

Adoptive immunotherapy for pancreatic cancer: Cytotoxic T lymphocytes stimulated by the MUC1-expressing human pancreatic cancer cell line YPK-1

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Abstract. MUC1 is a tumor-associated antigen that is overexpressed in invasive ductal carcinomas of the pancreas (PC). MUC1-specific cytotoxic T lymphocytes (CTLs) recognize MUC1 molecules in a HLA-unrestricted manner. In this study, we performed adoptive immunotherapy (AIT) in patients with PC with CTLs stimulated by the MUC1-expressing human PC cell line YPK-1. To induce CTLs, peripheral blood mononuclear cells (PBMCs) were cultured for 3 days with inactivated YPK-1 cells and then stimulated with interleukin (IL)-2 for 7 days. The cytotoxicity of these cells against human cancer cell lines was analyzed, and a variety of antibodies were evaluated for their ability to inhibit cytotoxicity. We treated 8 patients with unresectable PC and 20 patients with resectable PC postsurgically. CTLs were induced as described above, suspended in 100 ml saline and injected intravenously. Induced CTLs were cytotoxic against 5 MUC1-expressing PC cell lines and a breast cancer cell line, regardless of the HLA phenotype. Low cytotoxicity was observed in 7 MUC1-negative cancer cell lines. Anti-CD3 monoclonal antibody (mAb) or anti-CD8 mAb strongly inhibited cytotoxicity against YPK-1, whereas anti-class I mAb showed no inhibition. YPK-1 cells incubated with anti-MUC1 mAb also showed low cytotoxicity. Clinically, the median survival time was 5.0 months for patients with unresectable PC treated with AIT. None of the 5 patients without liver metastasis showed hepatic recurrence. The median survival time was 17.8 months for 18 out of 20

patients with resectable PC who underwent curative surgery, and the 1-, 2- and 3-year survival rates after surgery were 83.3, 32.4, and 19.4%, respectively. Liver metastasis was found in only one patient and no side effects of AIT were observed. CTLs stimulated by a MUC1-expressing human pancreatic cancer cell line showed a strong tumor cytotoxic activity in a MUC1-specific and MHC-unrestricted manner. AIT with stimulated CTLs significantly suppressed the post-surgical hepatic recurrence of PC. Adjuvant immunotherapy with CTLs may be useful in the postsurgical treatment of PC.

Introduction

Pancreatic cancer (PC) has the poorest prognosis among gastrointestinal cancers because of its low resectability, malignant behavior and low sensitivity to anticancer agents (1). Treatments for PC, including surgery, chemotherapy and radiotherapy, have failed to improve its prognosis (2,3). Metastasis to the liver is the most important factor underlying poor prognosis. New treatment strategies are therefore necessary.

Immunotherapy has an advantage over radiation therapy and chemotherapy because it can act specifically against the tumor without damaging normal tissue. Immunotherapeutic approaches to PC have included the use of monoclonal antibodies (4-7), cytokines (8), vaccine (9) and lymphokine-activated killer (LAK) cells (10). We reported on the efficacy of adoptive immunotherapy (AIT) with cytotoxic T lymphocytes (CTLs), induced from autologous pancreatic tumors but not from AIT with LAK cells (11). Although these immunotherapies have a potential as alternative treatments for PC, the effects have been limited.

Mucins are large glycoproteins that are expressed by a variety of normal and malignant epithelial cells (12). Although at least 16 different mucin polypeptides (apomucins), MUC1-16, have been identified in human tissues (13), MUC1 is unique in its transmembrane expression at the cell surface (14). Cancer-associated MUC1 is incompletely glycosylated, with truncated carbohydrate chains composed largely of 1-6 sugar units (15). As a result of incomplete glycosylation, cancer-associated MUC1 possesses exposed internal sugar units and

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naked peptide sequences that are cryptic in the normal mucin molecule (16). CTLs against MUC1 have been induced in patients with pancreatic, breast or ovarian cancer (17-19). Notably, unlike conventional CTLs, these CTLs recognize MUC1 molecules in a HLA-unrestricted manner. It has been proposed that normally cryptic CTL epitopes in the MUC1 core protein are unmasked by underglycosylation in tumor cells and that highly multivalent epitopes of tandemly repeated peptides on a single MUC1 molecule crosslink the T cell receptors (TCRs) of anti-MUC1 CTLs (16). Such immune responses against MUC1 may be useful for immunotherapy. It has been reported that MUC1 apomucin is the major type of mucin in invasive ductal carcinoma of the pancreas (20-23). Therefore, MUC1 may serve as a target antigen for the treatment of PC. We have reported on the use of AIT with CTLs induced from a MUC1-expressing human PC cell line, YPK-1, for unresectable PC (24,25). The objective of this study was to further analyze CTLs stimulated by YPK-1 cells and to evaluate the clinical efficacy of AIT with this type of CTL for unresectable and resectable PC.

Materials and methods

Cell lines. Human cancer cell lines used in the experimental study were: PC cell lines YPK-1, -2, -3 and -4, Panc 1 and MIA PaCa 2; the breast cancer cell line YMMK-1 and the esophageal cancer cell lines YES-1, -2, -3, -4, -5 and -6. YPK-1, -2, -3 and -4; YMMK-1 and the 6 esophageal cancer cell lines were established in our department. Panc1 and MIA PaCa 2 cell lines were obtained from the Health Science Research Resources Bank, Osaka, Japan. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) in 5% CO₂. MUC1 expression and HLA typing for each cell line are detailed in Table I.

CTL induction. We used peripheral blood mononuclear cells (PBMCs) obtained from a healthy volunteer, with HLA-A phenotype 24/26. Cells were cultured in serum-free medium (A1M-V) (Gibco, Paisley, Scotland) with the MUC1-expressing pancreatic cancer cell line YPK-1 (HLA-A phenotype 24/02) inactivated with 0.2 mg/ml mitomycin C (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan). The effector to target cell ratio was 1000:1. After 3 days, the cells were cultured with 10 Japan Reference Unit (JRU)/ml interleukin (IL)-2 (Shionogi Pharmaceutical Co., Tokyo, Japan) in a CO₂ incubator for 7 days. The cells induced were used as effector cells.

Cytotoxicity assay. Target cells (1x10⁶/ml) were labeled for 60 min at 37°C with 100 µCi/ml radioactive sodium chromate (⁵¹Cr) (Amersham Japan, Tokyo, Japan). The cells were then washed 4 times in RPMI-1640 medium (Sigma-Aldrich). Labeled cells were resuspended in culture medium (1x10⁵/ml). Effector cells consisting of induced CTLs were suspended at 0.5, 1.0 or 2.0x10⁶/ml. Effector cell suspension (0.1 ml) was added to a microplate (Falcon Plastics, Cockeysville, MD) with 0.1 ml target cells, to yield an effector to target cell ratio of 5:1, 10:1 or 20:1. All experiments were performed in

triplicate. Plates were incubated for 4 h at 37°C in a CO₂ incubator. The amount of ⁵¹Cr released into each well was determined with a γ counter (Auto Well Gamma System ARC-202, Aloka, Tokyo, Japan). The percentage of cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}$$

To measure the spontaneous ⁵¹Cr release of target cells in the absence of effector cells, target cells were mixed with 0.1 ml culture medium. To obtain maximal ⁵¹Cr release, target cells were treated with 0.1 ml 0.1 N hydrochloric acid.

Antibody inhibition of cytotoxicity. Anti-CD3, -CD4, -CD8 and anti-class I monoclonal antibodies (mAb) (each diluted at 1:50) were used for blocking assays and were purchased from Dako Corp., Carpinteria, CA. The MUC1-expressing pancreatic cancer cell line YPK-1 was used as the target cell. Effector cells consisting of induced CTLs were incubated with mAb at the indicated concentrations for 45 min at 37°C, washed 3 times in RPMI-1640 medium and suspended at 2x10⁶/ml. Target cells were labeled with ⁵¹Cr as described above, washed 4 times and resuspended in culture medium (1x10⁵/ml). Effector cell suspension (0.1 ml) was added with 0.1 ml target cells to yield a 20:1 effector to target cell ratio for cytotoxicity assays as described above.

For anti-MUC1 mAb blocking, target cells were preincubated for 1 h at 37°C with anti-MUC1 mAb MY.1E12 (diluted at 1:200), kindly provided by Dr Tatsuo Irimura, Department of Cancer Biology and Molecular Immunology, Faculty of Pharmaceutical Sciences, University of Tokyo, Japan. Cytotoxicity assays were performed as described above.

Analysis of CTL subsets. Induced CTL subsets were analyzed with the use of mAbs against surface antigens of human lymphocytes. All mAbs were purchased from Coulter Immunology (Hialeah, FL). Fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (T3), -CD4 (T4), -CD20 (B1), -CD25 (IL-2R1), -CD56 (NKH-1), -HLA-DR (I2) and anti-CD11b (MO1) were used. Phycoerythrin (PE)-conjugated anti-CD8 (T8) and TQ1 (cluster unknown) were also used. A two-color analysis was performed with a combination of TQ1/CD4 (suppressor-inducer T and helper T cell) and CD8/CD11b (cytotoxic T and suppressor T cell). Samples were analyzed with an EPICS flow cytometer (Coulter Electronics, Inc., Hialeah, FL) at a fluorescence excitation wavelength of 488 nm at 200-500 mW. For each sample, 5000 lymphocytes were analyzed.

Patients with unresectable PC. During the period 1995-1997, we treated 8 patients with unresectable PC who had not undergone chemotherapy (Table II). The treatment was performed at the Department of Digestive Surgery and Surgical Oncology (Department of Surgery II), Yamaguchi University Graduate School of Medicine. Three patients showed liver metastasis and three showed peritoneal dissemination. Two patients showed lung metastasis but no liver metastasis or peritoneal dissemination. TNM staging according to the UICC (26) was IV for all patients, and all

Table I. Cytotoxicities of CTLs stimulated by the MUC1-expressing human pancreatic cancer cell line, YPK-1, against a variety of human cancer cell lines.

Cancer cell lines	MUC1- expression	HLA typing			Cytotoxicity (%) E:T = 20:1
		A	B	Cw	
Pancreas					
YPK-1	+	24/02	46/01	03/-	49.0
YPK-2	+	24/02	39/03	12/-	47.3
YPK-3	+	02/01	13/48	03/08	56.8
YPK-4	+	26/10	40/-	03/-	49.6
Panc-1	+	02/11	38/01	12/03	54.3
MIA PACA 2	-	24/02	46/01	03/-	10.0
Breast					
YMMK-1	+	NE	NE	NE	41.2
Esophagus					
YES-1	-	02/01	15/11	03/-	14.8
YES-2	-	24/-	40/01	03/07	18.4
YES-3	-	24/04	15/01	01/-	17.3
YES-4	-	24/02	39/01	07/-	7.2
YES-5	-	02/01	15/11	03/-	11.8
YES-6	-	24/02	15/01	03/04	8.1

CTLs induced from a healthy volunteer with the HLA-A phenotype 24/26 were used as effector cells. Cytotoxicity was assayed by a 4-h ⁵¹Cr release assay. The values represent the means of percentage-specific lysis from triplicate determinations at an effector to target ratio of 20:1. The probable error was in all cases <10%. NE, no expression.

Table II. Characteristics and survival of the patients with unresectable pancreatic cancer.

No.	Age (years)	Gender	Location	T	H	P	Cell no. of CTLs (x10 ⁹)	Survival M
1	53	M	Pbt	T4	-	+	28.0	5
2	50	M	Pb	T2	-	+	15.0	7
3	59	M	Pbt	T4	+	-	10.0	4
4	65	M	Pbt	T3	+	-	0.6	8
5	73	M	Ph	T2	+	-	5.0	3
6	49	M	Ph	T3	-	+	4.0	2
7	61	M	Ph	T3	-	-	18.0	8
8	62	M	Ph	T3	-	-	12.0	3

Ph, head of the pancreas; Pb, body of the pancreas; Pbt, body and tail of the pancreas; T, primary tumor stage; H, liver metastasis and P, peritoneal dissemination. TNM staging according to the UICC was IV for all patients. The HLA-A phenotypes of all patients were not checked.

showed histologically confirmed invasive ductal carcinoma of the pancreas.

Patients with resectable PC. During the period 1997-2004, we treated 20 patients with resectable PC postsurgically. Patient characteristics and treatment are detailed in Table III. TNM stages according to the UICC (26) for these patients were between IA and IIB, and their HLA-A phenotype varied.

Resection was performed in the absence of hematogenous metastases, peritoneal dissemination, gross retroperitoneal tumor infiltration and complex vascular infiltration. A limited invasion of the portal or superior mesenteric veins was regarded as an indication for portal vein resection. All patients received pancreatoduodenectomy (PD), pylorus preserving pancreatoduodenectomy (PPPD) or distal pancreatectomy (DP) with extended retroperitoneal lymphadenectomy. Of the

Table III. Characteristics and treatment of the patients with resectable pancreatic cancer.

No.	Age (years)	Gender	HLA-A	TNM stage	Operation	Resection of portal vein	IORT
1	66	F	24/02	IA	DP	-	+
2	73	F	02/01	IIA	PPPD	-	-
3	66	M	24/02	IIB	DP	-	-
4	45	F	24/02	IIA	PPPD	+	+
5	73	M	24/02	IIB	DP	-	-
6	69	F	26/01	IIB	PD	+	+
7	55	M	11/33	IIB	PPPD	-	-
8	72	F	02/-	IIB	PPPD	+	+
9	66	M	02/31	IIB	PD	-	+
10	59	M	24/31	IIB	PD	-	+
11	60	M	02/11	IIB	DP	+	+
12	68	M	02/31	IIB	PD	+	+
13	67	F	11/26	IIB	PD	-	+
14	67	M	26/-	IIB	PD	+	+
15	54	M	02/24	IIB	DP	+	+
16	71	F	24/-	IIA	PD	+	-
17 ^a	78	M	02/-	IIB	PD	-	-
18	72	M	02/24	IB	PPPD	-	-
19 ^a	60	M	26/31	IB	DP	-	-
20	69	F	02/26	IIB	PD	-	-

^aTwo patients received non-curative resection because of the microscopic involvement of the resection margin (R1). PD, pancreatoduodenectomy; PPPD, pylorus preserving pancreatoduodenectomy; DP, distal pancreatectomy and IORT, intraoperative radiotherapy. TNM staging is according to the UICC.

patients who underwent resection of the portal vein, 7 out of 8 showed histological invasion. Eighteen patients underwent curative resection (R0). Two patients underwent non-curative resection, because the microscopic involvement of the resection margin (R1) was found. All patients had histologically confirmed invasive ductal carcinoma. Histological classifications were: well-differentiated adenocarcinoma for 4 patients, moderately for 13 patients, poorly for 1 patient and mucinous carcinoma for 2 patients. Intraoperative radiation (20-30 Gy) therapy was performed in 11 cases in which retroperitoneal tumor infiltration was suspected.

CTL induction. PBMCs were harvested with the COBE Spectra Apheresis System (COBE BCT, Inc., Lakewood, CO). CTLs were expanded from these PBMCs as described above, and cultures were checked for bacterial contamination. Induced CTLs were washed once, suspended in 100 ml saline and administered intravenously.

Schedule of AIT using CTLs: Patients with unresectable PC. CTLs were administered 1-11 times and the total number of CTLs administered were between 0.6×10^9 and 2.8×10^{10} (Table II). This study protocol was approved by the Institutional Review Board for Human Use at the Yamaguchi University School of Medicine in 1995. Written informed

consent was obtained from all patients. Tumors were evaluated by computed tomography (CT), magnetic resonance imaging (MRI) or autopsy.

Patients with resectable PC. CTLs were induced before surgery and administered intravenously within a week after surgery. After the patient recovery from surgery, CTLs were re-induced and administered several times. The total number of CTLs administered are shown in Table IV. Eight patients were treated twice; six, 3 times; two, 4 times; two, 5 times; one patient was treated 6 times and one, 8 times. The total number of CTLs administered were between 5.0×10^8 and 2.5×10^{10} . The treatment of 16 patients was completed within 2 months after surgery, that of 2 patients was completed within 3 months after surgery and that of 2 patients was completed within 4 months after surgery. Recurrence was evaluated by CT, MRI or autopsy. None of the patients received chemotherapy. This study protocol was approved by the Institutional Review Board for Human Use at the Yamaguchi University School of Medicine in 1997. Written informed consent was obtained from all patients.

Statistical analysis. Changes in surface markers were assessed using Student's t-test for paired or unpaired means. A P-value of <0.05 was considered significant. Values are presented as

Table IV. Total number and times of CTL transfer, recurrence sites and survival for the patients with resectable pancreatic cancer.

No.	Total cell no. (x10 ⁹)	Times of transfer	Recurrence sites			Survival
			Liver	Local	Other	
1	2.7	3	-	+	-	27 M Deceased
2	25.0	4	-	+	-	24 M Deceased
3	15.0	2	-	-	Lung, bone	13 M Deceased
4	12.0	4	-	+	-	21 M Deceased
5	6.0	3	-	+	Lung, bone	20 M Deceased
6 ^a	0.7	2	-	+	-	4 M Deceased
7	2.4	8	-	+	Peritoneum	15 M Deceased
8	2.2	5	-	+	Lymph nodes	16 M Deceased
9	2.7	5	-	-	-	74 M Alive
10	1.1	2	-	-	-	35 M Alive
11	0.7	3	-	+	-	16 M Deceased
12	0.6	2	+	+	-	6 M Deceased
13	1.0	2	-	-	-	49 M Alive
14	0.8	2	-	+	Peritoneum	15 M Deceased
15	1.8	3	-	+	Peritoneum	9 M Deceased
16	0.5	2	-	-	Bone	15 M Deceased
17	3.6	3	-	+	-	9 M Alive
18	11.0	6	-	-	-	20 M Alive
19	3.6	2	-	+	Peritoneum	12 M Deceased
20	6.6	3	-	-	Lymph nodes	14 M Deceased

^aTumor differentiation of patient No.6 was poor. This was the only case of tumor differentiation found in 20 patients.

mean \pm SE. A Kaplan-Meier analysis was used to estimate the cumulative survival.

Results

CTL cytotoxicity. CTLs were cytotoxic against 5 MUC1-expressing PC cell lines and 1 breast cancer cell line regardless of the HLA phenotype (Table I). Low cytotoxicity was observed against 7 MUC1-negative cancer cell lines, including the PC cell line MIA PACA 2 and 6 esophageal cancer cell lines. CTL cytotoxicity against PC cell lines (YPK-1 and -3) and esophageal cancer cell lines (YES-1 and -2) is shown in Fig. 1. Cytotoxicity was MHC-unrestricted and clearly decreased with the decreasing effector cell number.

Antibody inhibition of cytotoxicity. Anti-CD3 mAb or anti-CD8 mAb inhibited CTL cytotoxicity against YPK-1 cells (Fig. 2). Anti-MUC1 mAb also inhibited cytotoxicity in these cells. Anti-class I mAb showed no inhibition of CTL cytotoxicity (E:T = 20:1; anti-CD3, 66.5%; -CD4, 26.9%; -CD8, 76.9%; anti-class I, 11.8% and anti-MUC1, 64.1%).

Patients with unresectable PC. AIT with CTLs did not improve the median survival time (MST) in patients with unresectable PC. MST was 5.0 months (Table II). None of

the 5 patients without liver metastasis developed liver metastasis and no side effects of AIT with CTLs were observed.

Patients with resectable PC. The survival rate of 18 out of 20 PC patients that underwent curative resection is shown in Fig. 3. The survival time ranged from 4 to 74 months, and MST was 17.8 months. One-, 2- and 3-year survival rates after resection were 83.3, 32.4 and 19.4%, respectively. No side effects of AIT with CTLs were observed. Liver metastasis was found in only 1 patient (5.0%) and local recurrence was found in 13 patients (65.0%) (Table IV).

Changes of lymphocyte subsets before and after the culture of PBMCs are detailed in Table V. The proportions of CD3⁺, CD4⁺, suppressor-inducer T and cytotoxic T cells increased significantly after treatment ($P < 0.05$), whereas the proportions of IL-2 receptor-positive, CD56⁺ (NK cells), HLA-DR⁺ and suppressor T cells decreased significantly ($P < 0.05$).

Discussion

Despite intensive efforts by many groups to improve the efficacy of conventional chemotherapy or chemoradiotherapy for PC, little progress has been made (2,3). In Japan, PC is the fifth leading cause of cancer death in men and the sixth in women, and the incidence increases yearly (27,28). The

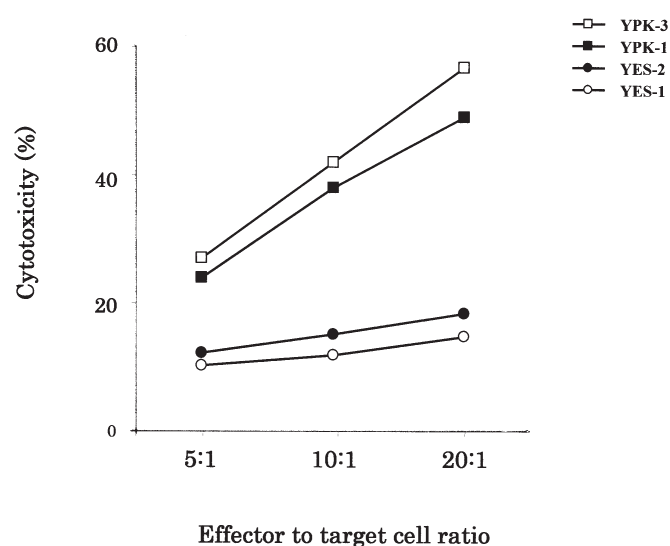


Figure 1. Cytotoxicity of CTLs induced from a healthy volunteer with the HLA-A phenotype 24/26. CTLs were stimulated by the MUC1-expressing human pancreatic cancer cell line, YPK-1 (HLA-A 24/02, MUC1-positive). Target cell lines were YPK-1, YPK-3 (pancreatic cancer, HLA-A 02/01, MUC1-positive), YES-2 (esophageal cancer, HLA-A 24/-, MUC1-negative) and YES-1 (esophageal cancer, HLA-A 02/01, MUC1-negative). Induced CTLs were cytotoxic against MUC1-expressing pancreatic cancer cell lines regardless of the HLA-A phenotype. Low cytotoxicity was observed in MUC1-negative esophageal cancer cell lines. Cytotoxicity was MHC-unrestricted and clearly decreased with the decreasing effector cell number.

prognosis is very poor, 80% of patients are not eligible for surgical resection because of local spread or metastatic disease at the time of diagnosis. Even with curative surgery, MST does not exceed 2 years, with a 5-year survival of 15-25% (29-32). New treatment strategies are necessary to improve the outcome of patients with PC.

We have reported on the partial effectiveness of AIT with CTLs induced from tumor-infiltrating (TILs) or peripheral lymphocytes stimulated by autotumor cells in unresectable and in recurrent PC, and on the ineffectiveness of AIT with LAK cells (11). However, AIT with CTLs stimulated by autologous tumors showed limited feasibility. It is technically difficult to isolate and expand autologous tumor cells of most histological tumor types, including PC. In addition, CTLs induced by autologous tumors are used as therapies for individual patients and cannot be generalized to all patients with the same type of cancer. To overcome these limitations, we developed an AIT with CTLs stimulated by a MUC1-expressing human PC cell line.

MUC1 is a target antigen for tumor-reactive CTLs from pancreatic, breast and ovarian cancers and multiple myeloma (17,33). MUC1-reactive T cells from patients with breast cancer, PC or multiple myeloma directly recognize MUC1 in an MHC-unrestricted manner (18,19,33-39). However, there is increasing evidence from mouse and human studies that T cells induced against the MUC1 protein can be MHC-restricted (40-45). We induced CTLs with a PC cell line expressing a high level of MUC1 and YPK-1 (25). Blocking assays showed that the anti-tumor activity of these cells was strongly inhibited by anti-CD3 or -CD8 mAbs, but not by anti-class I mAbs, indicating that these cells contain CTLs

Table V. Changes of lymphocyte subsets before and after the culture of PBMCs.

Subset	Before (%)	After (%)	P-values
CD3	36.7±10.1	77.0±15.0	P<0.05
CD4	42.1±9.9	52.1±10.6	P<0.05
CD8	28.2±9.0	28.5±11.6	NS
CD20	14.2±8.0	7.2±4.8	NS
CD25	20.1±8.3	14.0±8.7	P<0.05
CD56	24.0±10.6	14.3±6.6	P<0.05
HLA-DR	15.8±6.4	10.4±6.7	P<0.05
Tsi (TQ1+/CD4+)	32.7±7.6	41.7±10.0	P<0.05
Th (TQ1-/CD4+)	9.4 ±5.2	10.5±5.8	NS
Ts (CD8+/CD11b-)	7.2±4.0	1.5±1.6	P<0.05
Tc (CD8+/CD11b+)	21.1±8.1	27.1±10.8	P<0.05

Tsi, suppressor-inducer T ; Th, helper T ; Ts, suppressor T and Tc, cytotoxic T cells. Each specimen was 19 pairs derived from 6 patients who had undergone pancreatectomy. NS, non-significant.

and that the cytotoxicity was MHC-unrestricted. The stimulated cells showed a strong cytotoxicity against MUC1-expressing PC and the breast cancer cell lines but not against non-MUC1-expressing cell lines. Cytotoxicity against YPK-1 cells treated with anti-MUC1 mAb was greatly decreased. Taken together, these data indicate that induced CTLs kill target cells in a MUC1-specific and MHC-unrestricted manner. These results are consistent with those of Jerome *et al* (18). The mechanism of cytotoxicity in an MHC-unrestricted manner is poorly understood. Induced cells contain not only CTLs recognizing MUC1 but also LAK, natural killer (NK) and natural killer T (NKT) cells. However, a subset analysis of induced cells showed that the proportion of cytotoxic T cells was high. CTLs recognizing MUC1 may be a predominant cell type among induced cells.

We have reported that invading or metastatic PC cells express MUC1 all along the cell membrane (46). We therefore hypothesized that these CTLs may be used therapeutically for PC. We treated 8 patients with unresectable PC with AIT using these CTLs. Although AIT did not improve survival, none of the 5 patients with unresectable PC and without liver metastasis developed liver metastasis. No adverse effects were observed. It has been reported that the postoperative survival time for PC patients with hepatic recurrence is significantly shorter than that of patients with local recurrence (47,48). Our clinical results suggest that AIT with MUC1-specific and MHC-unrestricted CTLs may prevent the liver metastasis of PC. We therefore applied this

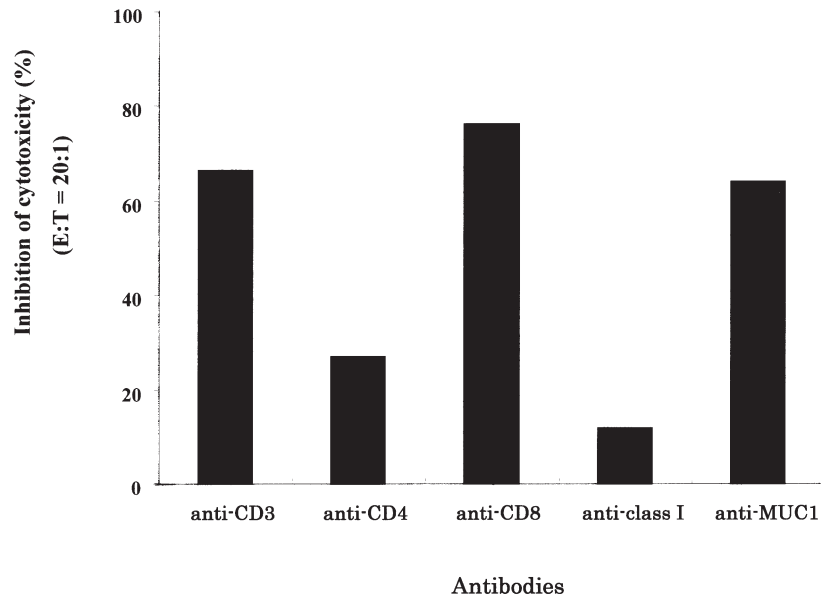


Figure 2. Inhibition of CTL-induced cytotoxicity by monoclonal antibodies (mAb). Anti-CD3 or -CD8 mAb strongly inhibited cytotoxicity against YPK-1 cells, whereas anti-class I mAb showed no inhibition. YPK-1 cells treated with anti-MUC1 mAb also showed a low cytotoxicity.

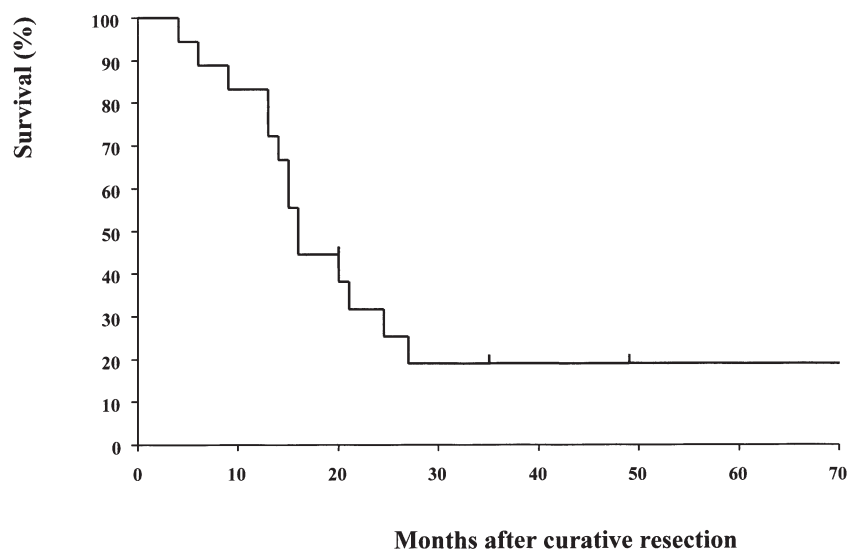


Figure 3. Kaplan-Meier estimates of the cumulative survival after curative resection in patients with pancreatic cancer (18 patients). The 1-, 2- and 3-year survival rates were 83.3, 32.4 and 19.4%, respectively.

AIT as an adjuvant therapy in PC patients who underwent curative resection because hepatic recurrence is an important factor for postsurgical survival. It has been reported that occult liver metastasis is often present in PC patients at the time of pancreatectomy (49,50), suggesting that an adjuvant therapy for PC should be performed in the early postoperative period. We therefore administered induced CTLs intravenously within 7 days after surgery. CTLs were re-induced and administered several times after the patient's recovery. Of the patients treated with this AIT, 19 out of 20 showed no hepatic recurrence regardless of the HLA phenotype. Kobari *et al* (51) reported suppressed liver metastasis with AIT with the

intraportal infusion of LAK cells after pancreatectomy. However, we did not find efficacy of LAK therapy for PC (11). It has been reported that intravenously injected peripheral blood lymphocytes and LAK cells are taken up mostly by the liver and spleen (52). Thus, CTLs may distribute mainly to the liver. The mechanism whereby intravenous administration of CTLs suppress liver metastasis remains to be elucidated.

In our clinical study, MST of adjuvant immunotherapy with induced CTLs was 17.8 months. The 1-, 2- and 3-year survival rates after resection were 83.3, 32.4 and 19.4%, respectively. Adjuvant immunotherapy proved to be better

than surgery alone, which our group had performed in our institute before 1996 (MST: 14.0 months, 1-, 2- and 3-year survival rates after resection were 60.6, 21.2 and 12.9%; data not shown). The MST of AIT with CTLs was similar to other adjuvant chemotherapies and chemoradiotherapies (153-56). Some clinical trials of adjuvant chemotherapy for patients with resectable PC using gemcitabine were reported (57,58), and it may be better than other chemotherapies. However, adjuvant chemotherapies and chemoradiotherapies including gemcitabine frequently cause severe toxicities. The advantage of AIT using CTLs is that treatment can be completed within 2 months after surgery and even after discharge from the hospital, treatment can be continued with no complications. New treatment strategies are necessary to suppress local recurrence and to further increase the survival time obtained with AIT.

In conclusion, CTLs stimulated by a MUC1-expressing human PC cell line show strong cytotoxicity in a MUC1-specific and MHC-unrestricted manner. AIT with these CTLs significantly suppresses the postsurgical hepatic recurrence of PC. Therefore, this immunotherapy may be useful as an adjuvant treatment.

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