

# Polysaccharide purified from *Ganoderma lucidum* inhibits spontaneous and Fas-mediated apoptosis in human neutrophils through activation of the phosphatidylinositol 3 kinase/Akt signaling pathway

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**Abstract:** *Ganoderma lucidum* has been widely used as a remedy to promote health and longevity in China. The polysaccharide component with a branched (1→3)-β-D-glucan moiety from *G. lucidum* (PS-G) has shown evidence of enhancement of immune responses and of eliciting anti-tumor effects. In this study, we investigated the effect of PS-G on neutrophil viability, which is manifested by spontaneous apoptosis. Annexin V staining and MTT assays reveal that PS-G is able to inhibit spontaneous and Fas-induced neutrophil apoptosis, and this effect of PS-G is enhanced by the presence of zVAD (a caspase inhibitor) and GM-CSF. The anti-apoptotic effect of PS-G is diminished by the presence of wortmannin and LY294002 (two PI-3K inhibitors), but is not altered by PD98059 (a MEK inhibitor). Western blotting indicates the stimulating effect of PS-G on Akt phosphorylation and its inhibition of procaspase 3 degradation, which occurs in neutrophils undergoing spontaneous apoptosis or triggered death by Fas. Taken together, PS-G elicitation of antiapoptotic effects on neutrophils primarily relies on activation of Akt-regulated signaling pathways. *J. Leukoc. Biol.* 72: 207–216; 2002.

**Key Words:** neutrophil apoptosis · PS-G · caspase activation · human

## INTRODUCTION

*Ganoderma lucidum*, an oriental fungus, has been widely used as a remedy to promote health and longevity in China and other Asian countries [1]. The fruiting bodies and cultured mycelia of *G. lucidum* are reported to be effective in the treatment of chronic hepatopathy, hypertension, hyperglycemia, and neoplasia. After component isolation and analysis, polysaccharide PS-G was purified from the hot-water extracts of the mycelium of *G. lucidum* followed by alcohol precipitation [2]. Structurally, PS-G is a branched (1→3)-β-D-glucan, which contains a backbone chain of (1→3)-linked D-glucose residues, 5 out of

16 D-glucose residues being substituted at 0–6 positions with single D-glucosyl units [3].

This major component of the hot-water-extractable fruiting body of *G. lucidum* was shown to possess anti-tumor activity in vivo [2–6] and to reduce tumor metastasis [6, 7]. When PS-G was given alone or in combination with cytotoxic, anti-tumor drugs, the lifespan of tumor-implanted mice was prolonged [8]. Further study has attributed the anti-tumor effect of PS-G to activated host immune responses [9]. The enhanced cytotoxic activity of natural killer cells and elevated tumor necrosis factor α and interferon-γ release, respectively, from macrophages and lymphocytes are the only documented evidence thus far to support the anti-tumor ability of PS-G [5, 6]. Whether PS-G possesses other mechanisms responsible for its anti-tumor effects is still unclear.

Neutrophils play a crucial role in host defense by phagocytosis and by killing invading microorganisms. The lifespan of human neutrophils released from bone marrow in circulation is about 6–10 h. This short half-life of neutrophils is a result of their undergoing spontaneous apoptosis [10–12]. This constitutively expressed program can be delayed by inflammatory mediators, such as granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-8, but enhanced by engagement of Fas receptors [13–16].

Programmed cell death, also known as apoptosis, plays a critical role in tissue morphogenesis and homeostasis and is characterized by cytoplasmic shrinkage, nuclear condensation, DNA fragmentation, and loss of cell membrane phospholipid asymmetry [17–19]. Initiation of apoptosis is controlled by regulation of the balance between life and death signals received by the cell [20]. Specific therapies designed to change the balance between life and death signals in neutrophils may influence the ability of host defense and the inflammatory state. It was therefore of great interest to investigate the effects of PS-G on neutrophil death.

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## MATERIALS AND METHODS

### Materials

RPMI 1640, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco-BRL (Grand Island, NY). Horseradish peroxidase-conjugated anti-rabbit antibodies, Ficoll-Hypaque, and the enhanced chemiluminescence (ECL) detection agent were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Rabbit polyclonal antibodies specific for procaspase 3 and Akt were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (Ser 473) polyclonal antibody was purchased from New England Biolabs (Beverly, MA). All reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories (Richmond, CA). Annexin-V-FLUOS was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). PD98059 and zVAD-fmk were purchased from Calbiochem (San Diego, CA). Anti-Fas antibody (clone CH-11) was purchased from Upstate Biotechnology (Lake Placid, NY). E5531 was kindly provided by Dr. Tsutomu Kawata (Tsukuba Research Laboratories for Drug Discovery, Japan). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

### PS-G purification from cultured *G. lucidum*

As in our previous study [5], fruiting bodies of *G. lucidum* were washed, disintegrated, and extracted with boiling water for 8–12 h. Hot-water extract of *G. lucidum* was fractionated into a polysaccharide fraction (alcohol insoluble) and nonpolysaccharide fraction (alcohol soluble, AS). The crude polysaccharide obtained was then passed through a gel-filtration Sephadex G 50 column (Pharmacia, Upsala, Sweden) and was further purified by anion exchange chromatography with a column of diethylaminoethyl-cellulose [2]. The PS-G we isolated was a protein-bound polysaccharide consisting of about 95% polysaccharide and 5% peptides. To rule out possible endotoxin lipopolysaccharide (LPS) contamination of PS-G samples, we determined LPS content by the chromogenic Limulus Amebocyte Lysate assay. We found that there was no detectable level of endotoxin (<0.10 endotoxin units/ml) in PS-G samples. Also in some experiments, the effect of PS-G was compared with *Escherichia coli* LPS (L8274, Sigma Chemical Co.) and was performed in the presence of the effective LPS antagonist E5531 [21].

### Neutrophil preparation

Neutrophils were isolated from citrate-anticoagulated venous blood (20–60 ml) obtained from healthy volunteers as described previously [22]. Briefly, cells were separated from whole blood by centrifugation, and the upper plasma layer was removed. Leukocytes were separated from erythrocytes in the cell pellet by differential sedimentation using 1.5% dextran. Granulocytes were then separated from monocytes and lymphocytes by centrifugation through a Ficoll-Hypaque gradient. Granulocytes were harvested from the interface of the gradient, and contaminating erythrocytes were removed by hypotonic water lysis. Neutrophil preparation contained >95% neutrophils, of which >99% were viable as determined by trypan blue dye exclusion. Freshly isolated neutrophils were resuspended in RPMI-1640 medium supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were cultured at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

### Cell viability assay

Cell viability was measured by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells (5×10<sup>5</sup> cells/well) were cultured in 200 µl medium in 96-well plates and incubated with vehicle or test compound for different time periods. After various treatments, 1 mg/ml MTT was added to culture plates and incubated at 37°C for an additional 2 h. Then cells were pelleted and lysed in 100 µl dimethyl sulfoxide. The absorbance at 550 nm was measured on a microplate reader. Each experiment was performed in duplicate and repeated five to six times.

### Analysis of neutrophil apoptosis

Annexin V is a protein that binds to phosphatidylserine (PS) residues, which are exposed on the cell surface of apoptotic, but not normal cells. In living cells, the distribution of the PS groups in the plasma membrane is asymmetrical such that the groups are directed toward the inside of the cell. During

apoptosis, this asymmetry is lost, and the PS groups are exposed to the exterior of the cell membrane. The binding of PS with annexin V is therefore an established biochemical marker of apoptosis. After neutrophils (5×10<sup>5</sup> cells/sample) were incubated as indicated with PS-G and/or CH-11, an agonistic anti-Fas immunoglobulin M, cells were pelleted (400 g, 5 min), washed twice with phosphate-buffered saline (PBS; pH 7.4), and resuspended in staining buffer containing 50 µg/ml propidium iodide (PI) and 0.025 µg/ml annexin V-fluorescein isothiocyanate (FITC). Double labeling was performed at room temperature for 15 min in the dark before flow cytometric analysis. Cells were filtered on a nylon mesh filter and immediately analyzed using FACScan and the Cellquest program (Becton Dickinson, San Jose, CA). Viable cells were determined from nonapoptotic and non-necrotic cell populations. Each experiment was repeated three to five times.

### DNA fragmentation and cell morphology

Cleavage of DNA into oligonucleosomal fragments, recognizable as a DNA ladder when electrophoresed on an agarose gel, is usually considered the biochemical hallmark of apoptosis. Genomic DNA was isolated using the Puregene kit #D-5000 (Gentra Systems, Minneapolis, MN). Briefly, following treatment with the test compound, cells were washed with PBS and lysed in cell lysis buffer containing Tris, ethylenediaminetetraacetate (EDTA), and SDS. After the addition of RNase A (0.6 U/ml), the mixture was incubated at 37°C for 30 min. Protein precipitation solution (ammonium acetate) was added to the samples to eliminate the contamination of proteins and was centrifuged at 2000 g for 10 min. Cell lysates were treated with 100% isopropanol to precipitate DNA. The DNA pellet was washed with 70% v/v ethanol and dissolved in DNA hydration buffer containing Tris and EDTA. The DNA concentration was determined at 260 nm by spectrophotometry. DNA (20 µg) was electrophoresed on a 1% w/v agarose gel containing 0.5 µg/ml ethidium bromide. DNA fragmentation bands were photographed under UV light. To examine the cell morphology, neutrophils were collected and plated on glass slides by cytopspin and then fixed with ice-cold, 95% v/v ethanol. The slides were rehydrated in PBS and incubated in hematoxylin solution (6 g/L hematoxylin, 52.8 g/L aluminum sulfate, 0.6 g/L sodium iodate) for 10 min. The stained cells were analyzed by light microscopy.

### Free radical scavenging capacity of PS-G and AS extracts of mycelium of *G. lucidum* (GLP-AS)

The free radical-scavenging capacity of PS-G and GLP-AS was measured with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) as described previously [23]. The DPPH radical has a deep, violet color as a result of its unpaired electron, and radical scavenging can be followed spectrophotometrically by absorbance loss at 517 nm when the pale, yellow, nonradical form is produced. A 5 µl aliquot of PS-G or GLP-AS (10–100 µg/ml) was mixed with 995 µl 100 µM DPPH solution (in ethanol) in a cuvette. Following a 30-min incubation in the dark, the change of absorbance at 517 nm was measured on a spectrophotometer (Model U-3200, Hitachi Instruments, San Jose, CA). The DPPH test was also done with vitamin E (50 µM) and ascorbic acid (50 µM) as positive controls.

### Western immunoblotting

To quantify the phosphorylated form of Akt at Ser 473 or degradation of procaspase 3, cells following incubation in the presence of various stimuli for different time periods were washed twice in ice-cold PBS and then lysed in buffer containing 20 mM Tris-HCl, 0.5 mM ethyleneglycol-bis(β-aminoethyl-ether)-N,N'-tetraacetic acid, 2 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin (pH 7.5). Samples of equal amounts of protein were subjected to SDS-PAGE on 10% (for Akt measurement) or 15% (for procaspase 3 measurement) polyacrylamide gels and transferred onto a nitrocellulose membrane, which was then incubated in 150 mM NaCl, 20 mM Tris, and 0.02% Tween (pH 7.4) containing 5% nonfat milk. The total and phosphorylated Akt or procaspase 3 bands were visualized by immunoblotting with specific antibodies and detected using an ECL technique and exposure to photographic film.

### Statistical evaluation

Values are expressed as the mean ± SEM of at least three experiments. Analysis of variance and Dunnett's tests were used to assess the statistical

significance of the differences, and  $P < 0.05$  was considered statistically significant.

## RESULTS

### PS-G antagonizes spontaneous neutrophil apoptosis

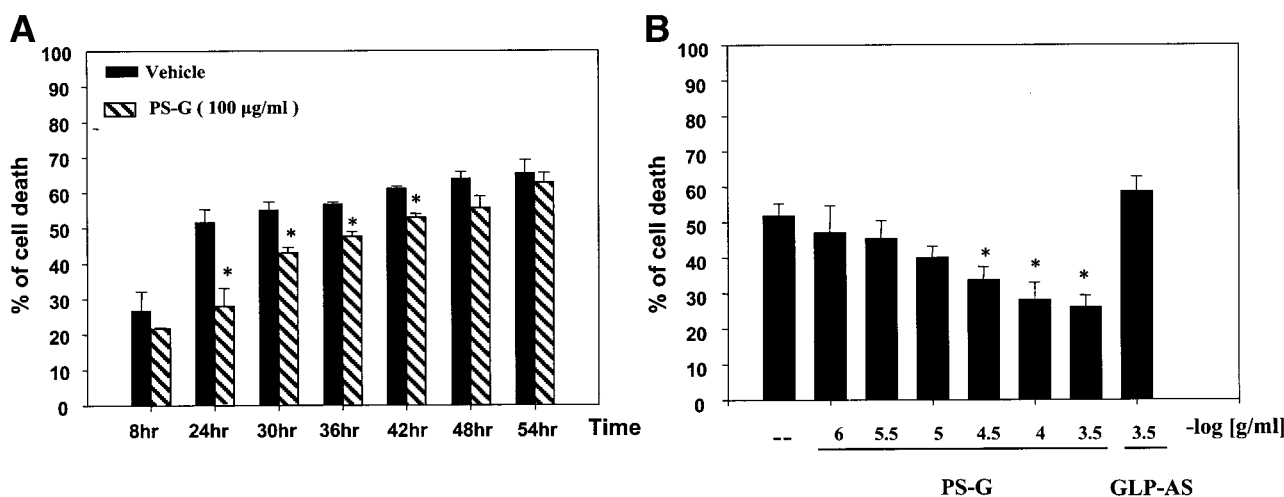
Isolated neutrophils progressively underwent cell death when cultured in medium alone in vitro. When using a mitochondria function-dependent MTT assay as an index of cell viability, time-dependent cell death of neutrophils was shown on in vitro culture (Fig. 1A). Loss of mitochondria activity was detected in about 50% of cells at 24 h. Incubation of freshly isolated neutrophils with 100  $\mu\text{g/ml}$  PS-G attenuated the population of dead cells within 42 h in in vitro culture, and its greatest effectiveness on cell protection was seen at 24 h of culture. At this time point, the population of dead cells was reduced by approximately 23%, from  $50.7 \pm 4.6\%$  ( $n=8$ ) of the control group to  $27.9 \pm 5\%$  ( $n=8$ ) of the PS-G-treated group (Fig. 1A). PS-G-treated neutrophils with longer in vitro culture, such as 48 h, eventually achieved only 9% cell protection. With prolonged culture of up to 54 h, a dead population equivalent to that of the control group was seen. These results suggest that PS-G can delay spontaneous neutrophil apoptosis. When examining the concentration-dependent effect of PS-G at 24 h of incubation, the results shown in Figure 1B indicate that concentrations higher than 30  $\mu\text{g/ml}$  PS-G are required for cell protection. In contrast to apoptotic delay caused by PS-G, constituents of *G. lucidum* extracted into the AS fraction failed to alter the lifetime of neutrophils at concentrations up to 300  $\mu\text{g/ml}$ .

In the early stages of apoptosis, loss of plasma membrane phospholipid asymmetry, resulting in the externalization of membrane PS, is a biochemical hallmark of apoptosis. We further used flow cytometry to investigate whether the loss of neutrophil viability as an index from MTT assays was a result

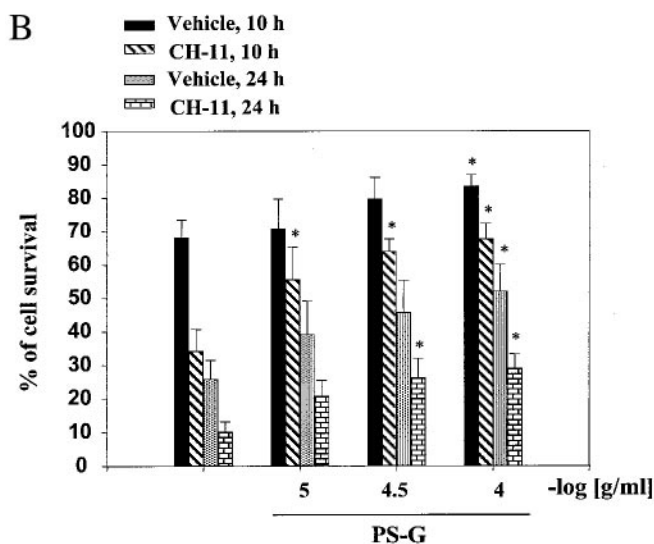
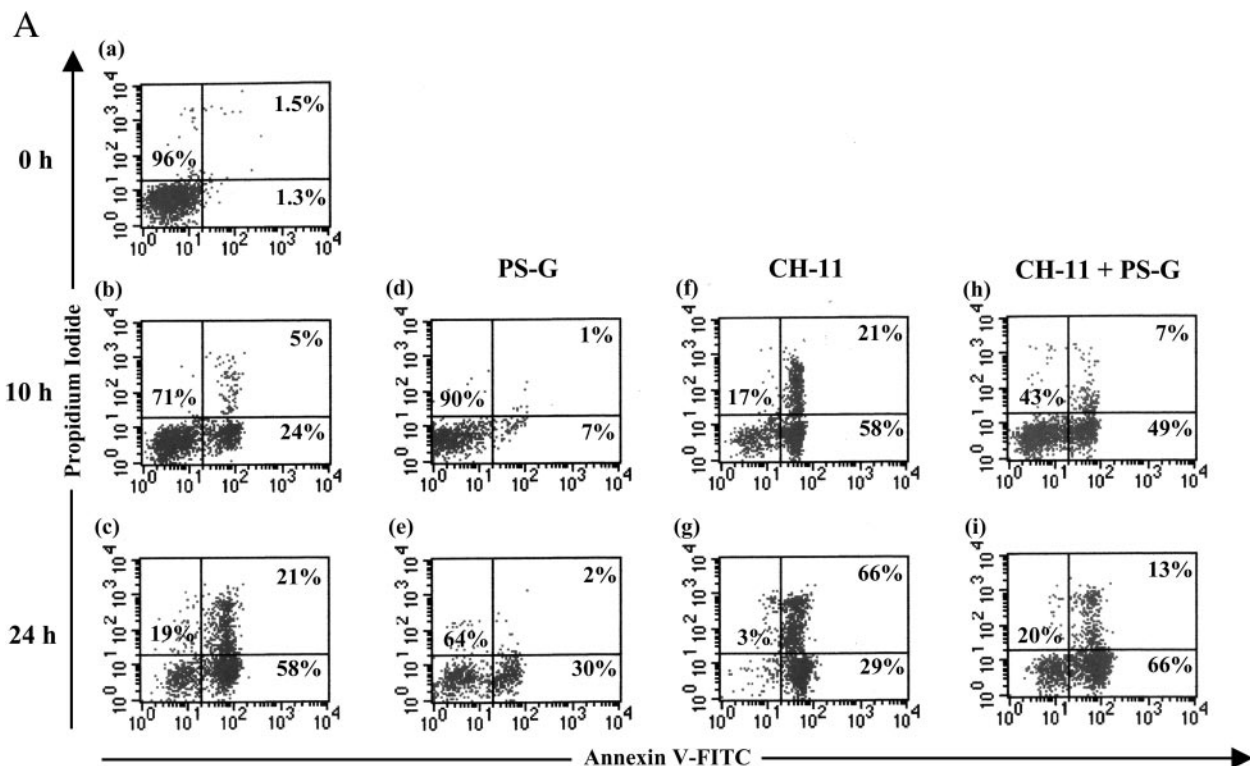
of apoptosis. The results from analyzing PI and annexin V labeling of neutrophils confirmed that neutrophils indeed died by apoptotic processes and were protected by the presence of PS-G (Fig. 2A). As compared with freshly isolated neutrophils (Fig. 2A, a), culturing in vitro for 10 h (Fig. 2A, b) and 24 h (Fig. 2A, c) produced increasing proportions of annexin V-positive cells, which displayed two types of labeling: annexin V-positive/PI-negative cells (lower right quadrant), corresponding to early apoptotic cells (24% at 10 h and 58% at 24 h), and annexin V-positive/PI-positive cells (upper right quadrant), corresponding to the advanced apoptotic cells and/or necrotic cells (5% at 10 h and 21% at 24 h). PS-G (100  $\mu\text{g/ml}$ ) treatment (Fig. 2A, d and e) significantly increased the percentage of living cells (lower left quadrant, annexin V-negative/PI-negative) as compared with control groups (90% vs. 71% at 10 h and 64% vs. 19% at 24 h). Calculating living cells, as indicated in the lower left quadrant, data from five experiments are summarized in Figure 2B and show that annexin V analysis is more sensitive than the MTT method to reflect cell viability; for example, 25% versus 50% cell survival after 24 h culture was, respectively, determined by annexin V and MTT assays.

### PS-G antagonizes Fas-mediated apoptosis

Neutrophil apoptosis not only progresses spontaneously, but also is accelerated by Fas stimulation [15, 24, 25]. To test the antiapoptotic ability of PS-G against Fas-mediated apoptosis, isolated neutrophils were stimulated with agonistic anti-Fas antibody (clone CH-11). In PI and annexin V measurements, percentages of living cells from CH-11 treatment groups (Fig. 2A, f and g) were significantly lower in comparison with those observed in cells cultured with vehicle (Fig. 2A, b and c; 17% vs. 71% at 10 h and 3% vs. 19% at 24 h). Likewise, PS-G treatment in the presence of CH-11 (Fig. 2A, h and i) significantly increased the percentage of living cells as compared with control groups treated with CH-11 alone (43% vs. 17% at 10 h and 20% vs. 3% at 24 h). The data from five experiments



**Fig. 1.** Effects of PS-G on spontaneous apoptosis in neutrophils. Isolated neutrophils were incubated with vehicle, PS-G [100  $\mu\text{g/ml}$  (A) and indicated concentrations (B)] or GLP-AS (300  $\mu\text{g/ml}$ ) for different time periods (A) or for 24 h (B). Following incubation, cell viability was determined by MTT assay. Each column represents the mean  $\pm$  SEM of at least three independent experiments performed in duplicate. \*,  $P < 0.05$  as compared with the control group without PS-G treatment.



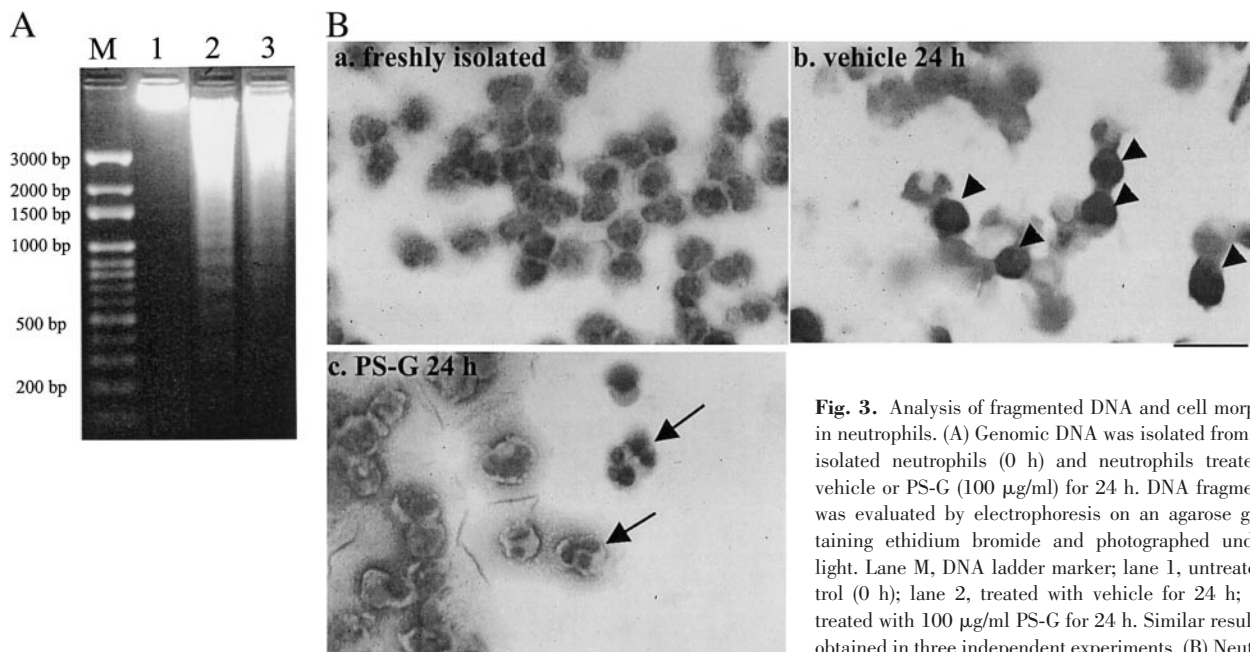
**Fig. 2.** Annexin V-FITC/PI flow cytometry of neutrophils. (A) Cells were incubated with vehicle (a–c), 100  $\mu\text{g/ml}$  PS-G (d and e), 250 ng/ml CH-11 (f and g), or PS-G in combination with CH-11 (h and i) for the indicated time periods. Cells were labeled by annexin V-FITC and PI following treatment and were analyzed by flow cytometry. The lower left quadrant (annexin V<sup>-</sup> PI<sup>-</sup>) of each panel shows viable cells. The lower right quadrant (annexin V<sup>+</sup> PI<sup>-</sup>) represents early apoptotic cells. The upper right quadrant (annexin V<sup>+</sup> PI<sup>+</sup>) contains advanced apoptotic and necrotic cells. (B) Data are representative of four independent experiments, which are summarized. Different concentrations of PS-G alone or in combination with CH-11 were added for 10 or 24 h, and then cell viability as assessed from flow cytometry was determined. Data represent the mean  $\pm$  SEM of four experiments. \*,  $P < 0.05$  as compared with the control group without PS-G treatment.

are summarized in Figure 2B. These results indicate that PS-G antagonizes not only spontaneous apoptosis but also Fas-induced apoptosis in neutrophils. Comparing both types of cell apoptosis, PS-G is more efficient in protecting cells from Fas action, as 10  $\mu\text{g/ml}$  PS-G is sufficient to trigger early protection at 10 h. Conversely, PS-G failed to reduce spontaneous cell death until concentrations up to 100  $\mu\text{g/ml}$ .

### DNA fragmentation and nuclear condensation

Degradation of DNA into a specific fragmentation pattern is a characteristic feature of apoptosis. In contrast to the random fragmentation with necrosis, apoptosis-associated DNA fragmentation is characterized by cleavage of the DNA at regular intervals, visualized on agarose gel electrophoresis as a DNA ladder consisting of multimers of approximately 200 base

pairs. Freshly isolated neutrophils were incubated with vehicle (Fig. 3A, lane 2) or 100  $\mu\text{g/ml}$  PS-G (Fig. 3A, lane 3) in vitro for 24 h, and the genomic DNA from these samples was subjected to agarose gel electrophoresis. A clear DNA fragmentation was seen in the 24 h vehicle treating group, and this pattern was less apparent in cells treated with PS-G. In addition, no fragmentation pattern was seen in freshly isolated neutrophils (Fig. 3A, lane 1). To detect morphological consequence in neutrophil apoptosis, we also performed light microscopy using hematoxylin-stained neutrophils that had been treated with vehicle or PS-G (Fig. 3B). We found that 24 h vehicle-treated neutrophils exhibited highly condensed chromatin and cytoplasmic disintegration (Fig. 3B, b). In contrast, freshly isolated neutrophils showed intact polymorphic nuclei (Fig. 3B, a). Moreover, in the PS-G-treating group, only a few



**Fig. 3.** Analysis of fragmented DNA and cell morphology in neutrophils. (A) Genomic DNA was isolated from freshly isolated neutrophils (0 h) and neutrophils treated with vehicle or PS-G (100  $\mu\text{g/ml}$ ) for 24 h. DNA fragmentation was evaluated by electrophoresis on an agarose gel containing ethidium bromide and photographed under UV light. Lane M, DNA ladder marker; lane 1, untreated control (0 h); lane 2, treated with vehicle for 24 h; lane 3, treated with 100  $\mu\text{g/ml}$  PS-G for 24 h. Similar results were obtained in three independent experiments. (B) Neutrophils treated with vehicle or PS-G (100  $\mu\text{g/ml}$ ) were plated on

glass slides by cytospin, and nuclear morphology was visualized using hematoxylin staining as described in Materials and Methods. For 24 h incubation, obvious nuclear condensation was seen in the vehicle treated group (b; arrowheads) but not in the PS-G-treated group. In the latter group, only a weak, nuclear condensation was seen (c; arrows). Original bar = 12  $\mu\text{m}$ .

neutrophils exhibited a much less obvious feature of nuclear condensation (Fig. 3B, c).

### Effect of PS-G on caspase activation

Biological and biochemical analyses of apoptosis have shown that caspases are key effectors of cell death [26, 27]. In neutrophil apoptosis in response to various noxious insults, caspase, with no exceptions, was an executioner for the death program [14, 15, 28, 29]. The effects of zVAD-fmk, a broad-spectrum, cell-permeable fluoromethylketone inhibitor of caspase, on spontaneous and Fas-induced apoptosis were then examined. In contrast to the protective effect of PS-G, the spontaneous apoptosis of neutrophils at 24 h was only slightly and not significantly reduced by zVAD-fmk, which also could not alter the protection afforded by PS-G (Fig. 4A). However, in 10- or 24-h culture of CH-11-treated cells, the population of surviving cells was significantly elevated in the presence of zVAD-fmk (30  $\mu\text{M}$ ; Fig. 4A). Combined treatment of cells with zVAD-fmk and PS-G led to a greater extent of cell protection as compared with treatment with each agent alone. These results suggest that although caspase activity, as previously demonstrated, increases during *in vitro* culture [15], it does not play a major role in spontaneous apoptosis. Furthermore, apart from the inhibitory action of PS-G on caspase (see below), PS-G appears to possess a caspase-independent mechanism, which contributes, at least in part, to antiapoptosis.

To verify the PS-G antiapoptotic mechanism, especially for Fas-induced apoptosis, related to caspase activity, we investigated whether PS-G affects the activation of caspase 3. Thus, in neutrophils progressing through spontaneous and Fas-induced apoptosis, we examined procaspase 3 degradation, which is known as the essential step triggering active caspase 3 release and functioning. As shown in Figure 4B,

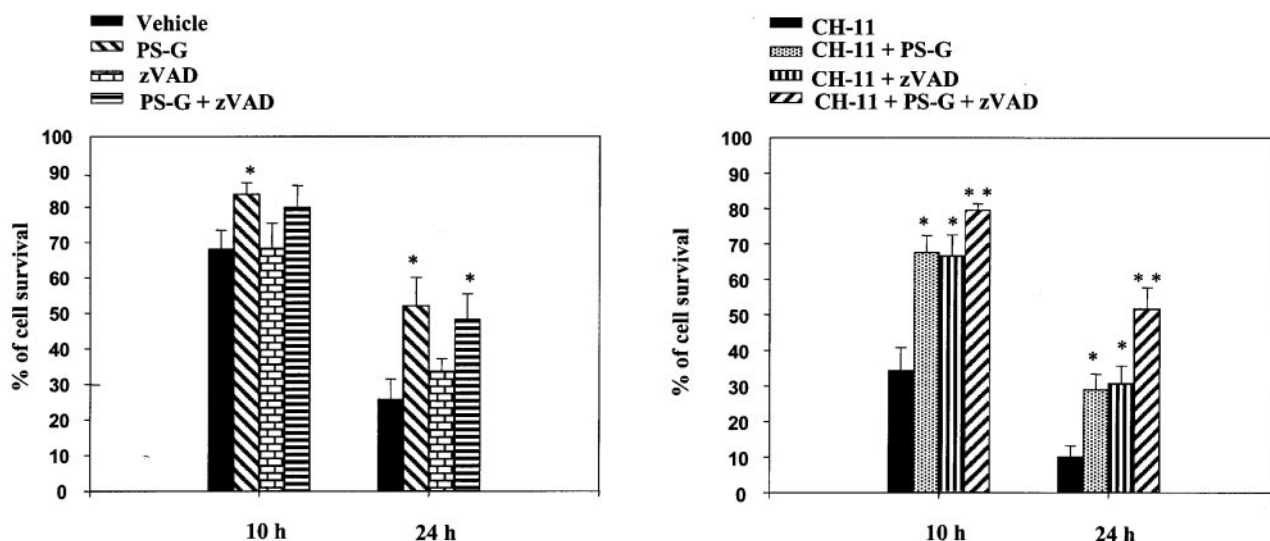
control neutrophils displayed a time-dependent loss of procaspase 3 within 20 h of culture, and a significant reduction by 40% was seen at 10 h. In contrast, in the presence of CH-11, procaspase 3 degradation was accelerated in onset and extent. After 10 h of culture, procaspase 3 was reduced to about 15% of the level of freshly isolated cells. In cells treated with 100  $\mu\text{g/ml}$  PS-G alone, the gradual decrease of procaspase 3 was diminished at 5, 10, and 20 h of culture. Similarly, PS-G obviously inhibited and delayed agonistic, Fas antibody-induced procaspase 3 loss at 5 h and 10 h of culture, and in the cells extended to 20 h of culture, only a slight elevation of procaspase 3 level was observed.

### Antioxidant-independent action of PS-G

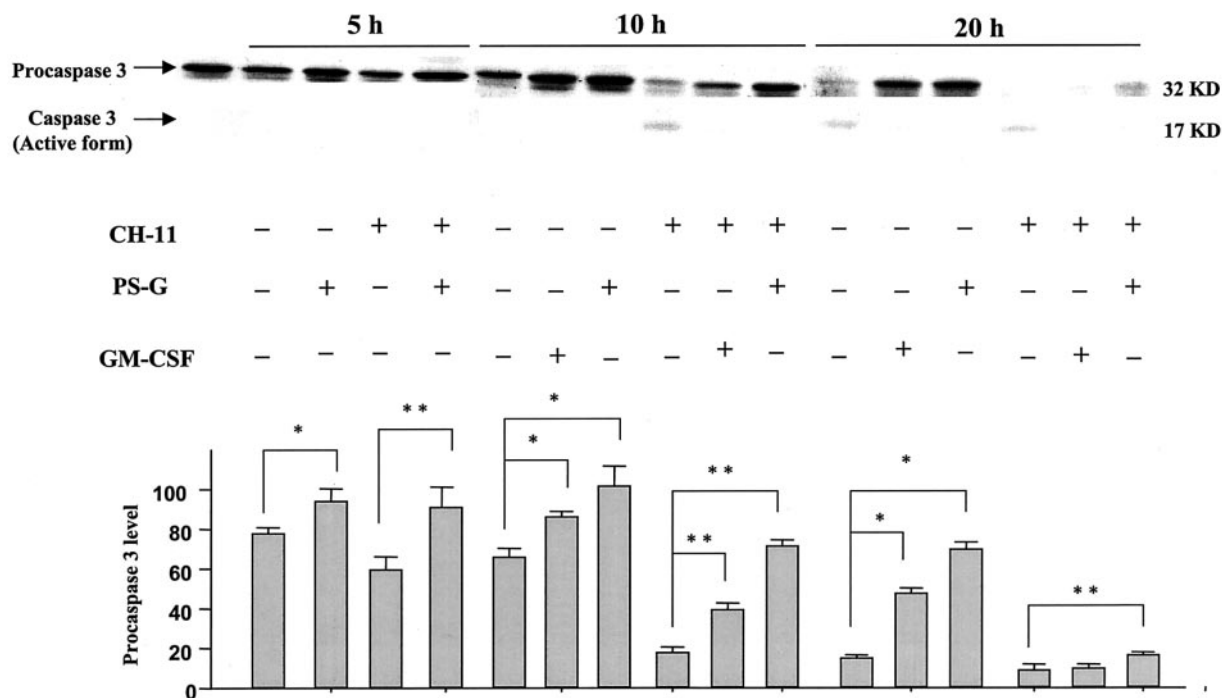
As demonstrated, reactive oxygen species (ROS) may contribute to the complex processes regulating cell apoptosis [14, 30]. To address whether ROS are relevant to PS-G action, the effects of glutathione (GSH), N-acetyl cysteine (NAC), and carboxyfullerene C60, a free radical scavenger as powerful as an antioxidant agonist [31, 32], were examined. Results from Figure 5A indicate that NAC (10 mM), GSH (10 mM), and carboxyfullerene (50  $\mu\text{M}$ ) cannot prevent spontaneous and Fas-induced apoptosis, even further increasing cell death in the cases of NAC and GSH. Using 2', 7'-dichlorofluorescein diacetate labeling to measure intracellular levels of ROS, particularly of  $\text{H}_2\text{O}_2$ , no significant changes following PS-G treatment were detected (data not shown).

To directly address the possible antioxidant ability of PS-G, DPPH assays were carried out. Figure 5B showed that in contrast to the antioxidant actions of vitamins E and C, PS-G at concentrations up to 100  $\mu\text{g/ml}$  possessed no antioxidant activity. However, the AS fraction of GLP did so at 30 and 100  $\mu\text{g/ml}$ .

A



B

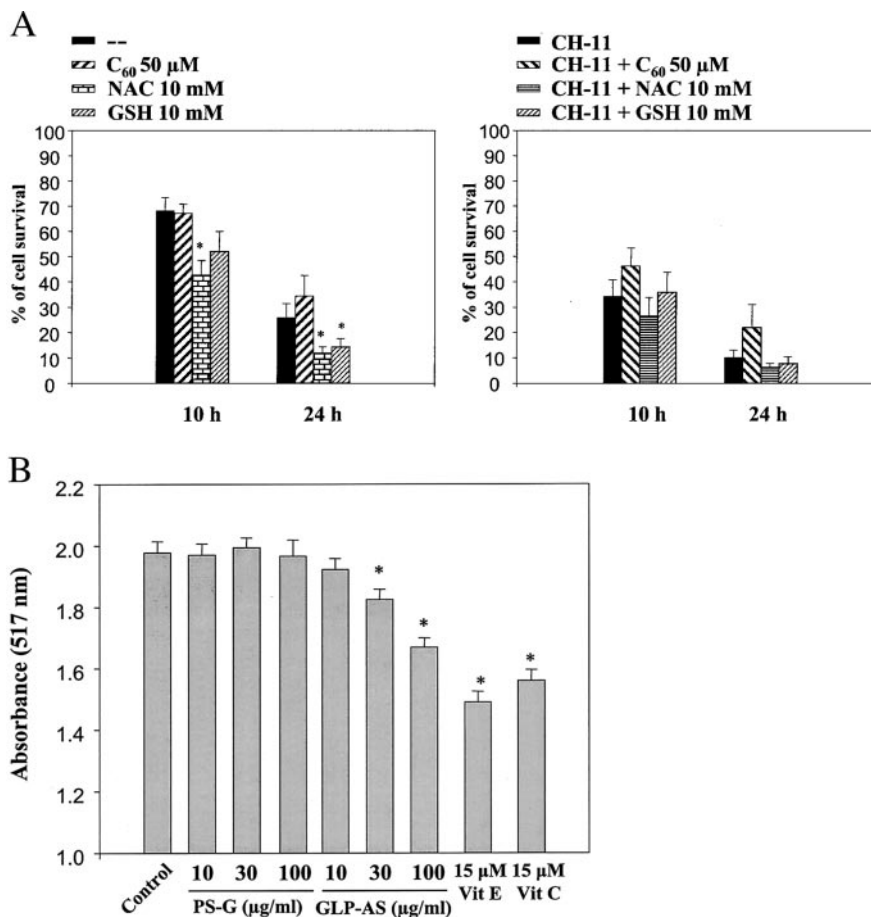


**Fig. 4.** Inhibition of caspase activation by PS-G. (A) Cells were pretreated with vehicle or zVAD-fmk (30  $\mu$ M) for 30 min before addition with vehicle, CH-11 (250 ng/ml), or PS-G alone or in combination as indicated for 10 or 24 h. Viable cells were analyzed by flow cytometry using annexin V-FITC/PI labeling. Data are presented as the mean  $\pm$  SEM of five experiments. \*,  $P < 0.05$  as compared with the control group without PS-G or zVAD treatment; \*\*,  $P < 0.05$  as compared with the protective action of PS-G and zVAD alone. (B) Cells were treated with vehicle, PS-G (100  $\mu$ g/ml), and/or CH-11 (250 ng/ml). At different time intervals, immunoblots of procaspase 3 were determined. The trace shown in the upper panel is from a representative experiment, and results from three independent experiments were calculated and shown in the lower panel. \*,  $P < 0.05$  as compared with the control group without PS-G or CH-11 treatment; \*\*,  $P < 0.05$  as compared with the control group with CH-11 treatment alone.

### PI 3 kinase (PI-3K)/Akt activation participates in PS-G protection

It has been established that several upstream protein kinases, such as PI-3K and extracellular regulated kinase (ERK), transduce signals suppressing apoptosis [33]. To elucidate the molecular mechanism for PS-G protection and the role of both kinases in PS-G action, we used selective pharmacological

inhibitors. In addition, here, we also compare the responses of PS-G with GM-CSF, which is known to effectively delay neutrophil apoptosis [25, 34, 35]. As shown in **Figure 6A**, the antiapoptotic effect of PS-G (100  $\mu$ g/ml) was unaffected by PD98059 (30  $\mu$ M) pretreatment, and the co-presence of wortmannin (300 nM) or LY294002 (30  $\mu$ M) significantly abolished the PS-G effect. Conversely, wortmannin and PD98059 re-



**Fig. 5.** Antioxidant-independent action of PS-G. (A) Cells were treated with 10 mM NAC, 10 mM GSH, 50 μM carboxyfullerene (C<sub>60</sub>), or CH-11 (250 ng/ml) as indicated for 24 h. Cell viability was determined by flow cytometry using annexin V-FITC/PI labeling. Data are presented as the mean ± SEM of four to five experiments. \*, *P* < 0.05 as compared with the control group. (B) Effects of PS-G and GLP-AS on DPPH scavenging activities. DPPH (100 μM) was incubated in the absence or presence of PS-G (10–100 μg/ml), GLP-AS (10–100 μg/ml), vitamin E (Vit E; 15 μM), or ascorbic acid (Vit C; 15 μM) at room temperature for 30 min, and then the absorbance at 517 nm was measured. Data represent the average from three independent experiments. \*, *P* < 0.05 as compared with the control group.

versed the antiapoptotic effect of GM-CSF. This suggests that PS-G might mimic GM-CSF action in rescuing neutrophils, at least partially by activating the PI-3K-dependent signaling pathway [16]. Concomitant to cell protection, GM-CSF reduced procaspase 3 activation in cells undergoing spontaneous or Fas-induced apoptosis (Fig. 4B). It is interesting that PS-G, in combination with GM-CSF treatment, further promoted cell survival as compared with cells treated with PS-G alone.

Bacterial endotoxin LPS is known to exert an antiapoptotic effect on neutrophils [36]. To rule out the possible LPS contamination of PS-G samples, we examined the effect of E5531, which is known to be a specific and potent antagonist of LPS [21]. The results indicated that E5531 at 1 μM was able to reverse the cell protection caused by LPS but had no effect on PS-G (Fig. 6A).

As wortmannin can eliminate the antiapoptotic function of PS-G, it was interesting to investigate if the downstream target of PI-3K, Akt, is involved. Phosphorylation of Akt at Ser 473 is required for Akt activation. Thus, the phosphorylated form of the Akt protein at Ser 473 was determined by immunoblotting with phosphorylated-specific antibody. As shown in Figure 6B, the phosphorylated form of Akt time-dependently increased after PS-G treatment and was prevented by the presence of wortmannin.

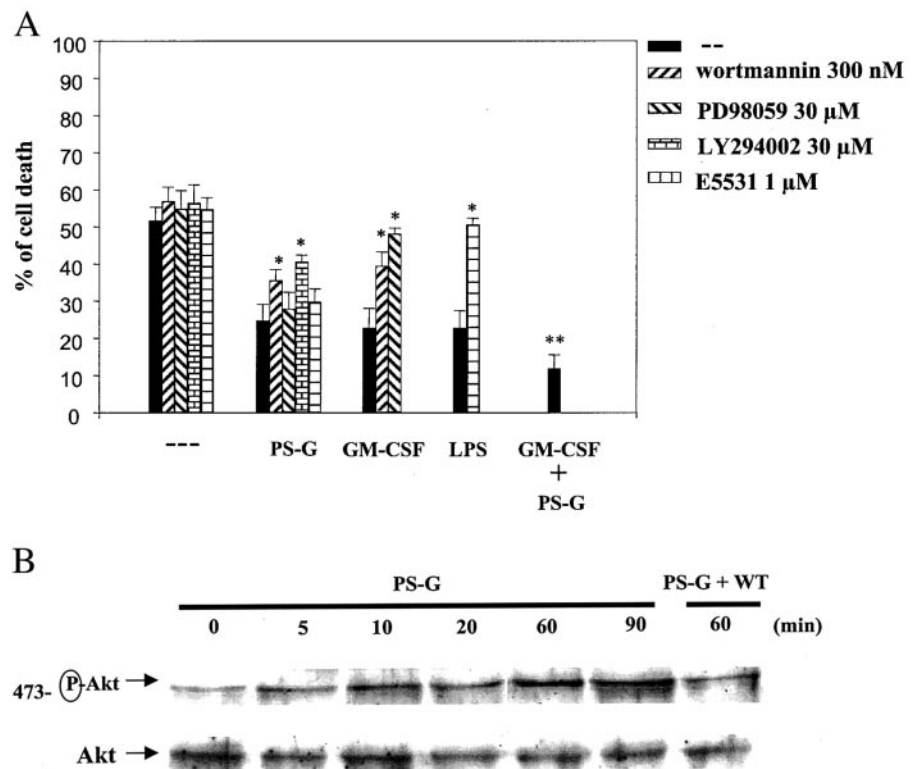
## DISCUSSION

Neutrophil apoptosis is a program for resolution of neutrophil-mediated inflammation, which may be delayed by inflammatory

mediators, while being accelerated by ligation of the Fas receptor [15]. In the present study, for the first time, we show that PS-G can significantly delay the neutrophil death program that is constitutively occurring or is induced by Fas engagement. This effect of PS-G displays a concentration-dependency within 10–300 μg/ml. Annexin V and PI staining, nuclear morphology, and DNA fragmentation all confirmed that neutrophil death, which is spontaneous or stimulated by Fas, displays apoptotic processes, with initial loss of membrane phospholipid asymmetry and externalization of PS followed by advanced apoptosis or necrosis with chromatin condensation, DNA cleavage, and membrane rupture.

Although the signaling pathway involved in spontaneous or stimuli-induced apoptosis in neutrophils has been studied by many investigators, the precise mechanism inducing neutrophil apoptosis is still not completely clear and might be distinguished depending on stimuli. Caspases are thought to play an important part in the execution phase of the death program, and the protease superfamily has been shown to be required for multiple pathways leading to apoptosis. Using a broad-spectrum caspase inhibitor, zVAD-fmk, we found that zVAD-fmk significantly protects neutrophils only from Fas-stimulated death. In contrast to Fas-stimulated apoptosis, caspases might not play a major role in spontaneous apoptosis as deduced from the noneffectiveness of the broad-spectrum caspase inhibitor zVAD-fmk on spontaneous apoptosis. In this respect, we found that PS-G not only protected cells from spontaneous apoptosis, but also delayed Fas apoptosis, which was further enhanced in the presence of zVAD. These results suggest that PS-G appears

**Fig. 6.** Effects of inhibitors on antiapoptotic function of GM-CSF and PS-G in neutrophils. (A) Cells were preincubated with vehicle, 300 nM wortmannin, 30  $\mu$ M LY294002, 30  $\mu$ M PD98059, or 1  $\mu$ M E5531 for 30 min and then incubated with LPS (1  $\mu$ g/ml), GM-CSF (10 ng/ml), PS-G (100  $\mu$ g/ml), or in combination for an additional 24 h. Following incubation, cells were harvested, and the percentage of dead cells was determined. \*,  $P < 0.05$  as compared with the antiapoptotic effect of PS-G, GM-CSF, and LPS without wortmannin, LY294002, PD98059, or E5531 pretreatment; \*\*,  $P < 0.05$  as compared with the control group with PS-G or GM-CSF treatment alone. (B) Cells pretreated or not with 100 nM wortmannin for 30 min were incubated with PS-G for the indicated time periods. After incubation, cells were lysed, and an equal amount of cell homogenates (100  $\mu$ g protein) was resolved on SDS-PAGE, followed by immunoblotting using antibodies specific to Akt or the Ser 473-phosphorylated form of Akt. Typical traces are representative of three experiments with similar results.



to possess a caspase-independent mechanism, which displays a major contribution to its antiapoptosis.

Although increased ROS in Fas-induced apoptosis was shown previously [14, 24], our present study indicates that neutrophil apoptosis, spontaneous or Fas-induced, is unaffected by GSH, NAC, or carboxyfullerene. Furthermore, we detect no significant changes in ROS production in the death process even in the presence of PS-G (data not shown). In contrast to PS-G, GLP-AS seems to have antioxidant ability as determined by the DPPH assay. Thus, it appears that ROS changes cannot be attributed to the antiapoptotic action of PS-G nor to the spontaneous apoptosis.

In addition to caspase 3, we next investigated whether PS-G uses the same pathway as GM-CSF, which delays neutrophil apoptosis by activating the PI-3K and ERK pathways [16]. We used pharmacological inhibitors of signal transduction to examine the possible mechanism used by PS-G to delay neutrophil apoptosis. Our data rule out the involvement of an ERK signal pathway in the antiapoptotic effect of PS-G, as mitogen-activated protein kinase kinase (MEK) inhibitor PD98059 failed to alter the response of PS-G. Conversely, present results further indicate that inhibitors of the PI-3K pathway, wortmannin and LY294002, attenuated the antiapoptotic effect of PS-G. Moreover, PS-G caused phosphorylation of Akt, a downstream target of PI-3K, as determined by immunoblotting. As previously reported, phosphorylated Akt as a prerequisite step for enzymatic activity may inhibit apoptosis by at least two mechanisms. First, Akt activation may phosphorylate Bad, a member of the Bcl-2 family. Phosphorylated Bad dissociates from Bcl-2, thereby increasing the antiapoptotic effects of Bcl-2 [37]. Second, Akt has been shown to phosphorylate and inactivate procaspase 9, an important member of the cell death

program [38]. Therefore, PS-G activation of Akt in neutrophils similar to that of GM-CSF may be attributed to its antiapoptotic function in neutrophils. Nevertheless, PS-G in combination with GM-CSF further delayed neutrophil apoptosis as compared with PS-G or GM-CSF treatment alone. Thus, in addition to activation of the PI-3K pathway similar to that of GM-CSF, it appears that PS-G may also promote neutrophil survival through an as-yet unknown mechanism.

Recently, the physiological and pharmacological roles of  $\beta$ -glucans have been studied intensively.  $\beta$ -Glucans derived from a variety of plant, fungal, and bacterial sources have been shown to produce a state of activation in leukocytes and thus activate the innate immune system [39–41]. In neutrophils, enhancement of oxidative burst response, phagocytosis, and microbicidal activity by  $\beta$ -glucans has been demonstrated [41, 42]. Furthermore, different  $\beta$ -glucan receptors located in leukocytes have been partially characterized, and delineating their ligand specificities as well as signal transduction is progressing [43–45]. In this study, we demonstrate for the first time the ability of  $\beta$ -glucan from *G. lucidum* to promote a neutrophil lifespan and provide another host defense mechanism for  $\beta$ -glucans. As signals regulating neutrophil apoptosis might be able to influence the inflammation state or host defenses, further clarifying PS-G actions in neutrophils will be helpful for the clinical application of PS-G in modulating immune responses. In this aspect, our recent work also indicates the ability of PS-G to accelerate neutrophil phagocytosis through a PI-3K-dependent signal pathway (our unpublished data).

To understand whether higher sensitivity for PS-G in Fas-induced apoptosis results from the alteration of Fas expression in neutrophils, we analyzed expressed Fas level by flow cytometry.

etry. The results indicate that Fas levels in plasma membranes of neutrophils are unchanged during 24 h of culture and that PS-G treatment fails to change them (data not shown).

In conclusion, we demonstrate that the polysaccharide component of *G. lucidum* elicits antiapoptotic effects on neutrophils, and this action primarily depends on the activation of Akt-regulated signaling pathways.

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