Table IV. Phenotypes and cytokine production profiles of ascites cells after *in vivo* OK-432 administration and *in vitro* IL-2 stimulation.

Phenotype and cytokine	Pt. 1	Pt. 2	Pt. 3
Phenotype (%)			
CD3	95	90	96
CD4	91	89	90
CD8	16	13	7
CD56	13	17	ND
Cytokine (pg/ml)			
TNF-α	3810	2710	883
IFN-γ	2000	1044	954
IL-4	8	12	<4
IL-6	3	20	11

Ascites cells were collected after OK-432 immunotherapy and further stimulated with IL-2 *in vitro*. Phenotypic analysis for activated cells was performed on Cytoron, and cytokine concentrations in culture supernatant of activated cells were determined by ELISA specific for Th1, Th2 cytokines as indicated. Pt., patient; ND, not determined.

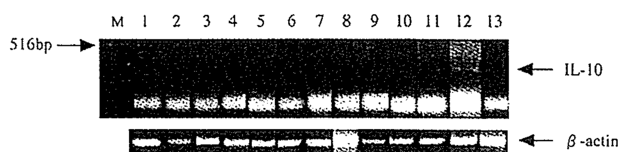

Figure 6. IL-10 mRNA expression in ascites cells before OK-432 immunotherapy. mRNA was extracted from ascites cells of gastric cancer patients before OK-432 immunotherapy, and IL-10 expression was evaluated by RT-PCR analysis using primers specific for human IL-10.

70% of patients were successfully treated by the locoregional administration of OK-432, even when chemotherapy had failed to regulate ascites. Although it has been reported that patients with malignant ascites have an extremely poor prognosis and that the mean survival period of patients with malignant ascites is approximately 78 days (16), there is considerable difference between the quality of the patients' limited time with and without malignant ascites, since these ascites can cause a full sensation in the abdomen, anorexia and dyspnea, all of which typically decrease patients' QOL (7,16). In the present study, approximately half of the patients who responded to the OK-432 immunotherapy showed an improvement in performance status, symptoms and oral food intake without notable side effects. We would like to emphasize that OK-432 locoregional immunotherapy is practical, effective and very significant in improving the QOL of patients with malignant ascites.

We have chosen to measure DTH skin reactions to OK-432 in order to address the characteristics of responder patients to OK-432 immunotherapy. It was observed that there was quite different responsiveness in the DTH reactions to OK-432 among the patients tested, indicating that each patient may show any of a fairly wide range of different reactions and clinical responses when administered OK-432. Interestingly, a positive relationship was demonstrated between the clinical efficacy of OK-432 immunotherapy and the DTH reactions. The DTH reaction has been previously reported to be one of the most popular parameters when establishing the immunological status of cancer patients (17,18). In a study of post-operative adjuvant chemoimmunotherapy, protein-bound polysaccaride PSK has been shown to have a survival benefit in gastric cancer patients who have positive DTH reactions to purified protein delivertives (19). We suggest that the DTH reactions reflect the sustained immune responsiveness levels of the host and that this sustained immune responsiveness is the minimal condition for the success of locoregional immunotherapy using OK-432 for malignant ascites from gastric cancer.

Because DTH reaction levels to OK-432 indicate patients' sustained immune responsiveness to OK-432 and because these levels differ among patients with malignant ascites, it may be possible to use the DTH reaction to determine the optimal dose of OK-432 for OK-432 immunotherapy for each individual patient. Our previous experiences with the locoregional administration of OK-432 in treating malignant ascites showed that fever elevation was often an adverse effect of this type of treatment. We hypothesized that patients who showed a significant adverse effect needed much less OK-432 than the empirical dose (5 KE), and that patients who had no response needed more. Therefore, we conducted a pilot study in which a DTH-oriented dose of OK-432 (1-10 KE) was administered to each patient. It was found that the DTH-oriented approach demonstrated significantly high efficacy in comparison with the empirical administration of OK-432. Moreover, significantly fewer adverse effects were observed in patients who received a DTH-oriented dosage of OK-432. Talmadge *et al* (20) have reported that the dose of IL-2 is critical and that a high dose of IL-2 does not always result in successful immunotherapy in a mouse tumor model. We suggest that there is an optimal dose of OK-432 for individual treatment of malignant ascites, and that the DTH-oriented administration of OK-432 is a highly effective treatment. If it is true that an optimal dose of therapeutic agents exists in immunotherapy for cancer, it is critical that it should not be decided uniformly, nor by patients' body weight or surface area, but by patients' responsiveness to the agents. This study provides a possibility of tailored immunotherapy for malignant ascites.

We next measured the cytokine production profiles of ascites cells by whole blood/ascites assay. It was demonstrated that the TNF-α production of ascites cells was stimulated *in vitro* with OK-432 and that these responses correlated well with clinical responses, indicating that not only the DTH reaction but also the measurement of *in vitro* TNF-α production of ascites cells with OK-432 is a good indicator for clinical responses to OK-432 immunotherapy. Cytokine production profiles have been studied in relation to patients' immunity and are usually measured with purified peripheral blood mononuclear cells (PBMCs) (21). It has been reported that a good correlation is obtained in comparing PBMC cultures with the whole blood system if the cell number is taken into account,

and that whole blood culture is a simple and reproducible method for the measurement of mitogen-induced cytokine production (22). Our data strongly suggest that the whole ascites assay using OK-432 is also both simple and reproducible to predict an *in vivo* situation for the purposes of locoregional administration of OK-432.

It should be noted that the TNF-α-producing potential of ascites cells by *in vitro* OK-432 stimulation correlated well with the DTH skin reaction levels to OK-432, indicating that it is possible to predict locoregional TNF-α response to OK-432 administration by the DTH reaction to OK-432. TNF-α may be effectively induced *in vivo* by the locoregional administration of OK-432 in responder patients who showed a strong DTH reaction to OK-432. The DTH reaction is a more convenient and practical method of determining the responsiveness, and does not require any exceptional equipment. It remains to be clarified why the DTH skin reaction levels correlate with the TNF-α production potential of ascites cells. One possible explanation is that both responses belong to the group of Th1 type immune responses (12). It is still unknown, however, why the DTH skin reaction did not correlate with the TNF-α production potential of peripheral blood cells.

It has been demonstrated that OK-432 up-regulates Th1 type cellular immune responses by stimulating IL-12 expression (10). In the present study, we attempted to understand locoregional responses to OK-432 according to the Th1/Th2 concept. We found that responders to locoregional immunotherapy for malignant ascites with OK-432 polarized on the Th1 axis when clinical responses were analyzed with Th1/Th2 dimensions on the basis of cytokine production profiles of ascites cells by *in vitro* OK-432 stimulation. Interestingly, *in vitro* cultivation with IL-2 of ascites cells of responder patients after OK-432 administration was able to stimulate $CD4^+$ cell expansion which did produce Th1 cytokines. These data strongly suggest that positive clinical responses can be obtained in patients in whom OK-432 up-regulates Th1 type responses. In other words, the Th1 dysfunction may exist at the level of the ascites microenvironment. Yoshino *et al* (23) have reported that the Th2 population is relatively dominant in gastric cancer patients even in the small tumor burden. Shibata *et al* (24) have also reported the decreased production of interleukin-12 and the dominance of Th2 immune responses in cachectic patients with colorectal and gastric cancer. Although the results of our study are consistent with these investigations on the Th1/Th2 concept of immunological status in gastric cancer patients, we would like to propose that the ascites microenvironment may be the Th1 dysfunction, rather than Th2 dominance.

If the ascites microenvironment is the Th1 dysfunction, the Th2 cytokine IL-10 may play some roles in patients who show a lack of response to OK-432 immunotherapy. In the present study, we detected IL-10 mRNA expression in the ascites cells of non-responders, but not in those of responders, prior to OK-432 immunotherapy. It has previously been reported that tumor cells of many histological types secrete IL-10 (25) and that IL-10 has immunosuppressive modulating actions, including down-regulation of HLA (26), inhibition of both CD40 expression and CD40-mediated dendritic cell function (27), and suppression of Th1 cytokine production (28), especially of IL-12 (29). Considering this evidence, we suggest that tumor-derived IL-10 may be involved, in part, in the Th1 dysfunction in the ascites microenvironment, and that ascites cells fail to produce Th1 cytokines even under stimulation with OK-432, which can stimulate IL-12 production. Previously, we have demonstrated with an IL-10-secreting murine tumor model that anti-IL-10 antibody enhances the anti-tumor activity of OK-432 (30). This type of approach may augment the clinical efficacy of OK-432 immunothetapy for patients with malignant ascites.

In conclusion, the locoregional immunotherapy using OK-432 is a simple and an effective treatment for malignant ascites in gastric cancer patients. The Th1 dysfunction may be present at the level of the ascites microenvironment, and a positive clinical response can be induced by the up-regulation of Th1 type immune responses by OK-432. IL-10 may be involved, in part, in the Th1 dysfunction. Finally, each patient has a different responsiveness to OK-432, and the DTH skin reaction to OK-432 is an excellent method not only for predicting clinical responsiveness but also for selecting the appropriate dose of OK-432 on an individual basis. This type of approach of the OK-432 immunotherapy improves the QOL even for terminally ill patients with malignant ascites from gastric cancer.

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Enhancing Effect of PS-K on IL-2-induced Lymphocyte Activation: Possible Involvement of Antagonistic Action Against TGF-beta

YOSHIYUKI YAMAGUCHI, KAZUHITO MINAMI, AKIKO OHSHITA,
YOSHIHARU KAWABUCHI, KOSUKE NOMA and TETSUYA TOGE

Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

Abstract. *Effects of protein-bound polysacchraide (PS)-K on interleukin (IL)-2-induced responses of peripheral blood mononuclear cells (PBMCs) were studied. PS-K (50 mcg/ml) was observed to enhance proliferative responses, cytotoxic activities against K562 and Daudi target cells, CD25+ cell population and telomerase activity of PBMCs stimulated with IL-2. The cytotoxic effector cells could be generated in the presence of PS-K even with a minimum amount of IL-2. The enhancing effect of PS-K on the IL-2-induced lymphocyte activation was more evident in PBMCs from cancer patients than in those from healthy volunteers, suggesting that PS-K may be beneficial if combined in the IL-2-based immunotherapy of cancer. TGF-beta inhibited the IL-2-induced lymphocyte activation of proliferative responses, cytotoxic activities and CD25+ cell population, the inhibitions of which were abrogated with PS-K. PS-K also abrogated the TGF-beta-induced anchorage-independent growth of normal rat kidney cells. Flow cytometric analysis using a labeled TGF-beta revealed that PS-K blocked the binding of TGF-beta at its receptor level on the surface of PBMCs. It is suggested that PS-K enhances IL-2-induced lymphocyte activation through, in part, an antagonistic action against TGF-beta.*

Polysaccharide (PS)-K, prepared from *Coriolus vesicolor* of the class Basidiomycetes, has a molecular weight of 50,000 to 100,000 and belongs to biological response modifiers (BRMs) (1, 2). The anti-tumor activity of PS-K was documented in experimental animal models (3, 4) and beneficial therapeutic effects were demonstrated in clinical studies of several types of tumors (5). Recently, it has been published that PS-K has survival benefit in patients with gastric (6, 7) and colorectal (8) cancers after surgery in combination with chemotherapy. The mechanisms of action by which PS-K modifies host biological immune responses have been addressed. It was reported that PS-K had immunopotentiating activities such as the augmentation of depressed natural killer activity in cancer patients (9), the maturation of defective dendritic cell function exposed to tumor-derived factors (10) and the up-regulation of HLA class I expression on tumor cells (11).

We have examined the immunomodulatory activity of PS-K and revealed its unique property of restoring the depressed immune responses in cancer patients (12). This activity was partly explained by its antagonistic action to soluble immunosuppressive factor(s), including immunosuppressive acidic protein (13). It is well established that transforming growth factor (TGF)-beta has a potent immunosuppressive activity (14, 15) and is involved in the regulation system of lymphocyte activation together with interleukin (IL)-2 (16, 17), although it was originally reported that TGF-beta transformed fibroblasts allowing them to grow on soft agar (anchorage-independent growth) (18, 19).

In this report, we attempted to clarify PS-K activities on the IL-2-induced lymphocyte responses and on the immunosuppressive activities of TGF-beta, in order to further understand the action mechanisms of PS-K. We will show its synergistic effect on IL-2-induced lymphocyte responses with up-regulation of IL-2R expression, the effect of which is possibly based on the antagonistic action against TGF-beta.

Materials and Methods

Reagents. PS-K was a kind gift from Kureha Chemical Industry, Tokyo, Japan. It was dissolved in RPMI-1640 medium, filter-sterilized and stored at -20°C until used. IL-2 (TGP-3) was purchased from Takeda Pharmaceutical Co. Ltd., Osaka Japan. A fluorescein isothiocyanate (FITC)-labeled anti-IL-2 receptor alpha chain (IL-2R, CD25) antibody and FITC-labeled TGF-beta (Fluorokine) were purchased from Becton-Dickinson Immune Systems, MP, USA.

Correspondence to: Yoshiyuki Yamaguchi, M.D., Ph.D., Kasumi 1-2-3-, Minami-ku, Hiroshima 734-8553, Japan. Tel: +81-82-257-5869, Fax: +81-82-256-7109, e-mail: shogo@hiroshima-u.ac.jp

Key Words: PS-K, IL-2, lymphocyte proliferation, IL-2R, TGF-beta.

Cells. The erythroleukemic cell line, K562 and Daudi cells (17) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium) in a humidified incubator with 5 % CO_2 at 37°C. Normal Rat Kidney (NRK) cells (18) were maintained in complete DMEM medium under the same conditions mentioned above and transferred twice a week using a standard trypsinization.

Isolation of PBMCs and proliferation assay. Heparinized venous blood was obtained from 5 healthy volunteers or 5 patients with unresectable cancer, including 3 gastric cancer patients with peritoneal metastasis and 2 colo-rectal cancer patients with liver metastasis. Peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll-Conray gradient and washed 3 times with RPMI-1640 medium. PBMCs were incubated in the medium supplemented with 10% heat-inactivated autologous serum, 2 - 2000 U/ml IL-2, in the presence or absence of PS-K. On day 4, cells were pulsed by ^{3}H-TdR and further incubated for 8 h. Cells were harvested and their radioactivity was determined by liquid scintillation counter (Beckmann).

Cytotoxicity assay. The cytotoxic activity of the activated lymphocytes was determined by standard ^{51}Cr releasing assay. In brief, 51Cr-labeled K562 or Daudi target cells (5 x 10^3) and effector lymphocytes (10^5) were cocultured in 96-well round-bottomed microtiter plates (Corning, No.25850) in a volume of 200 μl. After a 4-h incubation, the radioactivity of the supernatants was counted using an auto-gamma scintillation counter (500C, Packard, USA). Spontaneous release was determined in wells containing the target cells alone and maximun release was done by adding 100 μl of 1% Triton X-100 solution over the target cells instead of the effector cells. Cytotoxic activity was calculated from triplicate samples by the following formula: Cytotoxic activity (per cent) = (experimental release [cpm] - spontaneous release [cpm]) / (maximal release [cpm] - spontaneous release [cpm]) X 100.

ELISA specific for soluble IL-2R. Soluble IL-2R (p55) levels in the supernatants of lymphocyte cultivation by IL-2 with or without PS-K were detected by using an enzyme- linked immunosorbent assay (ELISA) kit specific for human soluble IL-2R (Immunothec, France). The assay procedure was performed according to the original instruction and the concentration of soluble IL-2R in the culture supernatants was calculated from the standard curve obtained by known control samples.

Flow cytometry. Fifty μl of the lymphocyte suspension (5 x 10^5) were incubated with a FITC-labeled anti-CD25 antibody at 4°C for 45 min. In some experiments, lymphocytes were also incubated with FITC-labeled TGF-beta in the presence or absence of PS-K. Cells were washed twice with RPMI-1640 medium and resuspended in the same medium. Flow cytometric analysis was performed on Cytron (Ortho Diagnostic Systems, USA). The argon ion laser was operated at 488 nm with 260 mw of power. After being adequately gated on lymphocytes by using forward and side scatter, FITC emission was collected with a 530/30 nm band-pass filter. Data collection was set up to stop when 10,000 events had been analyzed.

Bioassay for TGF-beta activity. TGF-beta activity was measured by the bioassay mentioned in detail elsewhere (20). In brief, NRK cells (80,000 cells / well) were plated in aliquots of 400μl of methylcellulose (1.2% w/v)-containing DMEM medium supplemented with 2% of FCS in 11-mm wells of a 48-well culture dish (Costar). Each well then received 10 ng/ml EGF (Wakunaga, Japan) and 40 μl of the TGF-β controls in the presence of varying concentrations of PS-K. After a 5-day incubation, 3H-TdR was added to a final concentration of 3 μCi / ml and the cells were incubated for an additional 24 h. The methylcellulose was then transferred to eppendorf tubes and diluted with at least 2 volumes of RPMI-1640 medium. The cells were pelleted by centrifugation and washed twice with the medium. DNA synthesis was determined from the incorporation of ^{3}H-TdR into TCA-precipitable materials.

Detection of telomerase activity (TRAP assay). Telomerase activity in PBMCs was measured by the telomeric repeat amplification protocol (TRAP) assay using the TRAPeze^™ Telomerase Detection Kit (Intergen Co., Purchase, NY, USA) (21). Cells (1 x 10^5) were lysed with 20 μl of CHAPS lysis buffer. The extracts were prepared for measurement of telomerase activity. Telomere elongation was conducted at 30°C for 30 min, and polymerase chain reaction (PCR) amplification was achieved with 28 cycles of incubation at 94°C for 30 sec and at 58°C for 30 sec in a PTC-100TM Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA). PCR products (10μl) were separated on 10% polyacrylamide gel electrophoresis. The gels were stained with SYBRTM Green I (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Densitometric measurements were made with the use of an FLA-2000 fluoro-image analyzer (Fuji Co. Ltd., Tokyo, Japan). Telomerase activity was evaluated with TPG units. One TPG unit was equivalent to the enzymatic activity that extended 600 molecules of TS primer with at least 4 telomeric repeats in a 10-min incubation at 30°C.

Statistical analysis. Statistical analysis was conducted by χ^2 test and paired or un-paired Student's *t*-test using StatView software (Version 5) on a Macintosh computer.

Results

PS-K enhances IL-2-induced lymphocyte proliferation and differentiation. First, we measured the proliferative responses of PBMCs stimulated with either 200 U/ml IL-2 alone or IL-2 plus PS-K (Figure 1a). The proliferative response of PBMCs from a healthy volunteer was 35,105±1422 cpm when stimulated with IL-2 alone. This was augmented in a dose-dependent manner by PS-K, and the maximum proliferative response of 38,544±1253 cpm was observed in the presence of 50 mcg/ml PS-K. There was a significant difference between the proliferative responses with and without PS-K ($p<0.05$).

We simultaneously measured the cytotoxic activity of PBMCs against K562 and Daudi target cells by stimulation with IL-2 alone or IL-2 plus PS-K (Figure 1b). Stimulation with IL-2 alone induced 39% cytotoxic activity against Daudi target cells at an effector-to-target ratio of 20. This was augmented in a dose-dependent manner by PS-K, similar to the proliferation experiments shown above, and the maximum cytolysis of 46% was observed in the presence of

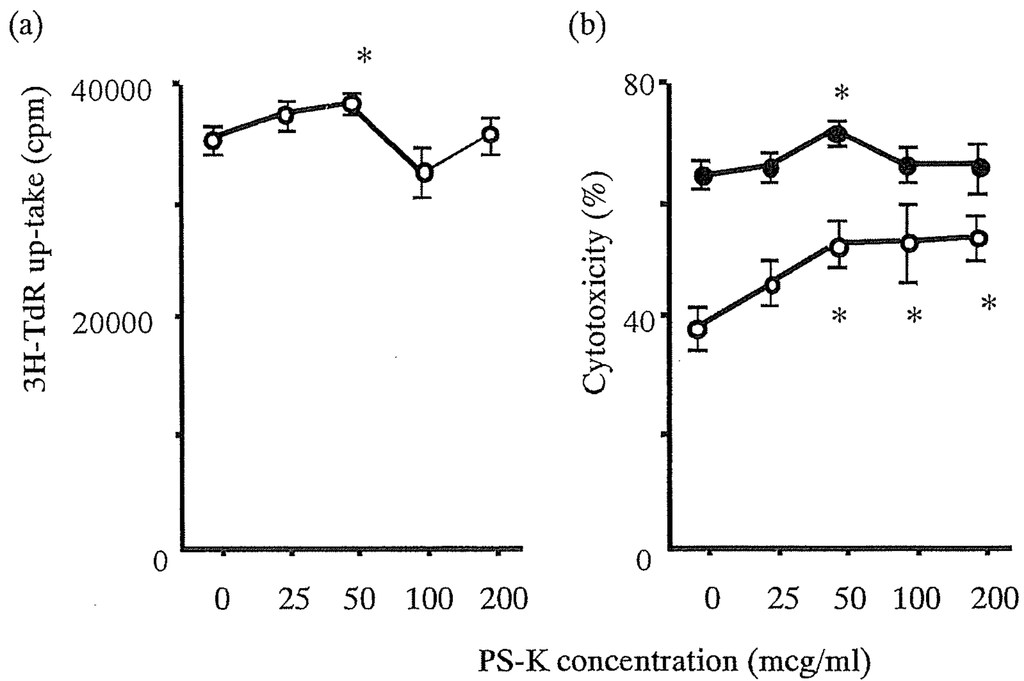

Figure 1. *Proliferative responses and cytotoxic activities of PBMCs stimulated with IL-2 in the presence of PS-K. (a): PBMCs were stimulated with 200 U/ml IL-2 in the presence of 0-200 mcg/ml PS-K and 3H-TdR uptakes of PBMCs were determined. (b): Cytotoxic activities against K562 (●) or Daudi (○) target cells were also analyzed. Significant differences form the value without PS-K, *p<0.05.*

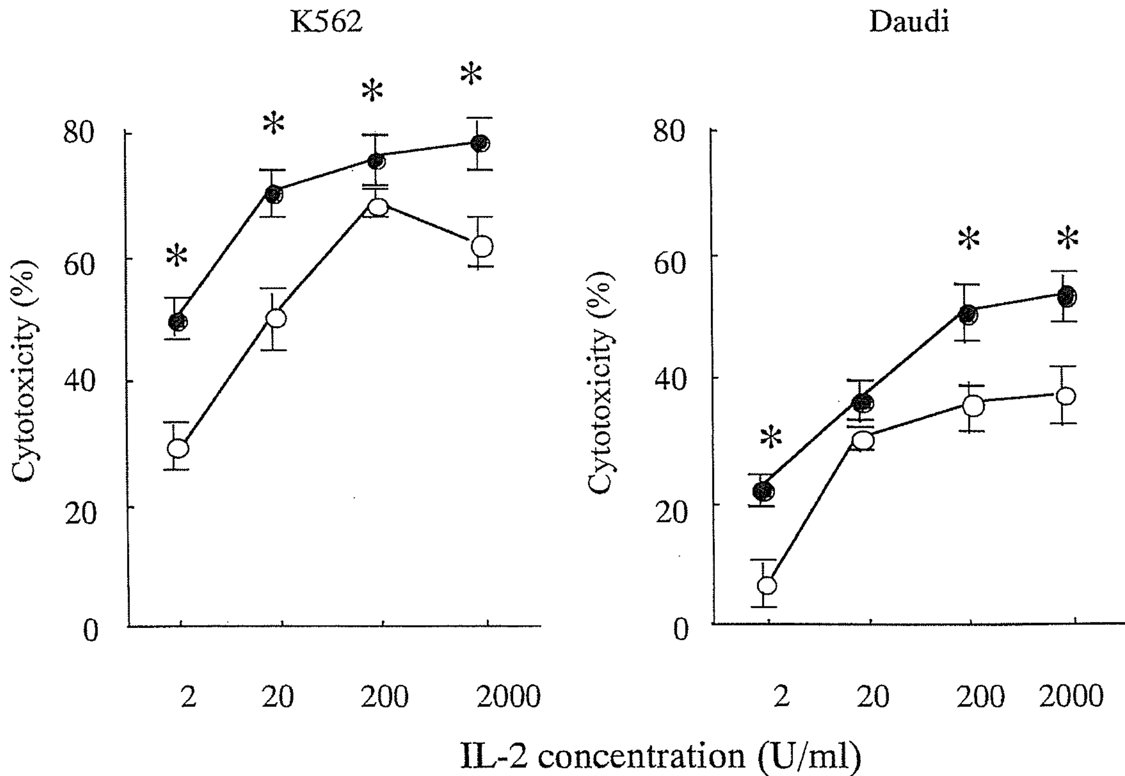

Figure 2. *Requirement of IL-2 for generating killing activity in PBMCs in the presence of PS-K. PBMCs were stimulated with 2-2000 U/ml IL-2 in the presence (●) or absence (○) of 50 mcg/ml PS-K, and cytotoxic activities against K562 or Daudi target cells were determined. Significant differences form the value without PS-K, *p<0.05.*

Table I. *Enhancing effect of CD25+ cell induction and soluble IL-2 Ra chain by PS-K.*

Days in culture		Healthy volunteer		Cancer patient	
		PSK		PSK	
		–	+	–	+
4	CD25+	14.2[a]	18.3 (28.9)	19.1	25.7 (34.8)
	sIL-2Rα	18.2[b]	46.9 (157.7)	4.3	34.5 (702.3)
8	CD25+	27.7	41.4 (49.5)	18.7	39.8 (112.8)
	sIL-2Rα	184.5	342.3 (85.5)	55.9	186.6 (233.8)

PBMCs were stimulated with 200 U/ml IL-2 alone or IL-2 plus 50 mcg/ml PS-K. CD25+ cell population of PBMCs was analyzed with flow cytometry and soluble IL-2Rα chain concentration in the culture of PBMCs was determined with ELISA.
a: CD25+ cell population (%), b: concentration of soluble IL-2Rα chain (pM). Numbers in parentheses indicate percentage of augmentation from values without PS-K.

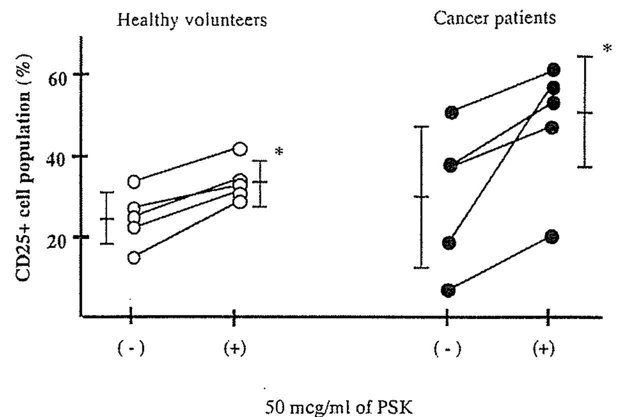

Figure 3. *Increase of CD25+ cell population in PBMCs stimulated with IL-2 plus PS-K. PBMCs from healthy volunteers and cancer patients were stimulated with 200 U/ml IL-2 alone or IL-2 plus 50 mcg/ml PS-K, and CD25+ cell populations were analyzed. Significant differences form the value without PS-K, *p<0.05.*

50 mcg/ml PS-K. There was a significant difference between the cytotoxic activities with and without PS-K ($p<0.05$). Similar results were observed in the cytotoxicity assay using K562 target cells. We decided that 50 mcg/ml PS-K was the optimum concentration for enhancing the proliferation and differentiation of PBMCs in the presence of IL-2.

Requirement of minimum IL-2 for generating killing activity in the presence of PS-K. We next determined the IL-2 concentration required to generate the killing activity in the presence of 50 mcg/ml PS-K (Figure 2). When culturing PBMCs with IL-2 alone, the cytotoxic activities against K562 and Daudi target cells increased in a dose-dependent manner of IL-2 and reached a plateau level at 200 U/ml IL-2. These cytotoxic activities generated with IL-2 alone were markedly enhanced in the presence of 50 mcg/ml PS-K. A significantly high cytotoxic activity was observed in the presence of PS-K even at a very low concentration of 2 U/ml IL-2 ($p<0.05$), which alone could not generate the killing activity of PBMCs against Daudi target cells.

IL-2R expression on PBMCs stimulated with IL-2 plus PS-K. To address the involvement of the IL-2/IL-2R system in the enhancing effect by PS-K of lymphocyte responses to IL-2, the CD25+ cell population of PBMCs and soluble IL-2Rα levels in the culture supernatant were investigated (Table I). On day 4 of the culture, the CD25+ population stimulated with 200 U/ml IL-2 alone was 14% and 19% in PBMCs from healthy volunteers and cancer patients, respectively. These were augmented by the addition of PS-K to 18% and 25% in PBMCs from healthy volunteers and cancer patients, respectively, showing 29 and 35 percent increases from those without PS-K. The augmentation seemed stronger in PBMCs from cancer patients than in those from healthy volunteers according to the percent increase basis. The augmentation of soluble IL-2Rα levels in the culture supernatant was more evident compared with the CD25+ cell population. The soluble IL-2Rα levels after IL-2 stimulation were enhanced in the presence of PS-K from 18 to 47 pM (158% increase) in PBMCs from healthy volunteers and 4 to 35 pM (702% increase) in PBMCs from cancer patients. Similar augmentations were observed in PBMCs stimulated for 8 days with IL-2 alone or IL-2 plus PS-K.

PBMCs from 5 healthy volunteers and 5 cancer patients were stimulated with IL-2 for 4 days and CD25+ cell populations were compared in the presence or absence of 50 mcg/ml PS-K (Figure 3). The CD25+ cell population increased from 24±5% with IL-2 alone to 34±5% with IL-2 plus PS-K in PBMCs from healthy volunteers. This augmentation of CD25+ cell populations by stimulating PBMCs with IL-2 plus PS-K was more evident in PBMCs from cancer patients. The CD25+ cell population was enhanced from 32±13% with IL-2 alone to 50±12% with IL-2 plus PS-K in PBMCs from cancer patients. There were significant differences between the values with and without PS-K in both healthy volunteers and cancer patients ($p<0.05$).

Telomerase activity of lymphocytes stimulated with IL-2 plus PS-K. PBMCs were stimulated with 200 U/ml IL-2 in the presence or absence of PS-K and telomerase activity was

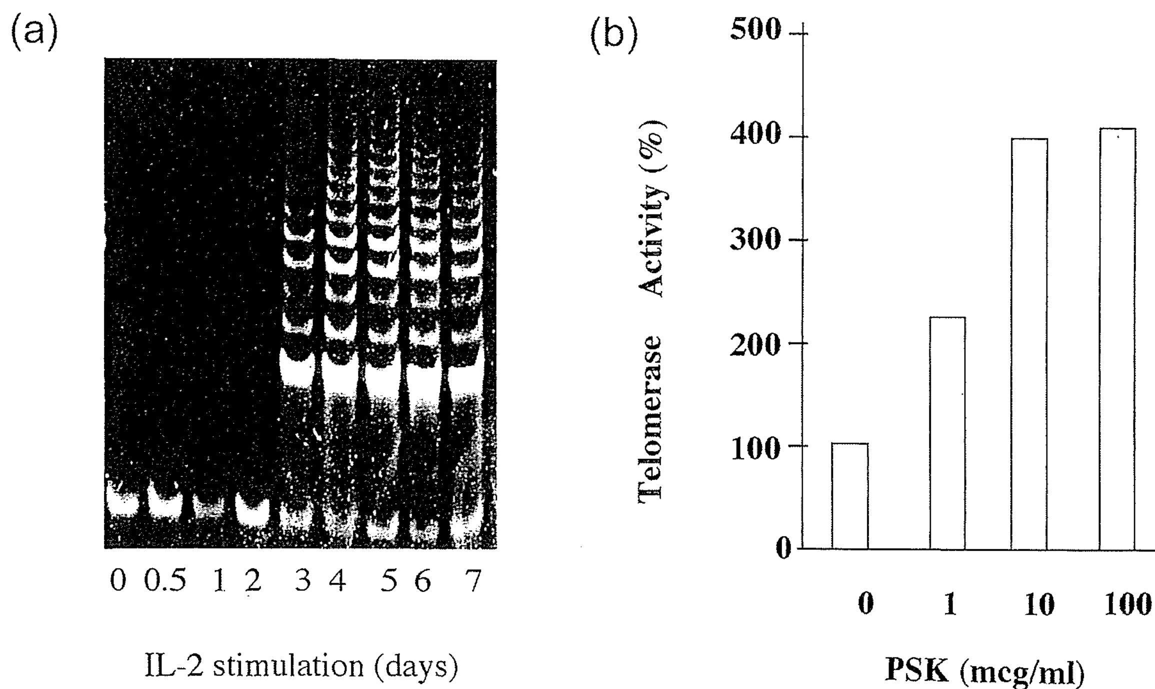

Figure 4. *Telomerase activity of PBMCs stimulated with IL-2 plus PS-K. PBMCs were stimulated with 200 U/ml IL-2 alone or IL-2 plus PS-K, and telomerase activity was determined with TRAP assay on days indicated. A representative result of PBMCs from a colon cancer patient with liver metastasis was shown.*

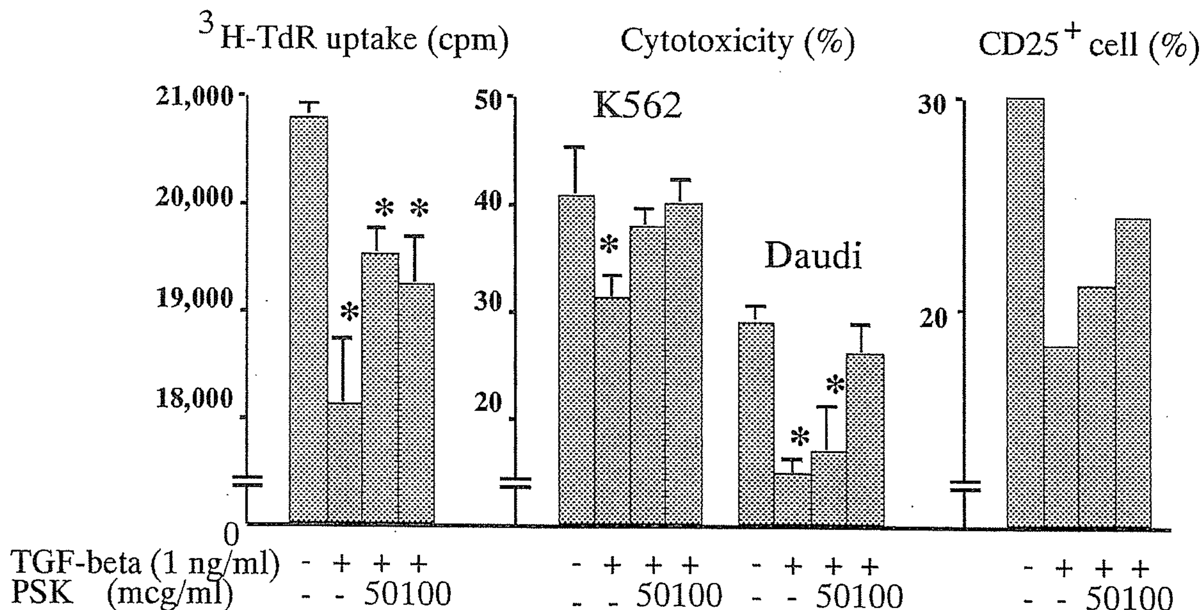

Figure 5. *Inhibitory effects of TGF-beta on IL-2-induced PBMC responses and its abrogation by PS-K. PBMCs were stimulated with 200 U/ml IL-2 in the presence or absence of TGF-beta and PS-K as indicated. Proliferative responses, cytotoxic activities and CD25+ cell populations of PBMCs were analyzed. Significant differences form the value with IL-2 alone, *p<0.05.*

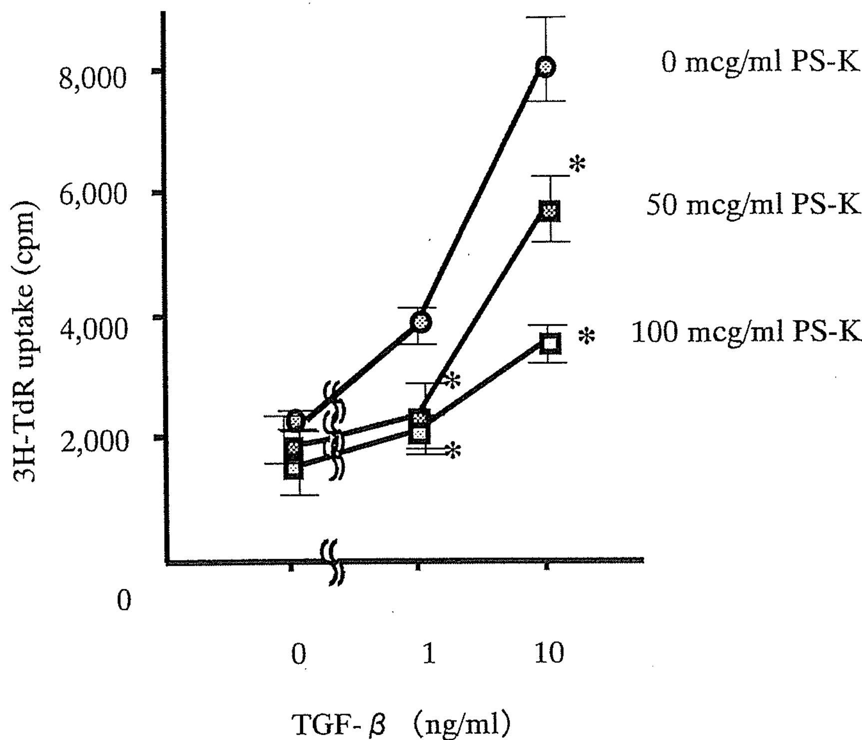

Figure 6. *Anchorage-independent growth of NRK cells in the presence of TGF-beta and PS-K. NRK cells were grown in a soft agar as described in Materials and Methods in the presence or absence of TGF-beta and PS-K, and 3H-TdR incorporation of NRK cells was determined. Significant differences from the value without PS-K, *p<0.05.*

determined by TRAP assay (Figure 4a, b). The telomerase activity of PBMCs stimulated with IL-2 alone was initially detectable between day 2 and day 5 at levels of 125 to 240 TPG units (Figure 4a). The addition of PS-K during the IL-2 stimulation augmented the telomerase activity in a dose-dependent manner of PS-K, and an almost 4-fold increase of the telomerase activity was observed in the presence of 10 mcg/ml PS-K when compared to IL-2 alone.

TGF-β-induced inhibiton of lymphocyte responses and IL-2R expression and their abrogation by PS-K. We next investigated the effects of TGF-beta on the IL-2-induced stimulation of PBMCs in the presence of PS-K (Figure 5). Proliferative responses of PBMCs were significantly depressed with the addition of 1 ng/ml TGF-beta into the culture. The expressions of cytotoxic activity against both K562 and Daudi cell targets as well as the CD25+ cell population of PBMCs were also suppressed with the addition of TGF-beta. These suppresssions were drastically abrogated with the addition of PS-K in a dose-dependent manner, and 100 mcg/ml PS-K almost completely restored the cytotoxic activity of PBMCs depressed by 1 ng/ml TGF-beta.

TGF-β-activity on anchorage-independent growth of NRK cells and its abrogation by PS-K. The effects of PS-K on the anchorage-independent growth of NRK cells were analyzed (Figure 6). The NRK indicator cells could grow on the soft agar when added with 10 ng/ml TGF-beta, and showed a 4-fold increase of ^{3}H-TdR uptake (8,129±766 cpm) compared to that without TGF-beta (2,147±106 cpm). This TGF-beta activity for the anchorage-independent growth of NRK cells was clearly inhibited by the addition of PS-K in a dose-dependent manner. DNA synthesis of NRK indicator cells in the presence of 100 mcg/ml PSK was 3,411±273 cpm even in the presence of 10 ng/ml TGF-beta. There was a significant difference between the values with and without PS-K ($p<0.05$). PS-K alone, however, did not show any effects on the anchorage-dependent growth of NRK cells (data not shown).

Inhibitory effect of PS-K on the binding of TGF-beta to its receptor on PBMCs. To understand the mechanisms by which PS-K inhibited the TGF-beta activities, a TGF-beta binding assay on IL-2-stimulated PBMCs was performed by using FITC-labeled TGF-beta in the presence of PS-K (Figure 7). The TGF-beta receptors were induced on the surface of PBMCs by stimulation with IL-2 for 4 days and were expressed on 15 to 34% of the IL-2-stimulated PBMCs. The binding of TGF-beta was clearly down-modulated when 100 mcg/ml PS-K was present at the staining of IL-2-stimulated PBMCs with FITC-labeled TGF-beta (Figure 7a). The down-modulation of TGF-beta

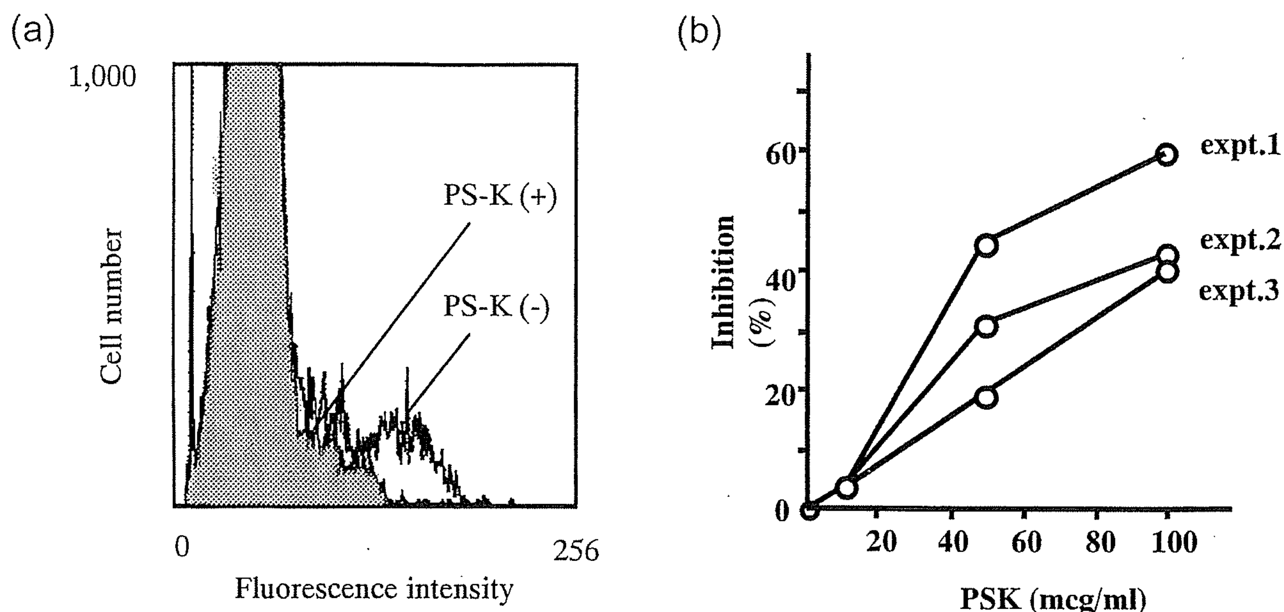

Figure 7. *Inhibition of binding of TGF-beta on PBMCs activated with IL-2. PBMCs were stimulated with 200 U/ml IL-2 and TGF-betaR was analyzed using FITC-labelled TGF-beta in the presence or absence of PS-K.*

binding on the IL-2-stimulated PBMCs was found to be dose-dependent of PS-K, and approximately a half or more inhibition of the binding was observed in the presence of 100 mcg/ml PS-K (Figure 7b).

Discussion

There are various reports investigating the immunopotentiating actions of PS-K. Kariya *et al.* (22) demonstrated that PS-K activated human NK cells independently of IFN and the IL-2/IL-2R system. Nio *et al.* (23) also reported that culturing lymphocytes from gastric cancer patients with PS-K alone induced up-regulation of IL-2R, but the immunomodulation by PS-K might be mediated by mechanisms independent of IFN and IL-2. Our study demonstrated, however, that PS-K augmented the activation of PBMCs including proliferative responses and expressions of cytotoxic activity, IL-2R and telomerase activity, when PBMCs were stimulated with PS-K in combination with IL-2. This augmentation was observed in PBMCs from cancer patients. It should be emphasized that PS-K could induce the cytotoxic activity of PBMCs even with low concentration of IL-2. Earlier clinical studies demonstrated that high-dose IL-2 exhibited considerable effects against solid tumors, but also had severe adverse effects, including a vascular leak syndrome (24, 25). The evidence that PS-K induced significant responses of PBMCs even with the minimal amount of IL-2 suggests that PS-K may advantageously be combined with IL-2-based immunotherapy for both clinical tumor responses and the reduction of adverse effects.

In order to understand the mechanism by which PS-K enhanced IL-2-induced lymphocyte responses, we focused the anti-immunosuppressive action of PS-K against TGF-beta, because it has been previously reported that PS-K has an antagonistic property against immunosuppressive acidic protein (IAP) (13). It was observed that PS-K abrogated the TGF-beta activity of the anchorage-independent growth of NRK cells and restored the TGF-beta-induced inhibition of lymphocyte proliferation, cytotoxic activity and IL-2R expression. It has been described that there is an IL-2/TGF-beta system which regulates lymphocyte responses (16, 17). During activation with IL-2, lymphocytes endogenously produce TGF-beta and express TGF-beta receptor on their surface in order to down-regulate their own responses triggered by IL-2. PS-K may enhance IL-2-induced lymphocyte activation by regulating the action of endogenous TGF-beta. Harada *et al.* (26) reported that PS-K reduced plasma TGF-beta1 levels in tumor-bearing mice. Habelhah *et al.* (27) showed that TGF-beta1 mRNA expression was suppressed in murine fibrosarcoma tissues treated with PS-K. Zhang *et al.* (28) also reported the down-regulation of TGF-beta1 by PS-K in a tumor invasion system. Collectively, PS-K may exert its immunopotentiating action by inhibiting both the production and the activity of TGF-beta.

Which are the molecular mechanisms by which PS-K inhibits TGF-beta activity? Matsunaga *et al.* (29) reported that PS-K specifically bound to the active form of TGF-beta itself. This is one explanation for the antagonistic action of PS-K against TGF-beta. Our data indicated that the receptor analysis using TGF-beta itself revealed that PS-K blocked the binding of TGF-beta to its receptor on the surface of PBMCs, suggesting that PS-K exerts its antagonistic action against TGF-beta at the level of TGF-betaR. We previously demonstrated that PS-K competed with soluble suppressor factors at the level of their receptors (30), which were recognized by a lectin wheat germ agglutinin (WGA) (31). Moreover, novel TGF-betaR was affinity-purified by using WGA, indicating that TGF-betaR has glycosilated sites recognized by WGA (32, 33). Taken together, we speculate that a lectin-like domain of protein-bound polysaccharide PS-K competes with TGF-beta, as well as WGA, at the glycosilation sites of TGF-betaR on the surface of responder cells. PS-K may affect the IL-2/TGF-beta system by competing with TGF-beta at TGF-betaR on activated lymphocytes, resulting in the restoration of the TGF-beta-induced inhibition of lymphocyte activation and, therefore, resulting in the augmentation of the IL-2-induced lymphocyte activation.

In conclusion, a polysaccharide preparation, PS-K, has an enhancing effect for lymphocyte activation in combination with IL-2, especially in cancer patients, the effect of which is partly based on an antagonistic action against TGF-beta at its receptor level. Thus, PS-K may be a beneficial agent to be combined in the IL-2-based immunotherapy of cancer.

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Feasibility Study of Adoptive Immunotherapy for Metastatic Lung Tumors Using Peptide-pulsed Dendritic Cell-activated Killer (PDAK) Cells

YOSHIYUKI YAMAGUCHI, KOJI OHTA, YOSHIHARU KAWABUCHI, AKIKO OHSHITA, RIKI OKITA, MAKOTO OKAWAKI, KATSUJI HIRONAKA, KAZUO MATSUURA and TETSUYA TOGE

Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan

Abstract. *We have established a novel culture system to generate effector lymphocytes designated as peptide-pulsed dendritic cell-activated killer (PDAK) cells using cultured dendritic cells (DCs), synthetic peptide, peripheral blood lymphocytes, and interleukin-2 plus immobilized anti-CD3 antibody. A feasibility study of an adoptive immunotherapy trial using PDAK cells was conducted on HLA-A2 and HLA-A24 cancer patients with antigen-positive lung metastasis that was defined by serological analysis or PCR analysis. Eleven patients with lung metastasis participated in the study: 6 with colorectal cancer, 2 with pancreatic cancer, 1 each with breast and lung cancer, and 1 with melanoma. The patients received either Muc-1, CEA, gp100, Her-2 or SART-3-PDAK cells generated in vitro, intravenously in combination with 350,000 U IL-2 weekly for 9 weeks, together with a planned dose-escalation schedule of three transfers each of $1x10^7$, $3x10^7$ and $1x10^8$ PDAK cells/kg for 6 patients, and with a uniform dose of $3x10^7$ PDAK cells/kg for the remaining 5 patients. Peptide/HLA-specific cytotoxic activity and TCRVβ gene usage of PDAK cells were analyzed. All transfers of PDAK cells, which showed peptide/HLA-specific lysis, were well-tolerated in all patients, and adverse effects (elevation of transaminase, fever, and headache) were observed primarily at grade 1, but in no case greater than grade 2. The generation of sufficient cells to treat the patients with $3x10^7$ PDAK cells/kg was feasible using our culture system, but we were able to generate and administer the dose of $1x10^8$ PDAK cells/kg in only one patient. One partial response (PR) of lung metastasis occurred in a pancreatic cancer patient who received $3x10^7$ Muc-1-PDAK cells/kg. The cytolytic units of PDAK cells in this patient appeared to be substantially higher compared to those in PD patients. TCR gene usage analysis on PDAK cells revealed preferential usage of TCRVβ segments. These results suggest that adoptive immunotherapy using PDAK cells for cancer patients with antigen-positive lung metastasis is safe and feasible, and tumor response should be examined in a future clinical trial.*

Abbreviations: CEA, carcinoembryonic antigen; CD, cluster of differentiation; CTL, cytotoxic T-lymphocyte; FCM, flow cytometry; GM-CSF, granulocyte-macrophage colony stimulating factor; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction; SART, squamous cell carcinoma antigen recognized by T cells; TCRV, T-cell receptor variable region; TNF, tumor necrosis factor.

Correspondence to: Yoshiyuki Yamaguchi, M.D., Ph.D., Kasumi 1-2-3, Minami-Ku, Hiroshima 734-8553, Japan. Tel: +81-82-257-5869, Fax: +81-82-256-7109, e-mail: shogo@hiroshima-u.ac.jp

Key Words: Adoptive immunotherapy (AIT), peptide-pulsed dendritic cell-activated killer (PDAK) cells, T-cell receptor (TCR), lung metastasis.

The discovery and molecular cloning of the crucial lymphocyte growth factor, interleukin-2 (IL-2) (1), has facilitated the clinical application of adoptive immunotherapy (AIT) for cancer using autologous lymphocytes activated *in vitro* with IL-2. Disease-associated immunosuppression in patients with cancer can disturb the effective emergence of anti-tumor responses *in vivo* (2). Therefore, the adoptive transfer of effector lymphocytes which have been educated and activated *ex vivo* to recognize tumor cells would, theoretically, provide an effective treatment for cancer. Of the techniques developed to date, the use of lymphokine-activated killer (LAK) cells (3), autolymphocyte therapy (ALT) (4) and tumor-infiltrating lymphocytes (TILs) (5) have been the best studied. While these approaches have not yet consistently shown great benefit for metastatic cancer (6), the conditioning chemotherapy regimen that enhances tumor responses of TIL therapy has recently been published (7).

We have conducted *ex vivo* cell therapy for cancer treatment using activated autologous lymphocytes, including

LAK cells, TILs and tumor-sensitized lymphocytes (8). The clinical results of trials using these activated lymphocytes, however, have demonstrated their limited tumor response in a fraction of patients with lung metastasis of renal cell carcinoma by systemic administration of effector cells (CR+PR=9%) (8). Locoregional administration of TILs has shown favorable results (77%) to reduce malignant effusions (8). These results suggest that effector cells, which express stronger and more specific cytotoxic activity, may result in better clinical efficacy, and that clinical trials of AIT using effector cells may be planned for metastatic lung tumors or locoregional administration. The success of adoptive cellular therapy depends on the ability to optimally produce cells equipped with the desired antigenic specificity, and then induce cellular proliferation while preserving the effector function and trafficking abilities of the lymphocytes (9).

In developing new approaches to AIT for patients with metastatic cancer, the increasing molecular understanding of antigen presentation and recognition has highlighted the use of professional antigen-presenting dendritic cells (DCs) (6, 9). In a previous work, we discussed a novel system for generating cytotoxic effector lymphocytes using antigenic peptides and cultured DCs, designated as peptide-pulsed DC-activated killer (PDAK) cells (10). In the present paper, we report on a clinical study of AIT using PDAK cells for patients with antigen-positive metastatic lung tumors which provides evidences in favor of the safety, feasibility and anti-tumor activity of this type of AIT.

Patients and Methods

Patients. Patients were eligible if they were HLA-A0201 or -A24 adults under 80 years old who had histologically-confirmed cancer with antigen-positive lung metastases that were refractory to standard therapy. Antigen expression of the tumor was ensured by serum carcinoembryonic antigen (CEA) levels, or reverse transcription-polymerase chain reaction using primers specific for Muc-1 (11), gp100 (12), Her-2 (13) and SART-3 (14) on the primary tumors and biopsy samples of metastatic lung tumors. They had Eastern Cooperative Group performance status 0 to 3 (15), and adequate bone marrow, hepatic and renal functions. Exclusion criteria included the following: uncontrolled infection; uncontrolled diabetes mellitus; overt autoimmune disease; concomitant use of corticosteroids; and history of interstitial pneumonia and pulmonary fibrosis. Patients who had received antitumor drugs within the preceding 4 weeks were also ineligible.

Study design. The study was an open-label, non-randomized, dose-escalation study, and was performed at Hiroshima University Hospital since 2000. The protocol was approved by the institutional review board, and all of the patients gave written informed consent. The patients received either Muc-1, CEA, gp100, Her2, SART-3-PDAK cells intravenously in combination with 350,000 U IL-2 (Shionogi, Japan) weekly for 9 weeks, together with a planned dose-escalation schedule of three transfers each of 1 x 10^7, 3 x 10^7 and 1 x 10^8 PDAK cells/kg for 6 patients, and with a uniform dose of 3 x 10^7 PDAK cells/kg for the remaining 5 patients. Adverse effects and tumor responses were carefully evaluated after every three transfers. Toxicity was assessed using the National Cancer Institute common toxicity criteria version 2.0. All patients were monitored clinically using imaging analysis such as chest X-ray and computed tomographic examinations and clinical efficacies were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) (16).

Table I. *Antigenic peptides used for PDAK cell induction.*

Antigen	HLA	Peptide sequence	Reference
Muc-1	A2	STAPPAHGV	19
	A24	GVTSAPDTRPAPGSTAPPAH	20
CEA	A2	YLSGADLNL	21
	A24	TYACFVSNL	22
gp-100	A2	VYFFLPDHL	23
Her-2	A2	KIFGSLAFL	24
SART-3	A24	AYIDFEMKI	25

DC preparation. DCs were induced using a modification of Romani *et al.* (17). Briefly, peripheral blood mononuclear cells (PBMCs) were collected from patients by the centrifugation of 20 ml heparinized venous blood samples on Ficoll-Conray gradients. PBMCs were allowed to adhere to culture flasks (Sumitomo Berclight, Akita, Japan) for 2 h at 37°C in RPMI-1640 medium containing 2% autologous serum. After removal of the non-adherent cells, adherent cells were cultured in RPMI-1640 medium supplemented with 2% autologous serum, 800 U/ml GM-CSF (IBL, Gunma, Japan), and 500 U/ml IL-4 (IBL). On day 5 of the culture, 100 U/ml TNF-α (IBL) was added and cells were cultured for another 2 days. The floating cells were collected as DCs. DCs were analyzed for quality assurance, and the release criteria of cultured DCs were defined with typical morphology (>95% non-adherent veiled cells) and phenotype (>85% HLA class I+, >75% HLA-DR+, >95% CD80+, >75% CD86+, >65% CD83+ and <20% CD14+) (10, 18).

Generation of PDAK cells. PDAK cells were generated as mentioned in detail elsewhere (10, 18). In brief, PBMCs were collected and fractionated into adherent and non-adherent cells. DCs, which had previously been prepared as mentioned above, were inactivated with 50 μg/ml mitomycin-C (Kyowahakko, Tokyo, Japan) and pulsed with antigenic peptide (40 μg/ml) for 2 h in RPMI-1640 medium containing 2% autologous serum supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The peptides used in this study are shown in Table I (19-25). The IFN-γ response of the patient's PBMCs to the peptide was confirmed before generating the PDAK cells (26). After 4 washes of DCs, the non-adherent fraction of PBMCs (10^7) was stimulated with peptide-pulsed DCs at a responder-to-stimulator ratio of 10, and maintained in RPMI-1640/2% autologous serum medium containing 10 U/ml IL-7 (IBL). Two days later, cells were washed and 80 U/ml IL-2 (Shionogi,

Table II. *Patients enrolled in the study.*

Case No.	Cancer	Age/Sex	Weight	HLA	P.S.	Metastasis	Prior therapy
1	pancreas	52/M	50	A24	3	lung, lymph nodes, pleuro-peritoneum	GEM
2	pancreas	36/M	78	A2	1	lung, liver	GEM
3	breast	45/F	54	A24	0	lung	CAF, TAX
4	colon	59/M	67	A2	2	lung, liver	LV, 5FU
5	melanoma	76/F	46	A2	2	lung, liver	-
6	colon	68/M	55	A24	1	lung, pleura, lymph nodes	LV, 5FU
7	colon	48/M	60	A24	3	lung, bone	LV, 5FU , RT
8	lung	24/M	55	A2	0	lung	CBDCA, TAX
9	colon	50/F	45	A24	0	lung	LV, 5FU, CPT-11
10	rectal	71/M	64	A24	0	lung	LV, 5FU
11	colon	78/M	58	A2	0	lung, lymph nodes	LV, 5FU, CPT-11

GEM, gemcitabine; CAF, cyclophosphamide + adriamycine + 5-fluorouracil; TAX, taxol; LV, leucovorin; 5FU, 5-fluorouracil; CBDCA, carboplatin.

Osaka, Japan) was added. Responder cells were re-stimulated with peptide-pulsed DCs on days 7 and 14 of the culture. Peptide-pulsed DC-activated killer (PDAK) cells (10^6 /ml) were further expanded on an anti-CD3 antibody (Janssen-Kyowa, Tokyo, Japan)-coated flask in the presence of 80 U/ml IL-2 (IL-2/CD3 system). The culture medium was half-changed with fresh medium containing IL-2 every 3-4 days. This culture system could usually yield more than 10^9 PDAK cells per flask. The above procedure was repeated for weekly administrations of PDAK cells in separate cultures. On day 21 or 28, the cells were counted, washed 3 times by saline, filtered through 50 μm mesh, and resuspended in 100 ml saline before administration. Bacterial and endotoxin examinations were made 3 days before and on the day of the administration.

Peptide/HLA-specific cytotoxic activity of PDAK cells. To examine the peptide/HLA-specific cytotoxic activity of PDAK cells, a conventional 4-h ^{51}Cr release assay was performed. The target cells used were T2 (10) and TISI (18) cells for HLA-A2 and A24 patients, respectively, maintained in RPMI-1640 medium supplemented with 10% FCS, 50 units/ml penicillin and 2 mM L-glutamine. Target cells were pulsed with or without 40 μg/ml peptide, which had been used for PDAK cell generation, for 18 h at 37°C and labelled with ^{51}Cr (100 μCi) for 2 h. Target cells were washed 3 times and plated onto round-bottomed, 96-well microtiter plates at a density of $1x10^4$ cells /0.1 ml. PDAK cells were added over the target cells at various densities in a final volume of 0.2 ml. After 4-h incubation at 37°C, release of ^{51}Cr in the supernatant was measured by an automated γ counter (Aloka, Tokyo, Japan). The mean percentage of the peptide/HLA-specific lysis of the triplicate wells was calculated by the following formula: ((release by PDAK against peptide-pulsed target) – (release by PDAK against peptide-un-pulsed target))/((maximum release) – (spontaneous release)) x100. The spontaneous release was obtained from the wells of peptide-pulsed target cells alone and was around 15% of the maximum release, which was obtained from wells added with 2% Triton X-100 over the peptide-pulsed target cells instead of PDAK cells. In some experiments, PDAK cells were incubated for 1 h at 4°C with 10 μg/ ml of anti-TCRαβ, TCRVβ6, 12 mAbs (Ortho Diagnostic System, Raritan, NJ, USA), then cytotoxicity assays were carried out. Killer units were the killing activity of PDAK cells multiplied by transferred cell numbers and was calculated at each transfer by the following formula: ((peptide/HLA-specific killing activity of PDAK at an E/T=20) x (transferred cell number) / (body weight $x10^7$).

Antibody and flow cytometry. The DCs (10^5) and PDAK cells ($5x10^5$) were stained with antibodies, washed, and then analyzed on FACSCan (Becton Dickinson, San Diego, CA, USA). The antibodies used were anti-class I, anti-HLA-DR, anti-CD80, anti-CD86, anti-CD-83, anti-CD14 antibodies for DCs, and anti-CD3, anti-CD4 and anti-CD8 antibodies for PDAK cells. All antibodies used were purchased from Becton Dickinson.

T-cell receptor gene usage analysis. Total RNA was extracted from 5 x 10^5 cells of PBMCs and PDAK cells and reverse-transcribed with random hexamer, as described previously (27). Aliquots of the cDNA were amplified by PCR in separate tubes, using Vβ-specific oligonucleotides and Cβ reverse primer on a DNA thermal cycler (Perkin Elmer, Norwalk, CT, USA). The amplified DNA was confirmed by Southern blot analysis using a Cβ probe with luminol reaction. The light output detected on X-ray film was quantified using NIH-imaging software and a Macintosh personal computer.

Diagnostic single-strand conformation polymorphism. To detect the clonotype of the complementarity determining region (CDR) 3 in the PCR product of each TCRβ band, the diagnostic single-strand conformational polymorphism (SSCP) technique was performed (27). In brief, 5 μl of the asymmetric PCR product, which was mixed with 5 μl of 95% formamide containing xylene cyanol and bromophenol blue, was heated at 95°C for 5 min, cooled on ice, and then loaded onto a 10% acrylamide gel. This was run at 100 V for 4 h in a cold room (4°C). The gel was then silver-stained (Silver Stain Plus, BioRad, Hercules, CA, USA).

Statistical analysis. Statistical evaluations for experimental values were analyzed using the non-parametric Student's *t*-test.

Table III. *Characteristics of PDAK cells, adverse effects and clinical responses.*

Case	Disease	Peptide	Step	Total cell No. (x 10^9)	CD4/CD8[a]	Killer unit (mean units in step 2)	Adverse effects (grade)	Response		
								lung (duration)	tumor marker[b]	total
1	pancreas	Muc-1	1,2	10.5	36/62	71, 65,–, 243, 237, 208, 189, 168, 204 (208)	–	PR[c](4[d])	stable	PR
2	pancreas	Muc-1	1,2	16.4	41/76	34, 28, 30, 72, 75, 54, 62, 57, 70 (65)	–	SD(3)	increase	PD
3	breast	CEA	1,2	11.3	25/81	25,–,–, 54, 66, 64, 57, 54, 69 (61)	hepatic (1)	SD(9)	stable	SD
4	colon	CEA	1,2	14.1	44/69	35, 24, 29, 73, 67, 77, 58, 64, 64 (67)	–	SD(3)	increase	PD
5	melanoma	gp100	1	0.9	–/–	–,–	–	NE	-	NE
6	colon	CEA	1,2,3	23.1	28/80	60,–, 54, 33, –,75, 169,–, 231(54)	–	SD(3)	decrease	PD
7	colon	CEA	2	5.4	35/42	6, 4, 3 (4)	–	PD	increase	PD
8	lung	Her-2	2	14.9	39/88	14, 21,–, –, 15, 18, 9, 11, 8 (14)	–	PD	-	PD
9	colon	CEA	2	12.2	48/36	7, 11,–, 10,–, 19,–, 25, 18 (15)	headache (1)	PD	increase	PD
10	rectum	SART-3	2	17.3	20/77	55, 62, 57,–, 83, 77, 72, 68, 66 (68)	–	SD(4)	decrease	SD
11	colon	CEA	2	15.7	32/86	93,–, 88, 73, 75, 82, 80, 93,–(83)	fever (1)	SD(4)	decrease	SD

Patients were administered intravenously with PDAK cells as indicated (step 1, $1x10^7$/kg; step 2, $3x10^7$/kg; step 3, $1x10^8$ PDAK cells /kg), and adverse effects and tumor responses were evaluated. Killer unit was measured at every transfer, as described in Materials and Methods.
[a], mean percentage of PDAK cell phenotypes in step 2.
[b], CA19-9 for pancreatic cancer, and CEA for colon and breast cancers.
[c], PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable.
[d], month for response duration.

Results

Patients. Eleven patients with various types of cancer (6 colon, 2 pancreatic, 1 breast, 1 lung and 1 malignant melanoma) participated in the present study (Table II). There were 8 males and 3 females, and their mean age was 55, with a range from 24 to 76. Five patients had HLA-A2 haplotype and 6 had HLA-A24 haplotype. The patients' Eastern Cooperative Oncology Group (ECOG) performance status was 0, 1, 2 and 3 in 5, 2, 2 and 2 patients, respectively. Seven patients had distant metastases in addition to lung metastases, and all but the melanoma patient had previously been treated with chemotherapy or radiotherapy, which had failed to inhibit tumor growth. Antigen peptides used for generating PDAK cells are shown in Table III. CEA was used for patients who had high serum CEA levels. The other antigen expression was confirmed by RT-PCR analysis for biopsy samples of lung metastasis from cases 1, 5 and 8, but not done in cases 2 and 10. Muc-1 peptide was chosen for case 2 because most pancreatic cancer has been shown to express Muc-1 antigen (11), and SART-3 peptide was chosen for case 10 because SART-3 has been reported to be ubiquitously expressed (14). Before generating PDAK cells, all peptides used were confirmed *in vitro* to stimulate IFN-γ production from patient's PBMCs (data not shown).

PDAK cells. PDAK cells could be generated in all patients enrolled (Table III). The total number of PDAK cells infused varied from 0.9 to $23.1x10^9$ cells depending on the patients' body weight and dose steps of the study. PDAK cells generated from 9 out of 10 patients tested showed predominant expansion of CD8 phenotype. In the dosage of step 2, PDAK cells from 7 patients expressed <50, but those from 3 patients showed ≥15 peptide/HLA-specific killer units, which mean peptide/HLA-specific activity of PDAK cells was connected with patient's body weight and cell numbers infused. Mean values of the killer units in the step 2 dosage varied from 4 to 208 among the patients tested.

Feasibility and toxicities. Feasibility and toxicity are also shown in Table III. Dose escalation of PDAK cell transfer was performed in 6 patients, with doses of $1x10^7$ and $3x10^7$ PDAK cells/kg given to 6 and 5 patients, respectively, but the planned dose of $1x10^8$ PDAK cells/kg could be administered to only 1 patient. The treatment was stopped in a melanoma patient with 2 transfers of gp-100-PDAK cells and in a colon cancer patient with 3 transfers of CEA-PDAK cells due to rapid disease progression. Grade 1 toxicities, including an increase of transaminase, headache and fever, were found in the breast cancer patient and 2 colon cancer patients who were treated with CEA-PDAK cells. No correlation was observed between toxicities and killer units or total numbers of PDAK cells transferred.

Tumor response. Tumor response is shown in Table III. When focused on lung metastasis, growth arrest of the lung tumor, including PR or SD responses, was observed in 7

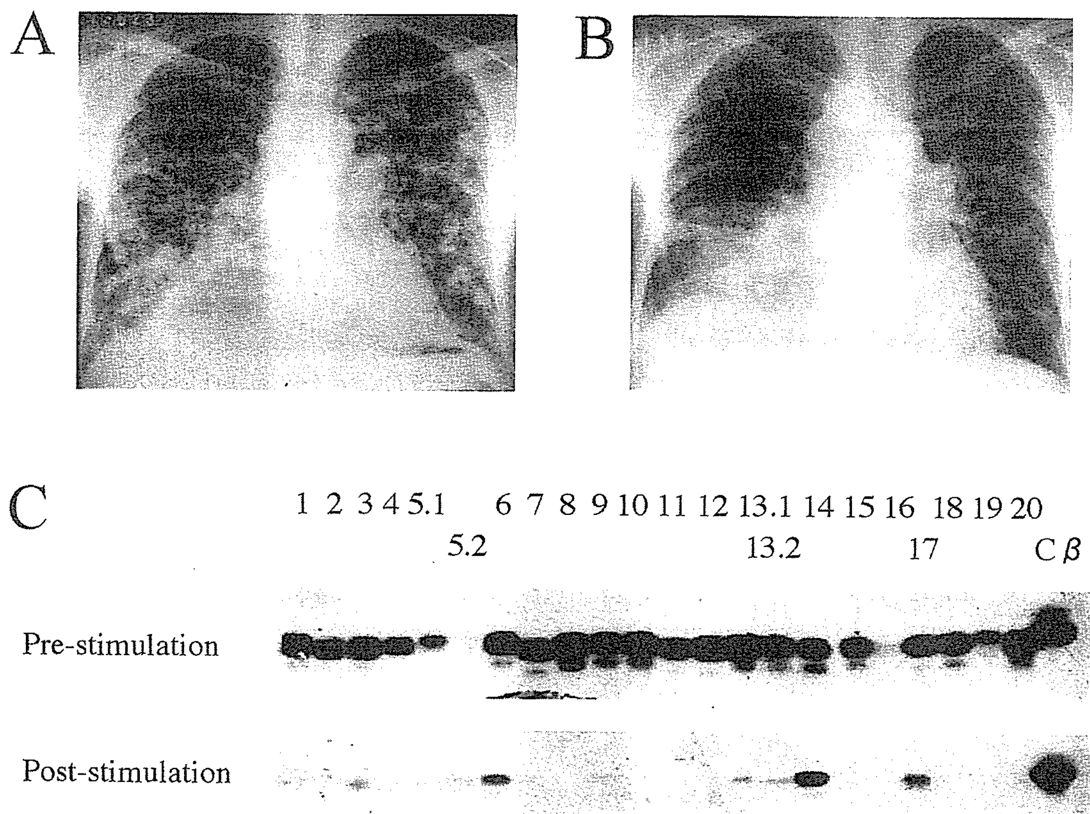

Figure 1. *A pancreatic cancer patient treated with Muc-1 PDAK cells. A 52-year-old male patient with pancreatic cancer who had lung, peritoneal, paraaortic lymph node metastases (case 1) was administered intravenously with Muc-1 PDAK cells according to the dose-escalation schedule, and chest X-ray examination was performed prior to (A) and after (B) the treatment. T-cell receptor gene analysis for Muc-1-PDAK cells was performed prior to and after the stimulation (C).*

out of 11 patients, although 4 of the 7 patients showed tumor progression in distant metastases other than the lung. A pancreatic cancer patient (case 1, Figure 1), who received $3x10^7$ Muc-1-PDAK cells/kg, showed <30% tumor reduction in the lung; in this patient, other distant metastases to the pleuro-peritoneum and lymph nodes were stable for a period of 4 months. A decrease of serum CEA levels was observed in 3 of the 7 colorectal cancer patients, one of whom (case 6, Figure 2) showed growth arrest of lung metastasis with $23.1x10^9$ CEA-PDAK cells, but showed no other tumor response in the pleura or lymph nodes. In an overall assessment, 1 PR (a pancreatic cancer patient), 3 SDs (1 breast and 2 colorectal cancer patients) and 6 PDs were observed.

There was no relationship between tumor response and total numbers of PDAK cells infused. Regarding the killer units, 7 patients with PR or SD response at lung lesions had mean value ± standard deviation of 87±54 activity, while 3 PD patients had that of only 11±6 activity, in the dose level of step 2 (Figure 3). There was a significant difference between these values ($p<0.05$).

T-cell receptor analysis. A 52-year-old male patient with pancreatic cancer (case 1) showed a partial response in lung metastasis by Muc-1-PDAK cell transfer (Figure 1A, B). The PBMCs of the patient demonstrated a diverse expression of TCRVβ gene usage before stimulation, while the transferred Muc-1-PDAK cells showed preferential usage of TCRVβ3, 6, 13.1, 13.2, 14 and 17 (Figure 1C).

A 68-year-old male patient with colon cancer (case 6) showed growth arrest of lung metastasis with a decrease in serum CEA level due to CEA-PDAK cell transfer (Figure 2A, B). TCRVβ gene usage analysis clearly demonstrated a difference in TCRVβ expression before and after stimulation, and the preferential usage of TCRVβ1, 2, 3, 5.2, and especially of TCRVβ6 and 12 was indicated (Figure 2C). The CEA-PDAK cells killed T2 target cells pulsed with the CEA peptide, whose cytotoxicity was significantly abrogated in the presence of anti-TCRVβ12 antibody ($p<0.05$), but this did not occur in the presence of anti-TCRVβ6 antibody or the irrelevant control antibody (Figure 2D). SSCP analysis showed clonotypic band pairs of the TCRVβ12 (Figure 2E).

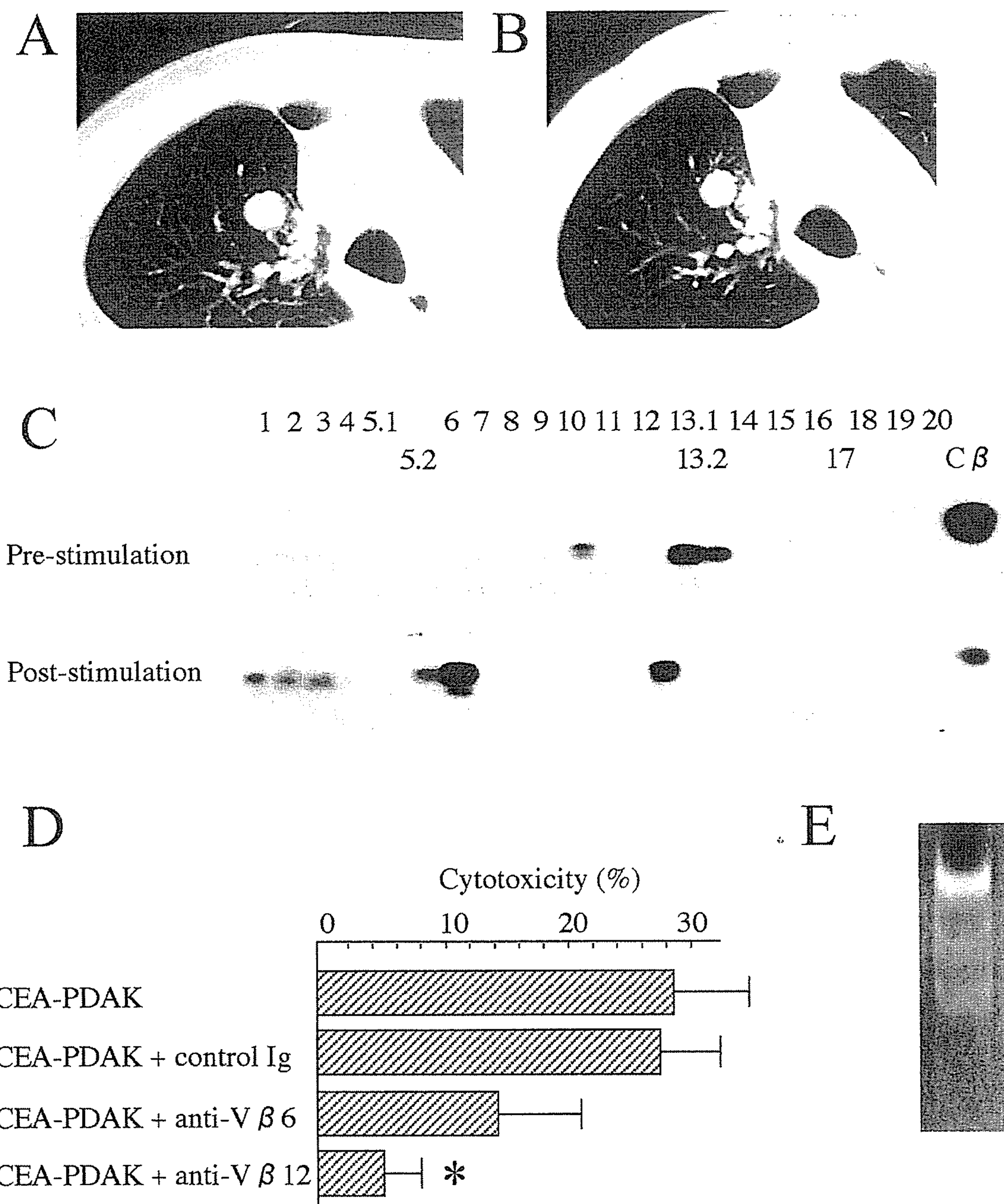

Figure 2. *A colon cancer patient treated with CEA-PDAK cells. A 68-year-old male patient with colon cancer who had lung, pleural and mediastinal lymph node metastases (case 6) was administered intravenously with CEA-PDAK cells, and CT scan examination was performed prior to (A) and after (B) the treatment. Serum CEA levels decreased from 40.5 to 21.8 ng/ml by the treatment. T-cell receptor gene analysis for CEA-PDAK cells was performed prior to and after the stimulation (C). The cytotoxicity of CEA-PDAK cells was determined against CEA peptide-pulsed T2 cells at an E/T ratio of 25 in the presence of the antibodies indicated (D). SSCP analysis was performed for detecting clonotypes of TVRVβ12 (E).*

Discussion

In the present study, we demonstrated the safety of AIT using PDAK cells for metastatic lung tumors. This is to be expected since AIT using activated lymphocytes has been shown to be essentially safe except in combined administration of high dose IL-2 (28, 29). We also showed that our method of AIT using PDAK cells was feasible at an administration of $3x10^7$ cells/kg, but not at a dose of $1x10^8$ PDAK cells/kg. Importantly, one partial response was observed in a

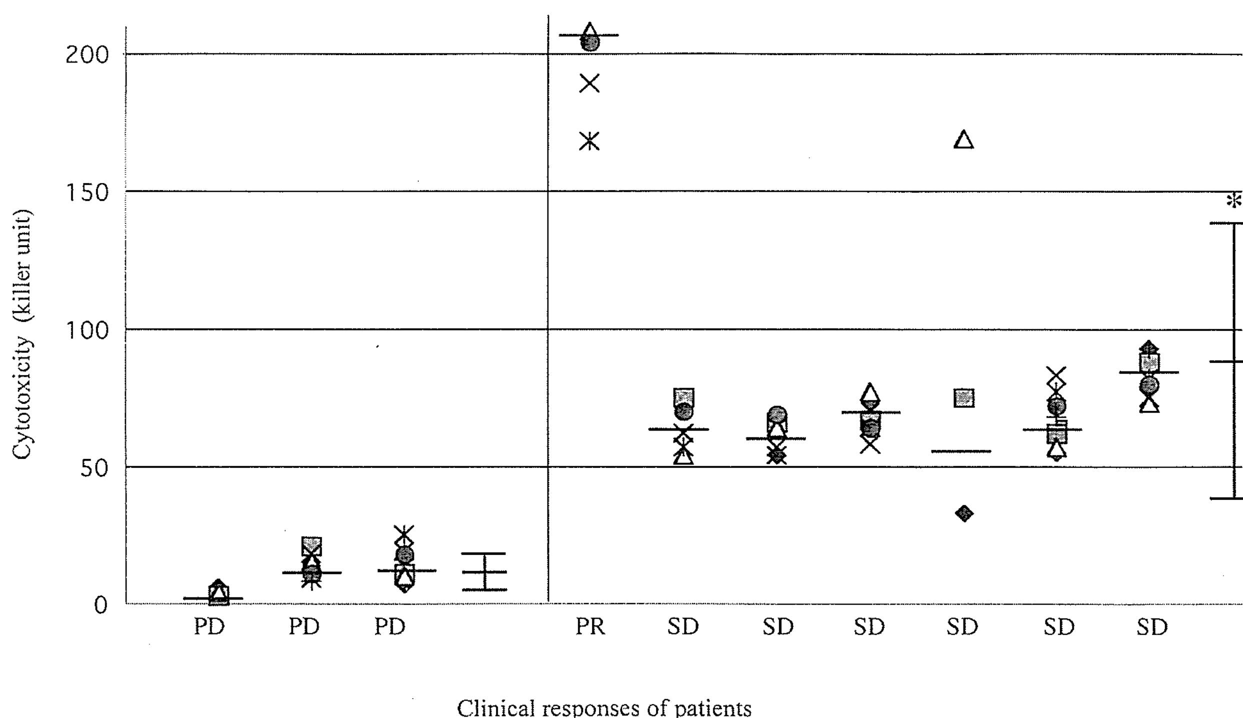

Figure 3. *Cytotoxic activity of PDAK cells in relation to the clinical responses. Killer units were determined before every transfer of PDAK cells, and those at the step 2 dosage of PDAK cells were plotted. Symbols indicate the killer units of PDAK cells at each transfer. A significant difference, *$p<0.05$.*

pancreatic cancer patient with a dose of $3x10^7$ Muc-1-PDAK cells/kg. Although Simon *et al.* state that dose-finding studies may not be necessary in cell therapy such as tumor vaccine trials (30), our results suggest that the dose of $3x10^7$ PDAK cells/kg is the limitation of our culture system and may be the least dose required for tumor responses.

We could observe only one PR and 6 SDs in metastatic lung lesions and no response in other lesions including liver, lymph node and effusion, by the intravenous systemic administration of PDAK cells. It has been reported that the trafficking of effector cells toward the tumor site is critical for tumor response (5-9). For example, other researchers have attempted to address hepatic tumors by arterial infusion of effector cells through the hepatic artery (31). By intravenous administration, 100% effector cells can reach lung lesions. Moreover, PDAK cells have been shown to express chemotactic chemokine receptor 5 (18), which is reported to be required for locoregional trafficking of effector lymphocytes (32). However, these may not explain the unsatisfactory results of lung lesions in this study by PDAK cell transfer. To augment the efficacies, we may pay more attention not only to generating the effector cells of high quality, but also to conditioning the host immune regulation systems before AIT using PDAK cells. Dudley *et al.* (7) reported the remarkable enhancement of the clinical efficacy of AIT against malignant melanoma using TILs by pre-treating patients with non-myeloablative lympho-depleting chemotherapy.

We measured the peptide/HLA-specific killing activity of PDAK cells and calculated the killer units upon transfer; the number of killer units indicates the total of peptide/HLA-specific killing activity of transferred PDAK cells. It is an interesting question whether or not this parameter of killing activity correlates with clinical responses to AIT using PDAK cells. In the present trial, the number of killer units in PR and SD patients was significantly higher than that in PD patients. In previous AIT trials using LAK cells, it has been reported that neither tumor reduction nor clinical toxicity correlates with dose or with the cytolytic activity of LAK cells, nor are there any correlations with other laboratory parameters including base-line lymphocyte count and IL-2-induced lymphocytosis (29). However, Kawakami *et al.* (23) have reported that tumor regression is correlated with the recognition of gp100 epitopes by the adoptively administered TILs in treating patients with melanoma. Like TILs, but unlike LAK cells, PDAK cells have been shown to recognize tumor cells in a peptide/HLA-specific manner (10, 18). Therefore, the peptide/HLA-specific killing activity of PDAK cells may be involved in clinical results of tumor responses. In addition,

measurement of peptide/HLA-specific killing activity is also important for the quality control of PDAK cells used in the trials (30). This issue for the quality control of the effector cells remains to be addressed in future clinical trials.

In order to generate PDAK cells of high quality, the selection of appropriate peptides may be critical. We have previously reported that the CEA peptide, which can stimulate CTL precursors to produce IFN-γ, differs in individuals among HLA-A24 healthy donors and colorectal cancer patients when tested with a whole blood assay using a CEA peptide panel (26), although CEA652 has been shown to have the most potent binding affinity for HLA-A24 molecules and to induce CEA-reactive CTLs (22). Kedl *et al.* (33) have reported the affinity maturation of a secondary T cell response and shown that high-affinity T cells out-compete lower affinity T cells during a response to antigenic challenge *in vivo*. This suggests that, when generating PDAK cells, inappropriate peptides may induce the affinity maturation of a secondary T cell response that may not be the best for an appropriate CTL generation. In a study by Mine *et al.*, notable tumor responses were realized in peptide vaccine trials, in which only those peptides that were able to stimulate patients' PBMCs to produce IFN-γ were administered (34). We suggest that the peptides to be used in cancer immunotherapy should be selected not only according to the HLA binding affinity of the peptide, but also depending on the patient's CTL precursor status. Thus, the host-oriented peptide evaluation (HOPE) approach may augment tumor responses to AIT using PDAK cells (26).

A more important issue to be addressed may be the identification of the TCRs that are involved in tumor eradication. We have shown preferential usage of TCRs in the PDAK cells in one patient (case 6) whose TCRVβ12 was involved in the recognition of peptides pulsed on experimental target cells. However, we failed to confirm whether or not the TCRs were involved in autologous tumor-specific recognition. In our communicating investigation using Her2-specific CTL clones, Her2 peptide-pulsed T2 cells, and Her2-expressing tumor cells, we have observed that there are 2 types of TCRs, one involved only in peptide/HLA recognition and one which is also involved in tumor recognition; the latter is more important in antigen-based immunotherapy (unpublished data). Identification of the TCR genes which are involved in tumor recognition permits us to produce TCR gene-modified effector cells that are rendered specifically to be reactive with antigen-expressing tumors (35). This approach may accelerate the development of tumor antigen-specific AIT.

In conclusion, AIT for antigen-positive metastatic lung tumors using PDAK cells was found to be both safe and feasible. Based on the present data on dosages, tumor response should be examined in a future clinical trial. Large-scale clinical trials are on-going to prove the efficacy of AIT using PDAK cells against metastatic lung tumors.

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