*Mem. Fac. Sci. Kyoto Univ. (Ser. Biol.),* 17: 41-54, Dec., 2001

# Protein-Bound Polysaccharide Inhibits Angiogenesis *in Vitro* and *in Vivo*

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(Recetved December 1, 2001)

**Abstract** The effect of protein-bound polysaccharide (PSK) on angiogenesis was investigated. Ten μg/ml of PSK inhibited 47% proliferation of human umbilical vein endothelial cells (HUVECs). On the other hand, 670 μg/mlof PSK inhibited 50% tube formation and 1000 μg/ml of PSK inhibited 30% cell adhesion. PSK inhibited proliferation of HUVECs more effectively than tube formation and cell adhesion. One μg/ml of PSK inhibited 40% cell proliferation in the presence of bFGF and 14% in the absence of bFGF, showing greater inhibition by PSK in the presence of bFGF than in the absence of it. Two hundred and fifty μg/ml PSK inhibited bFGF-induced increase in S phase population of the cell cycle and 100 μg/ml of PSK inhibited bFGF-induced phosphorylation of MAP kinase. Furthermore, direct binding of PSK to bFGF was demonstrated. In addition, PSK inhibited bFGF-induced angiogensis in vivo in a rat cornea assay. These results suggest that PSK binds to bFGF and interferes with bFGF-induced endothelial cell proliferation, leading to inhibition of angiogenesis.

**Key words**: Protein-bound Polysaccharide, Angiogenesis, bFGF, HUVEC

## Introduction

Angiogenesis is known to be important in developmental processes during embriogenesis and postnatal tissue growth. However, the angiogenic capability is reduced in adulthood except in the female reproductive system and during tissue repair (wound healing and cardiac ischemia). On the other hand, unregulated angiogenesis causes undesirable tissue growth. Therefore, regulation of angiogenesis is a useful target to develop new therapy. Angiogenesis stimulators may be used to treat wound healing or cardiac ischaemia. Actually, recombinant basic fibroblast growth factor (bFGF) has been applied to promote wound healing. On the contrary, angiogenesis inhibitors are useful to suppress tumor growth or diabetic retinopathy (Fan *et al.* 1995)

Folkman (1971) reported that solid tumors per se can only grow to a maximum of 1 to 2 mm in diameter and further growth requires induction of angiogenesis. Angiogenesis is also necessary for tumor metastasis (Fidler & Elis 1994). As mentioned above, anti-angiogenesis is an attractive new target to develop anti-cancer agents. Angiogenesis is a

complex process, involving basement membrane dissolution by protease, endothelial cell migration and proliferation, and formation of capillary network. Accordingly, there are many targets for developing anti-angiogenic agents, which may include proteolytic peptides, cytokines, hormones, chemically designed enzyme inhibitors, natural products, and clinically used anti-cancer agents (Kuiper *et al.* 1998). Herblin *et al.* (1994) reviewed the polysaccharides that have a potential to inhibit tumor-associated angiogenesis, and they include pentosan (a semi-synthetic sulfated polysaccharide), DS-4152, SCM-chitin-III, sulfated glycosaminoglycanoid, and protein-bound polysaccharide (PSK).

PSK extracted from the mycelium of *Coriolus versicolor* has been used as an anti-cancer agent in Japan. PSK is an immunomodulator (Nomoto *et al.* 1975; Ehrke *et al.* 1983; Suo *et al.* 1994; Harada *et al.* 1997; Hirose *et al.* 1990), and also inhibits tumor growth (Tsukagoshi *et al.* 1984) and metastasis (Kobayashi *et al.* 1995). Additionally, an anti-angiogenic effect of PSK has been demonstrated using a dorsal air sac assay (Kumar *et al.* 1992; Kanoh *et al.* 1994). However, the mechanism of the anti-angiogenic effect exhibited by PSK is unknown. It might be worthy to understand a novel mechanism of action different from the known actions of clinically available anti-cancer agents.

The present study suggests that direct binding of PSK to bFGF interrupts the action of bFGF and inhibits cell proliferation, thereby suppressing angiogenesis.

## Materials and Methods

### Cell lines

Human umbilical vein endothelial cells (HUVEC; Cell System corporation, Kirkland, WA) were grown at 37°C in 5% CO2 / 95% air in MCDB-131 medium (Sigma, St. Louis, MO) supplemented with 10 ng/ml human epidermal growth factor, 1 μg/ml Hydrocortisone, 10 μg/ml heparin, 50 μg/ml gentamycin, 0.25 μg/ml amphotericin B (Sigma), 10 μg/ml endothelial cell growth supplement (ECGS; Upstate, Lake Placid, NY) and 2% fetal bovine serum (GIBCO, Grand Island, NY). This is referred to as complete medium. In experiments examining basic fibroblast growth factor (bFGF; from bovine pituitary glands, Sigma), ECGS was omitted from the complete medium. HUVECs were used at passages two to six.

### Cell growth experiment

HUVECs were plated in 48-well plates ($1 \times 10^4$ cells/well) containing 0.5 ml complete medium per well. On the next day (day 0), the medium was changed to 0.5 ml complete medium with or without various concentrations of PSK. On day 3, 0.1 ml of MTT (5 mg/ml, Sigma) was added to each well and incubated for 4 h at 37 °C. The medium was aspirated and 0.5 ml dimethylsulfoxide was added, and the color was read in a plate reader (Model 3550, Bio-Rad, Richmond, CA) at 570 to 630 nm.

Using the same procedures as MTT assay, on day 3, the cells were pulsed with $^3$H-thymidine (37 kBq/ml, Amersham Pharmacia Bioteck UK Ltd., Buckinghamshire, England) for 5 h at 37 °C, and the radioactivity incorporated into cells was measured.

**Tube formation assay**

Two hundred and fifty μl per well of Matrigel (Collaborative Research Incorporated, Bedford, MA) was added to a 24-well plate and allowed to solidify for 2 h at 37°C. HUVECs ($9 \times 10^4$ cells/well) were plated in the Matrigel and incubated for 18 h at 37°C. After incubation, three random fields each in two cultures were observed with a phase-contrast microscope and photographed at x 25 magnification. The tube length was measured using an image analysis system, Video Plan (KONTRON Bildanalyse GmbH, Eching, Germany).

**Cell adhesion assay**

Forty-eight well flat-bottomed plates (non-tissue culture treated, Becton Dickinson, Franklin Lakes, NJ) were coated with 1 μg/well Matrigel, 0.2 μg/well human plasma fibronectin (GIBCO), or 0.2 μg/well human vitronectin (Promega, Madison, WI). Each plate was air dried overnight, washed with PBS (-), and incubated with MCDB-131 medium containing 1.5 % bovine serum albumin (BSA) for 1 h at 37°C. After incubation, the coated plates were washed with PBS (-). HUVECs ($4.5 \times 10^4$ cells/well) were plated and allowed to adhere for 1 h at 37°C. After incubation, the wells were washed with PBS (-) and adherent HUVECs were cultured in 0.5 ml of complete medium with 0.1 ml MTT (5 mg/ml) for 4 h at 37°C. The adherent HUVECs were determined by the MTT assay.

**Cell cycle analysis**

HUVECs were plated in T-175 flask ($2 \times 10^6$ cells/flask, Becton Dickinson, Franklin Lakes, NJ) containing 20 ml complete medium. On the next day (day 0), the medium was changed to 20 ml ECGS-free medium with or without 10 ng/ml bFGF and PSK. On day 3, the cells were harvested with 0.05 % trypsin and 0.53 mM EDTA (GIBCO). Dispersed cells were collected by centrifugation at 200 x g for 5 min, and the pellet was washed with PBS (-). The cells were resuspended in 0.5 ml PBS (-) containing 0.1 % Triton X-100 (Nacalai Tesque, inc., Kyoto, Japan). The cell suspension was passed through a metal-mesh (#150), and 50 μl ribonuclease A (50 mg/ml, Sigma), and 25 μl propidium iodide (1 mg/ml, Sigma) were added. Cell cycle distribution was analyzed by flow cytometry (FACScan, Becton Dickinson) and calculated by the CellFIT software (Beckton Dickinson).

**Detection of phosphorylated MAP kinase**

HUVECs ($2 \times 10^5$ cells/well) in ECGS-free medium were plated in 6-well plates and cultured for 24 h at 37°C. The cells were treated with bFGF (10 ng/ml) and PSK for 10 min. at 37°C, then washed with PBS (-) and lysed by RIPA buffer (Boehringer Mannheim GmbH, Germany). The protein concentrations were determined by a Bio-Rad DC-Protein assay. Forty μg protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Amersham). Phospho-threonine/tyrosine-phosphorylated MAP kinase (p44/42) was detected using a p44/42 MAPK antibody kit (New England Biolabs) and ECL system (Amersham). For analysis of total MAP kinase, the same membrane was stripped of bound antibodies and reprobed.

**Basic FGF binding assay**

A slightly modified Ishihara's method (Ishihara *et al.* 1992) was performed to determine the interaction of bFGF and PSK. Forty-eight-well flat-bottomed plates (non-tissue culture treated, Becton Dickinson) were coated with bFGF (125 ng / well) for 24 h at 4°C. Then MCDB-131 medium containing 1.5% BSA was added and incubated at 37°C for 1 h. After incubation, the coated plates were washed with PBS (-) and HUVECs ($1.2 \times 10^5$ cells/well) were plated and allowed to adhere for 1 h at 37°C. After the incubation, the wells were washed with PBS (-) and adherent HUVECs were cultured in 0.5 ml complete medium containing 0.1 ml MTT (5 mg/ml) for 4 h at 37°C. The adherent HUVECs were determined by the MTT assay.

**Gel filtration assay**

Four hundred thousand cpm of $^{125}$I-bFGF (4.81 MBq/mg, NEN Life Science Products, Inc., Boston, MA) and PSK (5 mg/ml) in 0.5 ml elution buffer [0.5 M NaCl, 0.1 % CHAPS (Sigma), 20 mM Tris-HCl (pH 7.4)] was incubated for 1 h at 37°C. The mixture was applied on a Sephacryl S-100 gel chromatography ( $\phi$ 1 cm x 50 cm, Amersham Pharmacia) equilibrated with the elution buffer. The radioactivity of the fractions was counted with a Minaxi $\gamma$ machine (Packard Instrument Co., Meriden, CT).

**Quantitation of sulfated glycosaminoglycan**

The quantity of sulfated glycosaminoglycan in PSK was evaluated according to the method of Franndale *et al.* (1986). Briefly, the dimethylmethylene blue color reagent was prepared by dissolving 1,9-dimethylmethylene blue (16 mg, Aldrich) in 1 l distilled water containing 3.04 g glycine, 2.37 g NaCl and 95 ml 0.1 M HCl (pH 3.0). Dimethylmethylene blue color reagent (2.5 ml) was added to 100 µl of each sample. After 10 seconds, absorbance at 525 nm was immediately read.

**Rat cornea assay**

A cornea micropocket was made approximately 1 mm from the center of the cornea of a male Wistar rat (6 week old, Clea Japan, Inc., Tokyo, Japan) anesthetized with Nembutal (Dainippon Pharm. Co., Osaka, Japan). Poly(ethylene-co-vinyl acetate) (Aldrich Chemical Co., Milwaukee, WI) pellets (2 µl) with or without 100 ng bFGF were implanted into the corneal pockets. Rats were immediately treated daily for 7 days with 1 or 5 mg/kg PSK (i.v.). Seven days later, India ink was injected into the ventricle of the rats, which were sacrificed. The corneal vessels were photographed. Responses were assessed by the method of Volpert *et al.* (1995). Briefly, no new vessels developing toward the pellet was graded as negative, new vessels reaching the pellet was graded as positive, and few vessels reaching the avascular cornea but not the pellet was graded as +/-.

**Statistical Analysis**

The Wilcoxon's U-test was used in statistical analysis. A level of $p<0.05$ was regarded as significant.

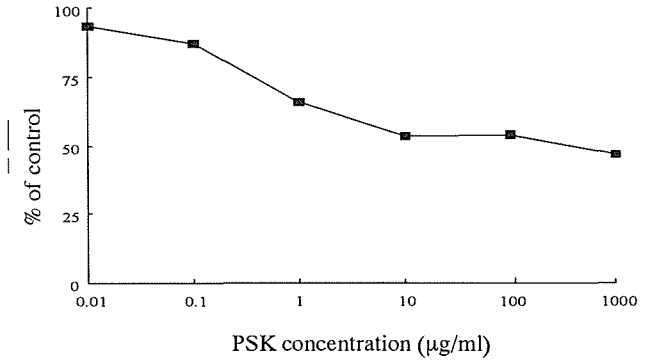


**Fig. 1.** Effect of PSK on the proliferation of HUVECs. HUVECs ($1 \times 10^4$ cells/ml) were cultured with PSK for 3 days. On day 3, MTT assay was performed. Each point represents the mean ± S.E.

## Results

### Effect of PSK on proliferation of HUVECs *in vitro*

To investigate the effect of PSK on endothelial cell proliferation, HUVECs ($1 \times 10^4$/0.5ml) were cultured with PSK for 72h. Inhibition of cell proliferation was estimated by MTT assay. HUVEC proliferation was inhibited by PSK in a dose-dependent manner. Percent inhibition was 34% at 1 μg/ml and 47% at 10 μg/ml of PSK (Fig. 1).

### Effect of PSK on tube formation

HUVECs form a tube-like structure when they are cultured on Matrigel (Fig. 2). The phenomenon of tube formation is characteristic of endothelial cells. The effect of PSK on tube formation was studied. HUVECs ($9 \times 10^4$/ml) were incubated in Matrigel-coated plates with or without PSK. After 18 h, the tube length was measured using an image analyzer. Six hundred and seventy μg/ml of PSK inhibited 50% of tube length (Fig. 2).

### Effect of PSK on the adhesion of HUVECs

To investigate the effect of HUVEC adhesion to extracellular matrixes, HUVECs ($4.5 \times 10^4$/well) were seeded in Matrigel-coated, fibronectin-coated or vitronectin-coated plate and incubated for 1 h at 37°C. Adherent HUVECs were evaluated by MTT assay. One thousand μg/ml of PSK inhibited 30.0% adhesion on Matrigel-coated plate, 19.9% on fibronectin-coated plate, and 11.9% of vitronectin-coated plate (Fig. 3).

### Effect of PSK on proliferation of HUVECs in the presence or absence of bFGF

Since PSK was more potent in inhibiting cell proliferation than tube formation or cell adhesion, the effect of PSK on cell proliferation was further studied. As the culture medium of HUVECs includes growth factors, we examined the possibility that PSK might interfere with the action of growth factors. Basic FGF is one of the most effective stimulators of HUVECs proliferation. The effect of PSK on bFGF-dependent cell proliferation

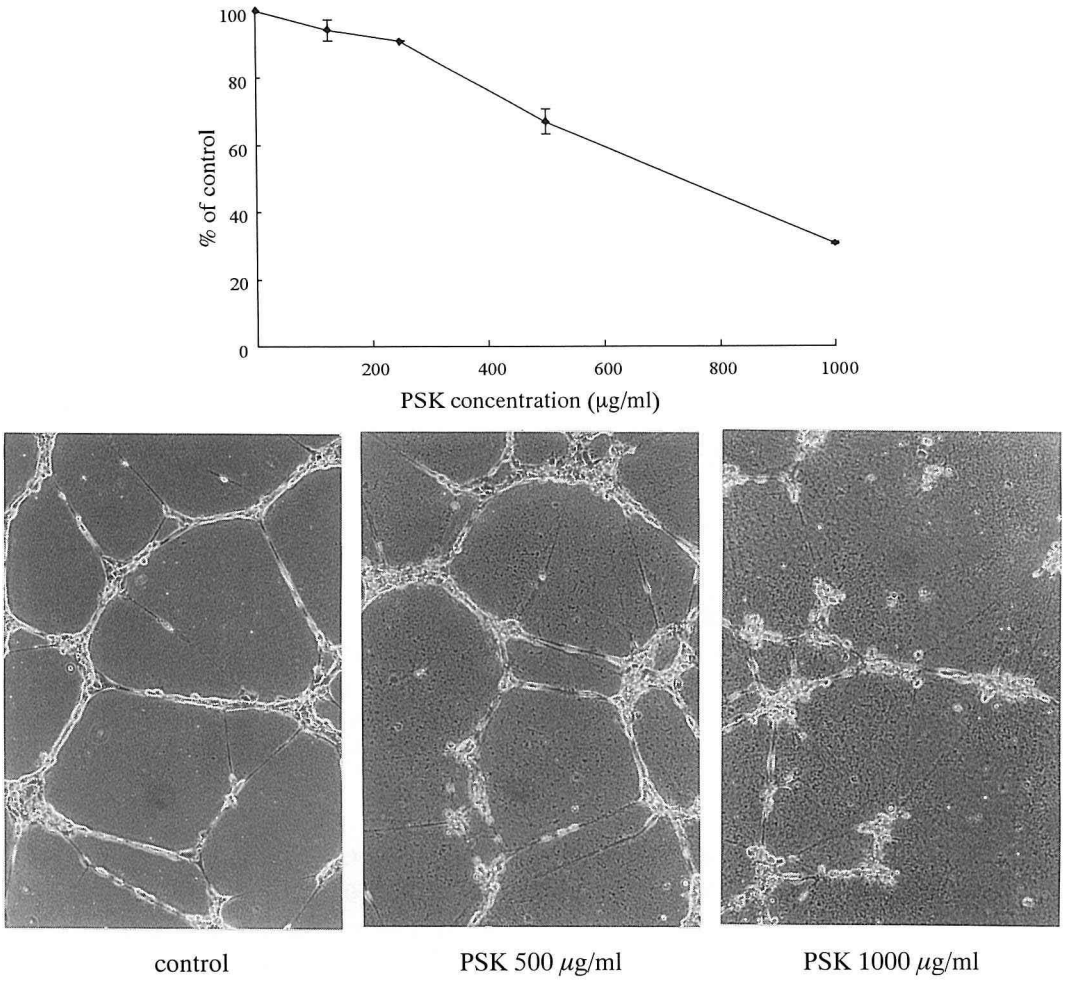


**Fig. 2.** Effect of PSK on tube formation. HUVECs ($9 \times 10^4$ cells/well) were plated on Matrigel-coated plates. HUVECs were incubated with PSK for 18 h. The tube length was measured by the image analysis system Video Plan. Each point represents the mean ± S.E. The photograph indicated the typical effect of PSK on tube formation (magnification × 25).

was investigated. PSK inhibited the proliferation of HUVECs both in the absence and presence of bFGF. However, the inhibition was 34.9% at 1 μg/ml and 76.6% at 5 μg/ml of PSK in the presence of bFGF (0.1 ng/ml), whereas the inhibition was 13.6% at 1 μg/ml and 45% at 5 μg/ml of PSK in the absence of bFGF. The effect of PSK was enhanced in the presence of bFGF (Fig. 4).

## Effect of PSK on cell cycle

We speculated that bFGF might increase the S phase population because bFGF is a strong mitogen. We investigated whether PSK suppresses the increase of S phase popula-

tion by bFGF. The cell cycle was analyzed by flow cytometry. When 10 ng/ml of bFGF was added to bFGF-free medium, the S phase population was increased from 15.9% to 24.5% and G0+G1 phase was decreased from 53.1 % to 37.8 % on day 3. Under this condition, 250 and 500 μg/ml of PSK reduced the proportions of S phase to 15.7% and 16.5 %, respectively, but 100 μg/ml of PSK had no effect (25.6%). The G0+G1 phase population was increased from 37.8% to 43.3% and 42% at 250 and 500 μg/ml, respectively, of PSK, but was unchanged at 100 μg/ml of PSK (Fig. 5).

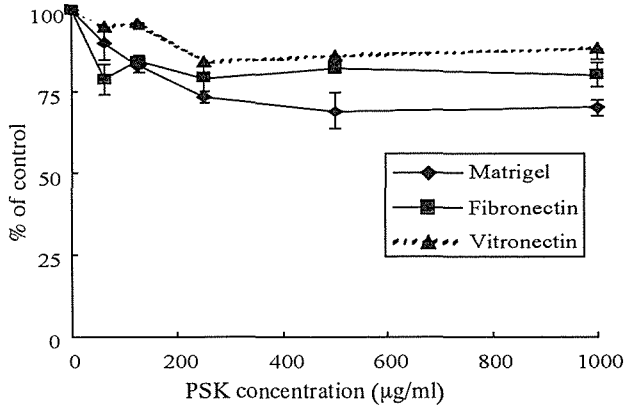


**Fig. 3.** Effect of PSK on the adhesion of HUVECs to extracellular matrixes. HUVECs (4.5 × $10^4$cells /ml) were incubated with PSK for 1 h to Matrigel- (1 μg/well), fibronectin- (0.2 μg/ml), vitronectin- (0.2 μg/well) coated plates. The adhesion of HUVECs was measured by MTT assay. Each point represents the mean ± S.E.

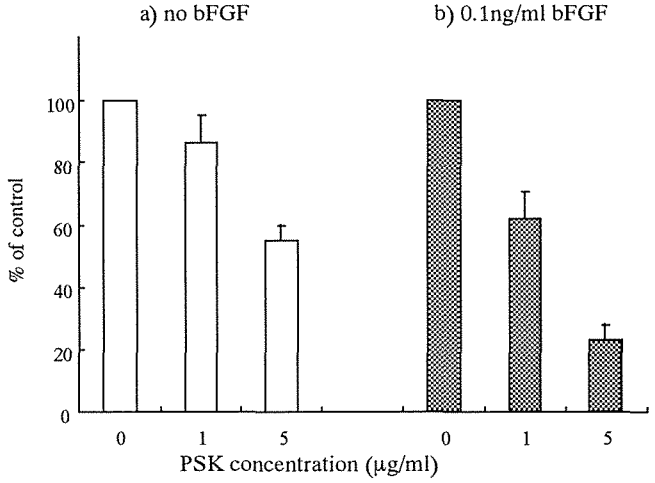


**Fig. 4.** Effect of PSK on the bFGF-dependent proliferation of HUVECs. HUVECs (1 × $10^4$ cells/ml) were cultured in ECGS-free medium with or without bFGF in the presence of PSK for 3 days. Cell proliferation was measured by $^3$H-thymidine (37 kBq/ml) incorporation. The vertical axis showed the percentage to non-PSK-treated controls in the respective bFGF and bFGF-free group. Each point represents the mean ± S.E.

## Effect of PSK on phosphorylation of MAP kinase

Basic FGF induced the phosphorylation of MAP kinase in cell proliferation. The effect of PSK on MAP kinase phosphorylation was studied by Western blot. When bFGF (10 ng/ml) was added to serum-free medium, phosphorylation of MAP kinase was observed. Under this condition, 100 μg/ml of PSK suppressed the phosphorylation of MAP kinase. Total MAP kinase was almost unchanged (Fig. 6).

## Binding of bFGF and PSK

To investigate the interaction of bFGF and PSK, bFGF-dependent cell adhesion assay was performed. When HUVECs are seeded in bFGF-coated (125 ng/well) plate, cells are bound to the plate via the FGF receptors. Under this condition, PSK decreased the adhesion of HUVECs in a dose-dependent manner; 100 μg/ml of PSK inhibited 55.5% of the adhesion (Fig. 7). This result suggests that bFGF may bind to PSK. Gel filtration was performed to investigate the direct binding of PSK to bFGF. When $^{125}$I-bFGF was subjected to gel filtration, a single peak was observed at fraction No. 46, and the radioactivity was 26,535 cpm. When a mixture of $^{125}$I-bFGF and PSK was subjected to gel filtration, the radioactivity of fraction No. 46 decreased to 22,039 cpm at 1 mg/ml of PSK, and 13,691 cpm at 5 mg/ml of PSK. Another peak appeared at fraction No. 31 with a higher molecular weight; the radioactivity at this peak was 14,111 at 1 mg/ml of PSK, and 44,465 cpm at 5 mg/ml of PSK (Fig. 8). These result indicated the possibility that bFGF binds directly to PSK.

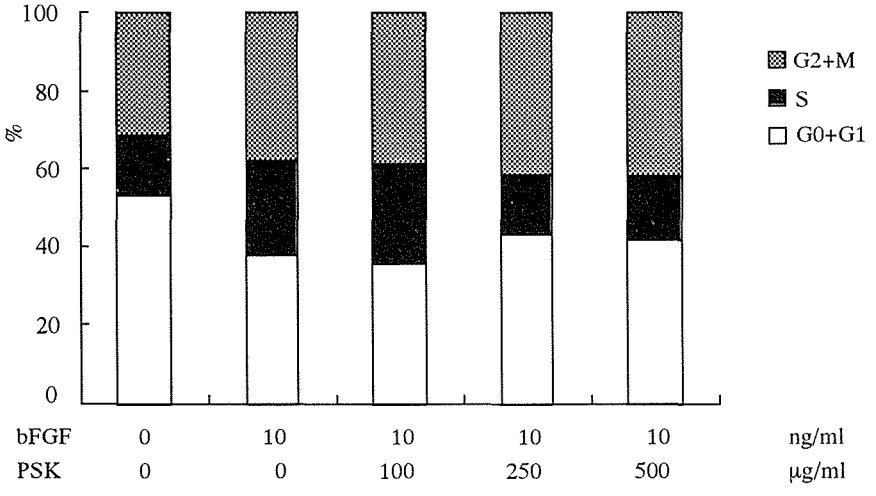


**Fig. 5.** Effect of PSK on the cell cycle of HUVECs. HUVECs ($2 \times 10^6$ cells/flask) were cultured with or without PSK in the presence of bFGF for 3 days. HUVECs were treated by propidium iodide and RNAase. Cell cycle distributions were determined by FACScan using CellFIT software.

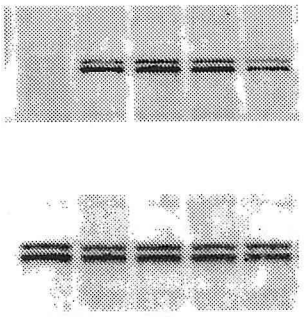


**Fig. 6.** Effect of PSK on MAP kinase phosphorylation. HUVECs ($2 \times 10^5$ cells/ml) were cultured in bFGF-free medium. After 24 h, cells were treated by bFGF (10 ng/ml) for 10 min with or without PSK. MAP kinase phosphorylation was detected by Western blot.

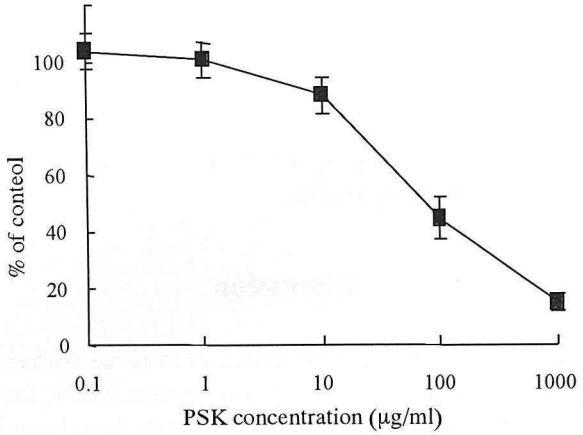


**Fig. 7.** Binding of PSK to immobilized bFGF. Basic FGF (125 ng/ml) was immobilized on 48 well-plate and HUVECs were incubated for 1 h at 37°C with or without PSK. After the incubation, plates were washed and adherent HUVECs were measured by MTT assay. Each point represents the mean ± S.E.

## Quantification of sulfated glycosaminoglycan

Basic FGF is known to bind to polysaccharide, and the binding site is sulfated glycosaminoglycan (Turnbull *et al.* 1992). It was investigated whether PSK contained sulfated glycosaminoglycan. Using heparin as control, PSK contained sulfated glycosaminoglycan and the amount was 1/100 that of heparin (Fig. 9).

## Effect of bFGF induced angiogenesis *in vivo*

A rat cornea assay was performed to study the effect of PSK on bFGF-induced angiogenesis *in vivo*. When pellets containing bFGF (10 ng) were transplanted into the corneas

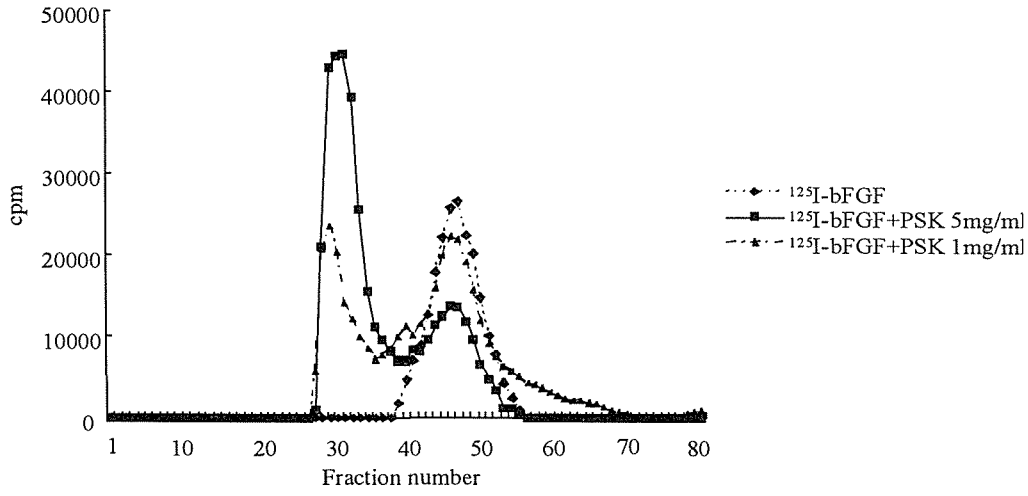


**Fig. 8.** Binding of PSK to $^{125}$I-bFGF. A mixture of $^{125}$I-bFGF and PSK in elution buffer was incubated for 1 h at 37°C, and gel filtration was performed. The radioactivity of the fractions was counted.

of rats, five of six rats in the non-PSK-treated group showed strong angiogenesis and one of six rats showed weak angiogenesis on day 7 after the transplant (Table 1). Of seven rats transplanted with bFGF pellets and given PSK (1 mg/kg i.v.) injection, four showed strong angiogenesis, two showed weak angiogenesis, and one showed no angiogenesis. In the other group of seven rats given PSK (5mg/kg, i.v.) injection, two showed strong, one showed weak, and four showed no angiogenesis. These results indicated that PSK inhibited the bFGF-induced angiogenesis *in vivo*.

## Discussion

Angiogenesis is regulated through a balance of various stimulators and inhibitors. The stimulators include vascular endothelial growth factor, FGFs, hepatocyte growth factor, IL-8, hormones. On the other hand, various inhibitors have been identified in the past decade, including angiostatin, endostatin, 16kDa prolactin peptide, tissue metaloproteinase inhibitor, interferons, interferon γ-inducible protein 10 (IP-10), IL-12, thrombospondin (Augustin 1998). Tumor cells release angiogenic stimulators, disrupting the balance and activating the angiogenic cascade (activation of endothelial cells).

PSK has been reported to suppress angiogenesis *in vivo* (Kumar *et al.* 1992; Kanoh *et al.* 1994). One possible mechanism for the anti-angiogenic action of PSK is an immunomodulating effect. Abe *et al.* (1990) reported that PSK activated immunocompetent cells and induced IFN-γ production. Therefore, PSK might induce the anti-angiogenic chemokine IP-10 in vivo (Angililo *et al.* 1995). Another possibility is a direct effect on endothelial cells. The present study investigated the effect of PSK on endothelial cells.

Ten μg/ml of PSK inhibited 47% HUVEC proliferation, while 670 μg/ml of PSK

**Table 1.** Effect of PSK on angiogenesis *in vivo* rat aornea assay.

| Treatment | + | +/- | - | Wilcoxon's U-test |
|---|---|---|---|---|
| None (control) | 5/6 | 1/6 | 0/6 | |
| PSK (1mg/kg i.v.) | 4/7 | 2/7 | 1/7 | N.S. (vs control) |
| PSK (5mg/kg i.v.) | 2/7 | 1/7 | 4/7 | $p<0.05$ (vs control) |

Pellet containing 100 ng bFGF was implanted in rat cornea micro-pocket. Rats were untreated or treated with PSK for 7 days, and cornea vessels were evaluated. +: new vessels reaching the pellet, +/-: new vessels reaching the avascular cornea but not the pellet, -: no new vessels. Figures are number of rats.

inhibited 50% tube formation, and 1000 μg/ml of PSK inhibited 30% HUVEC adhesion to Matrigel-, fibronectin-, and vitronectin-coated plates. The blood concentration of PSK was estimated to be 50 to 100 μg/ml, from the results of pharmacokinetics in an animal system using isotope-labeled PSK (Fujita *et al.* 1983). PSK appears to be most potent in inhibiting cell proliferation and this effect is observed at a physiologically attainable concentration in blood. We therefore focused on the inhibition of HUVEC proliferation by PSK. The complete medium contains growth factors. An anti-angiogenic polysaccharide pentosan was reported to inhibit heparin-binding growth factors (Zugmaier *et al.* 1992). The present study focused on the representative heparin-binding angiogenic factor, bFGF (Folkman & Klagsbrun 1987). When HUVECs were cultured in the presence or absence of bFGF, PSK inhibited cell proliferation to a greater extent in the presence of bFGF than in the absence of it. Basic FGF (10 ng/ml) increased the S phase population, and 250 μg/ml of PSK inhibited this increase. Friesel & Maciag (1995) and Tanaka *et al.* (1999) reported that MAP kinase was phosphorylated in bFGF-induced cell proliferation. In the

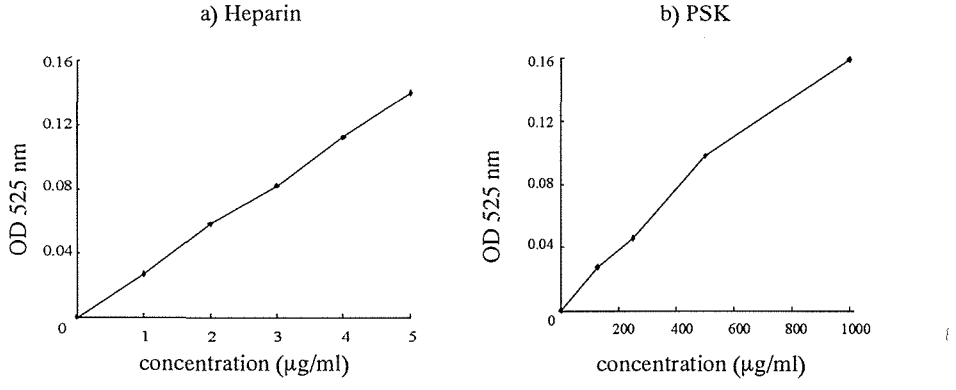


**Fig 9.** Quantitation of sulfated glycosaminoglycan. PSK was mixed with dimethylmethylene blue color reagent (see Materials and Methods) for 10 seconds. The absorbance at 525 nm was measured.

present study, 100 μg/ml of PSK suppressed phosphorylation of MAP kinase induced by bFGF. These results suggested that PSK interfered with the functions of bFGF. The possibility of interaction with bFGF was then investigated. When HUVECs were seeded in bFGF-coated plate, HUVECs adhered to the plate through interaction with bFGF. Under this condition, 100 μg/ml of PSK inhibited 55% of HUVEC attachment to bFGF-coating plate, which suggested that PSK interrupted the binding between bFGF and the bFGF receptors on cell surface. The binding of PSK to bFGF was further investigated by gel filtration, and direct binding of the two was demonstrated. Although the portion of PSK that binds with bFGF has not yet been elucidated, it is well known that bFGF binds to heparansulfate proteoglycan. Compared with the FGF receptors, the binding affinity of heparansulfate proteoglycan to bFGF is low, but it is an important mediator for the binding of bFGF and FGF receptor (Klagsbrun & Baird 1991). Turnbull *et al.* (1992) identified the binding site of heparansulfate proteoglycan to bFGF as sulfated glycosaminoglycan. PSK contained sulfated glycosaminoglycan, which indicated the capability for binding to bFGF. In the last experiment, rat cornea assay was performed to investigate whether PSK inhibits bFGF-induced angiogenesis *in vivo*. Five mg/kg of PSK significantly reduced bFGF-induced angiogenesis ($p<0.05$). This result indicated that PSK suppressed bFGF-induced angiogenesis *in vivo*.

In our *in vitro* system, the proliferation of HUVECs was inhibited in the absence of bFGF. The mechanism of this phenomenon is unknown. Yefenof *et al.* (1995) reported that PSK induced p53 expression and apoptosis. Therefore, PSK might directly act on endothelial cells *in vitro* and induce cell cycle arrest or apoptosis.

The present study suggests that PSK binds to bFGF and inhibits endothelial cell proliferation, thereby suppressing angiogenesis.

The goal of cancer treatment is to prolong survival. Cancer chemotherapy kills tumor cells and induces tumor reduction. The major problem of cancer chemotherapy is serious adverse drug reactions, furthermore there is no positive correlation between tumor reduction and survival time. After cancer chemotherapy, a steady state (no progression and no remission) is often observed. Clinicians have referred to this latent state as tumor dormancy (Uhr *et al.* 1997). Recently, strategies to prolong the dormant phase has been focused as another new approach to cancer treatment (Takahashi & Mai 2000). Folkman, *et al.* (1971) advocated the application of anti-angiogenesis therapy for tumor treatment, aiming to inhibit the induction of new vessels for tumor growth and stop the supply of nutrients. Anti-angiogenic agents are expected to induce tumor dormancy and reduce the adverse effects of conventional anti-cancer drugs. The strategy of prolonging tumor dormancy implies a possibility of living with cancer even though the cancer is not totally eliminated. The present results indicate an anti-angiogenic effect of PSK, and suggest that PSK might be useful in this new therapeutic approach.

## Acknowledgements

I wish to thank Drs. S. Yonei, T. Ando for valuable advice and fruitful discussions.

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