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Induction of gene expression for immunomodulating cytokines in peripheral blood mononuclear cells in response to orally administered PSK, an immunomodulating protein-bound polysaccharide

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Abstract The protein-bound polysaccharide extracted from a fungus, PSK, has been used as a biological response modifier in the treatment of cancer patients in Japan for over 16 years. The administration of PSK to tumor-bearing rodents inhibited tumor growth and modulated immune responses. Recently, an in vitro study has revealed that PSK is a strong inducer of cytokine gene expression and production in human peripheral blood mononuclear cells (PBMC). To establish whether PSK has cytokine-inducing activities in vivo, we have orally administered PSK (1 g, the clinical dose) to 12 healthy volunteers and 9 gastric cancer patients who had undergone gastrectomy, and assessed the gene expression for cytokines in PBMC of each subject. As determined by the reverse-transcribed polymerase chain reaction method, the induction of gene expression for both tumor necrosis factor α and interleukin-8 (IL-8) was detected in PBMC from 5 of the 12 healthy volunteers (42%) and 4 of the 9 patients (44%). Furthermore, the concentration of serum IL-8 was elevated in 5 healthy volunteers given PSK orally, who had shown induction of IL-8 gene expression, as detected by enzyme-linked immunosorbent assay. These findings indicate that responsiveness of PBMC to PSK, in terms of gene expression and production of cytokines, varies among individuals. Thus, when using PSK to treat cancer patients, it seems advisable to select patients on the basis of their responsiveness to PSK. We speculate that the cytokines induced by PSK might mediate the immunoenhancing action of this agent in vivo.

Key words PSK · TNF · IL-8 · PBMC · Gastric cancer

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Introduction

The protein-bound polysaccharide termed PSK has been isolated from *Corioulous versicolor* by hot-water extraction of the mycelia followed by saturated ammonium sulfate precipitation and desalting by dialysis against water [1]. PSK has been biochemically characterized and consists of a heterogeneous mixture of glycosylated protein with an average molecular mass of approximately 100 kDa [1]. The antitumor activities of PSK have been demonstrated in experimental animal models [1], and beneficial therapeutic effects have been noted in clinical studies of esophageal [2], colon [3, 4], and gastric cancer [5–7]. Although the mechanism of the immunoenhancing activity of this agent is not fully understood, it has been reported that PSK enhanced various immune responses in vivo as well as in vitro [1, 8–10]. It has been recently shown that PSK induced the expression of mRNA in human PBMC for interleukin-1α (IL-1α), IL-1β, IL-6, IL-8, tumor necrosis factor α (TNFα) and monocyte chemotactic and activating factor (MCAF) in vitro [11].

We demonstrate here the in vivo induction of gene expression and production of inflammatory and host-defensive cytokines, TNFα [12] and IL-8 [13], by oral administration of PSK, which might account for the immunomodulating activities of this agent.

Patients and methods

Patients and volunteers

Twelve healthy volunteers and 9 gastric cancer patients with gastrectomy were entered into this study. All patients were required to meet the following eligibility criteria: 2 weeks since curative surgical operation, a white blood cell count above 3000/mm^3, granulocyte count above 1500/mm^3, platelet count above 100000/mm^3, serum creatinine level above 1.5 mg/dl, bilirubin level below 1.5 mg/dl, and an albumin level above 3.0 g/dl. Exclusion criteria included the presence of hepatitis surface antigen or infections requiring antibiotic therapy.

Administration of PSK and preparation of peripheral blood mononuclear cells (PBMC)

Both groups (12 healthy volunteers and 9 patients) received orally, once, 1 g PSK (the clinical dose) (Sankyo Co. Ltd. Tokyo, Japan). Samples (10 ml) of whole blood were collected in heparinized tubes from volunteers before, and 1, 3, 6, 12, 24 and 48 h after PSK administration, and whole blood of patients was collected before and 24 h after PSK administration in the same way as from the volunteers. PBMC were isolated from whole blood by Ficoll-Hypaque gradient centrifugation.

RNA extraction and reverse transcription/polymerase chain reaction

Total cellular RNA of PBMC was prepared by guanidum isothiocyanate lysis followed by cesium chloride ultracentrifugation [14]. Reverse transcription (RT) of RNA (1 μg) was carried out in 10 μl final volume containing 0.75 μl 10 mM mixture of all four deoxynucleotide triphosphates; 1 μl random hexmers (100 ng/ml), 2 μl 5 × RT buffer (250 mM TRIS/HCl pH 8.3, 375 mM KCl, 15 mM MgCl), and 1 μl reverse transcriptase (200 units/ml) (Gibco-BRL, Bethesda, Md.). This solution (RT solution) was incubated at 37° C for 1.5 h and then heated at 98° C for 5 min. All polymerase chain reaction (PCR) primers (TNFα [15], IL-8 [13], β-actin [16]) synthesized by DNA synthesizer 381A (Applied Biosystems, Foster city, Calif.) and the expected size of the PCR product are described in Table 1. The following components were added to 1 μl RT solution: 8 μl 2.5 mM mixture of all four deoxynucleotide triphosphates, 10 μl 10 × PCR buffer (100 mM TRIS/HCl pH 8.3, 500 mM KCl, 15 mM MgCl, 1 mg/ml bovine serum albumin); 5 μl sense or antisense primer (10 μM), 70.5 μl distilled water; 0.5 μl Taq polymerase (5 units/μl; Perkin Elmer Cetus, Norwalk, Conn.). The RT-PCR assay [17] was performed in 26 cycles for both TNFα and IL-8, and 22 cycles for β-actin using a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, Conn.). Temperature cycling was initiated with each cycle as follows: (a) 94° C for 1 min (denaturation), (b) 55° C for 45 s (annealing), (c) 72° C for 2 min (primer extension). Samples comprising 10 μl PCR products and molecular mass marker (1000-base DNA ladder: Gibco-BRL, Bethesda, Md.) were electrophoresed on a 1% agarose gel and visualized under ultraviolet light.

Enzyme-linked immunosorbent assay (ELISA) for serum IL-8

Samples comprising 3 ml blood from volunteers, taken before and 1, 3, 6, 12, 24 and 48 h after PSK administration were clotted on ice and centrifuged for 10 min (500 *g*), and the serum was frozen at − 70° C until ELISA. Serum IL-8 was measured in triplicate using a double-ligand immunoassay based on a mouse monoclonal anti-IL-8 and rabbit polyclonal anti-IL-8 linked to alkaline phosphatase, which has been developed by our group in collaboration with Dr. K. Matsushima (Kanazawa University, Japan) [18]. The limit of detection was 25 pg/ml IL-8.

Results and discussion

The kinetics of gene expression for TNFα and IL-8 in PBMC from 5 of 12 volunteers receiving PSK (1 g, once) are shown in Fig. 1A. Both TNFα and IL-8 gene expression in PBMC of 5 volunteers (42%) were significantly induced 3 h after PSK administration and this induction remained for 24 h. The results from the other 7 volunteers did not show any induction of gene expression for these cytokines (data not shown). Expression of these cytokine genes was also assessed in PBMC of 9 gastric cancer patients with gastrectomy before and 24 h after PSK administration as shown in Fig. 1B. Among 9 patients, induction of gene expression for both TNFα and IL-8 was observed in PBMC of 4 patients (patients 1–4, 44%). However, gene expression for TNFα and IL-8 in PBMC of the other 5 patients (patients 5–9) could not be induced by PSK. Although gene expression for these cytokine in all of 12 volunteers was not detectable prior to PSK administration, 2 of 9 patients (8 and 9) showed the same level of gene expression for these cytokines before and 24 h after administration, which could be due to possible inflammation in these patients, since both TNFα and IL-8 belong to the inflammatory cytokine family. We found that all of the subjects in whom TNF gene expression was induced also showed induction of IL-8 gene expression in response to PSK administration. This is presumably explained by their gene expression having

Table 1 List of polymerase chain reaction (*PCR*) primers

Gene	Primer sequences	Expected size of PCR product (base pairs)
TNFα	5′ primer: 5′-CTTCTGCCTGCTGCACTTGGA	547
	3′ primer: 5′-TCCCAAAGTAGACCTGCCCAGA	
IL-8	5′ primer: 5′-GCTTCTAGGACAAGAGCCAGGAAG	351
	3′ primer: 5′-CTTGGATACCACAGAGAATGAATTTT	
β-Actin	5′ primer: 5′-ATGGATGATGATATCGCCGCCGCT	1067
	3′ primer: 5′-CGGACTCGTCATACTCCTGCTTG	

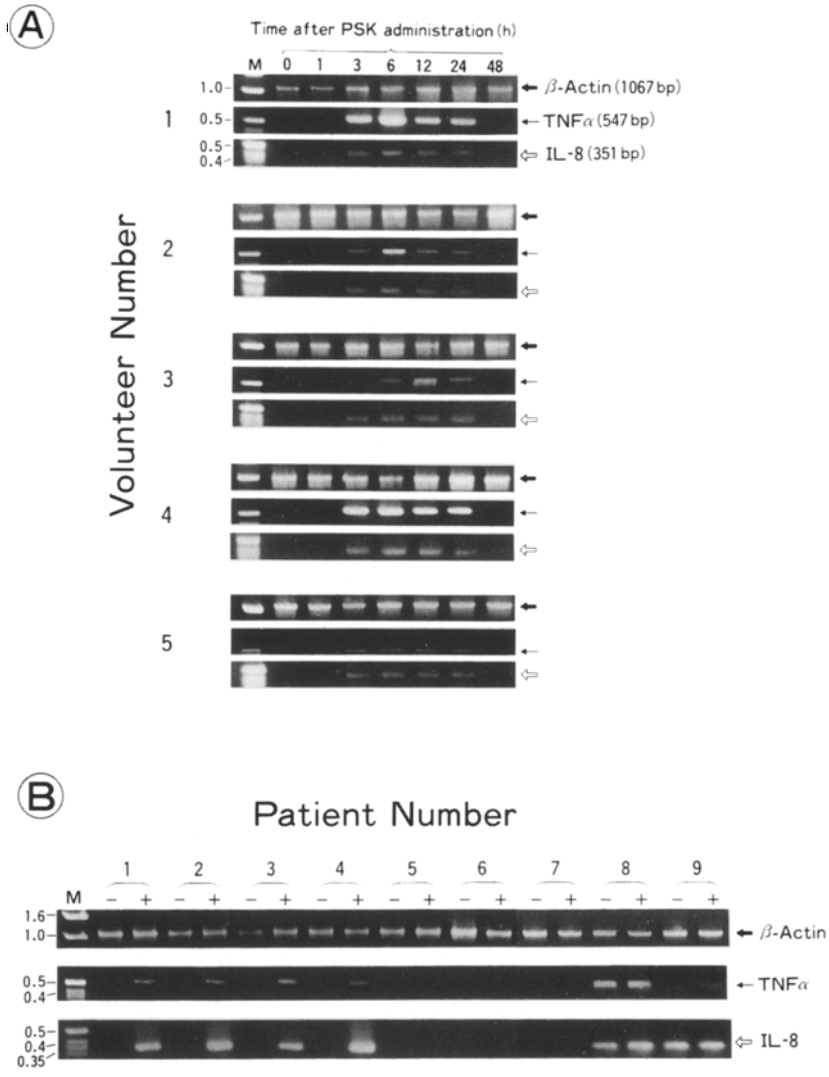

Fig. 1A, B Gene expression for cytokines and β-actin (control) in peripheral blood mononuclear cells (PBMC) of healthy volunteers (**A**), and gastric cancer patients (**B**) in response to orally administered PSK. **A** The total cellular RNA from PBMC of 5 healthy volunteers before, and 1, 3, 6, 12, 24 and 48 h after administration of PSK. **B** The total cellular RNA from 12 gastric cancer patients with gastrectomy before (−) and 24 h after (+) administration of PSK. RNA preparation and reverse transcriptase/polymerase chain reaction assays were performed as described in Patients and methods. *M* molecular mass marker (kilobase pairs). *TNF* tumor necrosis factor, *IL-8* interleukin-8

the same regulatory machinery [19]. Furthermore, the concentration of serum IL-8 was measured using ELISA [18]. All 5 volunteers whose gene expression for IL-8 in PBMC had been induced by PSK administration showed elevated levels of serum IL-8 6 h after PSK administration, and levels of IL-8 peaked at 24 h (Fig. 2). However, no serum IL-8 was detectable (< 25 pg/ml) in the other 7 volunteers (data not shown). These results were consistent with results of the gene expression assay as shown in Fig. 1A.

Some types of immunomodulating polysaccharide such as lentinan [20], sizofiran [21] and PSK are currently used for treating cancer patients in Japan. The immunoenhancing mechanism of these polysaccharides is still unclear, whereas the effect has been proposed to be mediated through host immune systems.

Ours is the first report describing the induction of cytokine gene expression in vivo by an immunomodulating polysaccharide, PSK. TNFα mediates multiple biological effects by directly stimulating target cells such as cytotoxic/cytocidal activities against tumor cells [22], enhancement of antibody production by B lymphocytes [23], and induction of IL-2 receptor expression on T lymphocytes [24]. The induction of TNFα by PSK would contribute, in part, to potent tumoricidal effects of this agent since the administration of neutralizing antibody against TNFα significantly attenuated the antitumor activity of PSK in murine experiments (unpublished results). Recently, it has been reported that TNF or IL-1 significantly induced mitochondrial manganese superoxide dismutase [25, 26], which was thought to be involved in possible protective effects of TNFα against cancer chemo- and radiation therapy [25]. In fact, overexpression of this enzyme has been reported by K. Hirose et al. [27] to promote the survival of cells exposed to doxorubicin and mitomycin C, and ionizing (γ ray) radiation, which are able to generate oxygen radicals [28, 29]. PSK has been shown to be protective against lethal doses of anticancer drugs and ionizing radiation in animal models [30], and we have observed the induction of Mn superoxide dismutase in human PBMC by treatment with PSK in vivo as well as in vitro [30]. Therefore,

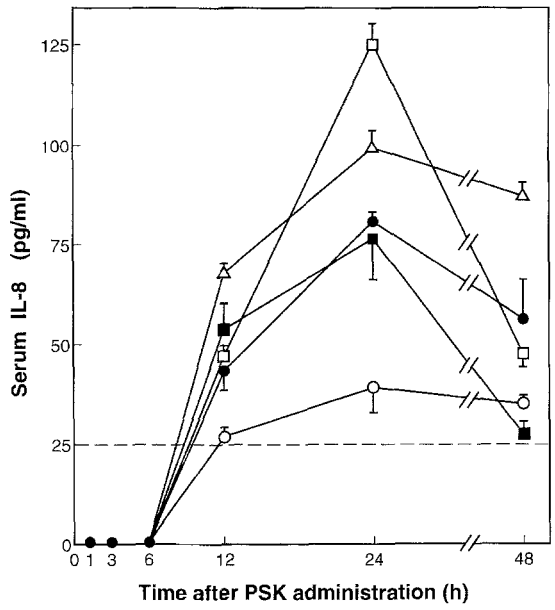

Fig. 2. The elevation of serum IL-8 levels in healthy volunteers in response to orally administered PSK. Preparation of serum and enzyme-linked immunosorbent assay were performed as described in Patients and methods. ■●△□○ Volunteers 1–5, respectively. Values represent the means ± SE from triplicate experiments. – – – Limit of detection for IL-8

the protective effects of PSK against chemo- and radiation therapy would be mediated through Mn superoxide dismutase induction by PSK-induced TNFα production.

IL-8 is a chemokine for neutrophils [13] and T lymphocytes [31]. It has been found to have anti-infectious [32] and tumoricidal activities in vivo (K. Hirose, unpublished results), mediated by recruitment and activation of neutrophils.

In this report, we have not mentioned the results on gene expression of the other inflammatory cytokines (IL-1, IL-6, and monocyte chemotactic and activating factor), which have been shown to be induced in human PBMC in response to PSK in vitro as previously reported [11]. We speculate that these inflammatory cytokines are probably induced by PSK in vivo, in addition to TNFα and IL-8, since the gene expression for each inflammatory cytokine is regulated in a similar manner.

In conclusion, the present study showing the induction of immunomodulating cytokines in PBMC by orally administered PSK could account, in part, for the multiple immunomodulating activities in vivo, bone marrow protection against chemo- and radiation therapy, and anti-infectious activities of PSK [33]. The induction of cytokine gene expression in response to oral administration of PSK was found in 5 of 12 volunteers and 4 of 9 gastric cancer patients. This strongly suggests the necessity to select patients who would clinically respond to PSK administration.

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