Extract of Sporoderm-Broken Germinating Spores of *Ganoderma lucidum* Activates Human Polymorphonuclear Neutrophils via the P38 Mitogen-activated Protein Kinase Pathway

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Background: *Ganoderma lucidum (G. lucidum)* has been used in traditional Chinese medicine for thousands of years because of its immunomodulatory properties. It is believed that *G. lucidum* enhances the human immune response by improving the function of human polymorphonuclear neutrophils (PMNs); nevertheless, the actual mechanism by which *G. lucidum* acts on human PMNs remains unknown. In this study, we investigated the molecular pathways through which *G. lucidum* activates human PMNs.

Methods: The phagocytic activity of PMNs was evaluated with and without treatment with the extract of sporoderm-broken germinating spores of *G. lucidum*. The same activity was measured after *G. lucidum* treatment with or without p38 mitogen-activated protein kinase (p38 MAPK) inhibitor. The activation of p38 MAPK was also evaluated with or without treatment with the extract of sporoderm-broken germinating spores of *G. lucidum*.

Results: In this study, we found that the extract of *G. lucidum* enhanced the phagocytic activity of PMNs in a dose-dependent manner, but this response was attenuated by treatment with SB203580, a p38 MAPK inhibitor. The extract of *G. lucidum* also enhanced activation of p38 MAPK in a dose-dependent manner.

Conclusion: These results clearly show that the extract of *G. lucidum* can modulate human immunity by activating human PMNs via the p38 MAPK pathway. These results may be of clinical importance to doctors of traditional Chinese medicine.

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Key words: traditional Chinese medicine, *Ganoderma lucidum* (*G. lucidum*), polymorphonuclear neutrophils (PMNs), p38 mitogen-activated protein kinase (p38 MAPK)

Ganoderma lucidum (G. lucidum), also called "Lingzhi", is an ancient Chinese herbal medicine that has been widely used in traditional Chinese medicine for thousands of years because of its

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immunomodulatory properties. The bioactivity of *G. lucidum* has been elucidated by many authors and includes such diverse properties as anti-HIV,[1] antiherpetic,[2] anticoagulant,[3] antiangiogenic,[4] antiandrogenic,[5] and antitumor activities.[6-9] The immunomodulatory function of *G. lucidum* is currently of great interest worldwide. Mechanistic studies have shown that the antitumor activity of *G. lucidum* is mediated through immune system effectors such as natural killer cells, macrophages, and T and B lymphocytes.[10-13]

Spores are the medically essential part of *G. lucidum* and are rich in polysaccharides, triterpenes and other bioactive substances. In our previous pilot study, we found that the sporoderm-broken germinating spores of *G. lucidum* modulated the phagocytic activity of polymorphonuclear neutrophils (PMNs). However, the mechanism underlying this activity remains unclear. Because human PMNs play an important role in inflammation and are the first line of defense against microbial invasion, we were interested in studying the effects of *G. lucidum* on human PMNs.

P38 mitogen-activated protein kinase (p38 MAPK) is an important mediator of PMN function in inflammation. It is the mammalian homologue of the yeast HOG kinase and participates in a cascade that controls cellular responses to cytokines and stress. Inflammatory stimuli such as lipopolysaccharide, tumor necrosis factor and interleukin-1 are the major inducers of p38 MAPK.[14]

In this study, we employed an in vitro system to evaluate the immunomodulatory effects of the extract of sporoderm-broken germinating spores of *G. lucidum* on human PMNs. We investigated the effects of *G. lucidum* and p38 MAPK inhibitors on the phagocytic activity of human PMNs and evaluated the influence of *G. lucidum* on the activation of p38 MAPK. These results will aid in elucidating the immunomodulatory mechanism of *G. lucidum* in human PMNs and in determining the clinical importance of *G. lucidum* in traditional Chinese medicine.

METHODS

Plants and spores

G. lucidum was donated by Chang Gung Biotechnology Co. Ltd. The sporoderm of the germinating spores was broken with an efficiency of up to 99.8% before the study. The spores of *G. lucidum* (15 g samples) were extracted with H_2O first at room temperature (RT) (400 ml x 3) and then at 70°C (400 ml x 3), separately. The filtrates were concentrated to yield brown syrups (2.31 g).

Isolation of PMNs from the peripheral blood of healthy individuals

Nine healthy volunteers, 6 women and 3 men, from 26 to 35 years old, without major systemic diseases, malignancy or hereditary diseases and without a history of drug or health food intake in the past 3 months, were enrolled in this study. Heparinized venous blood obtained from each individual was mixed with one-fourth volume of 4.5% dextran solution (molecular weight: 500,000) and incubated for 30 min at RT. The leukocyte-enriched supernatants were collected and diluted with equal volumes of Hanks' balanced salt solution. After Ficoll-Hypaque (specific gravity 1.077) density gradient centrifugation at 150 x g for 25 min, PMNs were obtained from the bottom. The PMNs were washed 3 times and suspended in 10% fetal bovine serum (FBS) in RPMI-1640 (10% FBS-RPMI). The cells were counted and the suspensions adjusted to 2×10^6 cells/ml. The viability of the cells was assessed by trypan blue dye exclusion and was > 95% for each experiment.

Measurement of phagocytic activity of PMNs with or without the extract of *G. lucidum*

The phagocytic activity of PMNs was measured as previously described.[15] Briefly, fluoresbrit carboxylate microspheres (0.75 μm, Polysciences Inc., Warington, PA, U.S.A.) were opsonized with fresh normal serum at 37°C for 45 min before use. One hundred microliters of freshly isolated PMN (2×10^6/ml) was incubated with 10 μl of opsonized beads (2×10^9/ml) at 37°C for 30 min in the presence of 40 μl of culture medium (as a negative control) or pretreated various concentrations of *G. lucidum* extract for 10 min.

The PMNs were collected by centrifugation at 300 x g for 10 min and washed 3 times to remove the non-phagocytosed beads. The percentage (%) and mean fluorescence intensity (MFI#) of bead-engulfing PMNs (phagocytosis-positive) were measured by flow cytometry on a FAXS 440 instrument (Becton-Dickinson, San Jose, CA, U.S.A.).

Measurement of phagocytic activity of PMNs treated with the extract of *G. lucidum* with or without p38 MAPK inhibitor

The same method was used to measure the phagocytic activity of PMNs treated with various concentration of *G. lucidum* extract with or without SB203580 as an inhibitor of p38 MAPK. The concentration of the inhibitor used was specific for the kinase as previously reported.[16]

Western blot analysis of p38 MAPK activation

The isolated PMNs were treated with various concentrations of *G. lucidum* extract for 7.5 min. A pretest study had been done with another 7 healthy volunteers who did not have systemic diseases, malignancy or hereditary diseases and no history of drug or health food intake in the past 3 months. This study revealed the best effect on p38 MAPK activation was at 7.5 min under the same concentration of *G. lucidum* treatment. Totally, 3 of the original 9 volunteers participated in the phagocytosis study (2 woman 28 and 33 years old and 1 man 29 years old) were excluded from this experiment because the amounts of isolated PMNs were not high enough in these 3 individuals. Total protein concentrations of the extracts were measured by BCA protein assay (Beyotime, Jiangsu, China), according to the manufacturer's instructions. Equal amounts of leukocyte extracts (50 μg protein) were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 10% gel. The separated proteins were blotted onto a polyvinylidene fluoride membrane. The membrane was blocked at RT for 1 h in 5% (w/v) dry skim milk and incubated at 4°C overnight with a 1:1000 dilution of the desired primary antibody. Primary antibodies used were rabbit antibodies specific for p38 MAPK, phospho-p38 MAPK, and β-actin (Cell Signaling, Beverly, MA, U.S.A.). The addition of the primary antibody was followed by incubation at RT for 1 h with a 1:1000 dilution of anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Beyotime). The peroxidase reaction was visualized by an enhanced chemiluminescence method with an EZ-ECL chemiluminescence detection kit (Biological Industries, Kibbutz Beit Haemek, Israel). The relative intensities of the protein bands were quantified by scanning densitometry in a Bio-Rad (Shanghai, China) Fluor-S multiimager using the Quantity One program. Relative expression levels of the target protein were expressed as target protein (p38 MAPK or phosphorylated p38 MAPK)/β-actin ratios.

Statistical analysis

Results were presented as median (inter-quartile range). The differences between groups were analyzed using the non-parametric Wilcoxon signed-rank test, and a "*p*" value of less than 0.05 was considered statistically significant.

RESULTS

Phagocytic activity of PMNs with or without the extract of *G. lucidum*

The PMNs were pretreated with various concentrations of *G. lucidum* extract for 10 min before incubation with the opsonized beads at 37°C for 30 min. The phagocytic activities of PMNs with or without *G. lucidum* treatment were determined by flow cytometry using MFI# as a measurement of PMN phagocytic activity. The PMN phagocytic activity was significantly enhanced after treatment with *G. lucidum* at concentrations of 40 mg/ml and 80 mg/ml (both $p < 0.05$) (Table 1). When the concentration of

Table 1. Effects of the Extract of *G. lucidum* with or without P38 MAPK Inhibitor on PMN Phagocytic Activity among 9 Healthy Volunteers (6 women and 3 men from 26 to 35 years old)

Concentration of *G. lucidum*	Phagocytic activity (MFI#)% N = 9, median (inter-quartile range)	Phagocytic activity (MFI#)% with p38 MAPK inhibitor treatment	*p value	†p value
0 mg/ml	42.92 (10.25)	42.88 (19.06)		
40 mg/ml	54.02 (16.875)	50.07 (6.705)	0.021	0.015
80 mg/ml	57.22 (12.27)	54.12 (11.79)	0.015	0.008
100 mg/ml	59.16 (8.9)	48.15 (9.67)	0.086	0.011

Abbreviations: *G. lucidum: Ganoderma lucidum;* PMN: polymorphonuclear neutrophil; MFI#: mean fluorescence intensity; *: by Wilcoxon signed-rank test, comparing PMN phagocytic activities using various concentrations of *G. lucidum* with 0 mg/ml of *G. lucidum* as the negative control. †: by Wilcoxon signed-rank test, comparing PMN phagocytic activities of *G. lucidum* using the same concentrations with or without p38 MAPK inhibitor.

G. lucidum was increased to 100 mg/ml, the PMN phagocytic activity was not altered relative to the negative control.

Phagocytic activity of PMNs treated with *G. lucidum* extract with or without p38 MAPK inhibitor

The phagocytic activity of PMNs treated with *G. lucidum* extract with or without an inhibitor of p38 MAPK was determined using MFI# as a measurement of PMN phagocytic activity. The PMN phagocytic activity was significantly attenuated by p38 MAPK inhibitor treatment at *G. lucidum* extract concentrations of 40 mg/ml and 80 mg/ml (both $p < 0.05$) (Table 1).

Activation of p38 MAPK as shown by western blot analysis

Western blot analysis was performed to measure p38 MAPK activation. The activation rate was determined by calculating the ratio of phosphorylated p38 MAPK to p38 MAPK for each sample. Under the same concentration of *G. lucidum* treatment, the best effect on p38 MAPK activation was at 7.5 min (Table 2). By using this assay, we found that treatment with *G. lucidum* extract for 7.5 min at concentrations of 40 mg/ml and 80 mg/ml significantly increased p38 MAPK activation ($p < 0.05$) (Table 3, Fig. 1).

Table 2. Time-course Effects of the Extract of *G. lucidum* on p38MAPK Activation among 7 Healthy Volunteers (4 women and 3 men from 24 to 41 years old)

Treatment time of *G. lucidum* (40 mg/ml)	Activation ratio (p-p38MAPK/p38MAPK) N = 7, median (inter-quartile range)	*p value
0 min	0.777 (1.034)	
1.0 min	0.813 (0.882)	0.611
2.5 min	0.811 (0.881)	0.865
5.0 min	0.853 (1.174)	0.235
7.5 min	0.879 (1.228)	0.018
10 min	0.925 (0.914)	0.396
15 min	0.863 (1.032)	0.175

Abbreviations: p-p38MAPK: phosphorylated- p38 mitogen-activated protein kinase; *: by Wilcoxon signed-rank test, using 0 min of *G. lucidum* treatment as the negative control.

DISCUSSION

G. lucidum, a traditional Chinese medicine, has long been used in Asian societies to promote health and treat various diseases. Many studies have been performed on the bioactivities of this herbal medicine, especially on its immunomodulatory activity. The bioactive substances from *G. lucidum* had previously been shown to modulate the immune response by promoting cytokine production by immune

Table 3. Effects of the Extract of *G. lucidum* on p38MAPK Activation among 6 of the 9 Original Healthy Volunteers

Concentration of *G. lucidum*	Activation ratio (p-p38MAPK/p38MAPK) N = 6, median (inter-quartile range)	*p value
0 mg/ml	0.468 (0.556)	
40 mg/ml	0.496 (0.687)	0.028
80 mg/ml	0.506 (0.746)	0.046
100 mg/ml	0.507 (0.708)	0.463
120 mg/ml	0.475 (0.878)	0.249
140 mg/ml	0.487 (0.730)	†0.249

Abbreviations: p-p38MAPK: phosphorylated- p38 mitogen-activated protein kinase; *: by Wilcoxon signed-rank test, using 0 mg/ml of *G. lucidum* as the negative control; †: negative value.

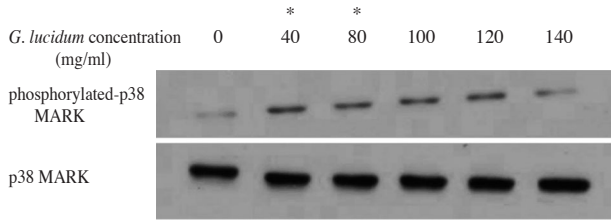

Fig. 1 Effects of the Extract of *G. lucidum* on P38 MAPK Activation. Western blot analysis performed to measure p38 MAPK activation by treatment with various concentrations of *G. lucidum*. p38 MAPK has significantly increased activation with *G. lucidum* treatment for 7.5 min at concentrations of 40 mg/ml ($p = 0.028$) and 80 mg/ml ($p = 0.046$). The activation rate was determined by calculating the ratio of phosphorylated p38 MAPK to p38 MAPK for each sample. Abbreviations used: *G. lucidum: Ganoderma lucidum*; p38 MAPK: p38 mitogen-activated protein kinase; *: $p < 0.05$

cells,[17,18] preventing oxidative injury,[19] enhancing the function of dendritic cells,[20] and activating lymphocytes.[10,13] Signaling pathways influenced by these bioactive substances have also been investigated. Polysaccharides from *G. lucidum* can induce the activation and maturation of human dendritic cells by activating the NF-κB and p38 MAPK pathways,[21] and can inhibit Fas-mediated apoptosis by activating Akt-regulated signaling pathways,[22] whereas triterpenes from *G. lucidum* exert anti-inflammatory and anti-proliferative effects by inhibiting NF-κB and AP-1 signaling in lipopolysaccharide-activated macrophages.[23]

In recent years, many chronic diseases such as allergic rhinitis,[15] arthritis,[14,24] diabetes mellitus,[25] heart diseases,[26] hypertension[27] and cancer,[28] have been reported to be related to inflammation. Human PMNs are well known to mediate inflammation and play an important role in the host defense against microbial infection. The functional activities of PMNs include chemotaxis, phagocytosis, secretion of anti-microbial agents, and generation of reactive oxygen intermediates.[29] Because the immunomodulatory function of *G. lucidum* is of great interest owing to its relevance to many diseases, we wished to study the effects of *G. lucidum* on human PMNs.

Previous reports had shown that polysaccharides from *G. lucidum* enhance the phagocytic activity of human PMNs and increase PMN migration by chemotactic assay.[30] p38 MAPK was found to be an important activator of PMN chemotaxis and phagocytosis.[16,31] In addition, exposure to *G. lucidum*-derived polysaccharide enhanced the activities of some immune mediators such as protein kinase C, p38 MAPK, Hck, and Lyn in a time-dependent manner.[30]

In this study we focused on the effects of *G. lucidum* on human PMNs via the p38 MAPK pathway. Our data indicated that the extract of *G. lucidum* at concentrations of 40 mg/ml and 80 mg/ml enhanced the phagocytic activity of PMNs in a dose-dependent manner. Furthermore, the increased phagocytic activity of PMNs treated with *G. lucidum* at 40 mg/ml and 80 mg/ml was significantly attenuated by treatment with p38 MAPK inhibitor (both $p < 0.05$).

For further confirmation of the effects of *G. lucidum* on the p38 MAPK pathway, we measured the *G. lucidum*-mediated activation of p38 MAPK by western blot analysis. The activation of p38 MAPK was also enhanced in a dose-dependent manner at *G. lucidum* concentrations of 40 mg/ml and 80 mg/ml, consistent with the concentrations that enhanced phagocytic activity of PMNs.

The above data show that brief treatment (in our study, *G. lucidum* treatment for 7.5 min had the best effects) with the sporoderm-broken germinating spores of *G. lucidum* activates p38 MAPK in a dose-dependent manner. The results indicate that *G. lucidum* acts on the p38 MAPK pathway in a dose-dependent manner.

The data presented support the hypothesis that *G. lucidum* exerts dose-dependent stimulatory effects on human PMNs and acts via the p38 MAPK pathway. These results confirm the results of previous studies in which *G. lucidum* enhanced PMN phagocytic activity via the p38 MAPK pathway. We hope that these results can be applied in people with chronic diseases related to inflammation in our future research. However, the optimal concentration of *G. lucidum* may be related to the method of extraction and the relative proportions of the many components in the extract. Furthermore, it is not clear that *G. lucidum* has the same effect when administered orally to humans.

The major disadvantage in this study was that our sample may not have been representative, because the sample size was small and limited to young healthy adults. Hence, selection bias is inevitable. Owing to the limited case number, this study can only serve as a preliminary study. Further more comprehensive randomized studies with larger sample sizes are needed. The time-course effects of *G. lucidum* on PMN phagocytic activity, studies of the potential mechanisms of the action of *G. lucidum* via other protein tyrosine kinases such as the Src family kinases, studies of more purified components of *G. lucidum* extract, and in vivo studies, will also be considered in our future research.

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靈芝破壁孢子萃取物藉由 p38 絲裂原活化蛋白激酶路徑活化嗜中性白血球

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背 景： 靈芝 *(Ganoderma lucidum, G. lucidum)* 在傳統中醫被用來調節人體免疫已有數千年的歷史，一般認爲靈芝可藉由活化人體嗜中性白血球來達到增強免疫的功能，然而對於靈芝作用在嗜中性白血球上的確切機轉並不明確。本實驗在於研究靈芝活化嗜中性白血球的分子生物機轉。

方 法： 評估靈芝破壁孢子的萃取物對人體嗜中性白血球吞噬作用強度的影響，然後加入 p38 絲裂原活化蛋白激酶 (p38 mitogen-activated protein kinase, p38 MAPK) 抑制劑於存在靈芝破壁孢子萃取物的嗜中性白血球中，探討 p38 絲裂原活化蛋白激酶抑制劑對嗜中性白血球吞噬功能的影響，接著評估靈芝破壁孢子萃取物對 p38 絲裂原活化蛋白激酶功能的影響。

結 果： 從本實驗中我們證實了靈芝破壁孢子的萃取物對嗜中性白血球吞噬功能有增強的效果，且與劑量相關，在加入 p38 絲裂原活化蛋白激酶抑制劑後吞噬的功能會被減弱。此外，靈芝破壁孢子的萃取物對 p38 絲裂原活化蛋白激酶的功能也有增強的效果，一樣與劑量相關。

結 論： 從以上的實驗結果證實了靈芝破壁孢子的萃取物可藉由 p38 絲裂原活化蛋白激酶路徑活化嗜中性白血球以調節免疫功能，這些結果可幫助傳統中醫醫師在臨床上的使用。

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關鍵詞： 傳統中醫，靈芝，嗜中性白血球，p38 絲裂原活化蛋白激酶

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