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Signaling mechanisms of enhanced neutrophil phagocytosis and chemotaxis by the polysaccharide purified from *Ganoderma lucidum*

[1]Ming-Jen Hsu, [2]Shiuh-Sheng Lee, [3]Sho Tone Lee & *,[1]Wan-Wan Lin

[1]Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan; [2]Department of Biochemistry, National Yang-Ming University, Taipei, Taiwan and [3]Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan

1 The polysaccharide from *Ganoderma lucidum* (PS-G) has been reported to enhance immune responses and to elicit antitumor effects. In our previous study, we found that PS-G efficiently inhibited spontaneously and Fas-enhanced neutrophil apoptosis when cultured *in vitro*. Since phagocytosis and chemotaxis play essential roles in host defense mediated by neutrophils, it is of great interest to know the effect of PS-G on these two cell functions, and the molecular events leading to these actions.

2 Using latex beads and heat-inactive *Escherichia coli* serving as particles for neutrophil engulfment, we found that PS-G is able to enhance phagocytic activity of human primary neutrophils and neutrophilic-phenotype cells differentiated from all *trans* retinoic acid-treated HL-60 cells.

3 Chemotactic assay using Boyden chamber also revealed the ability of PS-G to increase neutrophil migration.

4 Exposure of neutrophils to PS-G time dependently caused increases in protein kinase C (PKC), p38 mitogen-activated protein kinase (MAPK), Hck, and Lyn activities.

5 Results with specific kinase inhibitors indicate that phagocytic action of PS-G was reduced by the presence of wortmannin (Phosphatidylinositol 3-kinase, PI3K inhibitor), pyrazolpyrimidine 2 (Src-family tyrosine kinase inhibitor), Ro318220 (PKC inhibitor), and SB203580 (p38 MAPK inhibitor), but not by PD98059 (mitogen-activated protein/ERK kinase inhibitor). Moreover, chemotactic action of PS-G requires the activities of PI3K, p38 MAPK, Src tyrosine kinases and PKC.

6 All these results demonstrate the abilities of PS-G to enhance neutrophil function in phagocytosis and chemotaxis, and further provide evidence to strengthen the beneficial remedy of *G. lucidum* in human to enhance defense system.
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Abbreviations: ATRA, all *trans* retinoic acid; Bis X, bisindolylmaleimide X; dBcAMP, dibutyryl cAMP; ERK, extracellular signal-regulated kinase; fMLP, *N*-formyl-Met-Leu-Phe; GM – CSF, granulocyte – macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/ERK kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PP2, pyrazolpyrimidine 2; PS-G, polysaccharide from *Ganoderma lucidum*

Introduction

Chinese medical fungus, *Ganoderma* (*G.*) *lucidum*, has been a favorite remedy in oriental medicine for centuries. It was considered to maintain the vitality of human beings and to promote longevity (Shiao *et al*., 1994). This fungus has been reported to be effective in the treatment of hypertension, hyperglycemia, chronic hepatopathy, and neoplasia. The ethanol-precipitable fraction of hot-water extracts of *G. lucidum* was shown to possess antitumor activity *in vivo* (Miyazaki & Nishijima, 1981; Sone *et al*., 1985; Wang *et al*., 1993; 1997; Lee *et al*., 1995) and to reduce tumor metastasis (Hwang *et al*., 1989; Lee *et al*., 1995). The major active component was isolated, purified, and named as PS-G (polysaccharide from *G. lucidum*) (Miyazaki & Nishijima, 1981).

Structurally, PS-G is a branched (1→6)-β-D-glucan, which contains a backbone chain of (1→3)-linked D-glucose residues, five out of 16 D-glucose residues being substituted at O-6 positions with single D-glucosyl units (Sone *et al*., 1985). Studies have indicated the antineoplastic action of PS-G and attributed it to the activated host immune response (Won *et al*., 1989; Furusawa *et al*., 1992). PS-G was reported to enhance cytotoxic activity of natural killer cells, and to increase tumor necrosis factor-α and interferon-γ release, respectively, from macrophages and lymphocytes (Lee *et al*., 1995; Wang *et al*., 1997).

Mature human polymorphonuclear neutrophils are the most abundant leukocytes of the blood and are committed to death via apoptosis *in vivo* and *in vitro* within 72 h. Although these cells appear to have a very short life span (half-life 12 – 18 h), their survival can be extended significantly by certain growth factors, and proinflammatory cytokines such as granulocyte

*Author for correspondence; E-mail: wwl@ha.mc.ntu.edu.tw

colony-stimulating factor, granulocyte – macrophage colony-stimulating factor (GM-CSF), interleukin-2, and interleukin-8 (Leuenroth *et al*., 1998; Klein *et al*., 2000). In our previous study, we found that PS-G efficiently inhibited spontaneously and Fas-enhanced neutrophil apoptosis, and this effect is independent of the action through GM-CSF, TLR4, and *N*-formyl-Met-Leu-Phe (fMLP) receptors (Hsu *et al*., 2002). Neutrophils have a broad array of physical and biochemical attributes that distinguish them from other cells. Most of these pathways are adapted for one major purpose – to ingest and destroy invading pathogens. To achieve this purpose, chemotaxis of neutrophils to inflammatory site following concentration gradient of chemokine is the first step essential for host defense. Phagocytosis is then a process that involves binding and internalization of pathogens by neutrophils. Since neutrophils are greatly involved in inflammation and are the first type of cells to arrive at an inflammatory site, we are interested to elucidate whether PS-G affects the defense functions of neutrophils in terms of phagocytosis and chemotaxis.

Methods

Materials

RPMI 1640, fetal bovine serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, U.S.A.). Ficoll – Hypaque was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). PD98059, pyrazolpyrimidine 2 (PP2), SB203580, Ro318220, and bisindolylmaleimide X (Bis X) were purchased from Calbiochem (San Diego, CA, U.S.A.). Protein kinase C (PKC) activity assay kit was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). [γ-^{32}P]ATP (6000 Ci mmol^{-1}) and the enhanced chemiluminescence detection agent were purchased from NEN (Boston, MA, U.S.A.). Rabbit polyclonal antibody specific for p38 mitogen-activated protein kinase (MAPK), Lyn, protein A/G beads, and horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Mouse monoclonal antibody specific for Hck was purchased from Cell Signaling & Neuroscience (St Louis, MO, U.S.A.). All materials for SDS – PAGE were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Heat-inactivated fluorescein-labeled *Escherichia coli* particles and phalloidin-FITC were purchased from Molecular Probes (Eugene, OR, U.S.A.). Latex beads, wortmannin, fMLP, zymosan, GM-CSF, and all other chemicals were obtained from Sigma (St Louis, MO, U.S.A.).

PS-G purification from cultured G. lucidum

As our previous report (Wang *et al*., 1997), 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). 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 DEAE-cellulose (Miyazaki & Nishijima, 1981). PS-G we isolated was a protein-bound polysaccharide consisting of about 95% polysaccharide and 5% peptides. To rule out possible endotoxin lipopolysaccharide contamination of PS-G samples, we determined lipopolysaccharide content by the chromogenic limulus ameboctye lysate assay. We found that there was no detectable level of endotoxin (<0.10 EU ml^{-1}) in PS-G samples.

Neutrophil preparation

Neutrophils were isolated from citrate anticoagulated venous blood (20 – 60 ml) obtained from healthy volunteers. 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% viable as determined by trypan blue dye exclusion. Freshly isolated neutrophils were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin). Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

HL-60 cell culture and induction of differentiation

HL-60 cells were cultured at 37°C with 5% CO_2 in the following complete medium: 10% FBS, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin in RPMI 1640 medium. The cell density was maintained about 1.0×10^6 cells ml^{-1}. HL-60 cells were induced to differentiate by the addition of following agent: 1 μM all *trans* retinoic acid (ATRA), 200 μM dibutyryl cAMP (dBcAMP), or 1.25% DMSO, for a maximum 6 days, but normally 3 – 4 days sufficed to achieve the neutrophilic phenotype. To ascertain that ATRA produced the desired neutrophilic phenotype in HL-60 cells, cells were assayed for flow cytometric analysis of surface expression of differentiation-related antigens. Briefly, HL-60 cells were incubated with or without different inducers of differentiation for 96 h. Cells were then washed, centrifuged and resuspended in staining buffer (50 mM sodium phosphate pH 7.5, 100 mM KCl, 150 mM NaCl, 5% glycerol, 0.2% BSA) containing FITC-labeled anti-human CD11b antibody (Ancell Corporation, MN, U.S.A.) for 45 min on ice. After staining, cells were pelleted ($400 \times g$, 5 min), washed twice with PBS (pH 7.4), and immediately analyzed using FACScan and the Cellquest program (Becton Dickinson) with appropriate gating parameters.

Phagocytosis assay

Phagocytosis assay was performed using latex beads or heat-inactivated fluorescein-labeled *E. coli* particles for engulfment. Briefly, 200 μl aliquots of neutrophils suspended in RPMI medium (10^6 cells ml^{-1}) were added into the wells of a 96-well plate. Following treatment with the test compound as indicated for 30 min, cells were challenged with FITC – *E. coli* particles (10 μg ml^{-1}) or PE-latex beads (0.025% solids, 2 μm diameter) and stood at 37°C for another 15 min. Then the cells

were washed three times with PBS (pH 7.4), and suspended in PBS (pH 7.4). Fluorescence derived from FITC – *E. coli* or PE-latex beads ingested by neutrophils was immediately measured using FACScan and the Cellquest program (Becton Dickinson). The relative amount of ingested FITC – *E. coli* or PE-latex beads was calculated by subtraction of the mean fluorescence intensity of neutrophils alone from that of each test samples.

PKC activity assay

PKC activity was assayed by quantification of ^{32}P incorporation from [γ-^{32}P]ATP into the substrate, QKRPSQRSKYL peptide. Briefly, cells following incubation in the presence of PS-G for different time periods were washed twice in ice-cold PBS and then lysed in buffer containing 20 mM Tris-HCl, 0.5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg ml^{-1} leupeptin (pH 7.5). Assays were then performed at 30°C in a total volume of 60 μl containing 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM $CaCl_2$, 500 μM QKRPSQRSKYL peptide, 2 μM PKA inhibitor (PKI) peptide, 20 μM compound R24571 (calmodulin inhibitor), 0.5 mg ml^{-1} phosphatidylserine, 0.05 mg ml^{-1} diacylglycerol and 10 μCi [γ-^{32}P]ATP. Reactions were initiated by the addition of [γ-^{32}P]ATP and terminated after 10 min by transfer of the reaction mixture onto a P81 phosphocellulose paper. The papers were then washed with 0.75% phosphoric acid for three times. After washing, acetone was added and washed for two more minutes, removed and the paper stuffed into the scintillation vial containing 5 ml scintillation cocktail. Incorporation of ^{32}P into the substrate was then quantified by scintillation counter (Beckman).

Immunoblotting

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 (pH 7.5), 0.5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin. Samples of equal amounts of protein were subjected to SDS – PAGE on 10% 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 membranes were subsequently probed with primary antibody. The antibody – antigen complexes were detected by IgG peroxidase conjugates, followed by use of an enhanced chemiluminescence kit according to the manufacturer's instruction and exposure to photographic film.

Immunoprecipitation and protein kinase assay

Cells were washed twice with ice-cold PBS, lysed in 1 ml of lysis buffer containing 20 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol, 1% NP40, 1 mM sodium orthovanadate, 1 mM *p*-methylsulfonylfluoride, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, and centrifuged. The supernatant was then immunoprecipitated with respective specific antibodies against p38 MAPK, Lyn or Hck in the presence of A/G-agarose beads overnight. The beads were washed three times with lysis buffer and two times with kinase buffer (40 mM HEPES, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 200 μM sodium orthovanadate, 2 mM dithiothreitol). Then the beads were equally divided into two parts, respectively, for kinase assay and immunoblotting. The kinase reactions were performed by incubating immunoprecipitated beads with 20 μl of kinase buffer supplemented with 20 μM ATP and 3 μCi of [γ-^{32}P]ATP at 30°C for 30 min. For p38 MAPK assay, myelin basic protein (MBP, 1 μg per reaction) was added as a substrate, and for tyrosine kinase assay, acid-denatured enolase (12.5 μg per reaction) was used. The reaction mixtures were analyzed by SDS – PAGE followed by autoradiography. The precipitated protein levels of p38 MAPK, Lyn and Hck were assayed by immunoblotting as an internal control.

Neutrophil transmigration assay

The neutrophil transmigration assays were performed in a Boyden chamber using a 3 μm polyvinylpyrrolidone-free polycarbonate membrane. The chemotactic stimuli, fMLP (1 μg ml^{-1}) or PS-G (100 μg ml^{-1}) diluted in RPMI medium at different concentrations were added to the bottom wells of the chamber. Cells suspended in RPMI medium (5×10^5 cells ml^{-1}) were added to the top wells of the Boyden chamber and allowed to migrate for 30 min at 37°C in a 5% CO_2 atmosphere. In some experiments, neutrophils were pretreated with kinase inhibitors or vehicle for 30 min at 37°C and allowed to migrate in the Boyden chamber. Neutrophils that had migrated to the lower surface of the membrane were quantified by crystal violet staining and measuring the absorbance at 550 nm. In some experiments, the membranes were removed from the chambers, washed twice with PBS, and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were then permeabilized for 5 min in 0.1% Triton X-100. After blocking with 5% nonfat milk for 30 min, cells were labeled with phalloidin – FITC (200 ng ml^{-1}) for 1 h at room temperature. To observe stained cells, membranes were washed, mounted and examined under fluorescence microscope ($\times$400 amplification).

Statistical evaluation

Values were expressed as the mean $\pm$ standard error of the means (s.e.m.) of at least three experiments. ANOVA and Dunnetts tests were used to assess the statistical significance of the differences, with '*P*' values of less than 0.05 being considered statistically significant.

Results

PS-G dose dependently enhanced neutrophil phagocytic activity

Freshly isolated neutrophils were measured for their ability to phagocytose latex beads. Phagocytosis of latex particles was demonstrated by a progressive fluorescence shift on a logarithmic scale comparing FL2 of latex beads with control group (Figure 1a). When using mean fluorescence intensity as an index of neutrophil phagocytic activity, latex particles phagocytosed by neutrophils treated with vehicle alone increased time dependently (Figure 1b). This basal phagocytic

activity reached a steady-state level of two-fold increase within 60 – 90 min. When neutrophils were 30 min pretreated with PS-G (100 or 300 $\mu g\,ml^{-1}$) before latex beads exposure, their phagocytic activity was significantly enhanced at 15 and 30 min (Figure 1b). However, with longer challenge with latex particles up to 60 min, the phagocytic index of PS-G-treated neutrophils was equivalent to that of the cells without priming. When examining the concentration-dependent effect of PS-G on phagocytic activity of neutrophils, the results indicated that concentrations higher than 100 $\mu g\,ml^{-1}$ of PS-G were required to increase the rates of phagocytosis.

In addition, we also compared the response of PS-G with GM-CSF and zymosan, which are known to effectively enhance phagocytic activity of neutrophils. Cells with GM-CSF (10 $ng\,ml^{-1}$) or zymosan (100 $\mu g\,ml^{-1}$) pretreatment resulted in increases of phagocytic rate and maximal level.

In addition to latex beads, we also investigated if PS-G affects the engulfment of *E. coli* particle by neutrophils. Flow cytometric analysis indicated that within 15 min incubation, fluorescein-labeled *E. coli* particles were phagocytosed in the PS-G primed neutrophils with higher levels than in cells without priming. This effect of PS-G to enhance bacteria uptake by neutrophils displayed the concentration dependency, and an increase of about 45% was observed at 100 $\mu g\,ml^{-1}$ PS-G treatment (Figure 2a). In contrast to latex particles whose uptake by neutrophils showed the time dependency and reached maximum at 60 – 90 min, when cells challenged with *E. coli* particles for different time periods, a constant uptake amount was kept within 15 – 60 min incubation (Figure 2b). After that, the amount of *E. coli* particle uptake by neutrophils was declined. Interestingly, we found that the enhanced phagocytic ability of PS-G-treated neutrophils only occurred transiently at 15 min, and then returned to control level. Zymosan at 100 $\mu g\,ml^{-1}$ possessed similar effect as PS-G in bacteria phagocytosis, at least both in the uptake amount and kinetic pattern. In contrast, the ability of GM-CSF (10 $ng\,ml^{-1}$) to increase bacteria phagocytosis was greater and more sustained than PS-G and zymosan.

PS-G-enhanced phagocytic activity of differentiated HL-60 cells

In addition to primary neutrophils, we also examined the effect of PS-G on another granulocyte system, the differentiating HL-60 promyeloid cells. First, we verified this granulocyte differentiation process by adding the well-known differentiation agent and detecting the expression of CD11b (or Mac -1), the α subunit of $\beta 2$ integrin, CR3. CD11b is absent on immature promyeloid cells and has been widely used to reflex the initiation of myeloid differentiation into a more mature phenotype (Collins *et al*., 1978; Testa *et al*., 1993). In these experiments, cells were incubated for 96 h in culture medium plus 1 μM ATRA, 200 μM dBcAMP, or 1.25% DMSO. They were then stained with anti-CD11b antibody, and flow

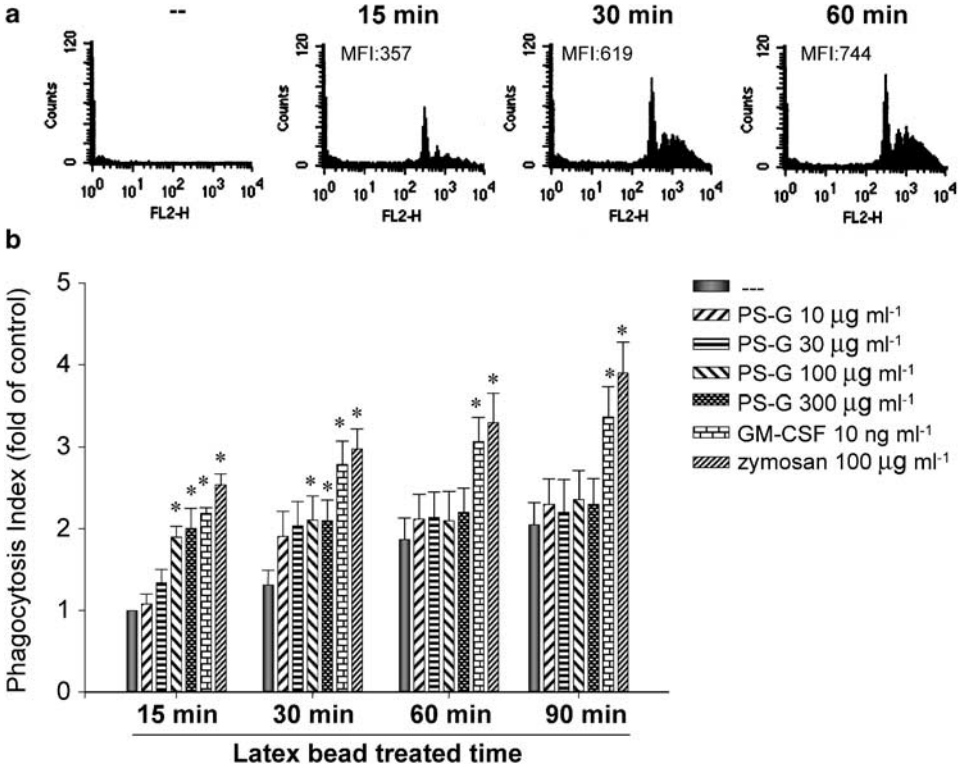

Figure 1 Effects of PS-G on phagocytosis of latex beads in neutrophils. Isolated neutrophils were preincubated with vehicle, PS-G (at indicated concentrations), GM-CSF (10 $ng\,ml^{-1}$), or zymosan (100 $\mu g\,ml^{-1}$) for 30 min. Following incubation, cells were challenged with latex particles and stood at 37°C for different time periods. Phagocytosis of latex particles by neutrophils was then analyzed using flow cytometry. The data in (a) indicate the time-dependent phagocytotic ability of neutrophils. The mean fluorescent intensity after drug treatment was compared with control and represents the phagocytosis index (b). Each column represents the mean ± s.e.m. of at least three independent experiments. *$P<0.05$ as compared to the control group without PS-G, GM-CSF, or zymosan treatment.

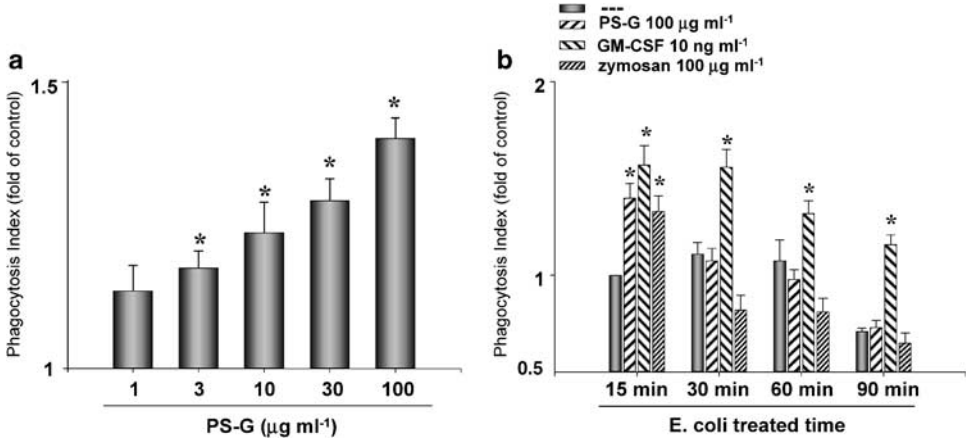

Figure 2 Effects of PS-G on phagocytosis of heat-inactivated *E. coli* particle in neutrophils. Isolated neutrophils were preincubated with vehicle, PS-G (at indicated concentrations), GM-CSF (10 ng ml^{-1}), or zymosan (100 μg ml^{-1}) for 30 min. Following incubation, cells were challenged with *E. coli* particles and stood at 37°C for another 15 min (a) or different time periods (b). Phagocytosis of *E. coli* particles by neutrophils was then examined using flow cytometry. Each column represents the mean ± s.e.m. of at least three independent experiments. *$P<0.05$ as compared to the control group without PS-G, GM-CSF, or zymosan treatment.

cytometric analysis was performed. A representative result of flow cytometric analysis of CD11b expression was shown (Figure 3a, upper panel). Since different stimuli may obtain various degrees of differentiation in HL-60 cells, and the highest percentage of CD11b positive cells was seen in ATRA-treated group, we choose ATRA-treated cells for the following experiments. The kinetic of expressed level of CD11b in the ATRA-treated cells was shown in Figure 3a (bottom panel). Despite the expression of surface marker like CD11b, we further determined whether the differentiated cells were able to engulf latex particles, which is a characterized feature of mature neutrophils. Results from Figure 3b demonstrated that HL-60 cells after 4-day differentiation into granulocytes obtained the ability to phagocytose latex beads.

We next examined the effects of PS-G, GM-CSF, or zymosan on the ability of these ATRA-treated cells to engulf latex particles. Results shown in Figure 3c revealed that all these three agents time dependently increased the engulfment of latex particles. However, compared to the more sustained stimulation by GM-CSF and zymosan for 60 – 120 min, the response of PS-G was weak and active for short-period. These results again confirm the stimulating pattern of PS-G, GM-CSF, and zymosan in latex bead phagocytosis by human neutrophils.

Effects of kinase inhibitors on phagocytic stimulation

To dissect the molecular mechanism involved in PS-G-induced phagocytic stimulation, we used several selective inhibitors. The concentrations of these inhibitors used are specific for the kinases as previously reported in neutrophils (Kodama *et al*., 1999; Klein *et al*., 2000; Tohru *et al*., 2000; Nijhuis *et al*., 2002) and we reported in other cell systems (Chen & Lin, 2001; Huang *et al*., 2002). As shown in Figure 4a, when latex beads were used, the stimulated effects of PS-G (100 μg ml^{-1}) and zymosan (100 μg ml^{-1}) were unaffected by mitogen-activated protein/ERK kinase (MEK) inhibitor (PD98059, 30 μM), while the presence of phosphatidylinositol 3-kinase (PI3 K) inhibitor (wortmannin, 300 nM) and PKC inhibitor (Ro318220, 10 μM) significantly diminished their effects. In contrast, only wortmannin reversed the enhanced effect of GM-CSF. When PS-G was cotreated with GM-CSF or zymosan, no further enhancement with statistical significance in phagocytosis was seen. These results suggest that PS-G might partially mimic GM-CSF and zymosan action in enhancing phagocytic activity of neutrophils, through activating the PI3K-dependent signaling pathway. Since the concentration of wortmannin employed might be high and probably nonselective, lower concentration of wortmannin was used to exclude this possibility. In addition, we also examined p38 MAPK inhibitor SB203580 within different concentrations between 1 and 10 μM. As shown in Figure 4b, lower concentrations of wortmannin (30 – 300 nM) still retained the inhibitory effect on PS-G action. Similarly inhibition of the p38 MAPK pathway using SB203580 (3 or 10 μM) abolished the potentiating effect of PS-G on neutrophil phagocytosis.

On the other hand, we further used *E. coli* particle for engulfment to assess and compare the mechanism of PS-G with zymosan and GM-CSF. Similar to the response using latex beads (Figure 4a and b), Ro318220, wortmannin, and SB203580 effectively inhibited the responses of PS-G and zymosan. In addition, we also tested PKC inhibitor Bis X and Src inhibitor PP2. As shown in Figure 4c, treatment with Bis X (10 μM) and PP2 (100 nM) significantly eliminated PS-G and zymosan actions on the engulfment of *E. coli* particle by neutrophils. Moreover, PS-G in combination with zymosan had no additive priming effect as compared to PS-G or zymosan individually (Figure 4c). This again implies that PS-G, GM-CSF, and zymosan may share similar downstream signaling pathways involved in the priming effect on neutrophil phagocytosis. In contrast, the effect of GM-CSF was inhibited by PP2, wortmannin and SB203580, but not sensitive to Ro318220.

PS-G-activated PKC, Src-family protein kinases and p38 MAPK

Since PKC inhibitors, such as Ro318220 and Bis X, Src family kinase inhibitor PP2 and p38 MAPK inhibitor

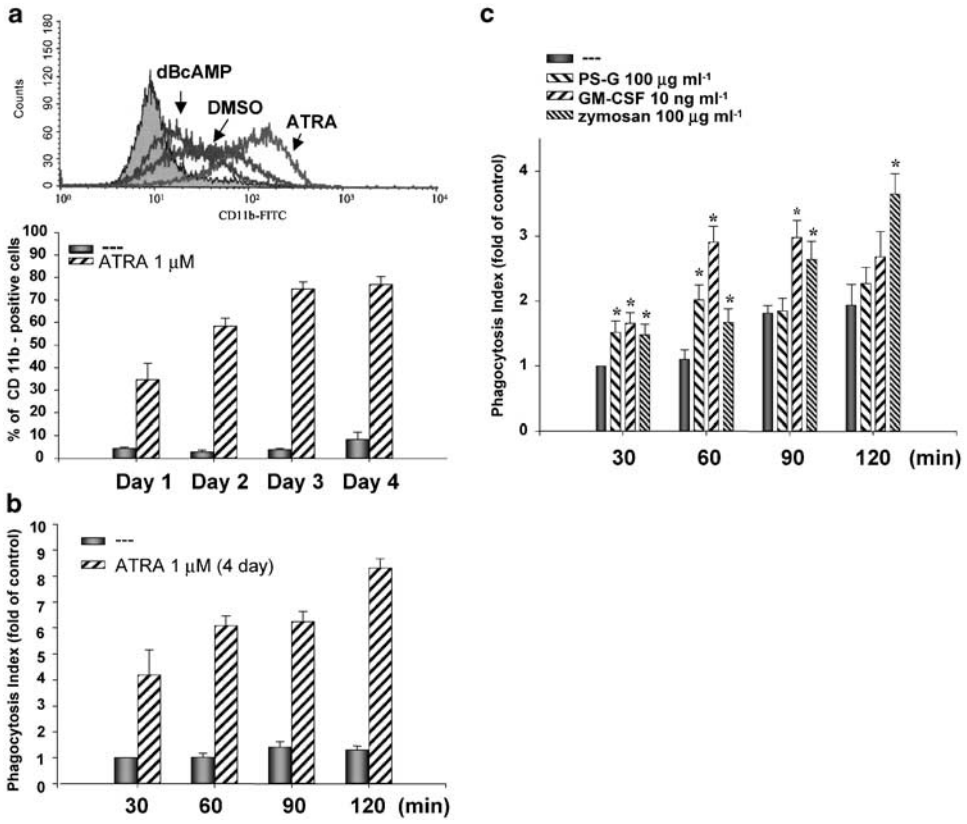

Figure 3 Assessment of HL-60 cells differentiation and phagocytosis. HL-60 cells were incubated with vehicle or different inducers of differentiation (1 μM ATRA, 200 μM dBcAMP, and 1.25% DMSO) for 96 h, except otherwise indicated. (a) Cells were then incubated with antibody against CD11b antigen and counted by flow cytometry. Shaded areas in top panel represented undifferentiated HL-60 cells. Bottom panel-showed the level of CD11b expression in HL-60 cells, incubated with or without ATRA for 1 – 4 days. (b) HL-60 cells by 4-day induction culture with ATRA or not was examined for phagocytic ability, using latex beads for engulfment. (c) ATRA-differentiated HL-60 cells were preincubated with vehicle, PS-G (100 μg ml^{-1}), GM-CSF (10 ng ml^{-1}), or zymosan (100 μg ml^{-1}) for 30 min. Following incubation, cells were treated with latex particles and stood at 37°C for different time periods. Phagocytosis of latex beads was then analyzed using flow cytometry. Each column represents the mean $\pm$ s.e.m. of at least three independent experiments. *$P<0.05$ as compared to the control group without stimulant treatment.

SB203580 could diminish the priming effect of PS-G on phagocytosis, PKC, Src family members and p38 MAPK involved in PS-G action were considered. Figure 5a demonstrated that PS-G (100 μg ml^{-1}) could time dependently increase PKC activity within 30 min incubation. In human neutrophils, we found that Hck and Lyn are two major tyrosine kinases of Src family and their activities, using *in vitro* enolase as a substrate, were rapidly and transiently stimulated by PS-G (Figure 5b). p38 MAPK activity was also assessed using *in vitro* phosphorylation of MBP as an index. As shown in Figure 5c, the activity of p38 MAPK was increased upon PS-G treatment.

PS-G enhanced neutrophil chemotaxis

Results from the above experiments suggest that PS-G could enhance neutrophil phagocytosis. To further ascertain the physiological implication in stimulating neutrophil function, we determined whether PS-G affects neutrophil chemotaxis. Figure 6a showed that PS-G at 100 μg ml^{-1} was active in eliciting chemotaxis as examined from fluorescence microscopy. Moreover, fMLP (100 ng ml^{-1}) has comparable efficacy in this effect (Figure 6a and b). These results suggest that PS-G might function as a chemoattractant to recruit neutrophils. In an attempt to elucidate signaling pathways involved in this response of PS-G, we tested several pharmacological inhibitors. As shown in Figure 6b, the presence of wortmannin (100 – 300 nM), Ro318220 (10 μM), PP2 (100 nM), and SB203580 (1 – 10 μM) significantly abolished the effect of PS-G effect. In contrast, the presence of PD98059 (30 μM) did not significantly alter the effect of PS-G.

Discussion

Neutrophils are the principal effectors of the initial host response to injury or infection and constitute a significant threat to invading bacterial pathogens (Bharadwaj *et al*., 2001; Power *et al*., 2001). Extravasation or the migration of neutrophils from the vascular system to sites of pathogenic exposure is a key event in immune defense (Tani *et al*., 2001; Ostermann *et al*., 2002). Neutrophils also carry potent destructive enzymes that can destroy invasive bacteria or damage normal tissues (Shi *et al*., 2001). Several reagents are known to alter the functions of neutrophils (Bharadwaj *et al*.,

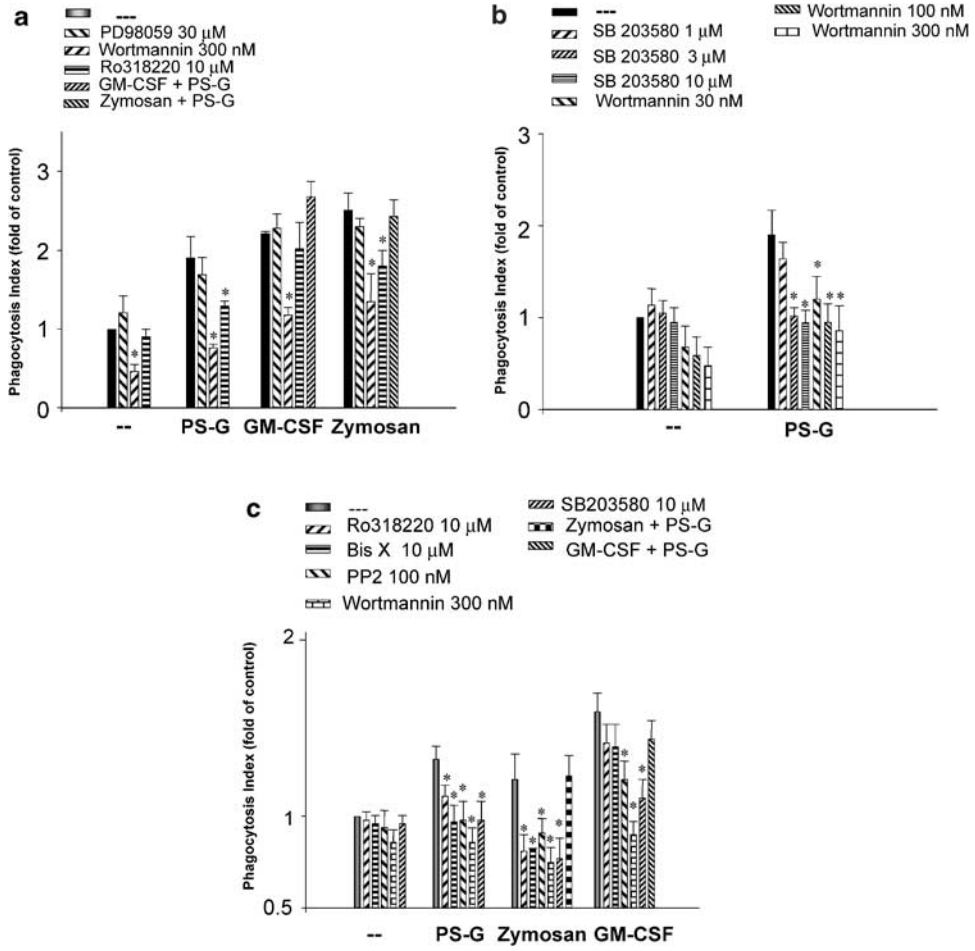

Figure 4 Effects of inhibitors on enhanced phagocytic functions of PS-G, GM-CSF, and zymosan in neutrophils. Neutrophils were preincubated with vehicle, 30 – 300 nM wortmannin, 1 – 10 μM SB203580, 30 μM PD98059, 10 μM Ro318220, 10 μM Bis X, or 100 nM PP2 for 30 min and then incubated with GM-CSF (10 ng ml^{-1}), PS-G (100 μg ml^{-1}), and/or zymosan (100 μg ml^{-1}) for an additional 30 min. Following incubation, cells were challenged with latex beads (a, b) or *E. coli* particles (c) and stood at 37°C for another 15 min. Phagocytosis was then determined using FACScan. $^*P<0.05$ as compared to the enhanced phagocytic effect of PS-G, GM-CSF, or zymosan alone.

2001; Marino *et al*., 2001; Nishimura *et al*., 2001). Cytokine like GM-CSF or microbial products such as yeast glucan and zymosan might modulate the function of neutrophils (Bober *et al*., 1995; Kumaratilake *et al*., 1996; Richardson & Chung, 1997; Bharadwaj *et al*., 2001; Wanten *et al*., 2001). In the present study, we demonstrated that the polysaccharide component of Chinese medicine *G. lucidum* possesses capability for enhancing neutrophil functions in phagocytosis and migration.

To dissect the signaling events involving in PS-G effects on neutrophil phagocytosis and migration, we used pharmacological inhibitors. Our data ruled out the involvement of extracellular signal-regulated kinase (ERK) signal pathway in phagocytic and chemotactic effects of PS-G, as inhibitor of the upstream activating molecule PD98059 failed to alter the response of PS-G. On the other hand, inhibitors of PKC (Ro318220, Bis X), PI3K (wortmannin), Src tyrosine kinase (PP2), and p38 MAPK (SB203580) attenuated both biological activities of PS-G. Accordingly, our previous study showing PI3K/PKB stimulation by PS-G (Hsu *et al*., 2002) as well as here for PKC, Src and p38 MAPK activation all together support the requirement of multiple signaling cascades for cell phagocytosis and migration. In this aspect, accumulated evidences have linked the crucial roles of these signaling cascades in granulocyte phagocytosis and migration. Defects in chemotaxis and loss of directionality of neutrophils isolated from PI3Kγ (−/−) mice have been demonstrated (Tohru *et al*., 2000; Hannigan *et al*., 2002). PKC inhibition has been reported to prevent phagocytosis in human monocytes and PMN (Fallman *et al*., 1992; Greenberg *et al*., 1993; Zhou & Brown, 1994). It appears that Src kinases can regulate PKB activation and thus modulate cytokine or chemoattractant-controlled neutrophil function (Korade-Mirnics & Corey, 2000; Nijhuis *et al*., 2002). Additionally, several lines of evidence suggested the involvement of p38 MAPK in phagocytosis and chemotaxis in neutrophils (Heuertz *et al*., 1999; Tohru *et al*., 2000).

β-Glucans have been recently studied for their ability to activate host defense mechanisms against tumors and microbial infections. Although heterogeneity of β-glucans derived from a variety of plant, fungal, and bacterial sources have been

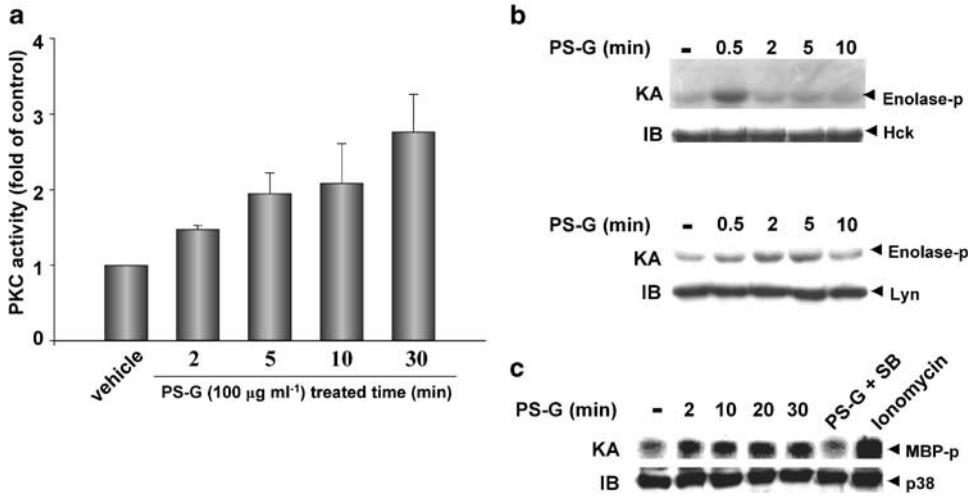

Figure 5 Effects of PS-G on PKC, Src-family tyrosine kinases and p38 MAPK activities in neutrophils. (a) Cells following incubation with PS-G (100 μg ml^{-1}) for different time periods as indicated were harvested. PKC activity was then determined using PKC activity assay kit, according to the manufacturer's instruction. Each column represents the mean $\pm$ s.e.m. of at least three independent experiments. Hck or Lyn tyrosine kinases activity (b) or p38 MAPK activity (c) of cells treated with PS-G for different time periods was assessed by immune complex kinase assays (KA) using enolase or MBP as substrates as described in 'Methods.' After SDS – PAGE, ^{32}P-labeled enolase or MBP was visualized by autoradiography and normalized for the amount of immunoprecipitated Hck, Lyn and p38 MAPK by immunoblotting (IB) with specific antibodies. Results are representative of three independent experiments.

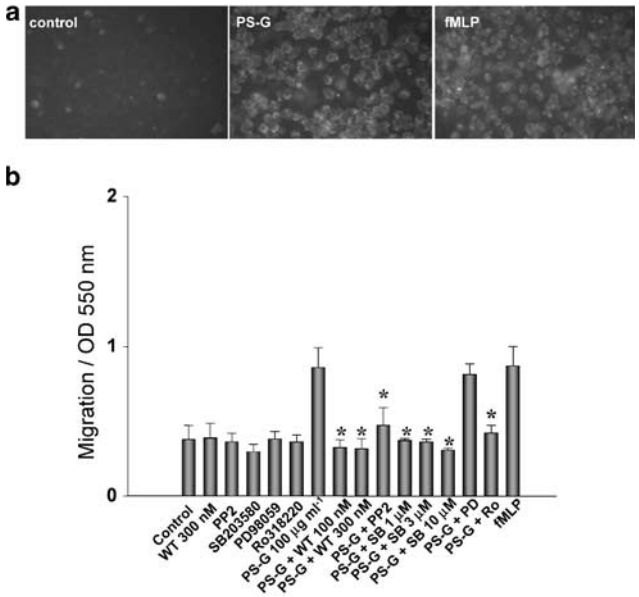

Figure 6 Effects of PS-G on neutrophil migration. Neutrophils were preincubated with vehicle or inhibitors (100 or 300 nM wortmannin, 100 nM PP2, 10 μM Ro318220, 30 μM PD98059, or 1 – 10 μM SB203580) for 30 min, and then allowed to migrate for 30 min toward fMLP (100 ng ml^{-1})- or PS-G (100 μg ml^{-1})-containing medium as indicated. Neutrophils that had migrated through the membrane were stained with phalloidin – FITC and shown under fluorescence microscopy (a). In (b), migrated neutrophils were stained with crystal violet and data were expressed as the absorbance at 550 nm. *$P<0.05$ as compared to the control group with PS-G treatment alone.

identified, they were shown to produce a state of activation in leukocytes and thus activate the innate immune system (Kokoshis *et al*., 1978; Bohn & Bemiller, 1995; Wakshull *et al*., 1999). Furthermore, different β-glucan receptors located in leukocytes have been partially characterized, and delineation for their ligand specificity as well as signal transduction is currently progressing (Ross *et al*., 1985; Thornton *et al*., 1996; Liang *et al*., 1998; Linehan *et al*., 2000). Analysis of the response of human leukocytes to β-glucans has shown that the integrin CR3 is primarily responsible for the phagocytic responses mediated by β-glucans (Vetvicka *et al*., 1996; 1997; Xia *et al*., 1999). In this study, we for the first time demonstrate the ability of β-glucan from *G. lucidum* to enhance phagocytosis and induce migration of neutrophils. Similar phagocytic results obtained from neutrophilic-phenotype cells differentiated from HL-60 as freshly isolated neutrophils may also suggest that this cell line provides an excellent tool for determining the function of neutrophils. Moreover, PS-G in combination with zymosan, a yeast β-glucan, which is commonly studied, had no additive priming effect as compared to PS-G or zymosan individually. Accordingly, zymosan has been reported to stimulate neutrophils by activating PKC and protein tyrosine kinase pathways (Sergeant & McPhail, 1997; Welch & Maridonneau-Parini, 1997; Wanten *et al*., 2001). This implies that PS-G appears to share common signaling pathways to accelerate neutrophil functions as zymosan does.

In conclusion, we demonstrated that the polysaccharide from *G. lucidum* might stimulate the functional activity of mature neutrophils. Together with ability to prolong life span of neutrophils by PS-G, it can thus exert a variety of important regulatory controls of neutrophil function during bacterial infections and is a good remedy to promote host immune response.

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