Protein-bound Polysaccharide-K (PSK) Induces Apoptosis and Inhibits Proliferation of Promyelomonocytic Leukemia HL-60 Cells

NORIYUKI HIRAHARA 1, MASAKI FUJIOKA 2, TAKEO EDAMATSU 2, AYAKO FUJIEDA 2, FUJIO SEKINE 2, TSUTOMU WADA 2 and TSUNEO TANAKA 1

1Department of Digestive and General Surgery, Shimane University Faculty of Medicine, Izumo, Japan;
2Biomedical Research Laboratories, Kureha Corporation, Tokyo, Japan

Abstract. Protein-bound polysaccharide-K (PSK) is extracted from Coriolus versicolor (CM101), and clinically used in combination therapy for gastrointestinal cancer and small cell lung carcinoma. PSK is a biological response modifier (BRM), and its mechanism of action is partly mediated, by modulating host immune systems, such as the activation of immune effector cells and the neutralization of transforming growth factor-beta (TGFβ) activity. Direct inhibition of tumor cell proliferation has been reported as another mechanism, but how PSK induces such an effect remains to be elucidated. Here, the anti-proliferative activity of PSK was examined using seven different human malignant cell lines (WiDr, HT29, SW480, KATOIII, AGS, HL60 and U937), and PSK was found to inhibit the proliferation of HL-60 cells most profoundly. Therefore, HL-60 cells were used to clarify the mechanism of anti-proliferative activity. Caspase-3 activation followed by apoptosis are involved at least in part in the PSK-induced anti-proliferative activity against HL-60 cells.

Protein-bound polysaccharide-K (PSK) is extracted from cultured mycelia of Coriolus versicolor (CM101) and its average molecular weight is approximately 100,000. The main structure is polysaccharide with the main chain linked by β-D-(1-4) binding and the side chains by β-D-(1-3) and β-D-(1-6) binding (1). PSK is an anticancer agent used for postoperative adjuvant therapy with chemotherapeutic agent for gastrointestinal cancer and small cell lung carcinoma in Japan, and to some extent in Taiwan (1-4).

Correspondence to: Noriyuki Hirahara, MD, Ph.D., Department of Digestive and General Surgery, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane, 693-8501, Japan. Tel: +81 853 202 232, Fax: +81 853 202 229, e-mail: norinori_hirahara@yahoo.co.jp

Key Words: PSK, apoptosis, HL-60, cell proliferation, caspase-3 activation.

Materials and Methods

Cells. Colon cancer cell lines (WiDr, HT29 and SW480), gastric cancer cell lines (KATOIII and AGS), promyelomonocytic leukemia cell line (HL 60) and monocytic cell line (U937) were obtained from American type culture collection (ATCC, Manassas, VA, USA). WiDr was maintained in MEM (Gibco BRL, USA) supplemented with 10% FBS (Life Technologies, Milan, Italy) and non-essential amino acids (Sigma, St. Louis, MO, USA). HT29 and KATOIII were maintained in McCoy’s 5A (Gibco BRL, USA).
supplemented with 10% FBS. SW-480 was maintained in DMEM (Gibco BRL, Paisley UK) supplemented with 10% FBS. AGS was maintained in Ham’s F12 (Gibco BRL, USA) supplemented with 10% FBS. HL-60 and U937 were maintained in RPMI1640 (Gibco BRL, USA) supplemented with 10% FBS.

PSK. PSK was manufactured at the Kureha Corporation, (Tokyo, Japan) and dissolved in Dulbecco’s Phosphate-Buffered Salines (DPBS, Gibco BRL, USA). In each experiment, freshly prepared PSK solution was used.

Cellular proliferation assay. Cellular proliferation was determined using water soluble tetrazolium (WST-1) (Dojindo, WAKO, Tokyo, Japan), a water-soluble version of methyl thiazolyl tetrazorium (MTT), according to the manufacturer’s instructions. Briefly, in the case of adherent cells, such as WiDr, HT29, SW-480 and AGS, the cells were seeded in a 96-well plate at a density of 3×10^3 cells/well (WiDr and SW-480) or 3×10^3 cells/well (HT29 and AGS) and incubated overnight. The next day, PSK or DPBS (vehicle control) was added to each well. After the indicated period of incubation, the medium was changed to WST-1 containing medium. The cells were incubated for another 2 h (KATOⅢ) or 3 h (HL-60 and U937) and the optical density was measured as described above.

Evaluation of apoptosis. To determine whether PSK-induced growth suppression was attributable to apoptosis induction, three different assays were performed to evaluate PSK-treated HL-60 cells.

First, phosphatidylserine externalization and membrane integrity was evaluated using a TACS™ Annexin V-FITC Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA), which contains FITC-conjugated Annexin V (Annexin V-FITC) and propidium iodide (PI). The HL-60 cells and U937 cells as controls were suspended in medium containing PSK or DPBS and seeded in a 96-well plate at a density of 3×10^5 cells/well. After the indicated period of incubation, WST-1 was added to each well and incubated for another 2 h (KATOⅢ) or 3 h (HL-60 and U937) and the optical density was measured as described above.

Second, DNA fragmentation was analyzed. The HL-60 cells were treated with PSK (30 or 100 μg/mL) or DPBS as described above. After the indicated period of incubation, the cells were harvested and resuspended in RIPA Lysis Buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were lysed by rotating for 30 min at 4˚C. After cell lysis, clear cell lysates were obtained by centrifugation. The cell lysates were sequentially treated with RNase A (Roche, Indianapolis, IN, USA) at a concentration of 0.05 mg/mL and Proteinase K (Worthington Biochemical Co., Lakewood, NJ, USA) at a concentration of 0.1 mg/mL for 30 min at 37˚C each. After incubation, the cell lysates were extracted with phenol/chloroform and the DNA was obtained by ethanol precipitation. One microgram of DNA was loaded onto 2% agarose gel. After electrophoresis, the DNA was visualized with ethidium bromide.

Third, chromatin condensation was analyzed. The HL-60 cells were treated with PSK (100 μg/mL) or DPBS as described above. After the indicated period of incubation, the cells were harvested and stained with 4’,6-diamidino-2-phenylindole (DAPI) (Boehringer Mannheim GmbH, Mannheim, Germany). The stained cells were observed under a fluorescent microscope (Olympus BH2, Olympus Corp., Tokyo, Japan).

Detection of active caspase-3. To overview which protein(s) are involved in PSK-induced apoptosis, a comprehensive analysis of apoptosis-related proteins was performed using Proteome Profiler™ Arrays (R and D Systems Minneapolis, USA). HL-60 cells were suspended in medium containing PSK or DPBS, and seeded in 100 mm culture dishes at a density of 1×10^6 cells/dish. After the indicated period of incubation, the cells were harvested and resuspended in PBS containing 1% saponin at a density of 1×10^6 cells/mL. The active form of caspase-3 was detected using APO ACTIVE 3™ (antibody to active caspase-3, Cell Technology, Inc., Mineapolis, MN, USA) according to the manufacturer’s instructions. The stained cells were analyzed by flow cytometer.

Statistical analysis. Statistical analyses were performed by the Student’s t-test. All the values are expressed as the mean±standard deviation. P-values of 0.05 or less were considered significant.

Results

Effect of PSK on cell proliferation. As shown in Figure 1, PSK effectively inhibited cellular proliferation of the HL-60 cells and to a lesser extent that of the HT29 cells and SW480 cells. PSK markedly inhibited cellular proliferation at 30 μg/mL in the HL-60 cells (p<0.05). In contrast, PSK only slightly inhibited the cellular proliferation even at a high dose (300 μg/mL) in the HT29 cells and SW480 cells. PSK did not show any inhibition of cellular proliferation at any dose in the other cell lines including the U937 cells. HL-60 cells were used to analyze the mechanism of action in the following experiments and U937 cells were also used in some experiments for comparison.

Induction of apoptosis. As shown in Figure 2, Annexin V–FITC and PI stained cells, which would be expected for late apoptotic or necrotic cells, were observed 48 h after the initiation of PSK treatment and further increased after 72 h treatment in the HL-60 cells. In contrast, no obvious dead cells were observed in the U937 cells treated with PSK under the same conditions. Furthermore typical biochemical features of apoptosis, such as DNA fragmentation (Figure 3) and chromatin condensation (Figure 4), were observed 72 h after the initiation of PSK treatment in the HL-60 cells.

Activation of caspase-3. The apoptosis proteome assay indicated that several proteins were implicated in PSK-induced apoptosis (data not shown). Among these candidate proteins, caspase-3 was further investigated. Caspase-3-positive cells increased in a dose and time dependent manner. The active form of caspase-3 was detected 48 h after PSK treatment was initiated and further increased until 72 h (Figure 5).
Discussion

Although strong anti-proliferative activity of PSK against HL-60 cells was found at 30 μg/mL in this study, Hattori et al. (10) did not find any effect up to 1000 μg/mL in the same cell line. In contrast, no effect on AGS cell proliferation was found, even at a high dose (300 μg/mL), although Jimenez-Medina et al. (11) reported that PSK profoundly inhibited cellular proliferation of AGS cells at 100 μg/mL. These discrepancies might be due to differences of experimental conditions, whereas Hattori et al. (10) used 1×10^4 cells/well, 3×10^3 cells/well were used in the present study. Hattori et al. also found that PSK inhibited cellular proliferation of Namalwa cells (Burkitt lymphoma cell line) at a density of 0.5×10^4 or 1×10^4 cells/well, but not at a higher density of 5×10^4 cells/well. Similarly when we used a higher density of HL-60 cells, no growth inhibitory effect was found (data not shown).

Figure 1. Effect of PSK on cellular proliferation. Seven human cell lines (WiDr, HT29, SW480, KATOIII, AGS, HL-60, U937) were exposed to PSK or DPBS (control). Each time-point represents the mean±SD (n=5). Statistical significance; *<0.05.
Figure 2. Effect of PSK on PI(+)Annexin V(+) staining. Representative PI and Annexin V profiles in HL-60 and U937 cells analyzed by flow cytometry after incubation with 30 μg/ml PSK. Control: cells incubated in DPBS. U937 cells were used for the negative control.

Figure 3. Effect of PSK on DNA fragmentation of HL-60 cells. After incubation with PSK or DPBS (control) for 48 or 72 h, DNA was extracted and analyzed by electrophoresis.
Therefore the initial cell number influences the effect of PSK. Jimenez-Medina et al. (11) used a similar initial cell number, however, unlike the present study they replaced the culture medium containing fresh PSK at 48 h after the initiation of PSK treatment. This discrepancy may have influenced the obtained results.

The present PSK-induced anti-proliferative activity against HL-60 cells was due at least in part to the induction of apoptosis, which has already been reported by others (9-11), but not elucidated. Comprehensive analysis of apoptosis-related protein expression upon PSK treatment in the HL-60 cells showed that the expression levels of several proteins had been changed. Among these proteins, pro-caspase-3 was prominently up-regulated. Pro-caspase-3 is known to be activated by various stresses (17) and the active form of caspase-3 plays a key role in the execution of apoptosis. In fact, we and others (10) found that the active form of caspase-3 was increased upon PSK treatment. Therefore, caspase-3 plays an important role in PSK-induced apoptosis.

So far, how PSK activates caspase-3 and induces apoptosis are unknown. As shown in this and previous studies, the growth inhibitory effect of PSK is cell-type specific. This implies the presence of a PSK receptor, which mediates such an effect, although future study will be necessary to identify such a receptor. Meanwhile, HL-60 cells are known to differentiate to neutrophil-like or macrophage-like cells upon various treatments, and eventually undergo apoptosis. However, no signs of differentiation of the HL-60 cells, such as adherent phenotype, gain of nitro-blue tetrazolium reducing activity or c-myc down-regulation were found upon PSK-treatment (data not shown). Therefore, PSK could directly induce apoptosis against HL-60 cells, without inducing differentiation, but such a possibility cannot be completely ruled out. In either case, upstream signaling events, which induce caspase-3 activation upon PSK-treatment, remain to be elucidated. Further study will be necessary to clarify these events and may be helpful in finding PSK-sensitive cell types, and eventually in identifying patients who could be beneficially treated with PSK.

In conclusion, PSK has an apoptosis-inducing activity against certain cell types, which at least in part, is mediated via caspase-3 activation.

**Competing Interests**

The Authors declare that they have no competing interests.
Acknowledgements

This study was conducted at Shimane University, Shimane, Japan, and Kureha Corporation, Tokyo, Japan.

References