the patients' CTL precursor status. It is reported that inappropriate peptides induce the affinity maturation of inappropriate T-cell receptors, which can disturb the appropriate peptide signalings for generating CTL responses (24), possibly resulting in the failure of cancer immunotherapy using antigenic peptides. This host-oriented peptide evaluation (HOPE) approach to augment tumor responses in the peptide-based clinical trials deserves a good deal of attention.

The time required for detecting candidate peptides to generate CTLs is another important issue in the immunotherapy of advanced cancer. It was reported that it took 14 days to detect candidate peptides to generate CTLs (10, 23). In our whole blood assay, it took 4-5 days, which suggests that our method may be more convenient for screening the candidate peptides. Finally, we attempted to establish a more rapid assay for predicting candidate peptides for generating CTLs by using the IFN-gamma gene (25). It was observed that the IFN-gamma response could be detectable within 6 hours using real-time-PCR instead of within 4-5 days using IFN-gamma-specific ELISA. This approach is more expensive and laborious than that of IFN-gamma-protein analysis, but can conserve the patients' limited time by permitting them to start peptide-based cancer immunotherapy earlier. Researchers who work in the treatment of advanced cancer patients should give the patients' time the highest consideration. The use of real-time-PCR for detecting the IFN-gamma gene may be a useful tool in the HOPE approach to peptide-based cancer immunotherapy.

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References

1 Taniguchi T, Matsui H, Fujita T, Takaoka C, Kashima N, Yoshimoto R and Hamuro J: Structure and expression of a cloned cDNA for human interleukin-2. Nature *302*: 305-310, 1983.

2 Kiessling R, Wasserman K, Horiguchi S, Kono K, Sjoberg J, Pisa P and Petersson M: Tumor-induced immune dysfunction. Cancer Immunol Immunother *48*: 353-362, 1999.

3 Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber JM, Shiloni E and Vetto JT: Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med *313*: 1485-1492, 1985.

4 Osband ME, Lavin PT, Babayan RK, Graham S, Lamm DL, Parker B, Sawczuk I, Ross S and Krane RJ: Effect of autolymphocyte therapy on survival and quality of life in patients with metastatic renal-cell carcinoma. Lancet *336*: 994-998, 1990.

5 Rosenberg SA, Spiess P and Lafreniere R: A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. Science *233*: 1318-1321, 1986.

6 Hoffman DM, Gitlitz BJ, Belldegrun A and Figlin RA: Adoptive cellular therapy. Semin Oncol *27*: 221-233, 2000.

7 Yeh H and June CH: Use of cell-based therapies for modification of host immune responses. Dev Biol *112*: 99-104, 2003.

8 Ohta K, Yamaguchi Y, Shimizu K, Miyahara E and Toge T: Novel system for generating cytotoxic effector lymphocytes using carcinoembryonic antigen (CEA) peptide and cultured dendritic cells. Induction of peptide-pulsed dendritic cell-activated killer (PDAK) cells. Anticancer Res *22*: 2597-2606, 2002.

9 Nukaya I, Yasumoto M, Iwasaki T, Ideno M, Sette A, Celis E, Takesako K and Kato I: Identification of HLA-A24 epitope peptides of carcinoembryonic antigen which induce tumor-reactive cytotoxic T lymphocyte. Int J Cancer *80*: 92-97, 1999.

10 Hida N, Maeda Y, Katagiri K, Takasu H, Harada M and Itoh K: A simple culture protocol to detect peptide-specific cytotoxic T lymphocyte precursors in the circulation. Cancer Immunol Immunother *51*: 219-228, 2002.

11 Shimizu K, Yamaguchi Y, Ohta K, Miyahara E and Toge T: Analysis of T cell receptors reactive with squamous cell carcinoma antigen SART-1 presented by HLA-A24 molecule. Oncology Reports *9*: 599-605, 2002.

12 Yoshida K, Tanabe K, Fujii D, Oue N, Yasui W and Toge T: Induction mechanism of apoptosis by troglitazone through peroxisome proliferator-activated receptor-gamma in gastric carcinoma cells. Anticancer Res *23*: 267-274, 2003.

13 Wang J, Liu HL, Xu CM, Yang Y, Lv ZJ, Pang HZ and Zhang ZN: Detection of erythrocytes deficient in glycosylphosphatidylinositol anchored membrane proteins in patients with paroxysmal nocturnal hemoglobinuria by the toxin HEC secreted by Aeromonas hydrophila J-1. Hematology *8*: 41-46, 2003.

14 Yamaguchi Y, Ohta K, Shimizu K, Minami K, Noma K, Hihara J and Toge T: Generation of cytotoxic effector lymphocytes by MLTC using tumor cells genetically modified to secrete interleukin-2. Anticancer Res *21*: 669-678, 2001.

15 Laufer S, Greim C and Bertsche T: An *in-vitro* screening assay for the detection of inhibitors of proinflammatory cytokine synthesis: a useful tool for the development of new antiarthritic and disease modifying drugs. Osteoarthritis Cartilage *10*: 961-967, 2002.

16 Elsasser-Beile U, von Kleist S and Gallati H: Evaluation of a test system for measuring cytokine production in human whole blood cell cultures. J Immunol Methods *139*: 191-195, 1991.

17 Yamaguchi Y, Miyahara E, Ohshita A, Kawabuchi Y, Ohta K, Shimizu K, Minami K, Hihara J, Sawamura A and Toge T: Locoregional immunotherapy of malignant effusion from colorectal cancer using the streptococcal preparation OK-432 plus interleukin-2. Induction of autologous tumour-reactive CD4+ Th1 killer lymphocytes. Br J Cancer *89*: 1876-1884, 2003.

18 van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, van den Eynde B, Knuth A and Boon T: A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science (Wash DC) *254*: 1643-1647, 1991.

19 Kawakami Y, Eliyahu S, Sakaguchi K, Robbins PF, Rivoltini L, Yannelli JR, Appella E and Rosenberg SA: Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2 restricted tumor infiltrating lymphocytes. J Exp Med *180*: 347-352, 1994.

20 Ito M, Shichijo S, Miyagi Y, Kobayashi T, Tsuda N, Yamada A, Saito N and Itoh K: Identification of SART3-derived peptides capable of inducing HLA-A2-restricted and tumor-specific CTLs in cancer patients with different HLA-A2 subtypes. Int J Cancer *88*: 633-639, 2000.

21 Sadanaga N, Nagashima H, Mashino K, Tahara K, Yamaguchi H, Ohta M, Fujie T, Tanaka F, Inoue H, Takesako K, Akiyoshi T and Mori M: Dendritic cell vaccination with MAGE peptide is a novel therapeutic approach for gastrointestinal carcinomas. Clin Cancer Res *7*: 2277-2284, 2001.

22 Itoh T, Ueda Y, Kawashima I, Nukaya I, Fujiwara H, Fuji N, Yamashita T, Yoshimura T, Okugawa K, Iwasaki T, Ideno M, Takesako K, Mitsuhashi M, Orita K and Yamagishi H: Immunotherapy of solid cancer using dendritic cells pulsed with the HLA-A24-restricted peptide of carcinoembryonic antigen. Cancer Immunol Immunother *51*: 99-106, 2002.

23 Mine T, Gouhara R, Hida N, Imai N, Azuma K, Rikimaru T, Katagiri K, Nishikori M, Sukehiro A, Nakagawa M, Yamada A, Aizawa H, Shirouzu K, Itoh K and Yamana H: Immunological evaluation of CTL precursor-oriented vaccines for advanced lung cancer patients. Cancer Sci *94*: 548-56, 2003.

24 Kedl RM, Schaefer BC, Kappler JW and Marrack P: T cells down-modulate peptide-MHC complexes on APCs *in vivo*. Nature Immunol *3*: 27-32, 2002.

25 Provenzano M, Mocellin S, Bettinotti M, Preuss J, Monsurro V, Marincola FM and Stroncek D: Identification of immune dominant cytomegalovirus epitopes using quantitative real-time polymerase chain reactions to measure interferon-gamma production by peptide-stimulated peripheral blood mononuclear cells. J Immunother *25*: 342-351, 2002.

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Locoregional immunotherapy of malignant ascites from gastric cancer using DTH-oriented doses of the streptococcal preparation OK-432: Treatment of Th1 dysfunction in the ascites microenvironment

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Abstract. Locoregional administration of the streptococcal preparation OK-432 is effective in treating malignant ascites from gastric cancer. In order to enhance the efficacy, we conducted a pilot study of locoregional immunotherapy for malignant ascites using host-oriented doses of OK-432. Moreover, action mechanisms of OK-432 were further explored in view of the T-helper type 1 (Th1)-Th2 concept. Gastric cancer patients with cytologically determined malignant ascites were locoregionally administered with OK-432. The dose of OK-432 was selected according to the delayed-type hypersensitivity (DTH) reaction levels to OK-432. Cytokine production profiles of ascites cells were determined using whole ascites assay by stimulation with OK-432. IL-10 mRNA expression was analyzed using RT-PCR. It was found that a positive clinical response was observed in 37 of the 51 (73%) patients with the DTH-oriented approach, showing a significantly higher efficacy than traditional dosage methods using empirical doses (31/58, 53%) ($p=0.0487$). The DTH-oriented administration of OK-432 produced adverse effects such as fever elevation ($p<0.0001$) and abdominal pain ($p=0.0013$) to a significantly lesser extent compared with the traditional treatment. Analysis of the action mechanism of OK-432 revealed that the DTH reaction in responders (19±6 mm) was stronger than that in non-responders (6±4 mm) ($p<0.0001$). Tumor necrosis factor (TNF)-α production of ascites cells was also higher in responders (3943±1247 pg/ml) than in non-responders (1217±939 pg/ml) ($p=0.0002$). There was a significant positive correlation ($p=0.0085$) between the levels of DTH reaction and TNF-α production of ascites cells, but not of blood cells. Responders appeared to polarize on the Th1 axis when clinical responses were plotted on Th1-Th2 dimensions according to the cytokine production profiles of TNF-α, IFN-γ, IL-4 and IL-6 of ascites cells. *In vitro* culture with IL-2 of ascites cells after OK-432 administration demonstrated an almost clonal expansion of $CD4^+$ lymphocytes, which produced TNF-α and IFN-γ, but did not produce IL-4 or IL-6. IL-10 mRNA expression was detectable in ascites cells from non-responders before treatment. These results suggest that the DTH-oriented locoregional administration of OK-432 may be both effective and less toxic in treating malignant ascites from gastric cancer, showing a possibility of the tailored immunotherapy for malignant ascites. Th1 dysfunction exists in the microenvironment of malignant ascites from gastric cancer, in which IL-10 may, in part, play a role. The up-regulation of Th1 responses by OK-432 may result in positive clinical responses. The DTH reaction to OK-432 may be a useful tool not only for predicting clinical response but also for selecting the optimal dose of OK-432.

Introduction

Malignant ascites often occurs as a principal clinical problem in primary or refractory cancer patients, and is associated with several objective and subjective symptoms such as anorexia, full sensation in the abdomen and dyspnea. If effective and practical treatments for malignant ascites were available for these terminally ill patients, it would offer them better quality of life and might possibly prolong the survival. Locoregional administration of various agents, including antineoplastic chemotherapeutic drugs (1,2), biological response modifiers for immunotherapy (2), and gene therapy agents (3) by paracentesis may be good clinical candidates for the treatment of malignant ascites due to its pharmacokinetic advances in drug delivery.

In the present study, we examine locoregional immunotherapy for malignant ascites using OK-432. OK-432 is a penicillin- and heat-inactivated lyophilized powder of *Streptococcus pyogenes* A3. In Japan, OK-432 has been approved for use in treating lymphangioma (4), in post-operative

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adjuvant therapy for several cancers in combination with anti-cancer chemotherapy (5,6), and in treating malignant effusions resulting from gastrointestinal and lung cancers (7). The standard approved dose of OK-432 for malignancies is 5 KE per body.

OK-432 has a capability to induce potent host-mediated immune responses and belongs to the category of biological response modifiers. The mechanism responsible for the anti-tumor activity of OK-432 has been studied both *in vitro* and *in vivo*. Uchida and Micksche (8) reported that intrapleural administration of OK-432 augmented ascites' natural killer (NK) cell activity and reduced ascites' NK suppressor cell activity. Katano and Torisu (9) demonstrated the induction of tumoricidal neutrophils by OK-432. In a previous study, we reported that the locoregional administration of OK-432 induced serial cellular infiltration of immunocompetent cells, including neutrophils, macrophages and lymphocytes, into the local cavity (7). Fujimoto *et al* (10) recently found that OK-432 stimulated IL-12 production to potentiate T-helper type 1 (Th1) responses. The terms Th1 and Th2 originally described in mouse model studies (11), and later were extended to studies on human immune systems (12,13). T-helper cells can be divided into two subpopulations, Th1 and Th2, according to the cytokine production profiles. Th1 produces interleukin (IL)-2, tumor necrosis factor (TNF) and interferon (IFN)-γ, and is involved in cellular immune responses including delayed-type hypersensitivity (DTH) reactions. On the other hand, Th2 produces IL-4, -5 and -6, and participates in humoral immune responses through IgE synthesis. Studies on the function of Th1 and Th2 have led to a greater understanding of many disorders, including autoimmune and neoplastic diseases (12,13).

We here conducted a pilot study of the locoregional immunotherapy for malignant ascites from gastric cancer using a host-oriented dose of OK-432. Further, we analyzed the clinical response in terms of Th1 and Th2 in order to address the mechanism of OK-432 immunotherapy. We will show that the up-regulation of Th1 responses by locoregional administration of OK-432 can result in a positive clinical response, and that the clinical response of patients with malignant ascites can be predicted with a DTH skin reaction, which may permit a host-oriented approach of determining an optimal dose of OK-432.

Materials and methods

Patients and locoregional immunotherapy using OK-432. Fifty-one gastric cancer patients with cytologically proven malignant ascites were enrolled in the study. The subjects were less than 80 years of age and had measurable ascites on sonographic examination and computed tomographic (CT) scans, an Eastern Cooperative Oncology Group performance status of 0-3, and a life expectancy of at least 3 months. Written informed consent was obtained from each patient before treatment. Ascitic fluid was removed by paracentesis before treatment, and a host-oriented dose of OK-432, described in detail below, was administered thereafter. OK-432 administration was once repeated on day 8 when no decrease of ascites was observed. Fifty-eight gastric cancer patients with malignant ascites who had been treated empirically with 5 KE of OK-432 served as a historical control. All of our subjects had been administered combination chemotherapy with 5-fluorouracil (350 mg/body, daily, continuously) and cis-platinum (5 mg/body, daily, intravenously), which had failed to reduce the ascites. Clinical responses were assessed on day 15 by cytological and sonographic examination together with CT scans. We then assigned our patients to one of two groups: responders, who showed disappearance or decrease of ascites with negative cytology for more than 1 month after the treatment; and non-responders, who had stable or increasing ascites even after the treatment. Toxicities associated with OK-432 immunotherapy were assessed according to the National Cancer Institute Common Toxicity Criteria Version 2.0, with the exception of fever elevation which was classified by degree: ≤37°C, 37-38°C, 38-39°C, and >39°C. Changes of performance status and subjective symptoms of full sensation in the abdomen and oral food intake were also evaluated before and after the treatment.

DTH skin reaction to OK-432 and its clinical dose. The DTH skin reaction test using OK-432 was performed in all patients prior to the treatment and doses of OK-432 were decided. In brief, 0.004 KE OK-432 dissolved in 0.02 ml saline was intradermally injected in the forearm and the diameter of redness was measured 24 h later. The dose of OK-432 for treatment was determined as follows: 10 KE for patients showing redness of 0-5 mm, 5 KE for those with 6-15 mm, 2 KE for those with 16-25 mm, and 1 KE for those with >26 mm of skin redness. One KE of OK-432 contains 0.1 mg of lyophilized streptococci.

Cytokine assay. Cytokine production potential was determined using whole blood/ascites assay in initial 20 patients consisting of 14 responders and 6 non-responders. Briefly, heparinized venous blood and ascites were collected prior to treatment and 0.5 ml of each sample was added to 5 ml of RPMI-1640 medium containing 0.1 KE/ml OK-432 (Chugai Pharmaceutical Company, Tokyo). Incubation was performed at 37°C in a 5% CO_2 incubator and supernatants were collected at 24 h, because preliminary experiments showed that cytokine production of blood cells and ascites cells peaked at 24 h when stimulated with 0.1 KE/ml of OK-432. IFN-γ and TNF-α were measured as Th1 cytokines, and IL-4 and -6 as Th2 cytokines by ELISA (R&D Systems, Minneapolis, MN).

Preparation of locoregional lymphocytes. Heparinized ascites cells of responder patients were obtained by paracentesis before and after treatment and pelleted. Cells were resuspended in RPMI-1640 medium and layered on 75/100% Ficoll-Conray gradient. After centrifugation at 400 g for 30 min, autologous tumor cells were collected from 75% interface and mononuclear cells from 100% interface. Mononuclear cells were washed 3 times and incubated in RPMI-1640 medium supplemented with 2% heat-inactivated autologous serum and 80 U/ml IL-2. Incubation was maintained with changing half the medium twice a week. On day 21, cells were harvested and subjected to flow cytometry, and culture supernatants were collected for measuring cytokine production by ELISA.

Table I. Patient characteristics.

	Empirical (n=58)	DTH-oriented (n=51)
Age	62±8	64±9
Gender (male/female)	41/17	32/19
P.S. (0/1/2/3)	4/21/28/5	4/19/24/4
Prior operation (yes/no)	52/6	46/5
Histology		
Differentiated	13	11
Undifferentiated	45	40
Concurrent metastases		
Liver	17	18
Lymph node	47	45

Flow cytometry. Fifty µl of the lymphocyte suspension ($5x10^5$) was incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-labeled monoclonal antibodies at 4°C for 45 min. Cells were washed twice and resuspended in RPMI-1640 medium. The monoclonal antibodies used were anti-Leu4a (CD3), -Leu3a (CD4), -Leu2a (CD8) and -Leu19 (CD56, Becton Dickinson, Mountain View, CA). Flow cytometric analysis was performed on Cytron (Ortho Diagnostic Systems, Inc., Raritan, NJ). After being adequately gated on lymphocytes using forward and side scatter, data collection was set up to stop when 10000 events had been analyzed.

IL-10 mRNA expression. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze IL-10 expression. In brief, total cellular RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction, and RNA samples were reverse-transcribed into cDNA with a random hexamer (14). PCR amplification of the cDNA was performed in a reaction mixture consisting of cDNA samples, Taq polymerase (Gibco-BRL, Grand Island, NY, USA) and the following primers: IL-10, 5'-AAGCTGAGAACCAAGA CCCAGACATCAAGGC-3' and 3'-AGCTATCCCAGAGCC CCAGATCCGATTTTGG-5' (15), and ß-actin (Stratagene, La Jolla, CA, USA). The reaction was carried out in a Perkin-Elmer Cetus thermal cycler (Perkin-Elmer Corporation, Eden Prairie, MN, USA) under conditions of 3 min denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. After amplification, 8 µl of the reaction mixture was removed and analyzed by electrophoresis through 2.0% agarose gels in Tris-borate-EDTA buffer, and the gels were then stained with ethidium bromide. The expected lengths of the amplified cDNAs were 328 and 514 bp for IL-10 and ß-actin, respectively.

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

Table II. Comparison of clinical efficacies between empirical and DTH-oriented OK-432 immunotherapies for malignant ascites in gastric cancer patients.

Response	Empirical (n=58)	DTH-oriented (n=51)
Yes	31 (53)	37 (73)
No	27 (47)	14 (27)

Locoregional immunotherapy of malignant ascites was performed in gastric cancer patients using DTH-oriented dose of OK-432, and its clinical responses were compared with those of empirical control treatments. A statistical value was significantly different, p=0.0487. Parentheses indicate percentages.

Results

Patient characteristics. The characteristics of the 51 patients in the present study are listed in Table I. The patients had a mean age of 64±9 years, and included 32 males and 19 females, and showed performance status (P.S.) values of 0-3. Forty-six patients had undergone prior resection of the primary tumor. Eighteen and 45 patients had concurrent liver and lymph node metastases, respectively, as well as ascites detectable by CT scan. Gastric cancer patients (n=58) with malignant ascites, who had been previously treated with an empirical dose of OK-432 (5 KE), are also shown in Table I as a historical control group. No significant differences were observed in the patients' characteristics between the DTH-oriented group and the control group.

Clinical efficacy of locoregional immunotherapy for malignant ascites using OK-432. Clinical efficacy was evaluated by sonographic examination and CT scan (Table II). Positive responses were observed in 37 of 51 (73%) patients in the DTH-oriented group and in 31 of 58 (53%) patients in the control group in treating malignant ascites by locoregional immunotherapy using OK-432. There was a significant difference in response rate between the control and the DTH-oriented groups (p=0.0487).

Toxicity of locoregional immunotherapy for malignant ascites using OK-432. The DTH-oriented administration of OK-432 was assessed in terms of toxicity (Table III). Hematological toxicity was not observed in any patients analyzed except as an increase in white blood cell counts at approximately 12000-18000/mm^3, which decreased spontaneously within one week (data not shown). The most common constitutional symptom was fever elevation with temperatures of >37°C observed in the control group in 90% of patients treated by OK-432, and most notably in 14% of the control group, who suffered a fever of >39°C. In the DTH-oriented group, 71% patients showed a fever elevation of >37°C and <38°C, and no patients suffered a temperature of >39°C. There was a significant difference of fever elevation levels between the control and study groups (p<0.0001). Abdominal pain, the second most frequently observed adverse effect, was also

Table III. Comparison of adverse effects between empirical and DHT-oriented OK-432 immunotherapies for malignant ascites in gastric cancer patients.

Adverse effects	Empirical (n=58)	DHT-oriented (n=51)
Fever elevation (°C)		
<37	6 (10)	5 (9)
≥37, <38	17 (29)	36 (71)
≥38, <39	27 (47)	10 (20)
≥39	8 (14)	0
Abdominal pain		
Grade 1, 2	8 (14)	14 (27)
Grade 3	12 (21)	0

Locoregional immunotherapy of malignant ascites was performed in gastric cancer patients using DTH-oriented dose of OK-432, and its adverse effects were compared with those of empirical control treatments. Statistical values were $p<0.0001$ and $p=0.0013$ for adverse effects of fever elevation and abdominal pain, respectively. Parentheses indicate percentages.

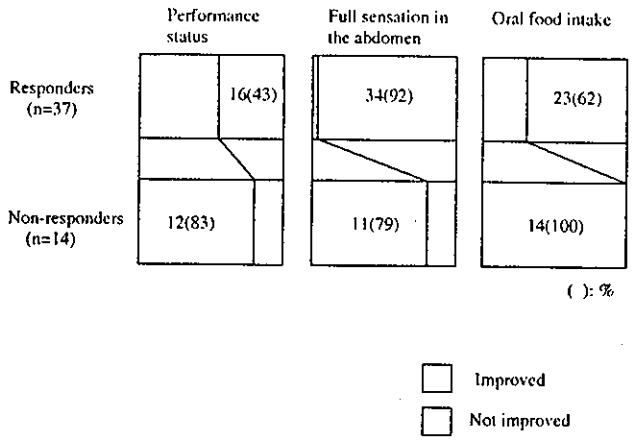

Figure 1. Clinical significance of locoregional immunotherapy for gastric cancer patients with malignant ascites. Gastric cancer patients with malignant ascites were treated with locoregional immunotherapy using OK-432, and improvement in the findings indicated, both subjective and objective, were compared between responders and non-responders.

examined. In the control group, 21% of patients showed grade 3 dull pain that disturbed their daily life for a couple of days after the treatment. In the DTH-oriented group, 27% of patients complained of grade 1 or 2 abdominal pain, but none suffered pain of grade 3. There was a significant difference in abdominal pain between the two groups ($p=0.0013$). Other toxicities experienced were of no more than grade 2 in the DTH-oriented group (data not shown).

Improvement of symptoms in responders. The significance of OK-432 immunotherapy was assessed by comparing performance status, full sensation in the abdomen and oral food intake between responders and non-responders (Fig. 1). Improvement in these three symptoms was found to be 43%, 93% and 64% in responders, and 17%, 17% and 0% in non-responders, respectively. There were significant differences between the presented values of responders and non-responders in full sensation of the abdomen and oral food intake, but not in performance status ($p<0.0001$, $p<0.0001$, $p=0.0986$, respectively).

DTH reactions to OK-432 and clinical responses. In order to address the background of responder patients to OK-432 immunotherapy, the DTH skin reaction to OK-432 was analyzed in patients with malignant ascites (Fig. 2). The redness of the DTH reaction differed from patient to patient within the range of 0-30 mm in diameter, with a mean ± SD of 15±8 mm. These skin reactions were clearly divided into two populations in view of clinical responses to OK-432 treatment. The redness diameters of the DTH reaction in responders and non-responders were 19±6 and 6±4 mm, respectively, and the DTH reaction of responders was significantly stronger than that of non-responders ($p<0.0001$).

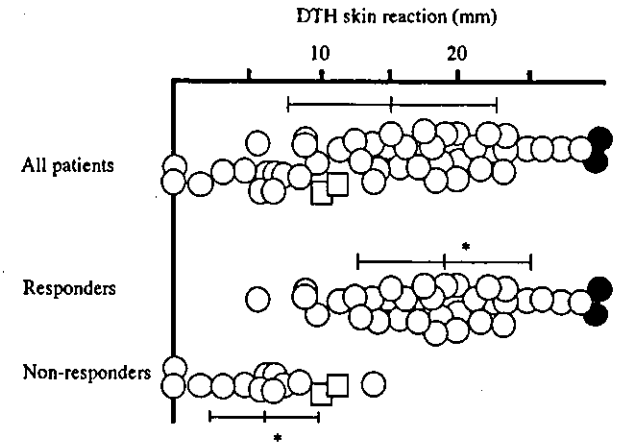

Figure 2. DTH skin reaction levels in gastric cancer patients with malignant ascites. A DTH skin reaction test using OK-432 was performed in gastric cancer patients with malignant ascites before OK-432 immunotherapy, and the diameter of skin redness was measured at 24 h and plotted. Patients who had skin redness with blister (●) and those with faint redness (○) are also shown. Significant difference from the value of responders, *$p<0.0001$.

Moreover, blister formation was observed in 2 responders, while it was rather difficult to determine the redness margin in 2 non-responders because of its faintness.

TNF-α production of blood cells and ascites cells by OK-432. The cytokine production of blood cells and ascites cells by *in vitro* stimulation with OK-432 was measured with the whole blood assay and the whole ascites assay in patients treated with OK-432 immunotherapy (Fig. 3). The blood cells of responder patients produced significantly higher TNF-α than those of non-responders (1418±907 pg/ml vs. 533±422 pg/ml) ($p=0.036$). The ascites cells produced more TNF-α than blood cells by OK-432 stimulation, and the difference in TNF-α production between responders and non-

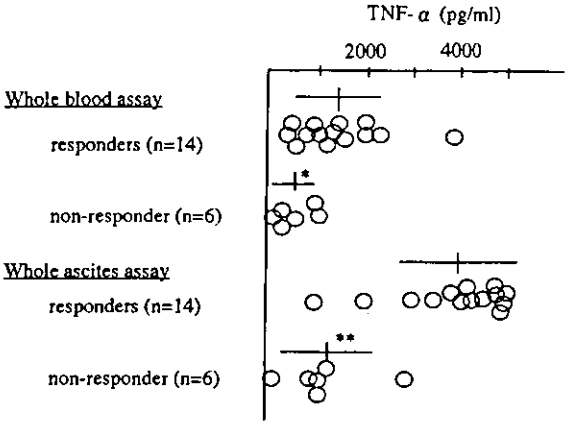

Figure 3. TNF-α production potential of blood and ascites cells stimulated with OK-432. Ascites fluids were collected from gastric cancer patients before OK-432 immunotherapy and diluted in medium, then stimulated with OK-432 for 24 h (whole ascites assay). TNF-α concentration in the supernatant was measured by ELISA. Values were significantly different between responders and non-responders, *p=0.036, **p=0.0002.

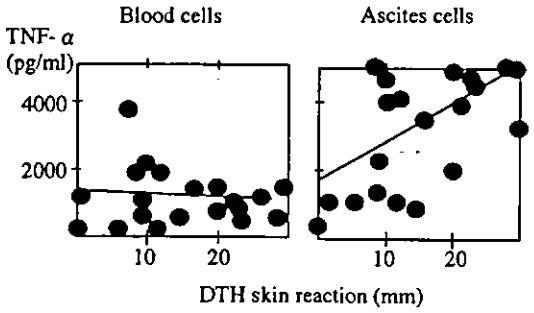

Figure 4. Relationship between DTH skin reaction levels and TNF-α production levels of blood cells or ascites cells. DTH skin reaction levels and TNF-α production levels of blood cells or ascites cells were measured in gastric cancer patients with malignant ascites before OK-432 treatment, and linear regression analysis was carried out to clarify relationship between both values. Regression coefficients were -0.036 (p=0.881) or 0.563 (p=0.0085) for relationships between DTH skin reaction levels and TNF-α production levels of blood cells or ascites cells, respectively.

responders was markedly augmented when evaluated by the whole ascites assay. Ascites cells from responder patients produced 3943±1247 pg/ml TNF-α, whereas those from non-responders produced 1217±939 pg/ml TNF-α. A significant difference was found between these two values (p=0.0002).

Relationship between levels of DTH response and TNF-α production. We next analyzed the correlation between levels of DTH reactions to OK-432 and TNF-α productions of blood cells or ascites cells stimulated with OK-432 *in vitro* (Fig. 4). We were unable to find any correlation between the DTH reaction and TNF-α production levels of blood cells stimulated with OK-432 (r=-0.036, p=0.881). We did, however, observe a significant positive correlation between the DTH reactions and TNF-α production levels of ascites cells by OK-432 stimulation, in which the correlation coefficient was calculated at 0.563 (p=0.0085).

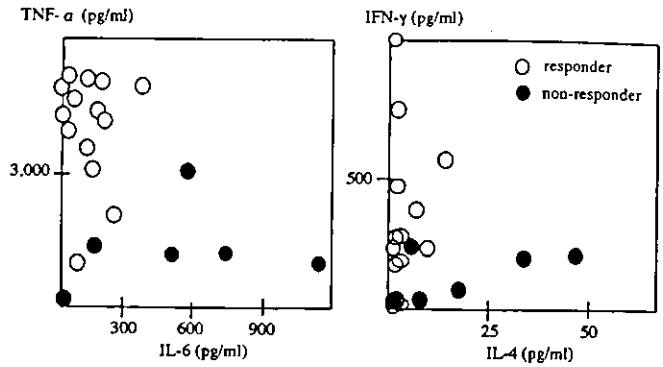

Figure 5. Comparison between clinical responses of OK-432 immunotherapy and Th1, Th2 cytokine production profiles of ascites cells by *in vitro* stimulation with OK-432. Cytokine production profiles, including TNF-α and IFN-γ as Th1 cytokines, and IL-6 and IL-4 as Th2 cytokines, of ascites cells from gastric cancer patients were determined by whole ascites assay using OK-432 stimulation before OK-432 immunotherapy. Clinical responses were plotted on a Th1-Th2 dimension as indicated.

Relationship between clinical responses and Th1/Th2 cytokines. Clinical responses were analyzed in detail in order to explore the mechanisms of OK-432 immunotherapy by using the Th1/Th2 concept based on cytokine production profiles of ascites cells of TNF-α, IFN-γ, IL-4 and IL-6, which were measured by means of the whole ascites assay using OK-432 (Fig. 5). When the clinical responses were plotted on a TNF-α/IL-6 dimension, responder patients appeared to polarize on the TNF-α axis, while the non-responder patients deviated on the IL-6 axis. A similar result was observed when the data were analyzed in the IFN-γ/IL-4 dimension, in which the responders polarized on the IFN-γ axis but the non-responders did not.

Preferential expansion of CD4⁺ Th1 lymphocytes. Ascites lymphocytes of 3 responders were stimulated *in vitro* with IL-2 and further characterized by the phenotype analysis and cytokine production analysis (Table IV). More than 89% of lymphocytes predominantly expressed CD3⁺ and CD4⁺ phenotypes, but CD8 and CD56 populations were around 15%. These CD3⁺CD4⁺ lymphocyte lines were found to produce predominantly TNF-α and IFN-γ but little or no IL-4 or IL-6.

IL-10 mRNA expression of ascitic cells. Finally, the expression of Th2 cytokine IL-10 was examined at the mRNA level (Fig. 6). Among the 13 patients examined, 10 of the 11 responders to OK-432 immunotherapy showed no expression of IL-10 mRNA in ascites cells before treatment (Fig. 6, lanes 1-10). However, both non-responders, as well as one responder, demonstrated positive bands of IL-10 mRNA expression in ascites cells prior to treatment (Fig. 6, lanes 11-13).

Discussion

We have demonstrated here the efficacy of locoregional immunotherapy for malignant ascites in gastric cancer patients using the streptococcal preparation OK-432. Approximately

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.

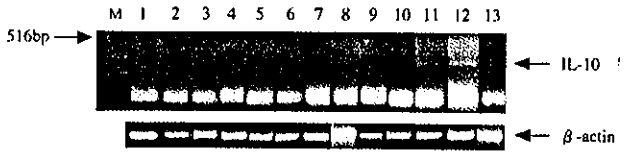

Figure 6. IL-10 mRNA expression in ascites cells before OK-432 immunotherapy. mRNA was extracted from ascites cells of gastric cancer patients before OK-432 immunotherapy, and IL-10 expression was evaluated by RT-PCR analysis using primers specific for human IL-10.

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 postoperative 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.

References

1. Kimura M, Konno T, Miyamoto Y, Kojima Y and Maeda H: Intracavitary administration: pharmacokinetic advantages of macromolecular anticancer agents against peritoneal and pleural carcinomatoses. Anticancer Res 18: 2547-2550, 1998.
2. Nio Y, Nagami H, Tamura K, Tsubono M, Nio M, Sato M, Kawabata K, Hayashi H, Shiraishi T, Imai S, Tsuchitani T, Mizuta J, Nakagawa M and Fukumoto M: Multi-institutional randomized clinical study on the comparative effects of intracavital chemotherapy alone versus immunotherapy alone versus immunochemotherapy for malignant effusion. Br J Cancer 80: 775-785 1999.
3. Hsiao M, Tse V, Carmel J, Tsai Y, Felgner PL, Haa M and Silverberg GD: Intracavitary liposome-mediated p53 gene transfer into glioblastoma with endogenous wild-type p53 *in vivo* results in tumor suppression and long-term survival. Biochem Biophys Res Commun 233: 359-364, 1997.
4. Ogita S, Tsuto T, Tokiwa K and Takahashi T: Intracystic injection of OK-432: a new sclerosing therapy for cystic hygroma in children. Br J Surg 74: 690-691, 1987.
5. Sakamoto J, Teramukai S, Nakazato H, Sato Y, Uchino J, Taguchi T, Ryoma Y and Ohashi Y: Efficacy of adjuvant immunochemotherapy with OK-432 for patients with curatively resected gastric cancer: a meta-analysis of centrally randomized controlled clinical trials. J Immunother 25: 405-412, 2002.
6. Sakamoto J, Teramukai S, Watanabe Y, Hayata Y, Okayasu T, Nakazato H and Ohashi Y: Meta-analysis of adjuvant immunochemotherapy using OK-432 in patients with resected non-small cell lung cancer. J Immunother 24: 250-256, 2001.
7. Yamaguchi Y, Satoh Y, Miyahara E, Noma K, Funakoshi M, Takashima I, Sawamura A and Toge T: Locoregional immunotherapy of malignant ascites by intraperitoneal administration of OK-432 plus IL-2 in gastric cancer patients. Anticancer Res 15: 2201-2206, 1995.
8. Uchida A and Micksche M: Intrapleural administration of OK432 in cancer patients: activation of NK cells and reduction of suppressor cells. Int J Cancer 31: 1-5, 1983.
9. Katano M and Torisu M: Neutrophil-mediated tumor cell destruction in cancer ascites. Cancer 50: 62-68, 1982.
10. Fujimoto T, Duda RB, Szilvasi A, Chen X, Mai M and O'Donnell MA: Streptococcal preparation OK-432 is a potent inducer of IL-12 and a T helper cell 1 dominant state. J Immunol 158: 5619-5626, 1997.
11. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA and Coffman RL: Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 136: 2348-2357, 1986.

12. Romagnani S: Human TH1 and TH2 subsets: doubt no more. Immunol Today 12: 256-257, 1991.
13. Maggi E, Parronchi P, Manetti R, Simonelli C, Piccinni MP, Rugiu FS, De Carli M, Ricci M and Romagnani S: Reciprocal regulatory effects of IFN-gamma and IL-4 on the *in vitro* development of human Th1 and Th2 clones. J Immunol 148: 2142-2147, 1992.
14. Miyahara E, Yamaguchi Y, Minami K, Hihara J, Noma K, Toge T, Takafuta T and Fujimura K: T-cell receptor V beta gene usage of human cytotoxic T-cell clones obtained from gastric cancer patients. Anticancer Res 19: 2057-2066, 1999.
15. Somoza N, Vargas F, Roura-Mir C, Vives-Pi M, Fernandes-Figueras MT, Ariza A, Gomis R, Bragado R, Marti M, Jaraquemada D and Pujol-Borrell R: Pancreas in recent onset insulin-dependent diabetes mellitus. Changes in HLA, adhesion moleculaes and autoantigens, restricted T cell receptor Vß usage, and cytokine profile. J Immunol 153: 1360-1367, 1994.
16. Mackey JR and Venner PM: Malignant ascites: demographics, therapeutic efficacy and predictors of survival. Can J Oncol 6: 474-480, 1996.
17. Van de Plassche-Boers EM, Drexhage HA, Kokje-Kleingeld M and Leezenberg HA: Parameters of T cell mediated immunity to commensal micro-organisms in patients with chronic purulent rhinosinusitis: a comparison between delayed type hypersensitivity skin test, lymphocyte transformation test and macrophage migration inhibition factor assay. Clin Exp Immunol 66: 516-524, 1986.
18. Brunet JL, Liaudet AP, Later R, Peyramond D and Cozon GJ: Delayed-type hypersensitivity and chronic fatigue syndrome: the usefulness of assessing T-cell activation by flow cytometry - preliminary study. Allerg Immunol 33: 166-172, 2001.
19. Nakazato H, Koike A, Saji S, Ogawa N and Sakamoto J: Efficacy of immunochemotherapy as adjuvant treatment after curative resection of gastric cancer. Study Group of Immunochemotherapy with PSK for Gastric Cancer. Lancet 343: 1122-1126, 1994.
20. Talmadge JE, Phillips H, Schindler J, Tribble H and Pennington R: Systematic preclinical study on the therapeutic properties of recombinant human interleukin 2 for the treatment of metastatic disease. Cancer Res 47: 5725-5732, 1987.
21. Laufer S, Greim C and Bertsche T: An *in vitro* screening assay for the detection of inhibitors of proinflammatory cytokine synthesis: a useful tool for the development of new antiarthritic and disease modifying drugs. Osteoarthritis Cartilage 10: 961-967, 2002.
22. Elsasser-Beile U, von Kleist S and Gallati H: Evaluation of a test system for measuring cytokine production in human whole blood cell cultures. J Immunol Methods 139: 191-195, 1991.
23. Yoshino S, Tabata T, Hazama S, Iizuka N, Yamamoto K, Hirayama M, Tangoku A and Oka M: Immunoregulatory effects of the antitumor polysaccharide lentinan on Th1/Th2 balance in patients with digestive cancers. Anticancer Res 20: 4707-4711, 2000.
24. Shibata M, Nezu T, Kanou H, Abe H, Takekawa M and Fukuzawa M: Decreased production of interleukin-12 and type 2 immune responses are marked in cachectic patients with colorectal and gastric cancer. J Clin Gastroenterol 34: 416-420, 2002.
25. Gotlieb WH, Abrams JS, Watson JM, Velu TJ, Berek JS and Martinez-Meza O: Presence of interleiukin-10 (IL-10) in the ascites of patients with ovarian and other intra-abdominal cancers. Cytokine 4: 385-390, 1992.
26. Matsuda M, Salazar F, Petersson M, Masucci G, Hansson J, Pisa P, Zhang QJ, Masucci MG and Kiessling R: Interleukin 10 pretreatment protects target cells from tumor- and all-specific cytotoxic T cells and downregulates HLA class I expression. J Exp Med 180: 2371-2375, 1994.
27. Shurin MR, Yurkovetsky ZR, Tourkova IL, Balkir L and Shurin GV: Inhibition of CD40 expression and CD40-mediated dendritic cell function by tumor-derived IL-10. Int J Cancer 101: 61-68, 2002.
28. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW and O'Garra A: IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol 146: 3444-3450, 1991.
29. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M and Trinchieri G: Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 178: 1041-1048, 1993.
30. Hihara J, Yamaguchi Y, Minami K, Noma K and Toge T: Down-regulation of IL-10 enhances the efficacy of locoregional immunotherapy using OK-432 against malignant effusion. Anticancer Res 19: 1077-1084, 1999.

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

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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.

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

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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

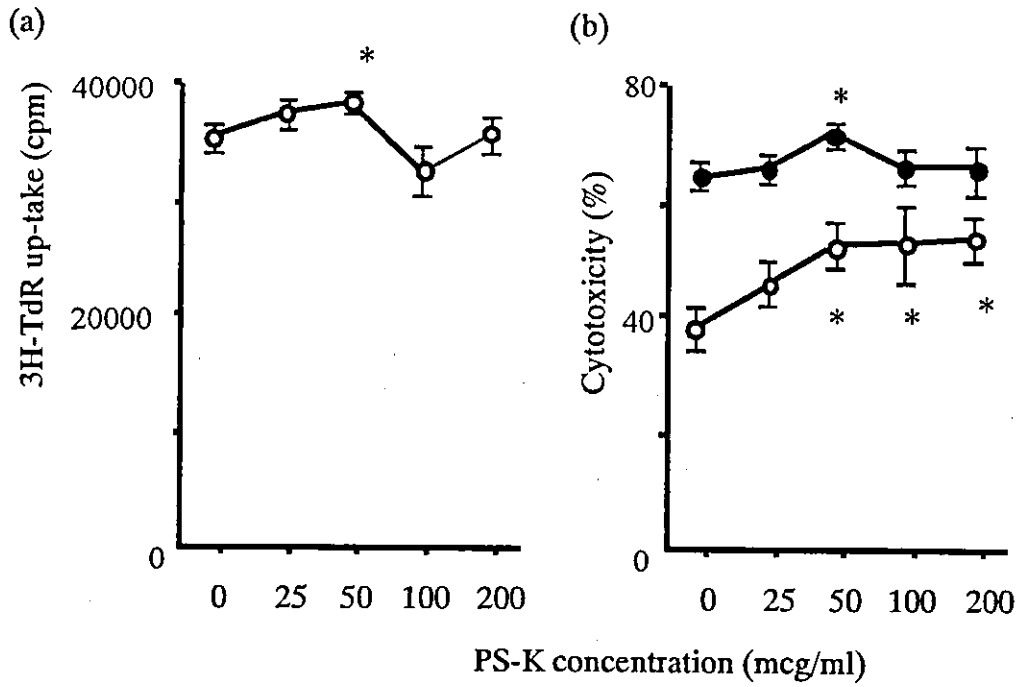

Figure 1. *Proliferative responses and cytotoxic activities of PBMCs stimulated with IL-2 in the presence of PS-K. (a): PBMCs were stimulated with 200 U/ml IL-2 in the presence of 0-200 mcg/ml PS-K and 3H-TdR uptakes of PBMCs were determined. (b): Cytotoxic activities against K562 (●) or Daudi (○) target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

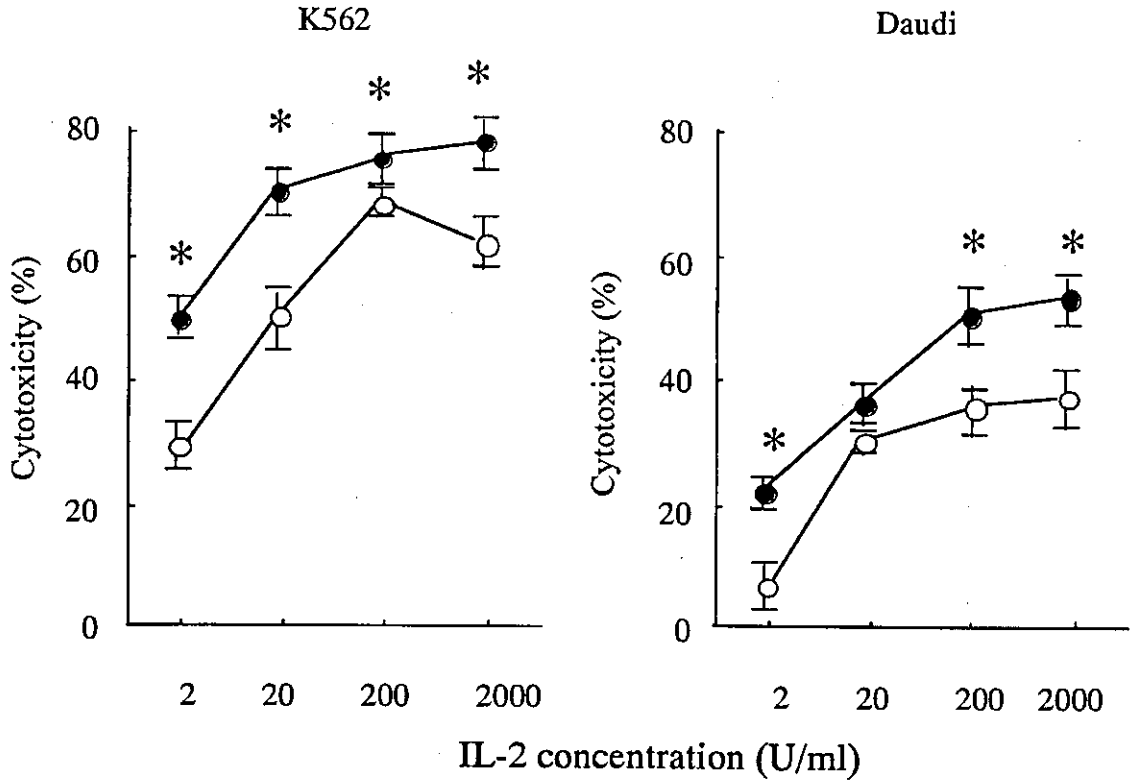

Figure 2. *Requirement of IL-2 for generating killing activity in PBMCs in the presence of PS-K. PBMCs were stimulated with 2-2000 U/ml IL-2 in the presence (●) or absence (○) of 50 mcg/ml PS-K, and cytotoxic activities against K562 or Daudi target cells were determined. Significant differences form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

Table I. *Enhancing effect of CD25+ cell induction and soluble IL-2 Rα 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.

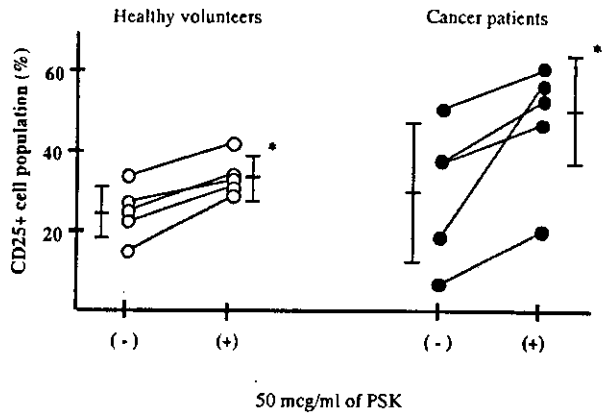

Figure 3. *Increase of CD25+ cell population in PBMCs stimulated with IL-2 plus PS-K. PBMCs from healthy volunteers and cancer patients were stimulated with 200 U/ml IL-2 alone or IL-2 plus 50 mcg/ml PS-K, and CD25+ cell populations were analyzed. Significant differences form the value without PS-K,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

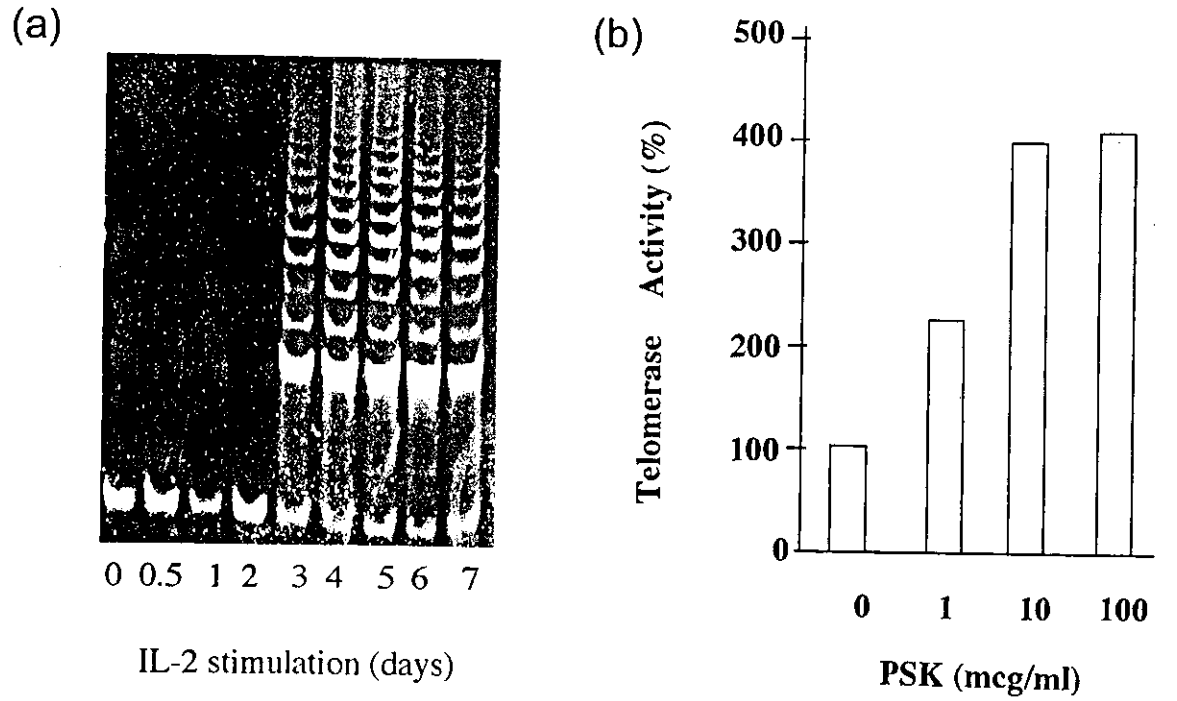

Figure 4. *Telomerase activity of PBMCs stimulated with IL-2 plus PS-K. PBMCs were stimulated with 200 U/ml IL-2 alone or IL-2 plus PS-K, and telomerase activity was determined with TRAP assay on days indicated. A representative result of PBMCs from a colon cancer patient with liver metastasis was shown.*

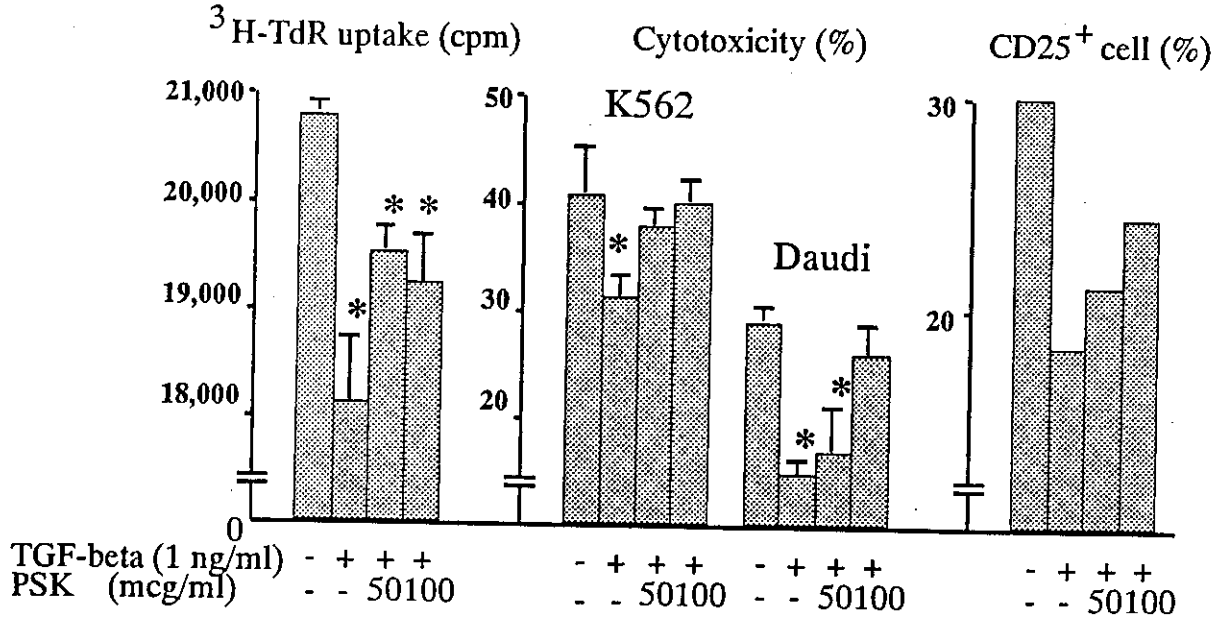

Figure 5. *Inhibitory effects of TGF-beta on IL-2-induced PBMC responses and its abrogation by PS-K. PBMCs were stimulated with 200 U/ml IL-2 in the presence or absence of TGF-beta and PS-K as indicated. Proliferative responses, cytotoxic activities and CD25+ cell populations of PBMCs were analyzed.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

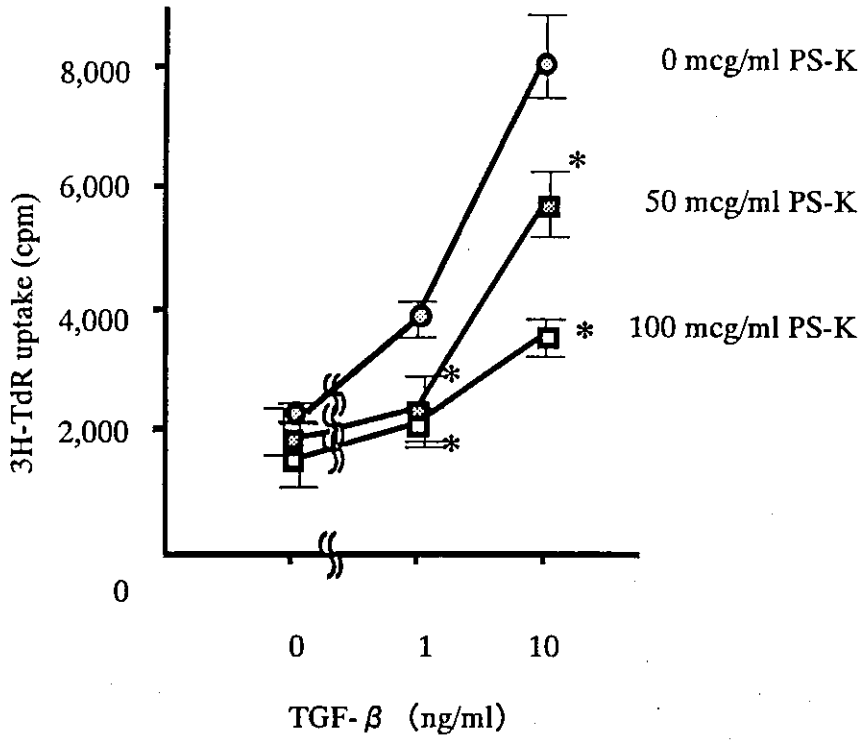

Figure 6. *Anchorage-independent growth of NRK cells in the presence of TGF-beta and PS-K. NRK cells were grown in a soft agar as described in Materials and Methods in the presence or absence of TGF-beta and PS-K, and 3H-TdR incorporation of NRK cells was determined. Significant differences from the 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

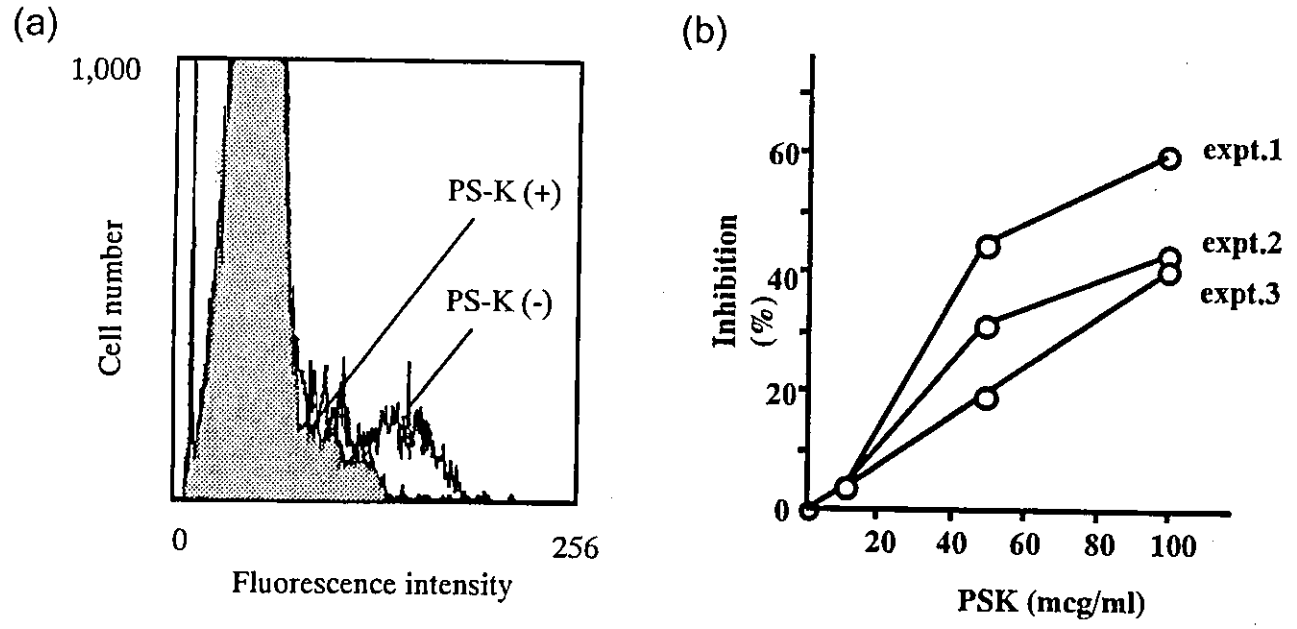

Figure 7. *Inhibition of binding of TGF-beta on PBMCs activated with IL-2. PBMCs were stimulated with 200 U/ml IL-2 and TGF-betaR was analyzed using FITC-labelled TGF-beta in the presence or absence of PS-K.*

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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References

1 Ooi VE and Liu F: Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. Curr Med Chem *7*: 715-729, 2000.

2 Fisher M and Yang LX: Anticancer effects and mechanisms of polysaccharide-K (PSK): implications of cancer immunotherapy. Anticancer Res *22*: 1737-1754, 2002.

3 Yefenof E, Gafanovitch I, Oron E, Bar M and Klein E: Prophylactic intervention in radiation-leukemia-virus-induced murine lymphoma by the biological response modifier polysaccharide K. Cancer Immunol Immunother *41*: 389-396, 1995.

4 Algarra I, Collado A, Garcia Lora A and Garrido F: Differential effect of protein-bound polysaccharide (PSK) on survival of experimental murine tumors. J Exp Clin Cancer Res *18*: 39-46, 1999.

5 Fukushima M: Adjuvant therapy of gastric cancer: The Japanese experiences. Semin Oncol *23*: 369-378, 1996.

6 Nakazato H, Koike A, Saji S, Ogawa N and Sakamoto J: Efficacy of immunochemotherapy as adjuvant treatment after curative resection of gastric cancer. Study Group of Immunochemotherapy with PSK for Gastric Cancer. Lancet *343*: 1122-1126, 1994.

7 Toge T and Yamaguchi Y: Protein-bound polysaccharide increases survival in resected gastric cancer cases stratified with a preoperative granulocyte and lymphocyte count. Oncol Rep *7*: 1157-1161, 2000.

8 Mitomi T, Tsuchiya S, Iijima N, Aso K, Suzuki K, Nishiyama K, Amano T, Takahashi T, Murayama N and Oka H: Randomized, controlled study on adjuvant immunochemotherapy with PSK in curatively resected colorectal cancer. The Cooperative Study Group of Surgical Adjuvant Immunochemotherapy for Cancer of Colon and Rectum (Kanagawa). Dis Colon Rectum *35*: 123-130, 1992.

9 Yunoki S, Tanaka N, Hizuta A and Orita K: Enhancement of antitumor cytotoxicity of hepatic lymphocytes by oral administration of PSK. Int J Immunopharmacol *16*: 123-130, 1994.

10 Okuzawa M, Shinohara H, Kobayashi T, Iwamoto M, Toyoda M and Tanigawa N: PSK, a protein-bound polysaccharide, overcomes defective maturation of dendritic cells exposed to tumor-derived factors *in vitro*. Int J Oncol *20*: 1189-1195, 2002.

11 Iguchi C, Nio Y, Takeda H, Yamasawa K, Hirahara N, Toga T, Itakura M and Tamura K: Plant polysaccharide PSK: cytostatic effects on growth and invasion; modulating effect on the expression of HLA and adhesion molecules on human gastric and colonic tumor cell surface. Anticancer Res *21*: 1007-1013, 2001.

12 Matsunaga K, Morita I, Oguchi Y, Fujii T, Yoshikumi C and Nomoto K: Restoration of immune responsiveness by a biological response modifier, PSK, in aged mice bearing syngeneic transplantable tumor. J Clin Lab Immunol *24*: 143-149, 1987.

13 Saji S, Sakamoto J, Teramukai S, Kunieda K, Sugiyama Y, Ohashi Y and Nakazato H: Impact of splenectomy and immunochemotherapy on survival following gastrectomy for carcinoma: covariate interaction with immunosuppressive acidic protein, a serum marker for the host immune system. Tumor Marker Committee for the Study Group of Immunochemotherapy with PSK for Gastric Cancer. Surg Today *29*: 504-510, 1999.

14 Mule JJ, Schwarz SL, Roberts AB, Sporn MB and Rosenberg SA: Transforming growth factor-beta inhibits the *in vitro* generation of lymphokine-activated killer cells and cytotoxic T cells. Cancer Immunol Immunother *26*: 95-100, 1988.

15 Chen W and Wahl SM: TGF-beta: receptors, signaling pathways and autoimmunity. Curr Dir Autoimmun *5*: 62-91, 2002.

16 Kasid A, Bell GI and Director EP: Effects of transforming growth factor-beta on human lymphokine-activated killer cell precursors. Autocrine inhibition of cellular proliferation and differentiation to immune killer cells. J Immunol *141*: 690-698, 1988.

17 Ruegemer JJ, Ho SN, Augustine JA, Schlager JW, Bell MP, McKean DJ and Abraham RT: Regulatory effects of transforming growth factor-beta on IL-2- and IL-4-dependent T cell-cycle progression. J Immunol *144*: 1767-1776, 1990.

18 Frolik CA, Dart LL, Meyers CA, Smith DM and Sporn MB: Purification and initial characterization of a type beta transforming growth factor from human placenta. Proc Natl Acad Sci USA *80*: 3676-380, 1983.

19 Nilsen-Hamilton M: Transforming growth factor-beta and its actions on cellular growth and differentiation. Curr Top Dev Biol *24*: 95-136, 1990.

20 Grotendorst GR, Smale G and Pencev D: Production of transforming growth factor beta by human peripheral blood monocytes and neutrophils. J Cellular Physiol *140*: 396-402, 1989.
21 Minami K, Yamaguchi Y, Yoshida K, Quan CP and Toge T: Dysregulation of telomerase activity and expression in lymphokine-activated killer cells from advanced cancer patients: possible involvement in cancer-associated immunosuppression mechanism. Oncol Rep *8*: 649-653, 2001.
22 Kariya Y, Inoue N, Kihara T and Fujii M: Activation of human natural killer cells by the protein bound polysaccharide PSK independently of interferon and interleukin-2. Immunology Lett *31*: 241-245, 1992.
23 Nio Y, Shiraishi T, Tsubono M, Morimoto H, Tseng CC, Imai S and Tobe T: *In vitro* immunomodulating effect of protein-bound polysaccharide, PSK on peripheral blood, regional nodes, and spleen lymphocytes in patients with gastric cancer. Cancer Immunol Immunother *32*: 335-341, 1991.
24 Rosenstein M, Ettinghausen SE and Rosenberg SA: Extravasation of intravascular fluid mediated by the systemic administration of recombinant interleukin 2. J Immunol *137*: 1735-1742, 1986.
25 Ballmer-Weber BK, Dummer R, Kung E, Burg G and Ballmer PE: Interleukin 2-induced increase of vascular permeability without decrease of the intravascular albumin pool. Br J Cancer *71*: 78-82, 1995.
26 Harada M, Matsunaga K, Oguchi Y, Iijima H, Tamada K, Abe K, Takenoyama M, Ito O, Kimura G and Nomoto K: Oral administration of PSK can improve the impaired anti-tumor CD4+ T-cell response in gut-associated lymphoid tissue (GALT) of specific-pathogen-free mice.Int J Cancer *70*: 362-372, 1997.
27 Habelhah H, Okada F, Nakai K, Choi SK, Hamada J, Kobayashi M and Hosokawa M: Polysaccharide K induces Mn superoxide dismutase (Mn-SOD) in tumor tissues and inhibits malignant progression of QR-32 tumor cells: possible roles of interferon alpha, tumor necrosis factor alpha and transforming growth factor beta in Mn-SOD induction by polysaccharide K. Cancer Immunol Immunother *46*: 338-344, 1998.
28 Zhang H, Morisaki T, Matsunaga H, Sato N, Uchiyama A, Hashizume K, Nagumo F, Tadano J and Katano M: Protein-bound polysaccharide PSK inhibits tumor invasiveness by down-regulation of TGF-beta1 and MMPs. Clin Exp Metastasis *18*: 343-352, 2000.
29 Matsunaga K, Hosokawa A, Oohara M, Sugita N, Harada M and Nomoto K: Direct action of a protein-bound polysaccharide, PSK, on transforming growth factor-beta. Immunopharmacology *40*: 219-230, 1998.
30 Toge T, Yamaguchi Y, Kegoya Y, Baba N, Yanagawa E and Hattori T: Blocking of lymphocyte surface binding sites for the soluble suppressor factor by protein-bound polysaccharide, PSK. Int J Immunopharmacol *11*: 9-12, 1989.
31 Greene WC and Waldmann TA: Inhibition of human lymphocyte proliferation by the nonmitogenic lectin wheat germ agglutinin. J Immunol *124*: 2979-2987, 1980.
32 Massague J: Subunit structure of a high-affinity receptor for type b-transforming growth factor. J Biol Chemistry *260*: 7059-7066, 1985.
33 O'Grady P, Kuo MD, Baldassare JJ, Huang SS and Huang JS: Purification of a new type high molecular weight receptor (type V receptor) of transforming growth factor beta (TGF-beta) from bovine liver. Identification of the type V TGF-beta receptor in cultured cells. J Biol Chem *266*: 8583-8589, 1991.

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ORIGINAL ARTICLE

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Paclitaxel probably enhances cytotoxicity of natural killer cells against breast carcinoma cells by increasing perforin production

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Abstract Paclitaxel, a semisynthetic taxane, is one of the most active chemotherapeutic agents for the treatment of patients with breast cancer. We focused on the effect of paclitaxel on the cytotoxicity of natural killer (NK) cells. NK cells were purified by negative selection with magnetic beads from peripheral blood mononuclear cells of healthy volunteers. A human breast carcinoma cell line BT-474 and an NK cell–sensitive erythroleukemia cell line K562 were used as targets. Cytotoxicity of NK cells was determined by ^{51}Cr-release assay with labeled target cells. Paclitaxel (1–100 nM) did not affect cellular viability, and significantly enhanced cytotoxicity of NK cells in a dose-dependent manner. Although paclitaxel did not affect Fas-ligand expression of NK cells, paclitaxel induced mRNA and protein production of perforin, an effector molecule in NK cell–mediated cytotoxicity. Concanamycin A, a potent inhibitor of the perforin-mediated cytotoxic pathway, inhibited paclitaxel-dependent NK cell–mediated cytotoxicity. Furthermore, paclitaxel induced activation of nuclear factor κB (NF-κB) in NK cells. NF-κB inhibitor pyrrolidine dithiocarbamate significantly suppressed both paclitaxel-induced perforin expression and NK cell cytotoxicity. Our results show for the first time that paclitaxel enhances in vitro cytotoxicity of human NK cells. Moreover, our results suggest a significant association between enhanced NK cell cytotoxicity, increased perforin production, and NF-κB activation.

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Keywords Natural killer cells · Nuclear factor κB · Paclitaxel · Perforin

Abbreviations

CMA	Concanamycin A
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IL	Interleukin
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
NF-κB	Nuclear factor κB
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PDTC	Pyrrolidine dithiocarbamate
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Introduction

Paclitaxel and docetaxel are two representative taxane-based chemotherapeutic agents for the treatment of patients with breast cancer [6, 10, 12, 30]. Paclitaxel is a semisynthetic taxane isolated from the Western yew, *Taxus brevifolia* [10, 12, 23, 30]. Paclitaxel is thought to induce apoptosis in carcinoma cells by binding to tubulin, inducing tubulin polymerization and microtubule formation, and blocking cell mitosis [22, 33, 43]. Judging from the results of recent studies, however, paclitaxel may induce apoptosis through different mechanisms, including c-Jun NH_2-terminal kinase activation, nuclear factor κB (NF-κB)[3] activation, p66 Shc phosphorylation, or mitogen-activated protein kinase pathway [3, 13, 21, 44, 46, 47], depending on cell lines and culture conditions. In addition, paclitaxel induces apoptosis in some cells and biological activation in other cells [24]. In murine models, paclitaxel has been shown