Anti-protein-bound polysaccharide-K monoclonal antibody binds the active structure and neutralizes direct antitumor action of the compound

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Abstract. Protein-bound polysaccharide K (PSK) is extracted and purified from *Coriolus versicolor* (CM101), and is used as an anti-cancer agent. In this study, focusing on the direct actions of PSK, we investigated whether PSK reaches tumor and immune tissues with its active structure remaining intact, and the direct action of PSK was evaluated by its antitumor effects against MethA fibrosarcomas implanted in immuno-deficient NOD/SCID mice. The results obtained suggest that PSK reaches the tumor tissue in its active form and exhibits antitumor effects against MethA cells.

Introduction

Protein-bound polysaccharide-K (PSK) is a protein-polysaccharide complex extracted from the cultured mycelia of *Coriolus versicolor* (strain CM101). In Japan, this compound is mainly used as an anti-cancer agent for postoperative gastric cancer and colorectal cancer in combination with chemotherapeutic agent. The molecular weight of PSK averages approximately 100,000, and varies over a wide range from 5,000 to 300,000. The main structural constituent is polysaccharide with the main chain linked by ß-D-(1→4) bond and the side chains by ß-D-(1→3) and ß-D-(1→6) bonds.

The antitumor effect of PSK is manifested by three major mechanisms of action: i) inhibition of cell proliferation (cytostatic effect); ii) neutralization of immunosuppressive substances; and iii) immunostimulatory activities (1-13).

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However, no data has hitherto demonstrated that the physiologically active structure of PSK is actually absorbed from bowel and reaches tumor and immune tissues. In addition, it remains unknown which moiety of the PSK molecule is responsible for these direct and indirect antitumor actions. One of the reasons is that PSK is not a compound with homogeneous structure, but is a mixture of glucan and protein complexes formed during the extraction process. Therefore isolation and structural determination of the active structure by conventional methods such as high performance liquid chromatography and mass spectral analysis are difficult. Furthermore, since the structure of its active site is unknown, there are difficulties in investigating the blood concentration and tumor tissue distribution of the compound after administration.

Methods for the detection of PSK include the Limulus test that detects ß1-3 glucan or lipopolysaccharide (LPS) (unpublished data). However, since the Limulus test is positive not only for PSK but also for other polysaccharides containing ß1-3 glucan (such as laminarin and yeast glucan), it is not a specific detection method for PSK (unpublished data). Production of antibodies against PSK and the evaluation of these antibodies have also been reported (14). However, the rabbit anti-PSK polyclonal antibodies produced recognize all molecules containing ß1-3 glucan, ß1-4 glucan and ß1-6 glucan structures, and is therefore relatively low specificity for PSK. These antibodies are not appropriate for the detection of PSK with physiological activities (unpublished data).

In the present study, we produced a monoclonal antibody against the active structure of PSK, and investigated whether PSK reaches tumor and other tissues with its active structure remaining intact.

Materials and methods

Animals experiment. All animal experiments were performed under the Institutional Guidelines for Care and Use of Laboratory Animals (Biomedical Research Laboratories, Kureha Corp.). The experimental protocol was approved by the Ethics Committee on Animal Experiments of the Biomedical Research Laboratories of Kureha Corp., and the

mice were handled in accordance with the guidelines of the committee. Five-week-old female NOD/scid mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan), and used in experiments at the age of 6 weeks after acclimatization under specific pathogen-free conditions. The mice were allowed free access to CE-2 diet (Oriental Yeast, Tokyo, Japan) and sterilized tap water. They were housed in an animal room conditioned at $25\pm2^{\circ}C$, $55\pm7\%$ humidity, laminar air flow and 12 h light/12 h dark cycle at 150-300 lux. To maintain a uniform environment, noise was carefully avoided, and only keepers and investigators were allowed into the animal room.

Materials. PSK (Kureha Corp., Tokyo, Japan) was dissolved in phosphate-buffered saline (PBS, pH 7.4). Cellulose [ß-(1,4)-D-glucan], laminarin [ß-(1,3)(1,6)-D-glucan], glycogen [α-(1,4)-D-glucan], dextran [α-(1,6)-D-glucan] were purchased from Sigma-Aldrich Japan (Tokyo, Japan).

Cell culture conditions. P2/0-Ag14 myeloma cell line (SP2/0 cells), mink lung epithelial cell line (Mv1Lu) and MethA fibrosarcoma cell line were obtained from the American Type Culture Collection (Rockville, MD, USA). Mouse macrophage-like cell line (J774.1) was obtained from RIKEN Bioresource Center (Tsukuba, Japan). Mv1Lu cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., San Diego, CA) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaill, France), 50 IU/ml penicillin, and 50 μg/ml streptomycin. SP2/0 cells, MethA fibrosarcoma cells and J774.1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen) supplemented with 10% FBS, 50 IU/ml penicillin and 50 μg/ml streptomycin. The cells were grown at $37^{\circ}C$ under 5% CO_2 in a humidified atmosphere and passaged before reaching confluency using 0.25% (w/v) trypsin solution containing 0.04% (w/v) EDTA.

Preparation of anti-PSK monoclonal antibodies. Anti-PSK monoclonal antibodies (mAbs) were prepared by immunizing BALB/c mice with PSK as follows. Two hundred micrograms of PSK was emulsified in an equal volume of Freund's complete adjuvant (Sigma-Aldrich Japan) and injected subcutaneously into female BALB/c mice (Charles River Laboratories Japan, Inc.). After 1 week, emulsion of PSK in Freund's incomplete adjuvant (Sigma-Aldrich Japan) was injected subcutaneously once a week for 6 weeks, followed by a mixture of PSK with TiterMax gold adjuvant (Interchim, Montluçon, France) once a week for 4 weeks. Seven days after the final booster, spleens were collected from the immunized mice. Freshly harvested spleen cells and myeloma cells (P2/0-Ag14 cells) were mixed at a ratio of 10:1, co-pelleted by centrifugation, and fused by adding PEG-1500 (Roche Applied Science, Indianapolis, IN, USA) solution to the pellet. The fused cells were centrifuged, resuspended in RPMI-1640 medium supplemented with 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin and $1x10^{-5}$ mol/l hypoxanthine-aminopterin-thymidine (HAT, Sigma-Aldrich Japan) and plated in 96-well plates. After 1 week of HAT selection, the medium was changed to 1X hypoxanthine and thymidine (HT) medium. The hybridomas were allowed to grow for 10-15 days, and the supernatants from the hybridoma

cultures were screened for anti-PSK mAb by an enzyme-linked immunosorbent assay (ELISA). The hybridomas that produce high-titer antibody to PSK were monocloned by limited dilution. After repeated cloning, two hybridoma clones, 2G9 mAb and 5G5 mAb, were selected for further characterization. The immunoglobulin subclass was determined with a mouse monoclonal antibody isotyping kit (IsoStrip; Roche Diagnostics, Indianapolis, IN). These two clones secreted immunoglobulins of subclasses IgM_{κ} and IgM_{λ}, respectively. 2G9 mAb and 5G5 mAb were purified by HiTrap SP Sepharose FF (GE Healthcare UK Ltd., Buckinghamshire, UK) and HiLoad Superdex 200 columns (GE Healthcare) from hybridoma serum-free culture supernatants and used in subsequent experiments. Purified anti-PSK mAbs were biotinylated using a Sulfo-OSu biotinylation kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

Competitive ELISA using 2G9 mAb and 5G5 mAb. A 96-well plate was coated with 50 μl of PSK (20 μg/ml) per well and kept at $4^{\circ}C$ overnight. After adding 0, 0.1, 0.5 or 1 μg/ml of 2G9 mAb (50 μl per well) as competitive antibody, 0.1 μg/ml of biotinylated 5G5 mAb (50 μl per well) was added to the 96-well plate and incubated at room temperature (RT) for 2 h. After washing each well with TBS containing 0.05% Tween-20 (TBS-T), 50 μl of horseradish peroxidase (HRP)-conjugated streptavidin (Zymed Laboratories, San Francisco, CA, USA; 0.1 μg/ml) was added and incubated at RT for 1 h. After washing, 100 μl of glycine-citric acid buffer containing 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) and hydrogen peroxide (ABTS Substrate Solution, KPL Inc., KPL, Gaithersburg, MD, USA) was added to each well. Color development was stopped by the addition of 100 μl of 5% sodium dodecyl sulfate solution (ABTS Peroxidase Stop Solution, KPL Inc.) and the optical density was measured at 405-630 nm.

Western blotting of PSK using anti-PSK mAb. PSK (1, 5, 10 μg) was dissolved in sodium Tris-HCl buffer (Daiichi Pure Chemicals Co., Ltd., Tokyo) containing sodium dodecyl sulfate (SDS), electrophoresed on 4-20% polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd.) after denaturing at $65^{\circ}C$ for 15 min, and transferred to Immobilon-P PVDF membrane (Millipore, Tokyo, Japan). The membrane was incubated in 10% Block Ace (DS Pharma Biomedical Co., Ltd., Osaka, Japan) at RT for 1 h. The membrane was then incubated with 0.5 μg/ml of anti-PSK mAb at RT for 2 h, washed with TBS-T and incubated with 0.1 μg/ml of HRP-conjugated goat anti-mouse IgM (Millipore) at RT for 1 h. After washing, the membrane was visualized using TMB membrane peroxidase substrate (KPL Inc.). The silver staining was visualized using a silver staining kit (Daiichi Pure Chemicals Co., Ltd.).

Binding of anti-PSK mAb to protein-degraded PSK. To investigate whether the anti-PSK mAbs (2G9 mAb and 5G5 mAb) recognize the glucan or protein moiety of PSK, competitive ELISA was performed using PSK with degraded protein as competitor. The peptide moiety of PSK was decomposed by dissolving in anhydrous hydrazine and heating at
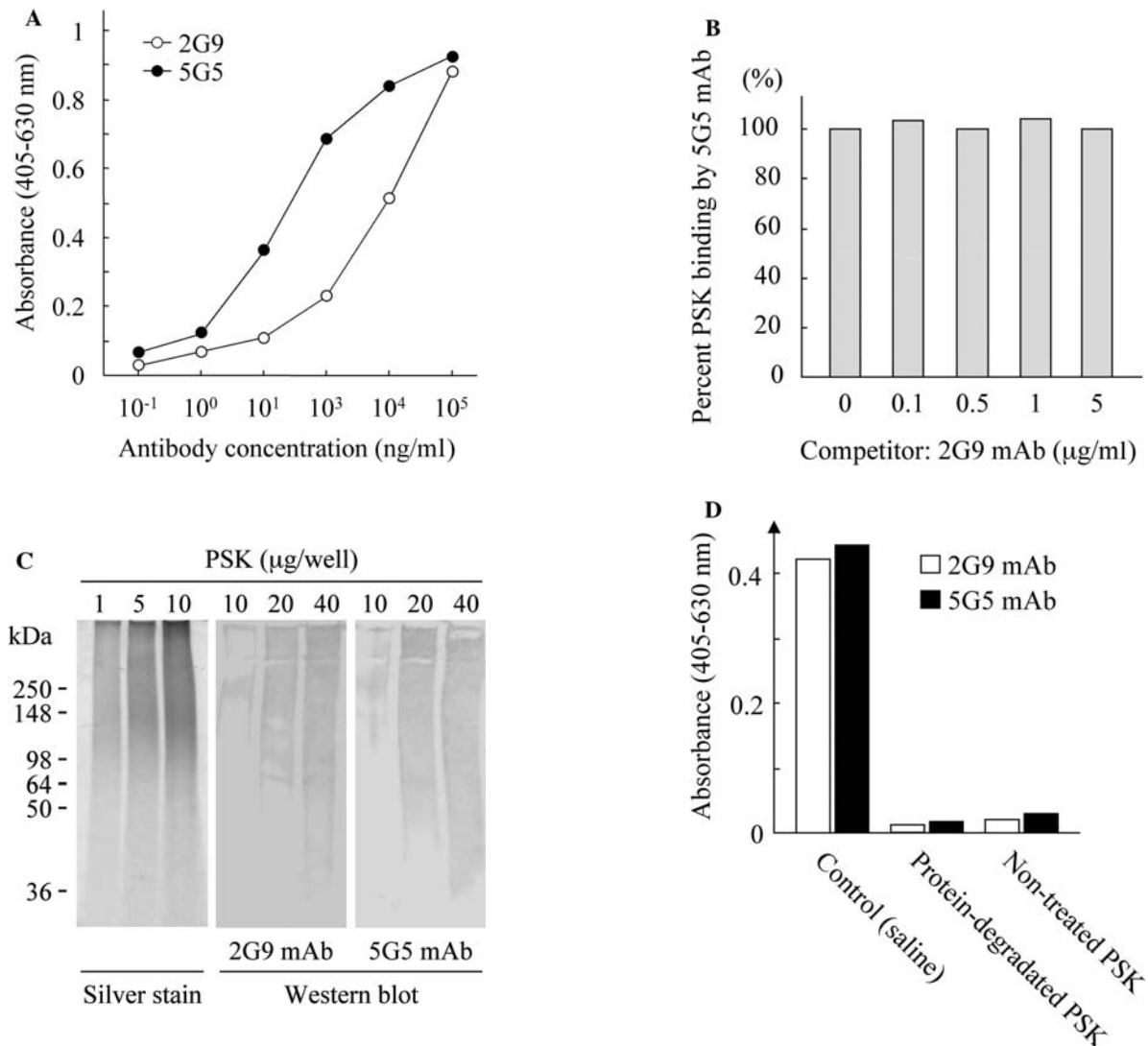
Figure 1. (A) Standard curve of anti-PSK monoclonal antibodies (mAb). Indirect ELISA was applied for estimation of the antibody binding capacity. Fifty microliters of 2G9 mAb and 5G5 mAb (0-4 μg/ml) were dispensed into each PSK-coated well and incubated at RT for 2 h. The titer of the antibody was determined by ELISA. The longitudinal axis and horizontal axis represent absorbance at 630 nm after subtraction of the background and dilution of the antibody, respectively. Each value represents the mean of three independent determinations. (B) Competitive ELISA to analyze the recognition site of anti-PSK mAb. The 2G9 mAb (0, 0.1, 0.5 or 1 μg/ml) was used as competitor, and biotinyled-5G5 mAb (0.1 μg/ml) was used for detection. ELISA was performed as described in Materials and methods. Each column represents the mean of triplicate determinations. (C) Western blots of PSK using anti-PSK mAb. PSK (1, 5, 10 μg) was subjected to SDS-PAGE and immunoblotted with anti-PSK mAbs (2G9 mAb or 5G5 mAb). These experiments were repeated at least thrice. Representative data are shown. (D) Competitive ELISA to evaluate binding of anti-PSK mAbs (2G9 mAb or 5G5 mAb) to hydrazine-treated (to decompose the protein moiety) and non-treated PSK. Each column represents the mean of triplicate determinations.

100˚C for 2 h in accordance with identification scheme (15). Competitive ELISA was performed according to the above procedures.

Competitive ELISA for PSK and other glucans using the anti-PSK mAbs. Competitive ELISA was performed to examine whether the anti-PSK mAbs (2G9 mAb and 5G5 mAb) recognize glucans having similar structures as PSK. Anti-PSK mAb (1 μg/ml) and 10 μg/ml of glucan (PSK, cellulose, laminarin, glycogen or dextran) were mixed and incubated at RT for 1 h. These mixtures were evaluated by competitive ELISA as described above.

Neutralization of PSK's cytostatic activity by anti-PSK mAbs. To examine whether the anti-PSK mAbs (2G9 mAb and

5G5 mAb) neutralize the cytostatic activity of PSK, proliferation of MethA fibrosarcoma cells was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. MethA fibrosarcoma cells were plated in a 96-well culture dish at a density of 1×10^3 cells/100 μl/well, and cultured in a 5% CO_2 incubator at 37˚C for 24 h. Anti-PSK mAb and PSK were mixed in various ratios in 50 mmol/l Tris-HCl buffer (pH 7.5) containing 0.2% bovine serum albumin and 0.2 mol/l sodium chloride and incubated for 1 h at 37˚C. The mixture was added to MethA fibrosarcoma cells in cultured dish, and cultured for another 72 h. Three h before the end of culturing, 1/10 volume of MTT (5 mg/ml in PBS) was added to each well. After incubation at 37˚C for 3 h, the supernatant was removed, and dimethylsulfoxide (DMSO; 100 μl/well) was added. The absorbance of formazan was

measured at 570 nm with reference at 630 nm using a Bio-Rad Microplate Reader 550 (Bio-Rad Laboratories Inc., Tokyo, Japan). Changes in cell number were expressed as mean ± SD percent cell proliferation compared with control cells.

Neutralization of PSK binding to transforming growth factor-β1 by anti-PSK mAbs. The binding of PSK to mouse transforming growth factor-ß1 (TGF-ß$_1$) (R&D Systems Europe Ltd., Abingdon, UK) was evaluated according to the modified method of Hoshi *et al* (16). Briefly, anti-PSK monoclonal antibody and PSK were mixed and incubated for 1 h at 37˚C. Recombinant mouse TGF-ß$_1$ was added to the anti-PSK mAb-PSK mixture and incubated for another 2 h at 37˚C. The TGF-ß$_1$-sensitive Mv1Lu cells were plated in a 96-well culture dish at a density of 1×10^4 cells/100 μl/well and cultured in a 5% CO$_2$ incubator at 37˚C for 24 h. The reaction mixture was added to the cells and incubated for another 24 h. Cell numbers were determined by MTT assay, and the changes were expressed as the mean ± SD percent cell proliferation compared with control cells.

Antitumor effect of PSK in NOD/scid mice. Exponentially growing MethA fibrosarcoma cells were dispersed with 0.25% (w/v) trypsin solution containing 0.04% (w/v) EDTA and resuspended in PBS at a density of 1×10^7/ml. MethA fibrosarcoma cells (1×10^6/mice) were implanted subcutaneously. into the flanks of severely-immunodeficient NOD.CB17-*Prkdc*^{scid}/J (NOD/scid) mice. PSK and anti-PSK mAb were dissolved in saline and administered intravenously at given doses three times a week for 17 days. The body weight and tumor volume of each mouse were measured at least once a week. To determine tumor volume, two bisecting diameters of a tumor were measured using a slide caliper, and the tumor volume was calculated using the following formula: tumor volume = length x (width)^2 x 0.5236 (17). At the end of the experiment, the tumor, spleen and liver was removed and weighed, and fixed in 10% formaldehyde neutral buffer solution. As a rule, each experimental group consisted of six mice, and the same experiment was repeated at least twice. Tumor growth curves were plotted as the mean volume ± standard error (SE).

Immunohistochemistry. Tumor tissues, liver and spleen were fixed in 10% formaldehyde neutral buffer solution and embedded in paraffin. Paraffin sections (4-μm) were deparaffinized with xylene and ethanol, treated with 3% hydrogen peroxide solution for 20 min to inactivate endogenous peroxidase and blocked with 2% porcine plasma in TBS for 15 min to reduce non-specific binding. Then biotinylated 2G9 mAb was added, and the sections were incubated at RT for 2 h. After washing three times with TBS, horseradish peroxidase-labeled streptavidin was added and incubated at room temperature for 1 h, followed by three washes with TBS. Immunoreaction was visualized using Liquid DAB Chromogen (Dako Japan Co. Ltd., Kyoto, Japan). The sections were counterstained with hematoxylin, mounted on glass slides with Entellan Neu (Merck) and examined under a microscope. At the same time, hematoxylin and eosin (H&E) staining was performed on serial sections for morphological evaluation.
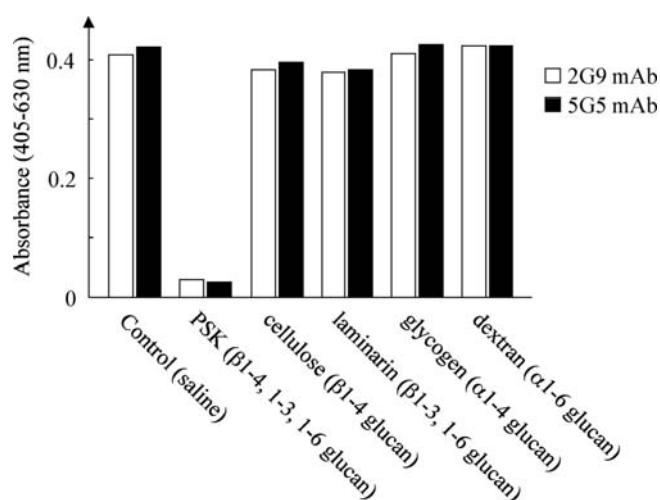

Figure 2. Binding of anti-PSK mAb to other glucans. To examine whether the anti-PSK mAbs (2G9 mAb or 5G5 mAb) recognizes glucans with similar structures as PSK, competitive ELISA was performed as described in Materials and methods. PSK, cellulose, laminarin, glycogen or dextran was used as competitor for anti-PSK mAb. Each column represents the mean of triplicate determinations.

Statistical analysis. All data are expressed as the means ± SD or SE. Statistical significance was determined by Student's t-test. P-values <0.05 were considered significant.

Results

Recognition site of anti-PSK mAbs. The standard curve of anti-PSK monoclonal antibodies (mAb) is shown in Fig. 1A. PSK is a complex of polysaccharide and protein, therefore competitive ELISA was performed to examine the recognition sites of the two anti-PSK mAb; 2G9 mAb or 5G5 mAb, in the glucan or polysaccharide moiety of PSK. First, we investigated whether 2G9 mAb and 5G5 mAb recognize the same epitope. After adding 2G9 mAb as a competitive antibody to PSK-coated 96-well plate, biotinyled-5G5 mAb was used to detect PSK in the plate. As shown in Fig. 1B, binding of 2G9 mAb was unaffected by competition with 5G5 mAb. The same result was obtained when 2G9 mAb was used as the competitive antibody and 5G5 as the detecting antibody (data not shown). These results show that 2G9 mAb and 5G5 mAb recognize different sites on PSK. Although the average molecular weight of PSK is ~100,000, it is consisted of a mixture of molecules with a broad molecular weight range, and the degree of polymerization ranges from a few thousands to hundreds of thousands. Western blotting was performed to investigate the molecular weight range of PSK with which the anti-PSK mAbs react. Since the staining patterns of the Western blots were similar to that of silver staining, the Western blots demonstrated that both mAbs recognize PSK for a broad molecular weight range (Fig. 1C). Next, we investigated whether the anti-PSK mAbs recognize the polysaccharide chain or the peptide of PSK. When the reactivity of the mAbs against PSK with proteins degraded by hydrazine was evaluated by ELISA, both mAbs bound the protein-depleted PSK similar to non-treated PSK (Fig. 1D). Therefore, the epitopes recognized by 2G9 mAb and 5G5 mAb are located in the polysaccharide moiety of PSK.
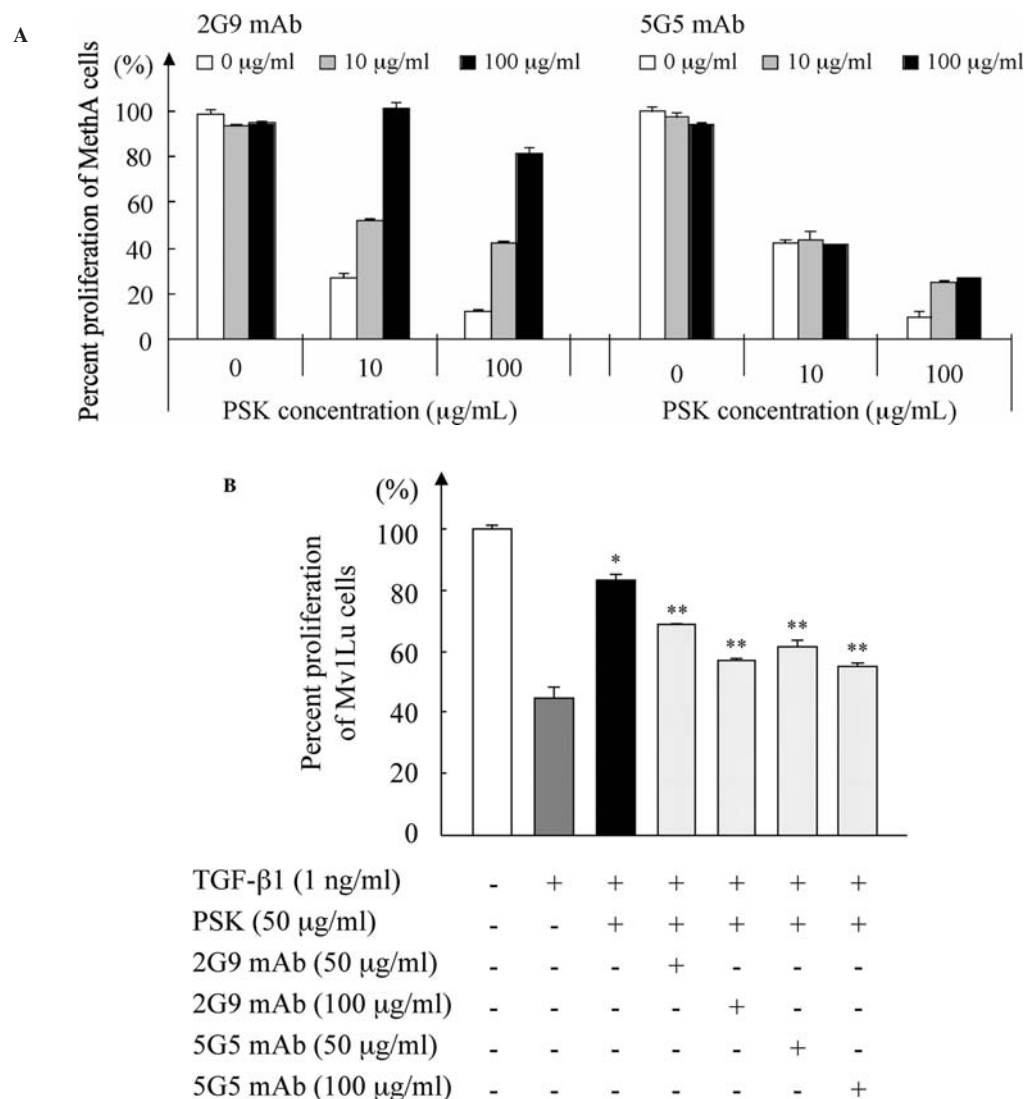
Figure 3. (A) Effect of anti-PSK mAb on cytostatic activity of PSK. MethA fibrosarcoma cells were plated in 96-well culture dish at a density of 1×10^3 cells/ 100 μl/well, and cultured for 24 h. Anti-PSK mAb and PSK were mixed at the indicated ratios, in 50 mmol/l Tris-HCl buffer (pH 7.5) containing 0.2% bovine serum albumin and 0.2 mol/l sodium chloride (binding buffer) and incubated for 1 h at 37˚C. The mixture was added to MethA fibrosarcoma cells and cultured for another 72 h. Viable cell number was determined by MTT assay, and expressed as percent cell proliferation compared with control cells without addition of PSK or mAb. Data are expressed as mean ± SD (n=3). (B) Effect of anti-PSK mAb on TGF-ß₁-binding activity of PSK. PSK and 2G9 mAb or 5G5 mAb were mixed in binding buffer at the indicated ratios and incubated for 1 h at 37˚C. Recombinant mouse TGF-ß₁ was added to the mixtures and incubated for another 2 h at 37˚C. The mixtures were added to TGF-ß₁-sensitive Mv1Lu mink lung epithelial cells, and incubated for 24 h. Viable cell number was determined by MTT assay, and expressed as percent cell proliferation compared with control cells. Data are expressed as the mean ± SD (n=3). *p<0.001 (vs. grey column), **p<0.01 (vs. black column).

Specificity of anti-PSK mAbs. To confirm that 2G9 mAb and 5G5 mAb specifically recognize the polysaccharide moiety of PSK, we investigated whether the mAbs also react with other ß-glucan structures. In a competitive ELISA assay using 10 μg/ml each of PSK [ß-(1,4)(1,3)(1,6)-D-glucan], cellulose [ß-(1,4)-D-glucan], laminarin [ß-(1,3)(1,6)-D-glucan], glycogen [ß-(1,4)-D-glucan] and dextran [ß-(1,6)-D-glucan], only PSK competed for binding to 2G9 mAb but not the other glucan structures (Fig. 2). The same results were obtained with 5G5 mAb. Since both mAbs recognize the PSK structure specifically and do not cross-react with other glucans, they are both highly specific for PSK.

Effect of anti-PSK mAbs on PSK-induced cytostatic effect. As a direct anti-cancer action, PSK is known to inhibit the growth of cancer cells (cytostatic effect). We investigated whether the anti-PSK mAbs neutralize the cytostatic effect of PSK. MethA cells known to be sensitive to PSK were used, and cell proliferation was examined in the co-presence of PSK and anti-PSK mAb. In the absence of 2G9 mAb, PSK dose-dependently inhibited MethA cell proliferation by 40% at a concentration of 10 μg/ml and 90% at 100 μg/ml. The addition of 10 μg/ml and 100 μg/ml of 2G9 mAb dose-dependently restored MethA cell proliferation. On the other hand, the addition of 5G5 mAb did not restore MethA cell proliferation (Fig. 3A). Since 2G9 mAb is capable of completely neutralizing the cytostatic effect of PSK, its epitope probably coincides with the active structure responsible for the cytostatic effect of PSK.

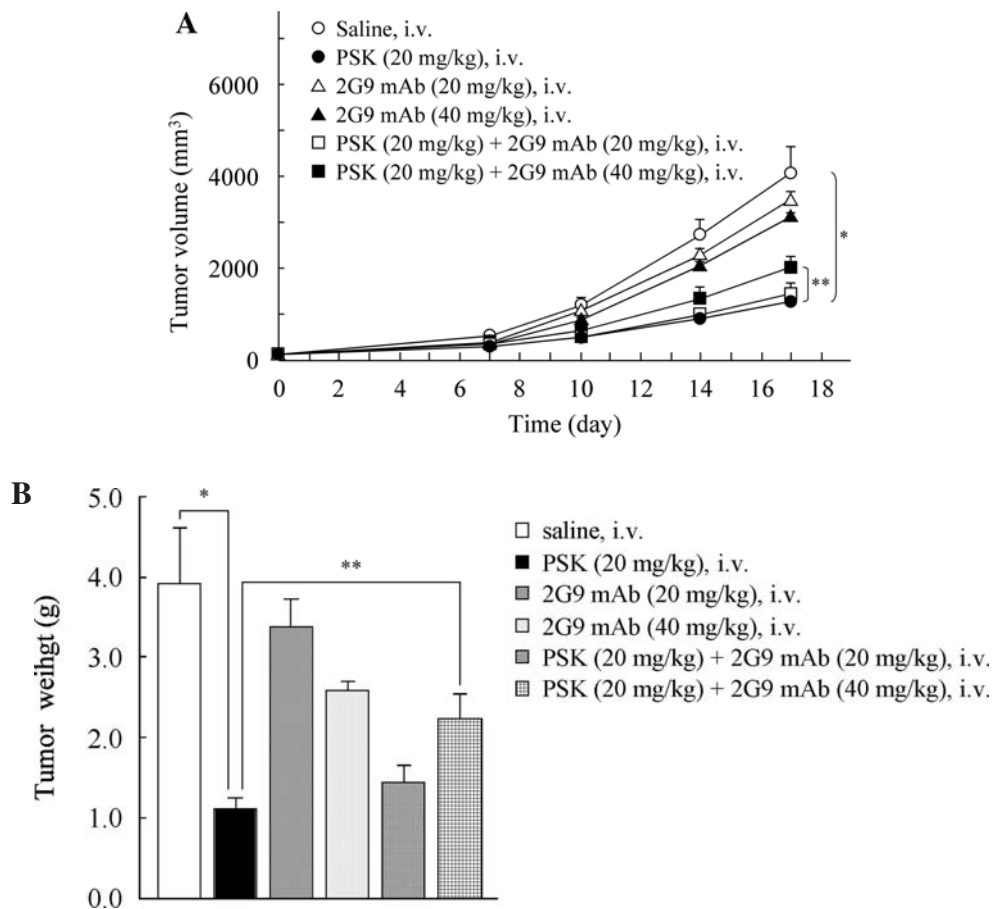*Effect of anti-PSK mAb on PSK-TGF-ß₁ binding.* TGF-ß₁ is a cytokine known to inhibit the growth and proliferation of

Figure 4. Effect of 2G9 mAb on *in vivo* antitumor activity of PSK. MethA fibroblast cells (1×10^6/mice) were implanted subcutaneously into the flanks of severely immunodeficient NOD.CB17-*Prkdc^{scid}*/J (NOD/scid) mice. PSK, 2G9 mAb or a mixture of the two were dissolved in saline and administered intravenously at thrice a week (A). Data are expressed as the mean ± SE (n=6). The experiments were repeated three times. Representative data are shown. (A) Changes in tumor volume over time. *p=0.001 (○ vs. ●) and **p=0.048 (● vs. ■). (B) Tumor weights on day 17 of implantation. *p=0.002 and **p=0.013.

immunocompetent cells, and its overproduction causes immunosuppression. A previous study confirmed that PSK binds TGF-ß₁ directly and inhibits its biological activity (6). Therefore, we examined whether the anti-PSK mAbs bind PSK and block the interaction between PSK and TGF-ß₁. Representative findings are shown in Fig. 3B. The addition of the PSK-TGF-ß₁ complex to an *in vitro* culture of Mv1Lu cells, a cell line sensitive to TGF-ß₁, promoted cell proliferation compared to the addition of TGF-ß₁ alone (p<0.001) suggesting that the PSK-TGF-ß₁ complex has a lower growth suppression activity than TGF-ß₁. When 2G9 mAb was added to PSK and TGF-ß₁, the *in vitro* proliferation of Mv1Lu cells was reduced compared to the addition of PSK-TGF-ß₁ complex (p<0.01). These results suggest that 2G9 mAb competes for the TGF-ß₁-binding site of PSK and prevents the formation of PSK-TGF-ß₁ complex. The same results were obtained for 5G5 mAb (p<0.01). Thus, it is possible that 2G9 mAb and 5G5 mAb inhibits the TGF-ß₁-blocking activity of PSK, and the mAb epitope may at least partially overlap with the TGF-ß₁-binding site of PSK.

Effect of anti-PSK mAbs on PSK-induced TNF-α production. Next, we investigated whether 2G9 mAb and 5G5 mAb

inhibit PSK-induced cytokine production by immune cells. A mixture of PSK and anti-PSK mAb was added to cultured monocytic J774.1 cells (a cell line that produces TNF-α by PSK), and TNF-α secreted in the supernatant was measured by ELISA. Both 2G9 mAb and 5G5 mAb did not inhibit PSK-induced TNF-α production (data not shown).

Antitumor effect of PSK evaluated in NOD/scid mice. The *in vitro* results obtained so far demonstrated that PSK exhibits direct action against tumor cells. Next we examined whether PSK also exhibits antitumor effect *in vivo* via direct actions. Using a tumor model of subcutaneous implantation of MethA tumor in severely immunodeficient NOD/scid mice (treated by asialo GM1 antibody), we investigated the therapeutic efficacy of PSK and the effect of 2G9 mAb. The mean tumor volume on day 17 after MethA implantation was 4566 mm³ in control mice compared with 1305 mm³ in PSK-treated mice, showing significant suppression of tumor growth in PSK-treated mice (p=0.001) (Fig. 4A). Since severely immunodeficient NOD/scid mice were used in this study, the antitumor effect can be considered to be a direct action of PSK. Furthermore, the mean tumor weight was 3.92 g in control mice and 1.09 g in PSK-treated mice, also showing significant suppression of tumor growth in PSK-treated mice (p=0.002) (Fig. 4B).
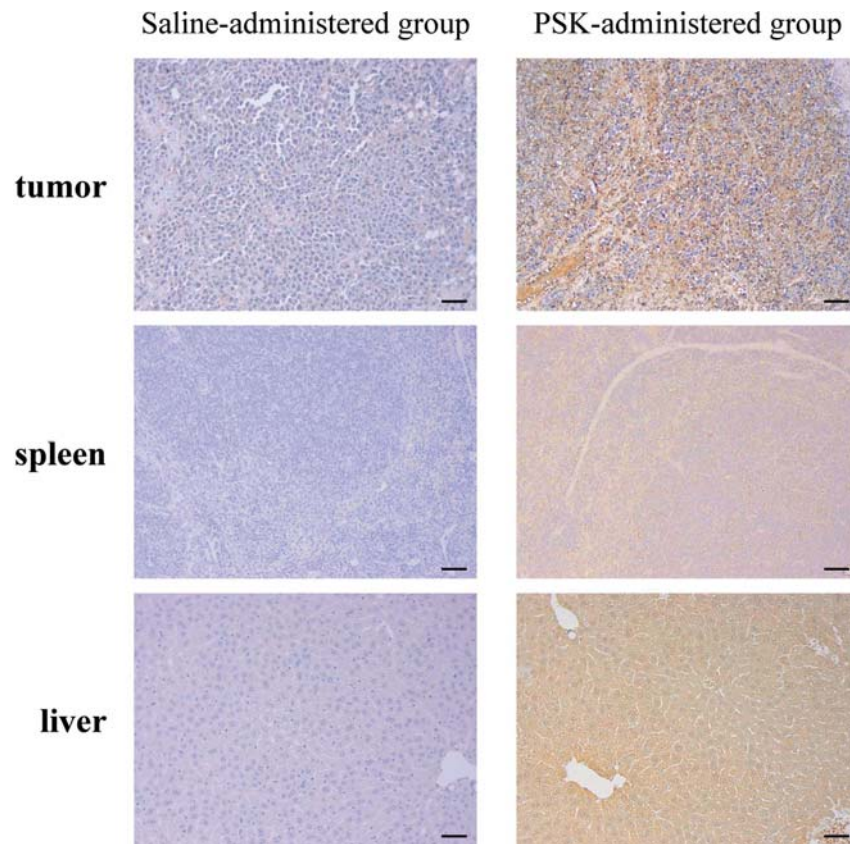
Figure 5. Immunostaining of active structure of PSK in various tissues. The tumor, spleen and liver were removed on day 17 after MethA tumor implantation. These samples were fixed with formalin, and paraffin sections were prepared, which were then stained with 2G9 mAb. Top, MethA fibroblast tumor. Middle, spleen. Bottom, liver. Bar, 50 μm. All experiments were repeated at least twice and representative micrographs are shown.

Effect of 2G9 mAb on in vivo antitumor effect of PSK. We investigated whether the neutralizing antibody 2G9 mAb neutralizes the effect of PSK *in vivo*. The mean tumor volume was 2137 mm³ in mice administered PSK (20 mg/kg) and 2G9 mAb (40 mg/kg), showing a significant reversal in tumor proliferation compared with 1305 mm³ in mice administered PSK alone (p=0.048) (Fig. 4A). In addition, when comparing tumor weight, the mean weight was 2.23 g in the group treated with PSK (20 mg/kg) and 2G9 mAb (40 mg/kg) and 1.09 g in the group treated with PSK alone (p=0.013) (Fig. 4B). These results indicate that 2G9 mAb suppresses the antitumor effect of PSK *in vivo*.

Tumor and tissue distribution of the active structure after intravenously administration of PSK. To determine whether PSK is absorbed into blood and reaches tumor cells and immune cells while maintaining its bioactive structure, we performed immunohistochemical studies using anti-PSK mAbs. NOD/scid mice implanted subcutaneously with MethA were given intravenous injection of PSK at a dose of 20 mg/kg on thrice a week, and were sacrificed on day 17. Histological specimens were prepared by the standard procedures and stained immunohistochemically using the 2G9 mAb, which is an antibody that neutralizes the direct actions of PSK. PSK was detected in the MethA tumor cells, spleen cells and liver cells (Fig. 5). These results suggest that PSK might come in contact with tumor cells and immune cells, while preserving the bioactive structure.

Discussion

Glucans are a structurally diverse group of polysaccharides containing D-glucopyranosyl units. They are involved in various physiological functions and have a wide variety of sources such as barley, oats, algae, bacteria, yeasts, fungi and mushrooms. Unlike low molecular weight substances, polysaccharides cannot be detected and quantitated by high performance liquid chromatography and mass spectral analysis. Therefore, the Limulus test and ELISA using specific antibody are the common detection methods. The Limulus test is generally used to detect LPS and ß1-3 glucan, but this test reacts with all the substances containing ß-D-glucan and is therefore not highly specific. A monoclonal antibody against proteoglycan (ß1-3 glucan, ß1-6 glucan) derived from *Grifola frondosa* cross-reacts with other compounds containing ß1-3 and ß1-6 branches, such as schizophyllan, lentinan, scleroglucan, pustulan, curdlan and laminarin, and is not a proteoglycan-specific antibody (18). Endoh *et al* (14) used rabbit anti-PSK polyclonal antibodies to examine the intra-tumoral distribution of PSK. Due to the relatively low specificity of polyclonal antibodies, efforts have been made to raise mouse monoclonal antibodies against PSK. However, since PSK is a protein-bound polysaccharide, the attempts so far have not produced monoclonal antibodies with high potency (unpublished data).

The objectives of the present study were to produce mouse monoclonal antibodies that are highly specific and recognize

the active structure of PSK, and to use this antibody to examine the intratumoral accumulation of PSK. In this study, we immunized Balb/c mice with a larger amount of antigen (200 μg) than is normally used for immunization, for a period of 12 weeks. By screening using neutralization of PSK activity as indicator, two clones were obtained: 2G9 mAb (IgM$_\kappa$) and 5G5 mAb (IgM$_\lambda$). Both mAbs recognize PSK molecules over a wide molecular weight range (Figs. 1 and 2) and do not crossreact with other glucan standard substances. A noteworthy finding is that although PSK contains ß1-4, ß1-6 and ß1-3 glucan structures, the 2G9 mAb and 5G5 mAb obtained in the present study do not react with a ß1-4 glucan (cellulose), a ß1-3, ß1-6 glucan (laminarin), an α1-4 glucan (glycogen) or an α1-6glucan (dextran). One explanation is that PSK is not a homogeneous glucan structure but possesses complex and intricate branch structures and the mAbs recognize this specific structure of PSK.

The 2G9 mAb and 5G5 mAb obtained from this study recognize different epitopes in the PSK molecule (Fig. 1D). Using these two mAbs, we examined their effect on the cytostatic effect of PSK. While 2G9 mAb completely abrogated the cytostatic effect, which is one of the direct actions of PSK, 5G5 mAb had almost no neutralizing effect on the cytostatic activity of PSK (Fig. 3A). These results suggest that the 2G9 mAb epitope is a structure associated with the cytostatic activity of PSK. Next, we investigated the TGF-ß$_1$-blocking effect of PSK. In this experiment, addition of 2G9 mAb or 5G5 mAb even in an excess amount did not completely inhibit the TGF-ß$_1$-blocking effect of PSK (Fig. 3B). A possible reason is that multiple TGF-ß$_1$-blocking sites are present in the PSK molecule and the mAb only recognizes one of them. To investigate the effect of PSK on immune cells, we examined the effect of the mAbs on PSK-induced TNF-α production. Both mAbs had absolutely no effect on this activity (data not shown). Production of antibodies that neutralize the cytokine-inducing activity of PSK should be attempted in the future.

Ikuzawa *et al* (19) produced ^{14}C-labeled PSK and studied the pharmacodynamics of PSK in SD rats. They detected ^{14}C-labeled low molecular weight substances in the blood at 1 h after oral administration, followed by the appearance of ^{14}C-labeled high molecular weight substances with time, and almost complete disappearance of labeled substance in the blood 72 h later. However, only ^{14}C was monitored in that experiment, and it remains unknown whether the absorbed substance was the bioactive structure of PSK. In the present study, we investigated whether PSK is distributed in tumor and immune tissues by immunohistochemistry using 2G9 mAb, which is an anti-PSK neutralizing antibody. Immunodeficient NOD/scid mice were implanted subcutaneously with MethA tumor, and PSK was administered by the intravenous route. On day 17 after MethA tumor implantation, tumor growth was significantly inhibited in the PSK-treated mice compared to control mice (Fig. 4). When the same experiment was conducted using Balb/c mice, tumor growth was almost absent and the tumor even disappeared in some mice (data not shown). However, using NOD/scid mice in the present study, PSK treatment reduced tumor growth to only 29% by volume compared with that in control mice on day 17 after tumor implantation (Fig. 4A). In this model animal almost completely deficient in immunological function, the residual tumor growth

not inhibited by PSK treatment is speculated to reflect the antitumor effect of PSK mediated by the immune system. To investigate the distribution of PSK in the body, tumor tissues and organs were obtained on day 17 after MethA implantation and examined by immunohistochemistry using 2G9 mAb. This experiment confirmed that PSK not only reaches the tumor tissue but also the spleen and liver (Fig. 5). Since 2G9 mAb used in this study is a neutralizing antibody, these results suggest that PSK with its bioactive structure intact reaches the tumor and directly attacks the tumor cells. However, the direct action to the cell of PSK is cytostatic, but not cytotoxic because PSK inhibits cell proliferation, but does not decrease in number of cells. So, it is suggested that PSK is not affecting any normal tissues other than tumor tissue (data not shown).

Considering the future application of the monoclonal antibody to patients, we have planned to develop an assay system for the detection and quantitation of PSK in blood using ELISA. When monitoring of blood PSK concentration becomes available, it may be possible to differentiate patients who are good or bad absorbers of PSK to predict the response to PSK therapy. Furthermore, individualized dose adjustment while measuring blood PSK concentrations may also be realized.

The anti-PSK monoclonal antibody; 2G9 mAb, produced in the present study is a PSK-specific antibody that does not cross-react with various glucans with structures similar to PSK. Furthermore, 2G9 mAb is capable of neutralizing the actions of PSK in inhibiting tumor cell growth and blocking TGF-ß$_1$, a strong immunosuppressive substance. This antibody that neutralizes the activity of PSK is expected to be a useful tool in the elucidation of the bioactive structure of PSK. Many attempts have been made to identify the active sites of PSK, but so far the active structure has not been elucidated. One of the reasons is that PSK is not a homogeneous structure but is consisted of diverse molecular structures. Consequently, cleavage, digestion and column chromatographic fractionation are difficult to perform, and evaluation of the activity of the fractions depends on assaying the biological activity. Because 2G9 mAb is a neutralizing antibody, the epitope is likely to coincide with or in close vicinity of the active structure of the molecule. Through further analysis of the epitope, it is possible that identification of the active structure and elucidation of the exact mechanism of action of PSK may be realized.

Finally, the use of this anti-PSK monoclonal antibody in the clinical setting may provide a simple and precise method of measuring blood concentrations of the active structure after PSK oral administration. Consequently, selection of patients who are good absorbers of PSK (PSK responders) and individualized dosage adjustment may be anticipated.

Acknowledgements

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