
HD5	NT	**1.6**	<0.1	-	<0.1	<0.1	NT	<0.1	<0.1
HD6	+	<0.1	<0.1	+	<0.1	<0.1	+	<0.1	<0.1
HD7	-	<0.1	<0.1	-	<0.1	<0.1	-	<0.1	<0.1
HD8	-	<0.1	**0.2**	-	<0.1	<0.1	-	<0.1	<0.1
HD9	-	<0.1	<0.1	-	<0.1	<0.1	-	<0.1	<0.1

a) The *KM-PA-4/CLP* was identified by the SEREX method using serum from this patient [16].

b) The OK-CTLs were established from this patient [4, 6].

c) NT: not tested.

d) The results of IgG and IgE antibodies binding to either the CLP 15-24, CLP 57-65 or CLP 104-113 peptide are shown by 10X absorbance values (10X OD unit) in ELISA. The experimental values, from which the background absorbance (10X OD unit) without peptide was subtracted, are shown. Serum samples were diluted at x100 for IgG and at x2 for IgE, respectively. Values judged as positive for peptide-specific antibody in ELISA are underlined.

e) The symbol represents successful induction of peptide-specific and HLA-A2$^+$ tumor-reactive CTL. The results are shown in Fig. 2.

Fig. 3A and B. A decrease in the absorbance unit of IgG against both the CLP 15–24 and CLP 104–113 peptides, but not against the CLP 57–65 peptide, was observed in serum dilutions in all 4 patients tested (Fig. 3A). As with IgE, sera from Pt5 and Pt7, and HD4 were evaluated as positive for IgE to both the CLP 15–24 and CLP 104–113 peptides, but not against the CLP 57–65 peptide (Fig. 3B). In the case of HD2, serum dilution resulted in a decrease in the absorbance of IgE against all three peptides (Fig. 3B). A competitive binding inhibition assay was performed to confirm the peptide specificity of IgG or IgE detected in this ELISA system. Detection of IgG or IgE antibody was inhibited by adding a corresponding peptide to the sample serum in a dose-dependent manner, but not by adding an irrelevant peptide (data not shown). These results indicate that an excess of a free peptide in serum samples showed a peptide-specific competition against the coating peptide, validating the peptide specificity of this ELISA system.

IgG and IgE specific to the CLP peptides were measured in sera of 12 HLA-A2$^+$ patients with cancer (Pt1–12) (8 pancreatic cancer and 4 colon cancer) and 9 HLA-A2$^+$ healthy donors (HD1–9), summarized in Table 1. IgG specific to the CLP 15–24 peptide was detected in the sera of all 12 cancer patients, and was also found in 5 of 9 healthy donors, while IgE specific to this peptide was detected in the sera of 8 of 12 cancer patients and of 4 of

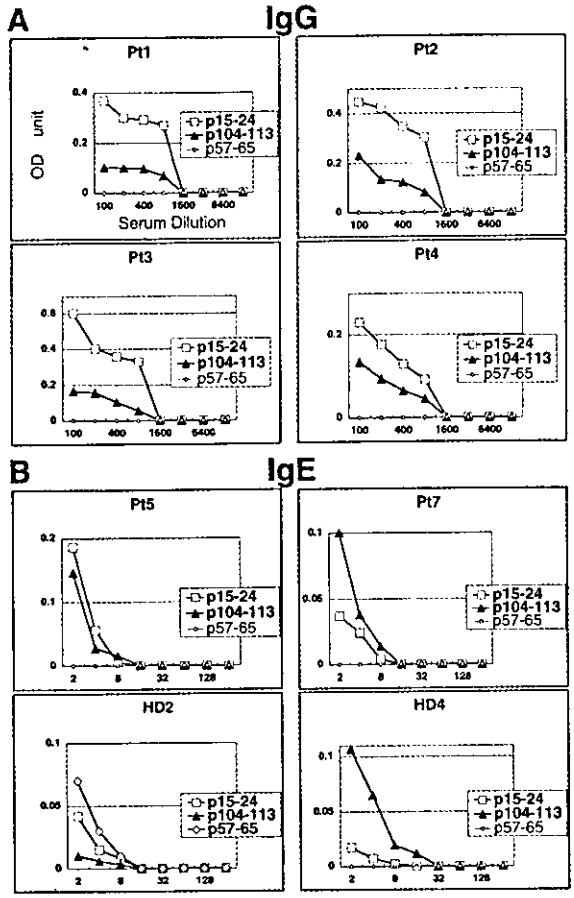

Fig. 3. Detection and quantification of serum IgG and IgE antibodies specific to the CLP peptides. One representative result of two highly reproducible experiments is shown. Peptide-reactive IgG and IgE in serially diluted serum samples from cancer patients and healthy donors were examined in (A) and (B), respectively.

9 healthy donors. IgG and IgE specific to the CLP 104–113 peptide were detected in the sera of 12 and 5 of 12 cancer patients, and in the sera of 0 and 2 of 9 healthy donors, respectively. In contrast, no IgG reactive to the CLP 57–65 peptide was detected in any of the donors tested. IgE reactive to the CLP 57–65 peptide was detected in only 1 healthy donor (HD2). Similar results were observed in HLA-A2$^-$ cancer patients and healthy donors for antibodies reactive to the CLP 15–24 and CLP 104–113 peptides, but IgG specific to the CLP 104–113 peptide was detected in the sera of 4 of 10 HLA-A2$^-$ healthy donors (data not shown). There was no significant association between the presence of the CLP 15–24 or CLP 104–113 peptide-specific IgG antibody in sera and successful induction of CTL from PBMC *in vitro* (p=0.546 and 0.222, respectively, evaluated by the χ^2 test). There was no inverse correlation between the existence of IgE antibody and successful CTL induction. CLP 15–24 or CLP 104–113 peptide-specific CTL were induced from PBMC, regardless of the detection of serum IgE specific to these peptides.

2.4 Type-I allergic reaction to the CLP 57–65 peptide

Type-I allergic reactions to some CTL epitopes were observed in the skin tests of cancer patients in the phase I clinical trial of peptide-based immunotherapy, and those patients who showed type-I allergic reaction against the peptide were not vaccinated with the peptide, because of the risk of systemic anaphylaxis (Gouhara et al., unpublished results). Because serum IgE reactive to the CLP peptides were detected in certain patients and healthy donors (Table 1), we investigated whether these CLP-derived CTL epitope peptides could elicit type-I allergic reactions in five HLA-A2$^+$ healthy and five HLA-A2$^-$ healthy volunteers. The CLP 57–65 peptide elicited the type-I allergy in all ten healthy volunteers tested, whereas the other two peptides failed to induce the type-I reaction in any donors.

The CLP 57–65 peptide and the analogue peptides in which individual amino acid residues were substituted by glycine (glycine scan) were prepared to gain a better understanding of the molecular basis of the type-I allergy, and the results of the skin test with the CLP 57–65 analogue peptides are listed in Table 2. The glycine substitution at positions 1 and 7 resulted in a decrease of the type-I allergic reaction, suggesting that position 1 (arginine) and position 7 (arginine) were important for the type-I allergic reaction to the CLP 57–65 peptide. On the other hand, substitution at position 6 resulted in an increase of the type-I allergic reaction in all cases tested. The results of IgE binding to analogues of the CLP 57–65 peptide are listed in Table 3. The glycine substitution at position 5, 6, or 9 in HD2 or at position 6, 7, and 9 in HD10 resulted in a complete or a moderate loss of IgE recognition, respectively. There was a discrepancy between important amino acid residues for the type-I allergic reaction and those for IgE antibody binding. These peptide analogues were also tested for the ability to stimulate the CLP 57–65 peptide-stimulated PBMC to produce IFN-γ. In contrast to the results of the type-I allergic reaction and antibody binding, recognition by peptide-specific CTL was lost in the majority of glycine-substituted analogue peptides (data not shown).

Table 2. Results of skin test with the CLP 57-65 analogue peptides [a]

CLP-derived peptides		Donors [b]			
		HD2 (HLA-A2$^+$, IgE$^+$)	HD6 (HLA-A2$^+$, IgE$^-$)	HD10 (HLA-A2$^-$, IgE$^+$)	HD11 (HLA-A2$^-$, IgE$^-$)
		Area of redness (cm^2)			
57-65	RLFAFVRFT	100	100	100	100
R57G	GLFAFVRFT	36.0[c]	17.4	39.1	16.7
L58G	RGFAFVRFT	114	100	156	100
F59G	RFGAFVRFT	114	100	56.3	52.9
A60G	RLFGFVRFT	114	156	66.0	82.6
F61G	RLFAGVRFT	64.0	156	56.3	67.0
V62G	RLFAFGRFT	128	278	352	186
R63G	RLFAFVGFT	16.0	17.4	31.6	13.2
F64G	RLFAFVRGT	114	178	66.0	151
T65G	RLFAFVRFG	100	50.2	100	20.7

a) The results of the skin test with analogues of the CLP 57-65 peptide are shown as relative percentages compared with those with the wild-type peptide.

b) Skin test was tested in 10 healthy volunteers, and the results of 4 donors (HLA-A2$^+$ or -A2$^-$, positive or negative of serum IgE specific to the CLP 57-65 peptide) are shown in the table.

c) Values showing more than 50% reduction, compared with those with the wild-type peptide, are underlined.

3 Discussion

The SEREX-defined KM-PA-4 gene was highly expressed at the mRNA level in pancreatic cancer cell lines as compared with normal pancreatic tissues in our previous study [16]. The sequence of the *KM-PA-4* gene was identical to that of the *CLP* gene (derived from human placenta) already registered in the GenBank (accession no. L54057). CLP was referred to because CLP protein product (142 amino acids) showed a significant homology to coactosin [17], a filamentous (F)-actin-binding protein from *Dictyostelium discoideum*, with 33.3% identity in amino acid sequence [18]. The mRNA of CLP is 1824 bp long and is expressed predominantly in placenta, lung, liver, and kidney, but not in the heart, brain, skeletal muscle, and pancreas [18]. Smith-Magenis syndrome (SMS), which involves the clinical symptoms of mental retardation, neuro-behavioral abnormalities, sleep disturbances, short stature, minor craniofacial and skeletal anomalies, congenital heart defects, and renal anomalies, is caused by deletion of the short arm of chromosome 17 in band p11.2. *CLP* gene is mapped to the SMS common deletion region [18]. This SMS critical region overlaps with a breakpoint cluster region associated with primitive neuroectodermal tumors, suggesting that CLP plays a role in DNA rearrangements of somatic cells [18]. CLP has also been demonstrated to interact directly with 5-lipoxygenase (5LO), which plays a pivotal role in cellular leukotriene synthesis [19, 20]. 5LO appeared to compete with F-actin for the binding of CLP. Further studies are needed to clarify the biological functions of CLP.

It has been reported that the same immunodominant myelin basic protein peptides were important for antibody binding and Th cell recognition in multiple sclerosis patients [21]. However, there has been no report of antibodies against class I-associated CTL epitopic peptides. In this study, we provided evidence of IgG and IgE antibodies against CTL epitopic peptides. We suppose that IgG and IgE antibodies detected by ELISA were specific to the CLP peptides based on the following: (1) serial dilution of serum resulted in a proportional decrease in antibody binding to the peptides; (2) addition of free peptides inhibited the peptide-specific binding of antibodies in serum to coating peptides, as shown in the competitive binding inhibition assay; and (3) peptide binding was significantly influenced by several analogue peptides with only one amino acid substitution. These results validate the specificity of IgG and IgE against the CLP peptides. On the other hand, IgG and IgE antibodies specific to the CLP-derived peptides were detected in the sera of HLA-A2-negative donors in similar proportion to that of both HLA-A2$^+$ cancer patients and healthy donors (data not shown). Determination of class II-associated epitopes of the CLP antigen is critically important to fully understand T cell response to the CLP.

Table 3. Binding of IgE antibodies to the CLP 57-65 analogue peptides[a)]

CLP-derived peptides		Donor HD2	HD10
		IgE bound to analogue peptides (OD unit) [b)]	
57-65	RLFAFVRFT	100 (0.067)	100 (0.113)
R57G	GLFAFVRFT	140 (0.094)	77.0 (0.087)
L58G	RGFAFVRFT	139 (0.093)	162 (0.183)
F59G	RFGAFVRFT	118 (0.079)	92.0 (0.104)
A60G	RLFGFVRFT	85.1 (0.057)	85.0 (0.096)
F61G	RLFAGVRFT	0[c)] (0)	85.0 (0.096)
V62G	RLFAFGRFT	0 (0)	15.9 (0.018)
R63G	RLFAFVGFT	85.1 (0.057)	10.6 (0.012)
F64G	RLFAFVRGT	97.0 (0.065)	61.9 (0.070)
T65G	RLFAFVRFG	0 (0)	29.2 (0.033)

a) The levels of IgE antibodies binding to analogues of the CLP 57-65 peptide are shown as relative percentages compared with those of the wild-type peptide.

b) Absorbance values (OD units) are shown in parentheses. The values were subtracted by the background absorbance (OD units) without peptides.

c) Values showing more than 50% reduction, compared with those bound to the wild-type peptide, are underlined.

Three patterns of existence of peptide-specific IgG antibodies were shown in the three CLP peptides. IgG antibodies against the CLP 15–24 peptide were detected in the sera from all of the cancer patients; these antibodies were also found in half of the healthy donors. Antibodies against the CLP 104–113 peptide were detected in the sera from all cancer patients, and they were found in none of the healthy donors. Antibodies against the CLP 57–65 peptide were not detected in the sera from any of the cancer patients or the healthy donors. In the present study, we found that CLP-derived peptide-specific and tumor-reactive CTL could be induced predominantly in cancer patients. In particular, CLP 104–113 peptide-specific and tumor-reactive CTL activity was induced in three of five cancer patients (60.0%), and in one of seven healthy donors (14.3%). Thus, the CLP 104–113 peptide may have the highest potential among the three peptides to induce tumor-specific cellular and humoral immunity in PBMC of cancer patients. On the other hand, we could not induce CTL against various peptides in some of the patients. We think that these variations may be partly due to the heterogeneity of tumor cells and T cell repertoires in each patient. There may be difference in precursor frequency of peptide-specific CTL in each individual. It is also possible that cellular immunity of some cancer patients may be depressed. The number of cancer patients who had CTL precursors reactive to EBV peptide was significantly lower than that of healthy donors (unpublished observation).

The type-I allergic reaction to the CLP 57–65 peptide was not restricted to HLA-A2$^+$ donors. This type of reaction to some CTL epitopes was also observed in prevaccinal skin tests of cancer patients in the phase I clinical trial of peptide-based immunotherapy (Gouhara et al., unpublished results). Allergy is classically defined as an immunological reaction to a foreign antigen [22]. However, anaphylactic shock to a self peptide was recently described in mouse EAE [23]. Injection of a myelin proteolipid protein 139–151 peptide in mice after immunization with the same peptide was reported to cause anaphylactic shock. The type-I allergic reaction to the CLP 57–65 peptide was observed in all donors tested, whereas detectable levels of IgE were only found in sera from two donors (HD2 and 10). The CLP 57–65-specific IgE may be trapped on the surface of mast cells by a high-affinity FcεR-I, and only a small amount of IgE, at levels too low to detect, is present in the circulation.

There was a discrepancy between important amino acid residues for the type-I allergic reaction and for IgE antibody binding. This observation may, in part, explain why IgE reactive to the CLP 57–65 peptide was not detected in the majority of donors who exhibited type-I allergic reaction. Glycine substitution at position 6 (V62G) resulted in an increase of type-I allergic reaction. This could be due to the molecular mimicry between microbial non-self peptides and V62G analogue of the CLP 57–65. *Escherichia coli, Mycobacterium tuberculosis*

and many other microbes have sequences sharing a six-amino acid sequence identity with V62G. On the other hand, the type-I allergic reaction and IgE binding activity was lost when glycine substitution was introduced in relatively localized amino acid residues, whereas CTL recognition was lost in the majority of the glycine-substituted analogue peptides. Both positions 2 and 9 of the peptides are considered to be anchor residues, and residues at other positions are thought to be TCR contact residues. The loss of CTL recognition in analogue peptides carrying a glycine substitution at position 2 or 9 is most likely due to the failure of peptide binding to HLA-A2, because glycine does not have a side chain. The loss of CTL recognition in their analogue peptides might be due to low affinity or to the lack of binding of CTL to TCR, because positions 1, 3, 4, 5, 6, 7, and 8 of the CLP 57–65 peptide have side chains of considerable size.

CLP is a self antigen, and its mRNA is expressed in some normal tissues [16, 19], as well as being overexpressed in pancreatic cancer cell lines, compared to normal pancreatic tissues. CTL induced by stimulation with CLP-derived peptides showed cytotoxicity against cancer cell lines, but not against two kinds of proliferating T and B lymphoblasts (Fig. 2). These results suggest that vaccination with these peptides were not associated with adverse effects on normal cells and tissues. Some self antigens expressed in certain normal tissues prove to be good candidates for cancer immunotherapy. For example, immunizing patients with normal self peptides derived from melanosomal proteins has resulted in dramatic tumor regression with only occasional vitiligo in some patients who had been prescribed immunotherapy with melanoma epitopes together with administration of IL-2 [24]. Further study of immune responses of self-antigen-recognizing and tumor-reactive CTL to normal cells and tissues is required.

NY-ESO-1 protein is thought to be the most immunogenic antigen, since it has been reported to have the ability to elicit both cellular and humoral immune responses to tumor cells. However, there are no reports on tumor-associated antigenic epitopes recognized by both cellular and humoral immune systems. We provide evidence that CLP can encode identical epitopes recognized by both the cellular and humoral immune systems. Further study is needed to clarify whether these peptides have the ability to induce orchestrated anti-tumor immune responses of not only CTL, but also Th cells and antibodies.

In conclusion, we identified three CLP peptides that were capable of propagating HLA-A2-restricted and tumor-reactive CTL from PBMC. One of these peptides, CLP 57–65, elicits a type-I allergic reaction. The HLA-A2 allele is found in 23% of Black Africans, 53% of Chinese, 40% of Japanese, 49% of Northern Caucasians, and 38% of Southern Caucasians [25]. These results indicate that the CLP 15–24 and CLP 104–113 peptides could be appropriate candidates in use for specific immunotherapy for a large number of cancer patients.

4 Materials and methods

4.1 HLA-A2-restricted CTL line

An HLA-A2-restricted and tumor-reactive CTL line, OK-CTL, was used to investigate whether SEREX-defined genes could encode antigens recognized by CTL. This cell line was established from the TIL of a colon cancer patient OK (HLA-A*0207/*3101, -B46/51, -Cw1) by incubation in medium supplemented with IL-2 (100 U/ml) alone for more than 50 days, the details of which have been described elsewhere [4, 6].

4.2 Analysis for antigen recognition by CTL

Eighteen candidate genes encoding pancreatic cancer antigens were identified using serum from Pt2 (HLA-A*0210/*2402, -B*5201/*4006, -DRB1*1502/*09012, -DQB1*0601/03) with pancreatic cancer through SEREX screening of a cDNA library generated from a human pancreatic adenocarcinoma cell line, CFPAC-1 [16]. Among them, five types of full-length cDNA clones encoding either KM-PA-4/CLP, KM-PA-5/HALPHA55, KM-PA-14/CGI55 protein, KM-PA-15/GIF, or KM-PA-18/hsp105, which were packaged in the EcoRI- and XhoI-digested pBluescript vectors, were inserted into the expression vector pCMV-SPORT-2 (Life Technologies, Rockville, MD). The cDNA of *HLA-A*0207* or *-A*2402* genes was obtained by RT-PCR and was cloned into the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA). cDNA (0–200 ng) of the of each of the SEREX-defined genes and 100 ng *HLA-A*0207* or *-A*2402* cDNA were suspended in 50 μl Opti-MEM (Life Technologies), mixed with Lipofectamine™ reagent (Life Technologies), and samples were cultured at room temperature for 30 min. A 50-μl aliquot of the mixture was then added to the COS-7 cells (5×10^3), which were then incubated for 6 h. Thereafter, the COS-7 cells were cultured for 2 days in RPMI 1640 medium supplemented with 10% FCS, followed by the addition of CTL (5×10^4 cells/well). After an 18-h incubation, 100 μl of the culture supernatant was collected and assayed in triplicate for IFN-γ production by ELISA.

4.3 Peptides

Eight different CLP-derived peptide candidates with the potential to bind to the HLA-A2 molecules [26, 27] were synthesized (Fig. 1B). The peptides and their analogues with a

single amino acid substitution to glycine were synthesized by Fmoc/PyBOP. These peptides were purchased from Sawady Laboratory (Tokyo, Japan), and their purity, estimated by HPLC, was >70%. For the additional studies, three peptides, including the CLP 15–24, 57–65, and 104–113 peptides, with a purity of >95%, were prepared. As a negative control, an HLA-A2-binding HIV-derived peptide (SLYNTYATL) with a purity of 90–98%, kindly provided by Dr. Kanaoka (Sumitomo Pharmaceutical, Osaka, Japan), was used.

4.4 Cancer cell lines

The cancer cell lines used in this study and their HLA-A alleles, shown in parentheses, were as follows: Panc-1 (HLA-A*0201/1101) pancreatic adenocarcinoma, YPK-3 (HLA-A*0201) pancreatic adenocarcinoma, PaCa-2 (HLA-A*2402) pancreatic adenocarcinoma, and RERF-LC-MS (HLA-A*1101) lung adenocarcinoma. HLA-A2$^+$ EBV-transformed B cell lines and HLA-A2$^+$ PHA-activated T lymphocytes were used as control cells.

4.5 *In vitro* sensitization of PBMC with the peptides

PBMC were isolated from 20 ml of heparinized blood of HLA-A2$^+$ cancer patient donors and healthy donors by Ficoll-Conray density gradient centrifugation, as reported previously [5]. Informed consent was obtained from all donors. HLA class I typing was performed on blood lymphocytes using the classical serological method [5]. A simple method was used to generate peptide-specific CTL from PBMC (Hida et al., unpublished results). In brief, PBMC (1×10^5 cells/well) were incubated with 10 μM peptide in 200 μl culture medium in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI 1640 medium, 45% AIM-V® medium (Gibco-BRL), 10% FCS, 100 U/ml of recombinant human IL-2, and 0.1 μM MEM nonessential amino acid solution (Gibco-BRL). Half of the medium was removed and replaced with fresh medium containing the corresponding peptide (20 μM) every 3 days for up to 12 days.

4.6 Assays for tumor antigen-reactive T cell responses

Peptide-stimulated PBMC were further expanded in the presence of feeder cells pre-pulsed with the indicated peptide for 21–28 days to obtain a relatively large number of cells. Thereafter, they were tested for their responses against various targets by a 6-h ^{51}Cr-release assay [5]. The surface phenotypes of the CTL were examined by direct immunofluorescence staining with FITC-conjugated anti-CD3, -CD4, or -CD8 mAb (Nichirei, Tokyo, Japan) [6]. To determine both effector cells and MHC restriction, 20 μg/ml anti-HLA-class I (W6/32, IgG2a), anti-HLA-A2 (BB7.2, IgG2b), anti-CD8 (Nu-Ts/c, IgG2a), anti-HLA-DR (H-DR-1, IgG2a), and anti-CD4 (Nu-Th/i, IgG1) mAb were added at the beginning of the cultures. Anti-CD13 (MCS-2, IgG2a) and anti-CD14 (JML-H14, IgG1) mAb were used as isotype-matched negative controls [6].

4.7 Skin test

Each peptide was dissolved in DMSO at 10 mg/ml, aseptically aliquoted, and stored at −80°C. Stock solutions were diluted with saline just before use. A sterility test was performed according to the method described in Section B-484 of the Japanese Pharmacopoeia 13th edn. Peptide solution (50 μl, 0.2 mg/ml) was injected intradermally into healthy volunteers and, 15 min later, flare and induration were inspected. Informed consent was obtained from all participants.

4.8 Detection of serum IgG reactive to the entire CLP antigen

SEREX-positive clones of CLP were subcloned and retested for serum reactivity, according to the following method. To determine the reactivity of other allogeneic sera samples (1:100 dilution) obtained from ten healthy blood donors (50–63 years old, mean 56.7 years, four men and six women) and ten patients with pancreatic ductal adenocarcinoma (43–79 years old, mean 62.1 years, four men and six women) against SEREX-positive clones, plates containing an equal number of sero-positive and sero-negative control clones were similarly processed. The immunoscreening method has been described previously [16].

4.9 Measurement of serum IgG and IgE reactive to the peptides

An ELISA was used to quantify serum levels of IgG and IgE reactive to the CLP-derived peptides. Immobilization of the peptides at their C termini to a 96-well Nunc Covalink flat plate (Fisher Scientific, Pittsburgh, PA) using disuccinimidyl suberate (Pierce, Rockford, IL) was performed according to the manufacturer's instructions. The plate of immobilized peptides (10 μg/well) was blocked with Block Ace (Yukijirushi, Tokyo, Japan), and washed with 0.05% Tween 20-PBS (PBST); 100 μl/well of either serum or plasma samples diluted with 0.05% Tween 20-Block Ace was then added to the plate. The plate was washed with PBST after 2-h incubation at 37°C, and further incubated for 2 h at 37°C with 1:1,000-diluted rabbit anti-human IgG antibodies (Dako, Glostrup, Denmark). The plate was washed nine times, and 100 μl of 1:100-diluted goat anti-rabbit Ig-conjugated horseradish peroxidase-dextran polymer (EnVision, Dako) was added to each well, and the plate was incubated at room temperature for 40 min. After washing, 100 μl/well of tetramethyl-benzidine substrate solution (KPL, Guildford, GB) was added, and the reaction was stopped by the addi-

tion of 1 M H_3PO_4. Absorbance was measured at 450 and 630 nm. As with the IgG-measurement procedure, levels of IgE were measured using 1:1,000-diluted rabbit anti-human IgE (Dako). Analogues of the CLP 57–65 peptide, in which the indicated positions were substituted by glycine, were used to determine amino acid positions crucial for the type-I allergic reaction, antibody binding, and CTL recognition.

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DETECTION OF PEPTIDE-SPECIFIC CYTOTOXIC T-LYMPHOCYTE PRECURSORS USED FOR SPECIFIC IMMUNOTHERAPY OF PANCREATIC CANCER

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The prognosis of pancreatic cancer is extremely poor with a 5-year survival of approximately 3%. Thus, the development of new treatment modalities, including a specific immunotherapy, is required. Our study investigated whether cytotoxic T-lymphocyte (CTL) precursors reacting to peptides with vaccine candidates (13 peptides for HLA-A2$^+$ or -A24$^+$ patients, respectively) were detectable in the prevaccination peripheral blood mononuclear cells (PBMCs) of 15 pancreatic cancer patients. Peptide-specific CTL precursors were detectable in the majority (11 of 15, 73%) of patients, with a mean positive number of 1.5 peptides (ranging from 0–5 peptides) per patient. Positive peptide profiles varied among patients. These results may provide a scientific basis for a new kind of cancer immunotherapy, namely, a CTL precursor-oriented peptide vaccine, for pancreatic cancer patients.

Key words: *peptide vaccine; monitoring; order-made therapy; HLA-A24; HLA-A2*

The prognosis of pancreatic cancer, the fifth-leading cause of death by cancer in both Japan and the United States, is extremely poor; the median period of survival after diagnosis is 3–4 months, with a 5-year survival rate of approximately 3%.[1,2] Therefore, the development of new treatment modalities, including specific immunotherapy, is of great importance for the treatment of pancreatic cancer. Recent advances in molecular biology and tumor immunology have enabled us to identify a large number of genes and antigenic peptides recognized by cytotoxic T-lymphocytes (CTLs) reactive to melanomas[3–7] and epithelial cancers,[8–15] thereby opening the door to peptide-based specific immunotherapies for cancer patients. However, clinical trials of peptide-based specific immunotherapies for advanced melanoma patients have rarely resulted in tumor regression.[16–19] This failure could be partly due to an insufficient induction of CTLs in the current regimes, so we should make more effort to induce peptide-specific CTL activity in each patient. Peptide-pulsed Langerhans cells in regional lymph nodes must come into contact with peptide-specific CTL precursor cells within 2 days in order to activate CTLs.[20] Therefore, determining the frequency of CTL precursors reacting to vaccinated peptides in peripheral blood mononuclear cells (PBMCs) will be an important feature of achieving antitumor immunity. We have recently established a new and simple method to detect peptide-specific CTL precursors in circulation (Hida *et al.*, unpublished data). With this new tool, our study has investigated whether CTL precursor cells reacting to peptides with vaccine candidates are detectable in PBMCs of pancreatic cancer patients. The new aspect of our study is the use of a new method to determine CTL precursor as a screening tool to determine those peptides to which the patient will most likely respond because reactive CTL precursor exist.

MATERIAL AND METHODS

Patients and cell lines

Ten HLA-A24$^+$ and 5 HLA-A2$^+$ pancreatic cancer patients were enrolled in our study after informed consent was obtained. None of these patients were infected with human immunodeficiency virus (HIV) or human T-lymphotropic virus type 1 (HTLV-1). Twenty milliliters of peripheral blood was obtained, and PBMCs were prepared by Ficoll-Conray density gradient centrifugation. PBMCs were also obtained from healthy volunteers (5 HLA-A24$^+$ and 5 HLA-A2$^+$). HLA class I typing was performed on PBMCs by classical serologic methods that have been reported previously.[9] Pancreatic cancer cell lines used for our study were Panc-1 (HLA-A0201/1101), paca-2 (HLA-A2402), YPK-1 (HLA-A2402), YPK-2 (HLA-A2402), YPK-3 (HLA-A0201), YPK-4 (HLA-A2601), HPAKII, SUIT2 and BxPC3. The KE4 esophageal SCC line (HLA-A2402/A2601), from which the SART1$_{259}$, SART2 and SART3 genes were cloned, was used as a positive control.[8] The HT1376 bladder carcinoma cell line (HLA-A2402), from which the ART4 gene was cloned, was used as a positive control.[12] The MKN-45 gastric adenocarcinoma cell line (HLA-A2402) was used as a negative control for SART2 expression. The SW620 colon cancer cell line (HLA-A2402/0201) and phytohemagglutinin (PHA)-blastoid T cells were also used as target cells, as reported previously.[13]

Detection of tumor antigens

Expression of SART1, SART2, SART3 and ART4 tumor antigens at the protein levels in pancreatic cancer cell lines, pancreatic cancer tissues and nontumorous pancreatic tissues were investigated by Western blot analyses using previously reported polyclonal antibodies.[8–10,12,22]

Peptides

The synthesized peptides used in our study are listed in Tables II and III. These peptides were purchased from Sawady Laboratory (Tokyo, Japan), and their purity was >95%. All peptides, except for Epstein-Barr virus (EBV)-derived peptide, were encoded by

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tumor-rejection antigens and have the ability to induce HLA-A24 or -A2-restricted CTLs specific to tumor cells in the PBMCs of cancer patients, as reported previously.[8-15] HIV-derived peptide with an HLA-A24-binding motif (RYLRQQLLGI)[13] and HTLV-1-derived peptide with an HLA-A2-binding motif (SLYNTYATL)[14] were used as a negative control.

Detection of peptide-specific CTL precursor cells

A simple method was used to detect peptide-specific CTLs in PBMCs. PBMCs (1×10^5 cells/well) were incubated with 10 μM of each peptide in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark) in 200 μl of culture medium. The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V® medium (GIBCO BRL, Walkersville, MA), 10% FCS, 100 U/ml of interleukin-2 (IL-2) and 0.1 mM MEM nonessential amino acid solution (GIBCO BRL). Half of the medium was removed and replaced with the new medium containing a corresponding peptide (20 μM) every 3 days for up 12 days. On the 12th day of the culture, 24 hr after the last stimulation, these cells were harvested, washed 3 times and then tested for their ability to produce IFN-γ in response to CIR-A2402 cells preloaded with either a corresponding peptide or HIV peptide (RYLRQQLLGI) as a negative control in HLA-A24$^+$ PBMCs. In HLA-A2$^+$ cases, T2 cells preloaded with a corresponding or HIV peptide (SLYNTYATL) were used as target cells. The target cells (CIR-A2402 or T2, 1×10^4/well) were pulsed with each peptide (10 μM) for 2 hr, and then effector cells (1×10^5/well) were added to each well with the final volume of 200 μl. After incubation for 18 hr, the supernatants (100 μl) were collected, and amounts of IFN-γ were measured by an ELISA (limit of sensitivity: 10 pg/ml). All experiments were performed in quadruplicate assays. A 2-tailed Student *t*-test was employed for the statistical analyses. Detectable levels of CTL precursors were judged as positive if the mean value of IFN-γ production by the peptide-stimulated PBMCs in response to a corresponding peptide was significantly ($p < 0.05$) higher than that in response to an HIV peptide.

Cytotoxicity assay

The peptide-stimulated PBMCs were further expanded in the presence of feeder cells for 21–25 days in order to obtain a relatively large number of cells as reported previously.[9,10] Cells were tested for cytotoxicity against various target cells by a 6 hr ^{51}Cr-release assay using a previously described method.[13] For an inhibition assay, the CTL activity was measured in the presence of 20 μg/ml of anti-CD8, -CD4, -HLA class I (W6/32), or -HLA class II (DR) monoclonal antibody (MAb). The surface phenotypes of the cells were measured by an immunofluorescence assay with FITC-conjugated anti-CD3, anti-CD4 or anti-CD8 MAb as reported previously.[9]

RESULTS

We initially investigated the expression of $SART1_{259}$, SART2, SART3 and ART4 tumor-rejection antigens at the protein levels in pancreatic cancers. Western blot analyses were carried out with polyclonal antibodies. Representative results are shown in Figure 1, and a summary is given in Table I. $SART1_{259}$ antigen was expressed in the cytosolic fraction of half of the pancreatic cancer cell lines and pancreatic cancer tissues, but it was not expressed in the nontumorous pancreatic tissues. It was not expressed in the nuclear fraction from any of the tested samples. These results are consistent with our previous results regarding other cancers reported elsewhere.[8] SART2, SART3 and ART4 tumor-rejection antigens were expressed in both the cytosolic and nuclear fractions from the majority of pancreatic cancer cells and cancer tissues tested. None of these antigens were expressed in either the cytosol or the nucleus of nontumorous pancreatic tissues. These results are also consistent with our previous results from studies of other cancers.[9,10,12] Expression of the other 6 tumor-rejection antigens, from which the peptides of vaccine candidates in our study were derived (Tables II, III), was not investigated in our study. These 6 antigens are nonmutated self-antigens, the biologic functions of

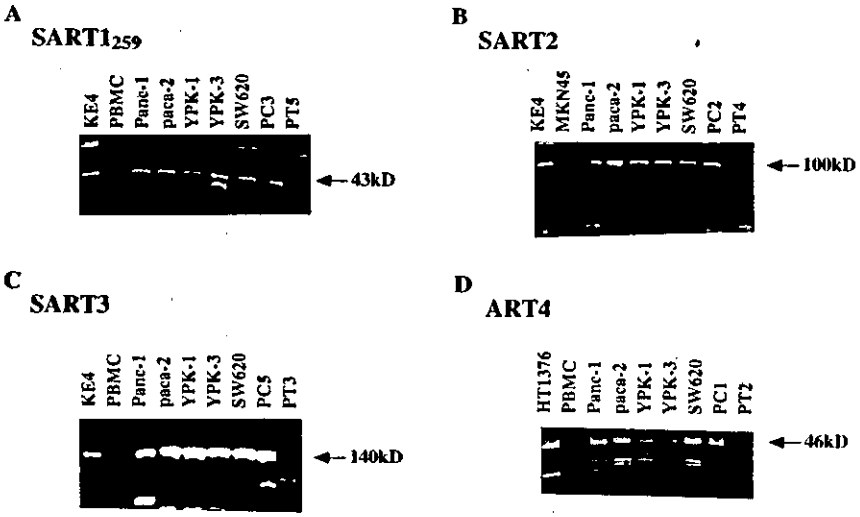

FIGURE 1 – Expression of the $SART1_{259}$, SART2, SART3 and ART4 antigens. Expression of $SART1_{259}$, SART2, SART3 and ART4 antigens in various samples of pancreatic cancer cell lines, pancreatic cancer tissues and nontumorous pancreatic tissues was investigated by Western blot analysis using polyclonal antibodies, as reported previously.[8-15] (*a*) Representative results of $SART1_{259}$ antigen expression in the cytosolic fraction are shown. Samples were KE4 (a positive control), PBMCs (a negative control), pancreatic cancer cell lines (Panc-1, paca-2, YPK-1 and YPK-3), a colon cancer cell line (SW620), pancreatic cancer tissue (PC3) and nontumorous pancreatic tissue (PT5). (*b*) Representative results of SART2 antigen expression in the cytosolic fraction are shown. Samples were KE4 (a positive control), MKN45 (a negative control), pancreatic cancer cell lines (Panc-1, paca-2, YPK-1 and YPK-3), a colon cancer cell line (SW620), pancreatic cancer tissue (PC2) and nontumorous pancreatic tissue (PT4). (*c*) Representative results of SART3 antigen expression in the cytosolic fraction are shown. Samples were KE4 (a positive control), PBMCs (a negative control), pancreatic cancer cell lines (Panc-1, paca-2, YPK-1 and YPK-3), a colon cancer cell line (SW620), pancreatic cancer tissue (PC5) and nontumorous pancreatic tissue (PT3). (*d*) Representative results of ART4 antigen expression in the nuclear fraction are shown. Samples were HT1376 (a positive control), PBMCs (a negative control), pancreatic cancer cell lines (Panc-1, paca-2, YPK-1 and YPK-3), a colon cancer cell line (SW620), pancreatic cancer tissue (PC1) and nontumorous pancreatic tissue (PT2).