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Possible mode of action of antiherpetic activities of a proteoglycan isolated from the mycelia of *Ganoderma lucidum* in vitro

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Abstract

A bioactive fraction (GLPG) was extracted and purified from the mycelia of *Ganoderma lucidum* by EtOH precipitation and DEAE-cellulose column chromatography. GLPG was a proteoglycan and had a carbohydrate:protein ratio of 10.4:1. Its antiviral activities against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) were investigated by the cytopathic effect (CPE) inhibition assay in cell culture. This kind of polysaccharide inhibited the development of the cytopathic effect in dose-dependent manner in HSV-infected cells, moreover did not show any cytotoxic effects on cells even when a concentration was as high as 2000 μg/ml. In order to study the possible mode of action of the antiviral activity of GLPG, cells were treated with GLPG before, during and after infection, and the viral titers in the supernatant of cell culture 48 h post-infection were tested by $TCID_{50}$ assay. The antiviral effects in pre-treated and treated during virus infection with GLPG were more remarkable than the treatment of post-infection. Although the precise mechanism has yet to be defined, our work suggested that GLPG inhibits viral replication by interfering with the early events of viral adsorption and entry into target cells. Thus, this proteoglycan seems to be a potential candidate for anti-HSV agents.

Keywords: *Ganoderma lucidum* proteoglycan (GLPG); Antiherpetic activity; Herpes simplex viruses; Cytopathic effect (CPE); $TCID_{50}$

1. Introduction

The pharmacology and clinical application of Traditional Chinese Medicine (TCM) has been well-documented for centuries in China. *Ganoderma lucidum* (Fr.) Karst, a well-known Chinese medicinal fungus, is an important source of material in TCM, and has been widely used as a remedy to promote health and longevity in East Asian countries. This old Chinese herb is a species of basidiomycetes that belongs to Polyporaceae (or Ganodermataceae) of Aphyllophorales (Yang et al., 2000). It is widely used for the prevention and treatment of various kinds of diseases, such as hypertension, bronchitis, arthritis, neurasthenia, hepatopathy, chronic hepatitis, nephritis, gastric ulcer, tumorigenic diseases, hypercholesterolemia, immunological disorders, and scleroderma in China and other countries in the Orient. Because of the potential medicinal value and wide acceptability of *Ganoderma lucidum*, it has attracted intense interest in the search for pharmacological compounds from these mushrooms. *Ganoderma lucidum* has no cytotoxicity and appears to be very safe because the oral administration of the extract does not display any toxicity (Sugiura and Ito, 1977; Kim et al., 1986), and merits investigation as a potential preventive agent in humans (Kim and Kim, 1999).

Herpes simplex virus (HSV) is capable of causing a widespread spectrum of mild to severe disorders. These include acute primary and recurrent mucocutaneous disease in the otherwise healthy adult. Moreover, HSV infections were reported to be recognized as a risk factor for human immunodeficiency virus (HIV) infection (Hook et al., 1992). HSV-1 causes several neuronal diseases; it spreads in sensory axons and infects sensory neurons in the ganglia of the pe-

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ripheral nervous system (Cook and Stevens, 1973; Townsend and Collins, 1986). HSV-2 is also known as oncogenic virus, which has the ability to convert cells into tumor cells (Lapucci et al., 1993). The various drugs with a clinically relevant activity against HSV infections include interferons (IFNs), acyclovir (ACV), vidarabine (ara-A), ganciclovir (DHPG) and phosphonoformic acid (foscarnet, PFA). However, these drugs have some undesirable complications, e.g., potentially toxic, mutagenic, and/or teratogenic for the host, and also induced emergence of drug-resistant viruses (Coen, 1991). Therefore, identification of efficacious new antiherpetic agents that lack such deleterious effects is very important.

Ganoderma lucidum was reported to have many biologically active components (Lee and Rhee, 1990; Kawagishi et al., 1993; Lin et al., 1995). Polysaccharide and triterpenes are two major categories of the bioactive ingredients. Some researches have reported that the triterpenes possess the bioactivities of antioxidation, hepatoprotection, cholesterol stasis, anti-hypertension, etc. (Lin et al., 2003). Previous studies suggested that *Ganoderma lucidum* polysaccharide (GL-PS), one of the main efficacious ingredient of *Ganoderma lucidum* Karst, had been under modern pharmacological research in recent 30 years, and been reported to be effective in modulating immune functions, inhibiting tumor growth, resisting the invasion of various virus (Lin, 1991, 2001; Kim et al., 2000). Miyazaki and Nishijima previously separated a heteroglycan having an antitumor effect from the fruit bodies of the fungus (Miyazaki and Nishijima, 1981). Moreover, Hikino et al. (1985) isolated several hypoglycemic glycans from another fraction of the same crude polysaccharide.

Though the fruit bodies and the spores of *Ganoderma lucidum* had been utilized as medicine for a long time, no data were reported about the antiviral activity of extracts from the mycelia. In this study, the antiherpetic activities of *Ganoderma lucidum* proteoglycan (GLPG) isolated from the mycelia of *Ganoderma lucidum* were investigated by the cytopathic effect (CPE) inhibition assay and virus yield inhibition assay. It was found that this kind of polysaccharide could efficaciously inhibit the HSV infection in vitro. Furthermore, we addressed in this work to find its possible mode of action of antiviral activity.

2. Materials and methods

2.1. Materials and reagents

Mycelia of *Ganoderma lucidum* (Fr.) Karst (Ganodermataceae) were preserved in our lab and authenticated by Dr Xiang-Dong Chen, Department of microorganism, College of Life Science, Wuhan University, Wuhan, People's Republic of China. Dulbecco's Modified Eagle's Medium (DMEM), trypsin, penicillin and streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), crystal violet and trypan blue were purchased from Sigma (St. Louis, MO, USA). 9-[(Hydroxyethoxy)methyl]-guanine (ACV) was purchased from Jahoo pharmaceutical Ltd. Co. Xi-An, China. Vero cells (African green monkey kidney cell, CCTCC GDC029) were obtained from China Center for Type Culture Collection (CCTCC, Wuhan, Hubei, China). Herpes simplex virus type 1 (HSV-1, No. SM40) and type 2 (HSV-2, No. 333) were kindly provided by Professor Zheng-Kui Gong, the center of disease control in Hubei province, People's Republic of China.

2.2. Extraction and purification of GLPG

Ganoderma lucidum (Fr.) Karst was grown in potato-agar-dextrose medium and fungal mycelia (130 g) were collected by filtration, dried and disrupted, and then the residue was extracted with 30- to 40-fold boiling water for 30 min. After centrifugation, the supernatant solution was concentrated to one tenth of the original volume under reduced pressure, intensively dialyzed against running water for three days and then double distilled water for one day. The retentate was added to three volumes of ice cold EtOH to precipitate the crude extracts. Then the sample was allowed to stand overnight at 4 °C. It was centrifuged and the precipitate obtained was lyophilized. The lyophilized products (6.5 g) were a dark brownish powder of water-soluble substance.

To purify crude products, a portion of crude polysaccharide fraction (1 g) was dissolved in 5 ml double distilled water and centrifuged to remove the insoluble materials. The supernatant was applied onto the DEAE-cellulose (Cl^--form, Sigma) column (bed volume = 50 ml), eluted with 0.1N NaCl. Consequently each corresponding peak was separately pooled, concentrated, dialyzed, and three volumes of ice cold EtOH was added to precipitate the polysaccharides. The polysaccharide content of each fraction was determined by phenol–sulfuric acid method (Dubois et al., 1956). The polysaccharide-enriched fraction 2 was lyophilized and designated as *Ganoderma lucidum* proteoglycan (GLPG, 68 mg), a hazel-colored and water-soluble powder. GLPG was dissolved in serum-free DMEM medium, filtered through a 0.22 μm filter and then stored at 4 °C. They were further diluted to indicated concentrations prior to each assay.

2.3. Cells and viruses

Vero cells were cultured with DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week. HSV-1 and HSV-2 were propagated in Vero cells as described previously (Cinatl et al., 1992) and quantified in terms of the 50% tissue culture infective dose ($TCID_{50}$) by endpoint dilution, with the infectious titer determined by the method of Reed and

Muench (Flint et al., 2000) and stored in small aliquots at −70 °C until use.

2.4. Cytotoxicity assay

2.4.1. MTT reduction assay

For cytotoxicity assay, Vero cells were seeded in 96-well plate (Falcon, NJ, USA) at a cell concentration of 2×10^3 cells per well in 100 μl of DMEM medium. After incubation of the cells for 12 h at 37 °C, various concentrations of GLPG were added, and the incubation was continued for 48 h, or at a given concentration the incubation time was prolonged for 96 h and viable cells yield was determined by MTT reduction assay according to a reported procedure (Mosmann, 1983). In brief, MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/ml and sterilized by filtration to remove a small amount of insoluble reside present in some batches of MTT. At the times indicated in the following, the MTT solution (25 μl) was added to each well, and plates were incubated again in 5% CO_2 at 37 °C for 4 h. Acid–isopropanol (100 μl of 0.04N HCL in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After 20 min at room temperature to ensure that all crystals were dissolved, the plates were read on a Perkin-Elmer ELISA reader (HTS 7000 plus), using a test wavelength of 570 nm and a reference wavelength of 620 nm.

2.4.2. Trypan blue exclusion method

The effects of GLPG on cell proliferation and viability were compared according to the commonly accepted method of staining uninfected cells with trypan blue. Vero cells were seeded in 96-well plates at a cell concentration of 2×10^3 cells per well in 100 μl of DMEM medium. The cells were grown at 37 °C in DMEM medium containing 10% of FBS and GLPG of various concentrations. Each day, the cells in triplicate from each treatment were trypsinized and the cells number in the collected suspensions were determined in a Neubauer hemacytometer using the trypan blue exclusion method, and the mean value was calculated.

Results were expressed as the ratio between the number of viable cells or the optical density in treated cultures and viable cells or optical density in the untreated control cultures. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration, which caused a 50% reduction in the number of viable cells or in the optical density.

2.5. Cytopathic effect (CPE) inhibition assay

The antiviral activity of GLPG was preliminarily determined by the CPE inhibition assay (Woo et al., 1997) with the following differences. Virus solution was diluted with serum free DMEM to 100-fold $TCID_{50}$/0.1 ml. Semi-confluent Vero cells in a 96-well culture plate were infected with the virus. After 1 h incubation the unadsorbed virus was removed, the cell monolayer was washed with PBS and then incubated with GLPG in DMEM containing 2% FBS. The plates were then incubated in 5% CO_2 at 37 °C for two days, the cell cultures were examined for evidence of the cytopathic effect. Controls consisted of Vero cells untreated alone and Vero cells infected with HSV. The inhibition of the cytopathic effect was assessed by light microscopy and measured by MTT reduction assay. After removing the culture medium, the MTT assay was carried out as described above. Antiherpetic activity for GLPG was finally evaluated by selectivity index (SI), the value of CC_{50} divided by 50% effective concentration (EC_{50}), and EC_{50} was calculated with the regression equation composed by percentage of inhibition to virus control (VC) group determined as follows:

$$[(OD_t)_v - (OD_c)_v]/[(OD_c)_{mock} - (OD_c)_v] \times 100$$

where $(OD_t)_v$ is the OD of the cell, treated with virus and GLPG, $(OD_c)_v$ is the OD of the cell, treated with virus (virus control), and $(OD_c)_{mock}$ is the OD of the mock infected cell only (cell control). ACV, which is clinically used for the treatment of herpetic disease, was used as a positive control under this assay system.

2.6. Virus yield inhibition assay

For the virus yield inhibition assay, semi-confluent Vero cell monolayer in 24-well plates (Falcon) were treated with GLPG before, during and after virus infection as described in the following section.

2.6.1. Pre-incubation of cell monolayer with GLPG before virus infection

GLPG was dissolved in DMEM with serum free and incubated with semi-confluent cell in 96-well tissue culture plates in increasing concentration from 10 μg/ml to 1000 μg/ml for 2 h at 37 °C and 5% CO_2. After removal of the unbound GLPG, the cells were washed with phosphate-buffered saline (PBS) and then infected with 100-fold $TCID_{50}$/0.1 ml of HSV-1 and HSV-2 corresponding to a multiplicity of infection (MOI) 0.1. After 1 h incubation the unadsorbed virus was removed, the cell monolayer was washed with PBS and further incubated in DMEM with 2% FBS. Controls consisted of Vero cells untreated alone and Vero cells infected with HSV-1 and HSV-2.

2.6.2. Incubation of virus with GLPG before virus infection

The assay was performed as described above, with the exception that GLPG was added together with the virus. Virus stock solution (100-fold $TCID_{50}$/0.1 ml) was mixed with various concentrations of GLPG in equal volumes, and incubated at 5% CO_2, 37 °C for 2 h. Then the mixtures were used for infecting cells. After an incubation time of 1 h, the solutions containing both GLPG and virus were removed, the cell monolayer was washed with PBS and further incubated in DMEM with 2% FBS.

2.6.3. Incubation of cell monolayer with GLPG after virus infection

The cell monolayer was infected with the virus (100-fold $TCID_{50}$/0.1 ml). After 1 h incubation the unadsorbed virus was removed, the cell monolayer was washed with PBS and then incubated with concentrations of GLPG from 10 μg/ml to 1000 μg/ml in DMEM with 2% FBS.

After 48 h incubation at 37 °C and 5% CO_2, the plates were frozen and thawed three times to release cell-associated virus into the supernatants. Semi-confluent cell monolayer, grown in 96-well plate, was inoculated with 10-fold dilutions of the supernatants for 1 h at 37 °C and 5% CO_2. After removal of the inocula, monolayer was washed once with PBS and then incubated in DMEM 2% FBS for 48 h. The inhibition of the cytopathic effect was assessed by light microscopy. Virus titer was determined by the endpoint dilution method and expressed as $TCID_{50}$/0.1 ml. According to Reed–Muench formula, the results were expressed as reduction ratio of virus titers by comparison with virus control. EC_{50}, the concentrations needed to restrain 50% virus infection compared to untreated infected cells, were determined directly from the curve obtained by plotting the inhibition of the virus yield against the concentration of the samples.

2.7. Statistical analysis

The data were expressed as mean ± S.D. The statistical significance of the difference between mean values was determined by Student's *t*-test. Data were considered different at a significance level of $P < 0.05$.

3. Results

3.1. Extraction and purification of GLPG

The technique about fed-batch fermentation of *Ganoderma lucidum* has been established in our lab and we were able to produce high quality mycelia (data not shown). We obtained the water-soluble substances (yield: about 5%, a brownish powder) from the mycelia by boiling water extraction and EtOH precipitation. The crude products were separated by ion-exchange chromatography on DEAE-cellulose column chromatograph and eluted with 0.1N NaCl. Consequently the corresponding fractions 1, 2, 3, 4 and 5 (yield: 17%) were obtained (Fig. 1). The polysaccharide-enriched fraction 2 (about 40%) was designated as GLPG, a hazel-colored water-soluble powder. GLPG was identified as consisting mainly of polysaccharides (approximately 86.4%) and proteins (approximately 8.3%) by the phenol–sulfuric acid method and the Lowry–Folin test, respectively. Our results showed a carbohydrate to protein ratio of 10.4:1, showing that GLPG is composed mainly of carbohydrate. The SephadexG-150 column chromatography (eluted by 0.1N NaCl) profile showed a single and symmetrically sharp peak (data not shown). PAGE electrophoresis (Tris–Cl buffer, pH 9.2 and visualized by thymol) result showed a single brown band (figure not shown), suggesting that GLPG is a homogeneous polysaccharide.

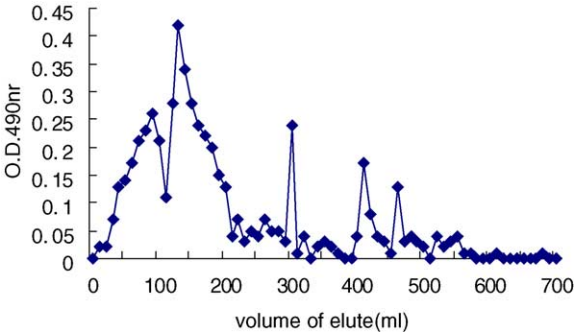

Fig. 1. Elution profile of the crude extracts of *Ganoderma lucidum* using DEAE-cellulose column chromatography. Fraction 2 was the polysaccharide-rich fraction (fraction 1: 40–110 ml; fraction 2: 110–240 ml; fraction 3: 240–340 ml; fraction 4: 340–450 ml; fraction 5: 450–510 ml; others: 510–700 ml). *Fraction 2.

3.2. Cytotoxicity

Cytotoxicity of GLPG was examined by means of trypan blue exclusion and MTT tests. Microscopic observations showed that no change occurred in cell growth and morphology in the presence of GLPG (data not shown), and MTT assay also approved that GLPG had no effect on the proliferation of cells, even up to a concentration of 2000 μg/ml. Trypan blue exclusion method showed that the total cell numbers were approximately 98% as compare with the control cell when the concentration is 2000 μg/ml (data not shown). Therefore, we could draw a conclusion that the CC_{50} was more than 2000 μg/ml (Tables 1 and 2).

3.3. Inhibition of HSV cytopathic effect (CPE) by GLPG

In the preliminary screening test for anti-HSV activity by the CPE inhibition assay, GLPG inhibited the appearance of CPE in HSV-1 and HSV-2-infected Vero cells with EC_{50} of 47.69 and 56.26 μg/ml, respectively (Table 1). At 48 h post-infection, HSV exposed Vero cells started to display signs of cytolytic infection characterized by rounding and clumping of cells. In the presence of GLPG, inhibition to these cytopathic effects was observed. GLPG exhibited effect in preventing cell detachment, rounding and clumping (figure not shown). The degree of inhibition showed to be proportional to the concentration of the GLPG in the wells. A concentration of 1000 μg/ml of polysaccharide almost provided full protection against the destruction of the cell monolayer by HSV during the period of the experiment (data not shown). The effect of the inhibition of cytopathic effect caused by HSV-2 was less than that obtained with HSV-1 in response to the polysaccharide treatment. With the same concentration, the efficiency of protection of GLPG to HSV-1 infected cells is higher than to HSV-2 infected cells. This conclusion also

Table 1
Antiviral activities of GLPG isolated from the mycelia of *Ganoderma lucidum* on herpes simplex viruses by cytopathic effect inhibition assay

Antiherpetic substances	Host cells	CC_{50}[a] (μg/ml)	EC_{50}[b] (μg/ml)		SI[c] (CC_{50}/EC_{50})	
			HSV-1[e]	HSV-2[f]	HSV-1[e]	HSV-2[f]
GLPG[d]	Vero	>2000	47.69	56.26	>41.94	>35.55
Acyclovir	Vero	819	1.6	2.0	511.88	409.50

[a] CC_{50} is the concentration of the 50% cytotoxic effect.
[b] EC_{50} is the concentration of the sample required to inhibit 50% of virus-induced CPE.
[c] Selectivity index (SI) = CC_{50}/EC_{50}.
[d] GLPG, *Ganoderma lucidum* proteoglycan isolated and purified from water-souble subatances of the mycelia of *Ganoderma lucidum.*
[e] HSV-1, herpes simplex virus type 1.
[f] HSV-2, herpes simplex virus type 2.

Table 2
Antiviral activities of GLPG isolated from the mycelia of *Ganoderma lucidum* on herpes simplex viruses by $TCID_{50}$ assay

Virus	CC_{50}[a] (μg/ml)	EC_{50}[b] (μg/ml)			SI[c]		
		A	B	C	A	B	C
HSV-1[d]	>2000	15.37	17.22	52.84	>130.1	>116.1	>37.85
HSV-2[e]	>2000	16.75	18.91	61.04	>119.4	>105.8	>32.77

A: GLPG was present before viral infection in Vero cells; B: GLPG was present during viral infection in Vero cells; C: GLPG was present after viral infection in Vero cells.
[a] CC_{50} is the concentration of the 50% cytotoxic effect.
[b] EC_{50} is the concentration of the sample required to inhibit 50% of virus-induced CPE.
[c] Selectivity index (SI) = CC_{50}/EC_{50}.
[d] HSV-1, herpes simplex virus type 1.
[e] HSV-2, herpes simplex virus type 2.

could be drawn from SI of 41.94 and 35.55 on HSV-1 and HSV-2 in Vero cells, respectively (Table 1).

3.4. Antiviral activity of GLPG by $TCID_{50}$ assay

The inhibition of virus yield by GLPG was evaluated by $TCID_{50}$ assay in Vero cells. GLPG showed strong antiviral activity against HSV-1 and HSV-2 when present before, during and after viral infection, especially when pre-incubation of cells with GLPG before virus infection. When the concentration is as higher as 1000 μg/ml, GLPG inhibited almost completely the virus yield.

We examined the antiviral activity of GLPG when GLPG was incubated with cells prior to infection with the virus, the virus titer of the supernatant dropped from $10^{4.6}$ $TCID_{50}$/0.1 ml to $10^{0.7}$ $TCID_{50}$/0.1 ml with HSV-1 when the concentration of GLPG was 40 μg/ml, and the inhibition rate was more than 84% (Fig. 2A). When the concentration of GLPG reached to 80 μg/ml the virus titer of the supernatant dropped from $10^{4.7}$ $TCID_{50}$/0.1 ml to $10^{0.7}$ $TCID_{50}$/0.1 ml with HSV-2, and the inhibition rate was 84% (Fig. 2A). In this case, the EC_{50} value of GLPG before viral infection was 15.37 and 16.75 with HSV-1 and HSV-2, respectively (Table 2).

HSV was mixed with various concentrations of GLPG and incubated at 37 °C for 2 h. Then, the mixtures were used for infecting cells, the result of virus yield inhibition was shown in Fig. 2B. GLPG significantly inhibited viral infection and the titration curves of the antiviral activity of GLPG showed a similar slope to Fig. 2A. GLPG caused a distinct reduction of virus yield at a concentration of 40 μg/ml. The EC_{50} value of GLPG during infection was 17.22 and 18.91 with HSV-1 and HSV-2, respectively (Table 2).

In order to study the antiviral activity after viral adsorption, GLPG was incubated with the infected cell monolayer after infection for 1 h. Results are shown in Fig. 2C. GLPG was less effective at low concentrations (<40 μg/ml) compared with GLPG was present before and during viral infection. However, when the concentration rinsed to 80 μg/ml, GLPG strongly inhibited viral multiplication. The EC_{50} value of GLPG after viral infection was 52.84 and 61.04 with HSV-1 and HSV-2, respectively (Table 2).

4. Discussion

Current chemotherapeutic antiviral drugs have been characterized as having in many cases limited clinical efficacy, suboptimal pharmacokinetics and toxic side effects (Patick and Potts, 1998). In response to this, it is necessary to identify and develop new antiviral agents with different targets from the standard therapy. The fungus *Ganoderma lucidum* has been used as a folk medicine for treating several diseases in China and has recently attracted much attention on account of its biological activities. It appears to be very safe because oral administration of the extract did not display any toxicity (Sugiura and Ito, 1977; Kim et al., 1986). However, *Ganoderma lucidum* normally takes 2 months to complete a fruiting body culture in solid-state fermentation, and it is also difficult to control the product quality during its cultiva-

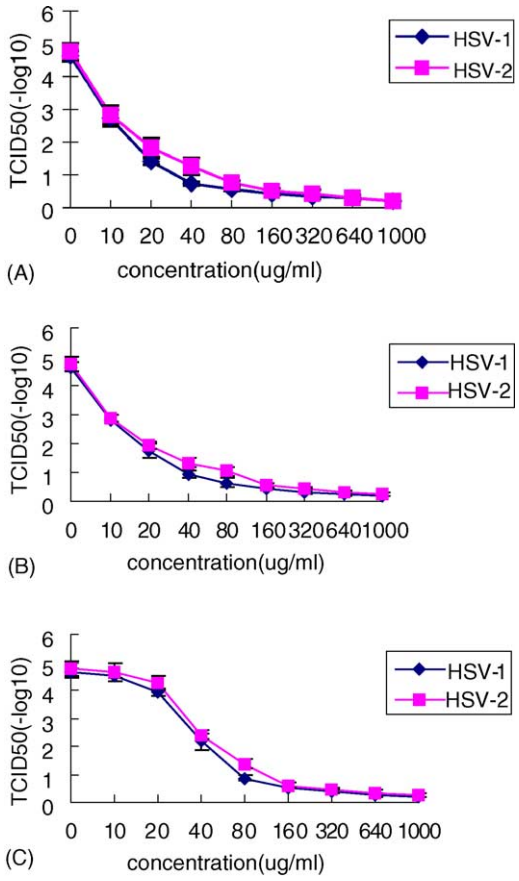

Fig. 2. Effect of increasing concentration of GLPG on the titer of HSV-1 and HSV-2 in infected Vero cells by $TCID_{50}$ assay. The multiplicity of infection was 0.1. GLPG was present in before (A), during (B) and after (C) HSV infection. The data were reported on the horizontal axis in −log 10 units as mean values ± S.D. for at least three separate experiments. Not significantly effective than VC; $^{*}P > 0.05$ (Student's *t*-test).

tion. Previous studies indicated that efficient production of bioactive polysaccharide could be processed by fed-batch fermentation of *Ganoderma lucidum* (Tang and Zhong, 2002). Besides it was reported that apart from exopolysaccharide, some components extracted from liquid-cultured mycelia were also demonstrated to be bioactive (Mizuno et al., 1995). Thus, we have an interest in the mycelium of fungus and try to find bioactive polysaccharide from it due to its short period of mycelial cultivation (i.e., usually less than 2 weeks).

In an attempt to find antiherpetic substances to reduce the adverse side effects associated with long-term therapy and limit the emergence of resistant virus, *Ganoderma lucidum* proteoglycan (GLPG) was isolated from water-soluble substances of the mycelia of *Ganoderma lucidum* by EtOH precipitation and DEAE-cellulose column chromatography. GLPG was a proteoglycan consisting of about 86.4% carbohydrate, and caused inhibition of HSV-induced cytopathic effects. In the preliminary screening test for anti-HSV activity by the CPE inhibition assay, GLPG inhibited the appearance of CPE in HSV-1 and HSV-2-infected Vero cells with EC_{50} of 47.69 μg/ml and 56.26 μg/ml, respectively (Table 1). GLPG had no cytotoxicity on Vero cells at a concentration of 2000 μg/ml. Therefore, GLPG exhibits a potent antiherpetic activity with SI of more than 35. This result is in consistent with the other previous reports (Eo et al., 1999; Kim et al., 2000), except that they used polysaccharides isolated from the carpophores of *Ganoderma lucidum*. Most of the antiviral or antitumor polysaccharides isolated from the hot water extract of *Ganoderma lucidum* were reported to be branched β-glucans with (1 → 3)-β-, (1 → 4)-β- and (1 → 6)-β-linkages and the average molecular weight was 1,050,000 (Mizuno et al., 1984). Therefore, protein-bound polysaccharide represented the inhibitory effect on the infection of HSV-1 and HSV-2 may be these β-glucans. It appears that the protein and polysaccharide were bound together since the protein moiety was not completely removed during the purification process, and the entity of binding is uncertain Also, these results suggest that the antiviral activity of protein bound polysaccharide is related to the net of electric charge. It is known that the antiviral activities of polysaccharides increase with the molecular weight or the degree of sulfation (Witvrouw et al., 1994). Therefore, the antiherpetic activity of GLPG would be expected to increase further by sulfating or partial digestion.

Comparing the effects of synthetic antiherpetic agent for clinical use, the SI value of GLPG seems to be much smaller than that of ACV. When one considers that most of the known compounds of the *Ganoderma lucidum* belong to polysaccharides whose average molecular weight is approximately above 50,000 (Mizuno et al., 1984), the SI value of GLPG is not too small. By using the average molecular weight, EC_{50} of GLPG is $0.95 \sim 1.13 \times 10^{-3}$ μM and that of ACV is $7.11 \sim 8 \times 10^{-3}$ μM in Vero cells. Therefore, EC_{50} of GLPG is comparable to this antiherpetic agent. The fact that the absence of toxicities in GLPG has great significance, since the CC_{50} of GLPG was above 2 mg/ml, and this mushroom has been used as folk medicine for treating several diseases in the Orient.

It is well-known that the antiviral activities of polysaccharides are linked to the anionic features of the molecules and that they inhibit the early stages of viral infection such as attachment and penetration (Marchetti et al., 1994). But the exact mechanism of antiviral activity of *Ganoderma lucidum* polysaccharide is still unclear. Therefore, our findings presented the first evidence on the possible mode of the action, such as the virus yield inhibition assay. Vero cell monolayer in 24-well plates was treated with GLPG before, during and after virus infection. On the basis of three ways of delivering drug experiments it all showed GLPG could inhibit the HSV infection in vitro. The efficiency of protection against virus infection by post-infection was somewhat lower than that achieved by pre-incubation and during viral infection with GLPG. And Fig. 2 indicated that GLPG showed a minimal inhibition at lower concentration presented after cells were infected, whereas the inhibition was evident when GLPG was present before and during infection. A significant reduction of the viral infection could only be found with higher concentrations when assayed post-virus infection. It is most possible in this case that GLPG inhibits progeny virus from the second

infection rather than inhibits the replication events of virus inside the cells.

It was queried that polysaccharide prevents virus infection by blocking virus adsorption onto the host cells, and if so, does it exert its effect by interacting either with the virus particles or with the host cells. Thus, to enable us to ascertain the site of interaction, we applied GLPG to the cells for 2 h and then removed the GLPG before the virus infection, or GLPG and virus were mixed and stand for a short time, then were brought into contact with the cells, as described on the Section 2. Our results showed a strong inhibition of viral infection in both situations (Fig. 2A and B). It could be explained by a strong or irreversible interaction between polysaccharide and the cell membrane, resulting in blocking the receptor for virus adsorption on the cell membrane. Several viral glycoproteins such as gB, gC, gD and the corresponding receptors present in the cell membrane are responsible for adsorption and penetration (Wudunn and Spear, 1989; Herold et al., 1991; Shieh et al., 1992; Trybala et al., 1994; Marchetti et al., 1996; Cocchi et al., 2001). The interaction between the polysaccharide and the envelope of HSV particles also may be presented. The binding of GLPG to glycoprotein gB, gC and/or gD of the virion, also interrupted the interaction between the cell receptor and the virus to inhibit viral infection. This might imply that the antiviral activity of GLPG is based on an interaction of this compound with such viral glycoproteins and their cell receptors. In other words, GLPG exerted its inhibitory effect by interacting with the positive charges on the virus or on the cell surface, thereby, preventing the adsorption and penetration of the virus into the host cells.

In conclusion, GLPG showed strong antiviral activity against HSV-1 and HSV-2 in Vero cells. These results imply that the effective antiviral concentrations of GLPG are far from the cytotoxicity threshold, and, consequently, this natural product possess good SIs and reflect the possibility of development as antiviral agent. The antiviral activity may be mainly due to the inhibition of the attachment of HSV to cells, and also the inhibitory effect on penetration is assumed to augment the antiviral activity of GLPG. The molecular entity of GLPG is currently in progress, and the exact step (steps) affected by GLPG during the viral replication cycle remains to be elucidated.

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