Protein-bound Polysaccharide-K (PSK) Induces Apoptosis *via* p38 Mitogen-activated Protein Kinase Pathway in Promyelomonocytic Leukemia HL-60 Cells

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Abstract. *Background/Aim: Protein-bound polysaccharide-K (PSK) is extracted from Coriolus versicolor (CM101) and is clinically used in combination therapy for gastrointestinal cancer and small-cell lung carcinoma. We have previously demonstrated that PSK induces apoptosis and inhibites proliferation of promyelomonocytic leukemia HL-60 cells, but the signaling pathway for this action remains to be elucidated. In HL-60 cells, the mitogen-activated protein kinase (MAPK) pathway has been reported to be involved in stimuli-induced apoptosis. Therefore, involvement of the p38 MAPK pathway in PSK-induced apoptosis was herein investigated. Materials and Methods: HL-60 cells were used in this study. Western blotting was performed to detect phosphorylated p38 MAPK. A p38 MAPK inhibitor, SB203580, was used to examine the roles of p38 MAPK in PSK-induced apoptosis and growth inhibition. Results: PSK induced p38 MAPK phosphorylation. Co-treatment with SB203580 blocked PSK-induced apoptosis, caspase-3 activation and growth inhibition. Conclusion: The p38 MAPK pathway plays an important role in PSK-induced apoptosis.*

Protein-bound polysaccharide-K (PSK) is derived from an extract of Coriolus versicolor (CM101), which is a species of mushroom, and its major components are compounds possessing the β-glucan structure (1). In Japan, PSK is an antitumor agent used in combination with other chemotherapeutic agents as postoperative adjuvant therapy for gastrointestinal cancer and small-cell lung carcinoma.

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Although several clinical trials have demonstrated the beneficial effects of PSK (2-6), such as prolongation of survival time, its mechanisms of action remains unclear. Several mechanisms have been proposed to explain its antitumor activity. Among them, its immunomodulatory effects have been extensively studied (7-9). A recent report revealed that PSK acts as an agonist for toll-like receptor 2 (TLR2) (10), and the stimulation of natural killer cells by PSK (11, 12) is dependent on TLR2 activation.

Besides its immunomodulatory effects, several reports have suggested that PSK possesses cytotoxic activity specific for certain cell types (13-15). Using non-obese diabetic/severe combined immune-deficient (NOD/SCID) mice treated with anti-asialo-GM1 antibody, Hoshi *et al*. (16) recently demonstrated that PSK continued to exhibit antitumor activity against subcutaneously implanted Meth A tumors. This finding suggests that direct tumor cell killing activity may also be another antitumor mechanism of PSK.

Our previous study demonstrated that PSK inhibits cellular proliferation in a cell type-specific manner and that the inhibition is most profound for promyelomonocytic leukemia HL-60 cells (15). While we have shown that this inhibition of cellular proliferation is due, in part, to caspase-3-mediated apoptosis, the signaling pathway remains unclear.

The present study investigated the role which p38 mitogen-activated protein kinase pathway (MAPK) plays in the activation of caspase-3 in PSK-treated HL-60 cells.

Materials and Methods

Cells. Promyelomonocytic leukemia cell line (HL-60) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and was maintained in RPMI-1640 (Gibco BRL, MD, USA), supplemented with 10% fetal bovine serum (Life Technologies, Milan, Italy).

Reagents. PSK was manufactured at Kureha Corporation (Tokyo, Japan), and was dissolved in Dulbecco's phosphate-buffered saline

(DPBS) at a concentration of 100 mg/ml (Gibco BRL). In each experiment, the PSK solution was freshly prepared and was further diluted with medium. The p38 MAPK inhibitor SB203580 was purchased from LC laboratories (Woburn, MA, USA), and dissolved in dimethyl sulfoxide (DMSO) at 100 mmol/l, to prepare a stock solution. Before use, the stock solution was diluted with medium. The final concentration of SB203580 was 30 μmol/l, and the final concentration of DMSO was less than 0.1%. Dibutyryl-cyclic AMP (DB-cAMP) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and was dissolved in DPBS at 100 mmol/l. Nitro-blue tetrazolium (NBT) was purchased from Wako Pure Chemical Industries, Ltd., and was dissolved in DPBS at 2 mg/ml. In each experiment, freshly prepared NBT solution was used. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), and was dissolved in DMSO at 100 μg/ml to prepare a stock solution. Before use, the stock solution was diluted with medium to 1 μmol/l. This solution was further diluted to 100 nmol/ml as described below, and the final concentration of DMSO was less than 0.1%.

Western blotting. HL-60 cells were suspended in medium containing DPBS (control) or PSK (100 μg/ml), and seeded into 100-mm culture dishes at a density of 3×10^5 cells/dish. After the indicated time of incubation, cells were harvested and lysed in RIPA Lysis Buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA, by rotation for 30 min at 4˚C. After cell lysis, a clear cell lysate was obtained by centrifugation and the protein concentration was determined. Twenty microgram of protein were used for western blotting. Antibodies against phosphorylated p38 MAPK and p38 MAPK (Cell Signaling Technology, Danvers, MA, USA) were used to detect the phosphorylated form of p38 MAPK and total p38 MAPK, respectively. Chemiluminescent signals generated by using the ECL Advanced™ western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK) were detected by Light-Capture II Cooled CCD Camera Systems (ATTO, Tokyo, Japan).

Evaluation of apoptosis. Phosphatidylserine externalization and membrane integrity were evaluated using the TACSTM Annexin V-FITC Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, USA), which contains Fluorescein isothiocyanate (FITC)-conjugated annexin V (annexin V-FITC) and propidium iodide (PI). HL-60 cells were suspended in medium containing DPBS (control) or PSK (100 μg/ml) in the presence or absence of SB203580 (30 μmol/l), and were seeded into 6-well plates at a density of 6×10^4 cells/well. After the indicated time of incubation, cells were harvested and stained using the kit, according to the manufacturer's instructions. Stained cells were analyzed by flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ, USA).

Detection of active caspase-3. HL-60 cells were suspended in medium containing DPBS (control) or PSK (100 μg/ml) in the presence or absence of SB203580 (30 μmol/l), and seeded into 175 cm^2 culture flasks at a density of 1.05×10^6 cells/flask. After the indicated time of incubation, 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., Minneapolis, MN, USA) according to the manufacturer's instructions. Stained cells were analyzed by flow cytometry.

Cellular proliferation assay. Cellular proliferation was determined using WST-8 (Dojindo, Kumamoto, Japan), a water-soluble form of methyl thiazolyl tetrazorium (MTT), according to the manufacturer's instructions. Briefly, cells were suspended in a medium containing DPBS (control) or PSK (100 μg/ml) in the presence or absence of SB203580 (30 μmol/l), and seeded into 96-well plates at a density of 3×10^3 cells/well. After 72 h of incubation, WST-8 was added to each well and incubated for another 3 h. WST-8 is reduced to an orange colored formazan, which has maximum absorption at 460 nm. Optical density (O.D.) at 450 nm and 630 nm were measured. The O.D. at 630 nm was then subtracted from the O.D. at 450 nm.

Evaluation of differentiation. HL-60 cells are known to differentiate into neutrophil-like or macrophage-like cells when stimulated by various differentiating stimuli, and eventually undergo apoptosis. Therefore, we examined whether PSK induced differentiation of HL-60 cells by these two methods.

Firstly, NBT-reducing activity, which is induced during differentiation and indicates superoxide-producing ability, was examined. Dibutyryl-cyclic AMP, a well-known differentiating stimulus, was used as positive control. HL-60 cells were suspended in medium-containing DPBS (control) or PSK (100 μg/ml) or DB-cAMP (200 μmol/l), and seeded into 100-mm culture dish at a density of 3×10^5 cells/dish. After 72 h of incubation, cells were harvested and resuspended in medium at 1×10^6 cells/ml. Cell suspension (400 μl) was mixed with 2 mg/ml of NBT solution (500 μl) and 1 μmol/l of PMA solution (100 μl), and was incubated for 15 min at 37˚C. After incubation, cells were recovered and the number of blue-stained cells and the total number of cells were counted under a microscope (IX71, Olympus, Tokyo, Japan). Photomicrographs were representative of three independent experiments. Cells with NBT-reducing activity (blue-stained cells) were expressed as a percentage of the total cells.

Secondly, several genes are known to be down-regulated upon differentiation. Among these genes, c-myc oncogene *(c-Myc)*, B cell lymphoma *(BCL-2)* and poly ADP-ribose polymerase1 *(PARP1)*, were evaluated. DB-cAMP was used as positive control. HL-60 cells were suspended in medium containing DPBS (control) or PSK (100 μg/ml) or *DB-cAMP* (200 μmol/l), and seeded into 75-cm^2 culture flasks at a density of 4.5×10^5 cells/flask. After 72 h of incubation, cells were harvested and total RNA was extracted using the FastPure RNA kit (Takara, Osaka, Japan). Complementary DNA was prepared using PrimeScript RT reagent kit (Takara). PCR was performed with SYBR Premix Ex Taq II (Takara), appropriate primers (Greiner Japan, Tokyo, Japan) and LightCycler (Roche Diagnostics, Meylan, France), under the following thermal cycling conditions: denaturation at 95˚C for 30 s; followed by 40 cycles of 95˚C denaturation for 5 s and 60˚C annealing/extension for 20 s. Primer sequences are described in Table I. Ct values were calculated from a standard curve. The calculated Ct values of each gene were normalized by subtracting the Ct values of *beta-actin*. Relative gene expression is expressed as a ratio relative to the vehicle control (DPBS-treated HL-60 cells).

Results

Phosphorylation of p38 MAPK. Several reports have indicated that p38 MAPK plays an important role in apoptosis of HL-60 cells induced by various stimuli (17-20). Therefore we examined the role of p38 MAPK in PSK-induced apoptosis. Firstly, protein expression of the phosphorylated form of p38

Table I. *Primers used for quantitative real-time polymerase chain reaction.*

c-Myc	Forward	ctccttgcagctgcttagac
	Reverse	ggtagaagttctcctcctcg
BCL-2	Forward	cacctggatccaggataacg
	Reverse	ggccaaactgagcagagtct
PARP1	Forward	ggatgggttctctgagcttc
	Reverse	gacttggcatactctgctgc
Beta-actin	Forward	catccgcaaagacctgtacg
	Reverse	gatcttcattgtgctgggtgc

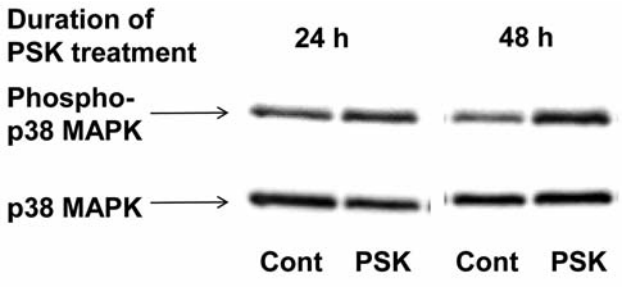

Figure 1. *Effect of PSK on p38 MAPK phosphorylation in HL-60 cells. After 24 or 48 h of incubation with Dulbecco's phosphate-buffered saline (control) or PSK, cell lysates were obtained and subjected to western blotting. Data are representative of three independent experiments.*

MAPK, which is believed to be activated, was increased after 48 h of PSK treatment (Figure 1).

Effect of p38 MAPK inhibitor on PSK-induced apoptosis. A p38 MAPK inhibitor, SB203580, was used to examine the role of p38 MAPK in PSK-induced apoptosis. PSK-induced apoptosis, as demonstrated by Annexin V-FITC and PI staining, was blocked by co-treatment with SB203580 (Figure 2). The active form of caspase-3 was increased upon PSK treatment and was reduced by co-treatment with SB203580 (Figure 3). Furthermore, PSK-induced growth inhibition was also blocked by SB203580 (Figure 4). These results suggest that p38 MAPK plays an important role in PSK-induced apoptosis and growth inhibition.

Evaluation of differentiation. HL-60 cells are known to differentiate into neutrophil-like or macrophage-like cells upon stimulation by various differentiating stimuli, and eventually undergo apoptosis (21, 22). Therefore we examined whether PSK induced differentiation of HL-60 cells. Firstly, NBT-reducing activity, which is induced during differentiation and indicates superoxide-producing ability, was examined. The well-known differentiating stimulus DB-cAMP induced NBT-reducing activity after 48 h of treatment, whereas PSK did not (Figure 5). Several genes are known to be down-regulated upon differentiation of HL-60 (23-25). Among such genes, *c-Myc BCL-2* and *PARP1* were evaluated. DB-cAMP caused down-regulation of these genes, but PSK had no effect (Figure 6). Furthermore, although another well-known differentiating stimulus, PMA, induced adhesion of HL-60 cells to the plastic dishes used, PSK showed no activity (data not shown).

Discussion

Using three different methods to evaluate differentiation, we observed no signs of differentiation on PSK-treated HL-60 cells. Therefore, we conclude that the apoptosis-inducing effect of PSK is due to direct, rather than indirect activities such as inducing differentiation first, followed by cell death.

In HL-60 cells, p38 MAPK has been reported to be involved in apoptosis induction by various stimuli (17-20). In apoptotic processes, p38 MAPK is reported to phosphorylate BCl-2 to inhibit its anti-apoptotic properties, such as prevention of cytochrome C release from mitochondria (26, 27). Cytochrome C release from mitochondria to the cytosol is a key event in mitochondria-dependent apoptotic processes, leading to activation of caspase-9, which in turn activates caspase-3/7 (28). Park and Kim (20) reported that auranofin induced p38 MAPK activation and apoptosis in HL-60 cells, and that co-treatment with SB203580 prevented cytochrome C release, caspase activation and apoptosis. Therefore PSK-induced p38 MAPK activation might trigger mitochondria-dependent apoptotic processes. In fact, we observed loss of mitochondrial transmembrane potential after PSK treatment (data not shown), which is purported to occur in tandem with mitochondrial cytochrome C release (29). However we cannot rule out the possibility that the loss of mitochondrial transmembrane potential is the result of apoptotic cell death, rather than the cause. In either case, PSK-induced p38 MAPK activation is an event upstream of caspase-3 activation and of apoptotic cell death in HL-60 cells.

Our results suggest that a rather prolonged treatment with PSK (48 h) is needed to achieve p38 MAPK activation in HL-60 cells. Ahn *et al*. (19) also found delayed p38 MAPK activation in 8-chloro-cyclic AMP-induced apoptosis of HL-60 cells. Therefore, p38 MAPK may be activated slowly in some cases.

How PSK activates p38 MAPK remains unknown. Recently, TLR2 has been reported to be a receptor for PSK (10). Besides the ability to induce inflammatory cytokines, TLR2 ligands also induce apoptosis (30-34). For some ligands, apoptosis is mediated by p38 MAPK (33, 34). In HL-60 cells, mycoplasmal or bacterial lipoproteins have been reported to induce apoptosis *via* TLR2 (31, 32). PSK might induce apoptosis in a similar manner. Differentiated HL-60

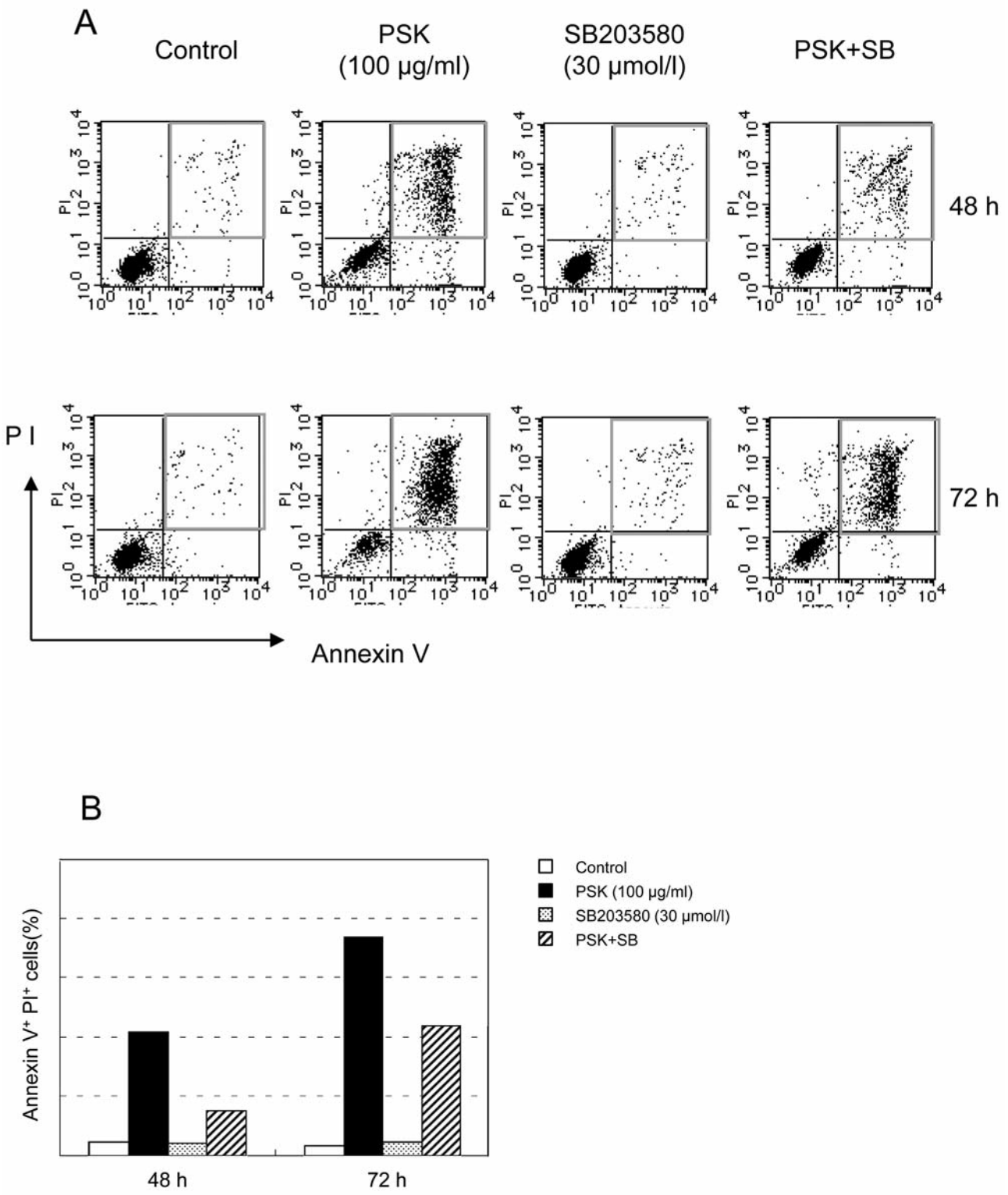

Figure 2. *Effects of a p38 MAPK inhibitor, SB203580, on PSK-induced apoptosis. HL-60 cells were treated with Dulbecco's phosphate buffered saline (control) or PSK for 48 or 72 h in the presence or absence of SB203580. Cells were stained with Fluorescein isothiocyanate (FITC)-conjugated Annexin V (Annexin V-FITC) and propidium iodide (PI), and were analyzed by flow cytometry. A: Flow-cytometric results; B: data after numerical conversion. Data are representative of two independent experiments.*

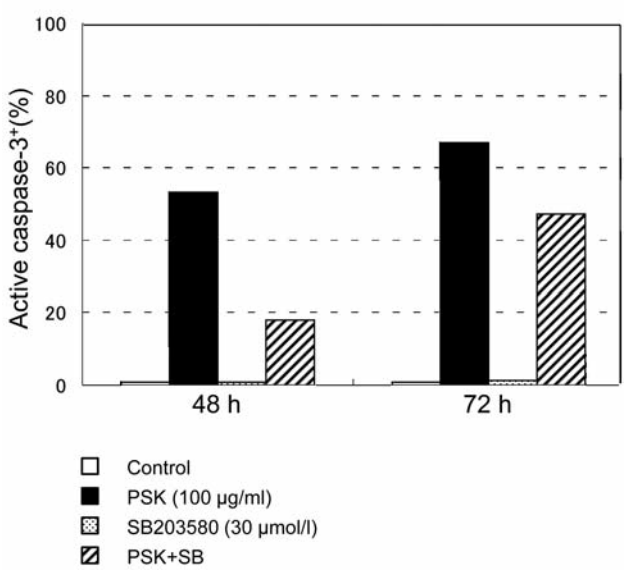

Figure 3. *Effects of a p38 MAPK inhibitor, SB203580, on caspase-3 activation. HL-60 cells were treated with Dulbecco's phosphate-buffered saline (control) or PSK for 48 or 72 h in the presence or absence of SB203580. Cells were stained with anti-active caspase-3 antibody, and analyzed by flow cytometry. Data are representative of two independent experiments.*

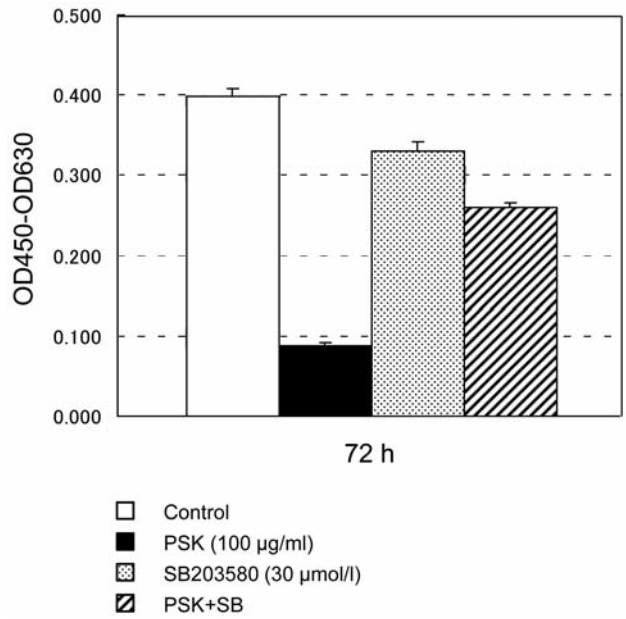

Figure 4. *Effects of SB203580 on PSK-induced growth inhibition. HL-60 cells were treated with Dulbecco's phosphate-buffered saline (control) or PSK for 72 h in the presence or absence of SB203580. Cellular proliferation was determined using WST-8, a water-soluble form of MTT. Data are expressed as the mean±SD (n=5). Data are representative of two independent experiments.*

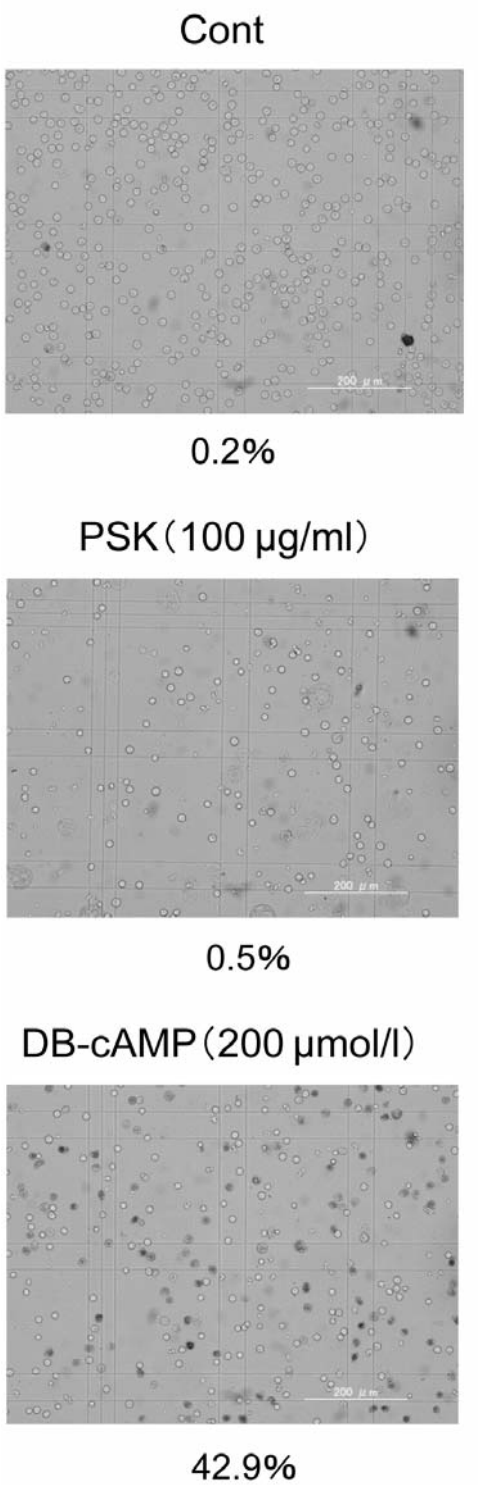

Figure 5. *Effects of PSK on neutrophil-like differentiation of HL-60 cells. After 72 h of incubation with Dulbecco's phosphate-buffered saline (control), PSK, or dibutyryl-cyclic AMP (DB-cAMP), the nitroblue tetrazolium (NBT)-reducing activity of these cells was evaluated. Photomicrographs are representative of three independent experiments. Cells with NBT-reducing activity (blue-stained cells) were counted and expressed as a percentage of the total cells.*

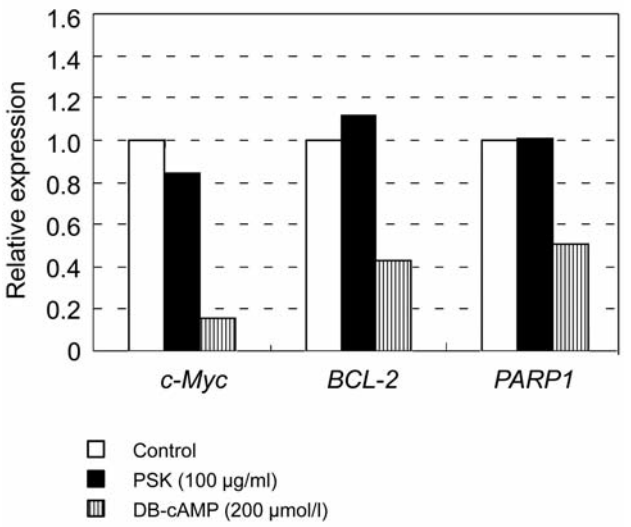

Figure 6. *Effects of PSK on differentiation-associated gene expression of HL-60 cells. After 72 h of incubation with Dulbecco's phosphate-buffered saline (control), PSK or dibutyryl-cyclic AMP (DB-cAMP), total RNA was extracted and subjected to real-time PCR. The expression of each gene was normalized to that of beta-actin expression and expressed as a ratio relative to that of the control. Data are representative of two independent experiments.*

cells, such as those used in the present study, express TLR2 at very low levels, and these cells have been reported to respond poorly to TLR2 ligands, compared to differentiated HL-60 cells (35, 36). Therefore PSK actions mediated by TLR2 require further investigation. Future studies are necessary to investigate the PSK receptor that mediates p38 MAPK activation and subsequently apoptosis.

In conclusion, this study demonstrates that PSK induces apoptosis of HL-60 cells without inducing differentiation, and p38 MAPK plays an important role in this process.

Competing Interests

KT and YT have no potential conflicts of interest. ET, AF, MF and TW are employees of Kureha Corporation, but the study was conducted with scientific integrity and presents no conflict of interest.

Authors' Contributions

NH was the lead Author. TE, AF, and MF participated in experiments and performed the assays. NH, TE, TW and YT designed the study and drafted the manuscript. All authors have read and approved the final manuscript.

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