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Possible Mechanism Underlying the Antiherpetic Activity of a Proteoglycan Isolated from the Mycelia of *Ganoderma lucidum in Vitro*

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GLPG (*Ganoderma lucidum* proteoglycan) was a bioactive fraction obtained by the liquid fermentation of the mycelia of *Ganoderma lucidum*, EtOH precipitation, and DEAE-cellulose column chromatography. GLPG was a proteoglycan with 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 using a cytopathic inhibition assay. GLPG inhibited cell death in a dose-dependent manner in HSV-infected cells. In addition, it had no cytotoxic effect even at 2 mg/ml. In order to study the mode of action of the antiviral activity of GLPG, cells were treated with GLPG before, during, and after infection, and viral titer in the supernatant of cell culture 48 h post-infection was determined using a $TCID_{50}$ assay. The antiviral effects of GLPG were more remarkable before viral treatment than after treatment. Although the precise mechanism has yet to be defined, our work suggests that GLPG inhibits viral replication by interfering with the early events of viral adsorption and entry into target cells. Thus, this proteoglycan appears to be a candidate anti-HSV agent.

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

Introduction

The pharmacology and clinical application of Traditional Chinese Medicine (TCM) has been documented for centuries in China. *Ganoderma lucidum* (Fr.) Karst, a well-known medicinal fungus, is an important source of material in TCM, and is widely used to promote health and longevity in East Asian countries. This novel Chinese herb is a member of the basidiomycetes species and belongs to Polyporaceae (or Ganodermataceae) of Aphyllophorales (Yang *et al*., 2000). It is widely used for the prevention and treatment of various diseases, such as hypertension, bronchitis, arthritis, neurasthenia, hepatopathy, chronic hepatitis, nephritis, gastric ulcer, tumorigenic diseases, hypercholesterolemia, immunological disorders, and scleroderma in China and in other Oriental countries. Because of its potential medicinal value and the wide availability of *G. lucidum*, it has attracted intense interest from those searching for compounds with useful pharmacological properties. Moreover, *G. lucidum* has no cytotoxicity and appears to be safe because its long history of oral administration has not associated it any toxicity (Kim *et al*., 1986; Sugiura and Ito, 1997). Thus, we considered that it merited investigation as a potential preventive agent in humans (Kim *et al*., 1999).

Herpes simplex virus (HSV) is capable of causing a widespread spectrum of mild to severe disorders. These include acute primary and recurrent mucocutaneous diseases in otherwise healthy adults. In addition, HSV infections have been reported to be 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 peripheral nervous system (Cook *et al*., 1973; Townsend *et al*., 1986). HSV-2 is a known oncogenic virus and has the ability to cause the onco-conversion of normal cells (Lapucci *et al*., 1993). Various drugs with 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., they are potentially toxic, mutagenic, and/or teratogenic, and may also induce drug-resistant virus emergence (Coen, 1991). Therefore, the identification of efficacious new antiherpetic agents that lack such side effects is of importance.

G. lucidum has been reported to contain many biologically active components (Lee and Rhee, 1990; Kawagishi *et al*.,

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1993; Lin *et al*., 1995). Previous studies have suggested that *Ganoderma lucidum* polysaccharide (GL-PS), one of the main efficacious ingredient of *G. lucidum* Karst, and has a history of being examined pharmacologically over the past 30 years. As a result it has been reported to be effective at modulating immune functions, inhibiting tumor growth, an at resisting invasion by various virus (Lin *et al*., 1999; Kim *et al*., 2000; Lin *et al*., 2001). Miyazaki and Nishijima previously separated a heteroglycan having an antitumor effect from the fruiting bodies of the plant (Miyazaki and Nishijma, 1981). Moreover, Hikino and coworkers isolated several hypoglycemic glycans from another fraction of the GL-PS (Hikino *et al*., 1985).

Though the fruiting bodies and the spores of *G. lucidum* have been used medicinally for some time, no reports are available data on the antiviral activities of mycelium extracts. In this study, the antiherpetic activities of *Ganoderma lucidum* proteoglycan (GLPG) isolated from the mycelium of *Ganoderma lucidum* were investigated using a cytopathic effect (CPE) inhibition assay and a virus yield inhibition assay. It was found that GLPG can effectively inhibit HSV infection *in vitro*. Further, we investigated the mechanism underlying it antiviral activity.

Materials and Methods

Materials and reagents Mycelium of *Ganoderma lucidum* (Fr.) Karst (Ganodermataceae) was preserved in our laboratory. Dulbecco's Modified Eagle's Medium (DMEM), trypsin, penicillin, and streptomycin were purchased from Gibco BRL (Grand Island, USA); and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), crystal violet and trypan blue from Sigma (St. Louis, USA). Vero cells (African green monkey kidney cells, CCTCC GDC029) were obtained from the Chinese 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, at the Center for Disease Control in Hubei province, Peoples Republic of China.

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. The residue obtained was extracted with 30-40 volumes of boiling water for 30 min. After centrifugation, the supernatant solution was concentrated to one tenth of its original volume under reduced pressure, intensively dialyzed against running water for three days, and then against doubly distilled water for one day. The retentate was added to three volumes of ice cold EtOH to precipitate the crude extracts. The sample was then allowed to stand overnight at 4, centrifuged, and the precipitate obtained was lyophilized to give a dark brown water-soluble powder referred to as the crude polysaccharide fraction (6.5 g).

To purify the crude products, a portion of the crude polysaccharide fraction (1 *g*) was dissolved in 5 ml of doubly distilled water and the centrifuged to remove insoluble materials. The supernatant was applied to a DEAE-cellulose (Cl^--form, Sigma, St. Louis, USA) column (bed volume = 50 ml), and eluted with 0.1 N NaCl. Each eluted peak was separately pooled, concentrated, dialyzed, and the polysaccharides they contained were precipitated by adding three volumes of ice cold EtOH. We harvest 100 mg polysaccharide and the content of each fraction was determined using the phenol-sulfuric acid method (Dubois *et al*., 1956). The polysaccharide-enriched fraction 2 was lyophilized and designated as *Ganoderma lucidum* proteoglycan (GLPG, 68 mg/100 mg), a hazel-colored and water-soluble powder. GLPG was dissolved in serum free Dulbeccos Modified Eagles Medium (DMEM) media, filtered through a 0.22 μm filter and then stored at 4°C. This GLPG stock material was diluted to the indicated concentrations for each assay.

Cells and viruses Vero cells were cultured with DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 I.U./ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere and subcultured 2~3 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, using the method of Reed and Muench (Flint *et al*., 2000), and stored in small aliquots at −70°C until required.

Cytotoxicity assay

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

Trypan blue exclusion The effects of GLPG on cell proliferation and viability were compared using trypan blue staining method, which stains uninfected cells. Vero cells were seeded in 96-well plates at a concentration of 2×10^3 cells per well in 100 ul of DMEM medium. The cells were then grown at 37°C in DMEM medium containing 10% FBS and various concentrations of GLPG. Cells from each treatment were trypsinized daily in triplicate and cells number in the collected suspensions were counted using a Neubauer hemacytometer using trypan blue exclusion; mean values were calculated.

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

Cytopathic effect (CPE) inhibition assay The antiviral activity of GLPG was determined initially using a CPE inhibition assay (Woo *et al*., 1997), with some modification. Briefly, virus solution was diluted with serum free DMEM by 100-fold $TCID_{50}$/0.1 ml. Semi-confluent Vero cells in a 96-well culture plate were infected with the virus. After incubation for 1 h, the unabsorbed virus was removed, cell monolayers were 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, and the cell cultures were examined for evidence of a cytopathic effect. Untreated Vero cells and Vero cells infected with HSV were used as controls. The inhibition of the viral cytopathic effect was assessed by light microscopy and quantified using MTT reduction assays, which were performed after removing the culture medium, as described above. Antiviral activity was finally expressed as a selectivity index (SI), the value of CC_{50} divided by 50% effective concentration (EC_{50}), which was calculated using a regression equation composed by percentage of inhibition to virus control (VC) group determined as follows:

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

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 mock-infected cells (control).

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

Pre-incubation of cell monolayer with GLPG before virus infection GLPG was dissolved in serum free DMEM and incubated with semi-confluent Vero cells in 96-well tissue culture plates in increasing concentration from 10 μg/ml to 1 mg/ml for 2 h at 37°C in 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) of 0.1. After incubating for 1 h unadsorbed virus was removed, the cell monolayer was washed with PBS, and then further incubated in DMEM containing 2% FBS. Controls consisted of untreated Vero cells or Vero cells infected with HSV-1 or HSV-2.

Incubation of HSV virus with GLPG before infection The assay was performed as described above, with the exception that GLPG was added with the virus. Virus stock solution (100 folds $TCID_{50}$/0.1 ml) was mixed with GLPG of 2-fold diluted concentrations in equal volumes, and incubated in 5% CO_2 for 2 h at 37°C. These mixtures were used to infect cells. After incubating for 1 h, the solutions containing GLPG and virus were removed, and the cell monolayer was washed with PBS and further incubated in DMEM containing 2% FBS.

Incubation of Vero cell monolayer with GLPG cells after HSV virus infection Cell monolayers were infected with virus (100 fold $TCID_{50}$/0.1 ml). After incubating for 1 h unadsorbed virus was removed, and cell monolayers were washed with PBS and then incubated with GLPG from 10 μg/ml to 1 mg/ml in DMEM containing 2% FBS. After incubating GLPG and infected for 48 h at 37°C in 5% CO_2, the plates were frozen and thawed three times to release cell-associated virus into the supernatant. Semi-confluent cell monolayers, grown in a 96-well plate, were inoculated with 10-fold dilutions of the supernatants for 1 h at 37°C in 5% CO_2. After removing the inocula, monolayers were washed once with PBS and then incubated in DMEM 2% FBS for 48 h. Inhibition of the cytopathic effect of HSV by GLPG was followed by light microscopy. Virus titers were determined using the endpoint dilution method and expressed as $TCID_{50}$/0.1 ml. According to Reed-Muench formula, the results were expressed as the ratio of versus the virus control. EC_{50}, the concentration needed to restrain virus infection by 50%, was determined directly from the curve obtained by plotting the inhibition of the virus yield against the concentration of the samples.

Statistical analysis Data are expressed as mean ± S.D. The statistical significance of the difference between mean values was determined using the Students *t*-test, and a P level of < 0.05 was considered significant.

Results

Extraction and purification of GLPG The fed-batch fermentation of *G. lucidum* technique was established on a laboratory scale and produces high quality mycelia (data not shown). We obtained water-solubles (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 a DEAE-cellulose column chromatograph and eluted with 0.1 N NaCl. Consequently the corresponding fractions 1, 2, 3, 4 and 5 (yield, 17%) were obtained (Fig. 1). The polysaccharide -enriched fraction 2 a hazel-colored water-soluble powder (about 40% of the crude product) was designated GLPG. GLPG was found to consist mainly of polysaccharides (approximately 86.4%) and proteins (approximately 8.3%) by the phenol-sulfuric acid method and by the Lowry-Folin test, respectively. Our results showed a carbohydrate to protein ratio of 10.4 to 1, showing that GLPG is composed mainly of carbohydrate. The SephadexG-50 column chromatography (eluted by 0.1 N NaCl) profile showed a single and symmetrical sharp peak (data not shown). PAGE electrophoresis (Tris-Cl buffer, pH 9.2, and visualized by thymol) showed a single brown band (figure not shown), suggesting that GLPG is a homogeneous polysaccharide.

Cytotoxicity Cytotoxicity of GLPG was examined by trypan blue exclusion and MTT testing. Microscopic observations showed that no change occurred in Vero cell growth or morphology in the presence of GLPG (data not shown), and an MTT assay demonstrated that GLPG had no effect on cell proliferation, even up to 2,000 μg/ml (Fig. 2A, B). Trypan blue exclusion showed that total cell numbers were approximately 98% of the control at a concentration of 2,000 μg/ml (Fig. 4). Therefore, we concluded that the CC_{50} of

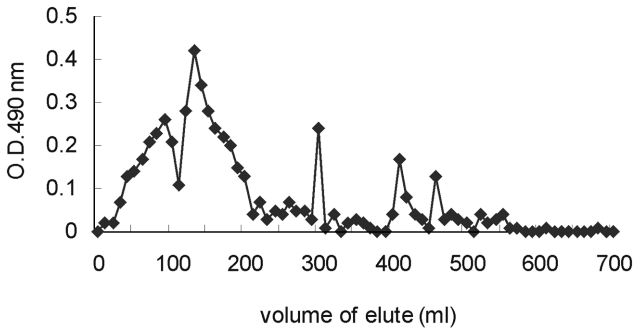

Fig. 1. Elution profile of the crude extracts of *G. lucidum* using DEAE-cellulose column chromatography. Fraction 2 was the polysaccharide-rich fraction. (fraction 1, 40-110 ml; fraction 2, 110-240 ml; fraction 3, 240-340 ml; fraction 4, 340-450 ml; fraction 5, 450-510 ml; others, 510-700 ml).

GLPG was >2 mg/ml (Table 1, 2).

Inhibition of the cytopathic effect (CPE) of HSV by GLPG In the primary screening test for anti-HSV activity using the CPE inhibition assay, GLPG was found to inhibit the appearance of CPE in HSV-1 and HSV-2-infected Vero cells with EC_{50}s of 48 and 56 μg/ml, respectively (Table 1). Twenty-four h after infection, HSV exposed Vero cells started to display signs of cytolytic infection characterized by cell rounding and clumping. In the presence of GLPG, these cytopathic effects were found to be inhibited. GLPG prevented cell detachment, rounding, and clumping (figure not shown). The degree of inhibition was found to be proportional to the GLPG concentration in the wells. A concentration of 1,000 μg/ml of polysaccharide almost provided full protection against the destruction of the cell monolayer by HSV during the experimental period for 2 h. The inhibition of the cytopathic effect of HSV-2 was less than that of HSV-1 in response to the polysaccharide treatment. At the same concentration, the protection afforded by GLPG to HSV-1 infected cells was higher than that afforded HSV-2 infected cells. This conclusion also could be drawn from SIs of 42 and 36 of HSV-1 and HSV-2 in Vero cells, respectively (Table 1).

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 cells were pre-incubated with GLPG before virus infection. At a concentration of 1 mg/ml, GLPG almost completely inhibited the virus.

We examined the antiviral activity of GLPG, when it was incubated with Vero cells prior to infection with HSV 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 at a GLPG concentration of 40 ug/ml, equivalent to an inhibition rate of >84% (Fig. 3A). When the concentration of GLPG reached 80 ug/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, an inhibition rate of 84% (Fig. 3A). In this case, the EC_{50} value of GLPG before viral infection was 15 and 17 μg/ml for HSV-1 and HSV-2, respectively (Table 2).

When HSV inocula were mixed and incubated with various concentrations of GLPG for 2 h 37°C, and then used to infect cells, we obtained the virus yield inhibition results shown in Fig. 3B, GLPG significantly inhibited viral infection and the titration curves of antiviral activity of GLPG showed a similar slope to that of Fig. 3A. GLPG caused a distinct reduction in virus yield at a concentration of 40 μg/ml. The EC_{50} value of GLPG during infection was 17 and 19 μg/ml for HSV-1 and HSV-2, respectively (Table 2).

In order to study antiviral activity after viral adsorption, GLPG was incubated with the infected cell monolayer after it had been infected for 1 h. Results are shown in Fig. 3C.

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

Virus	CC_{50} (ug/ml)	EC_{50} (ug/ml)	SI (CC_{50}/EC_{50})
HSV-1	>2000	48	>42
HSV-2	>2000	56	>36

EC_{50} is the concentration of the sample required to inhibit 50% of virus-induced CPE.
CC_{50} is the concentration of the 50% cytotoxic effect.
$SI=CC_{50}/EC_{50}$.

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

Virus	CC_{50} (μg/ml)	EC_{50} (μg/ml)			SI		
		A	B	C	A	B	C
HSV-1	>2000	15	17	53	>130	>116	>38
HSV-2	>2000	17	19	61	>119	>106	>33

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.
EC_{50} is the concentration of the sample required to inhibit 50% of virus-induced CPE.
CC_{50} is the concentration of the 50% cytotoxic effect.
$SI=CC_{50}/EC_{50}$.

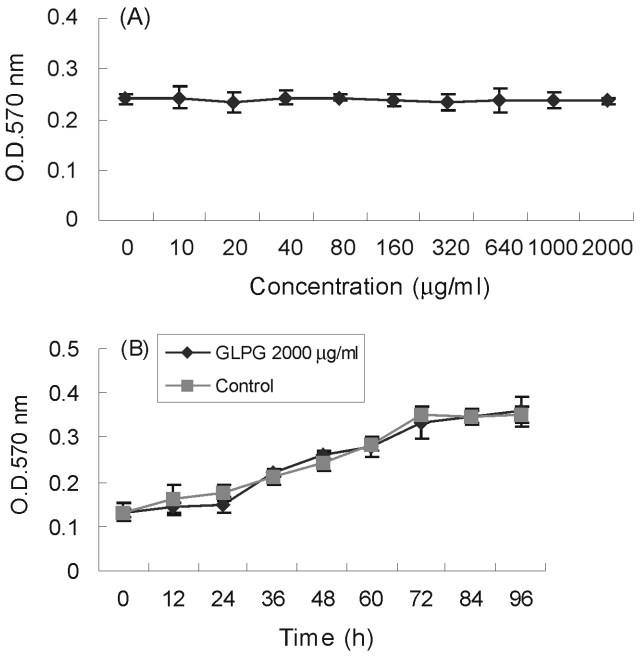

Fig. 2. The effects of GLPG on cell proliferation by MTT assay. (A) Vero cells were treated with GLPG at concentration range from 10 to 2,000 μg/ml for 2 d. (B) Vero cell were treated with GLPG at concentration of 2,000 μg/ml during 4 d. Viable cells yield were detected every 12 h. The cell untreated with GLPG was used as a control in the experiment. MTT assays were performed as described. Results shown represent the mean ± S.D. for at least three separate experiments.

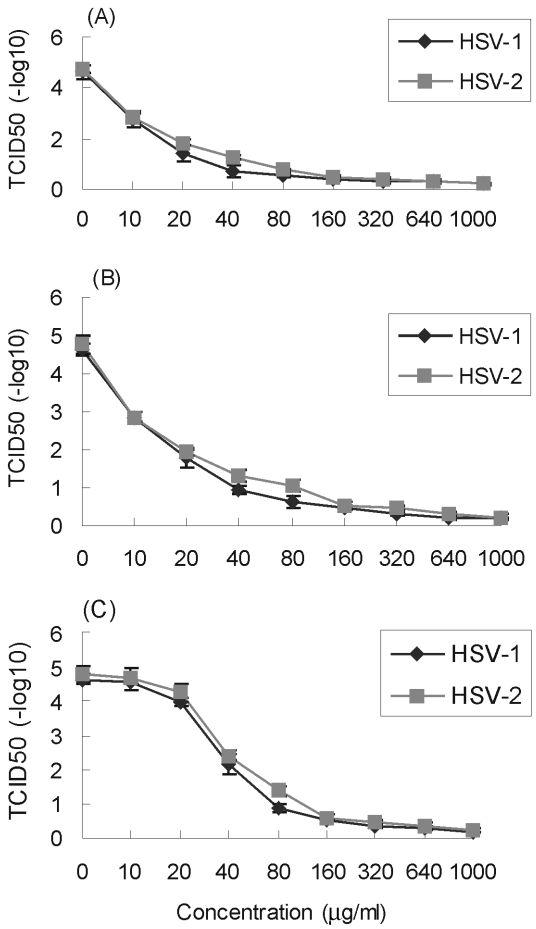

Fig. 3. Effect of increasing concentration of GLPG on the titer of HSV-1 and HSV-2 in infected Vero cells by $TCID_{50}$ assay. The multiplicity of infection was 0.1. GLPG was present in before (A), during (B) and after (C) HSV infection. The data were reported on the vertical axis in log10 units as a mean values ± S.D. for at least three separate experiments. Not significantly effective than VC; $p > 0.05$ (Student *t*-test).

GLPG was less effective at low concentrations (< 40 μg/ml) compared with that of GLPG was present before and during viral infection. However, when the GLPG concentration was raised to 80 μg/ml, GLPG strongly inhibited viral multiplication. The EC_{50} values of GLPG after viral infection were 53 and 61 μg/ml for HSV-1 and HSV-2, respectively (Table 2).

Discussion

In an attempt to find antiherpetic substances that reduce the adverse side effects associated with long-term therapy, and limit the emergence of resistant virus, *Ganoderma lucidum* proteoglycan (GLPG) was isolated from the water-soluble extract of the mycelia of *G. lucidum* by EtOH precipitation and DEAE-cellulose column chromatography. GLPG was found to contain a proteoglycan consisting of about 86.4% carbohydrate, and it inhibited the cytopathic effects of HSV. In the primary screening test for anti-HSV activity by CPE inhibition assay, GLPG inhibited the appearance of CPE in HSV-1 and HSV-2-infected Vero cells with EC_{50}'s of 48 and 56 μg/ml, respectively (Table 1). GLPG had no cytotoxicity on cells at 2,000 μg/ml. Therefore, GLPG exhibits a potent antiherpetic activity with an SI of more than 35. Previous reports have shown that most of the antiviral or antitumor polysaccharides isolated from the hot water extract of *G. lucidum* are branched β-glucans with (1→3)-β-, (1→4)-β- and (1→6)-β-linkages of average molecular weight ca. 1,050,000 (Mizuno *et al*., 1984). This suggests that β- glucans may be protein bound polysaccharides that exhibit antiherpetic activity. It appears that the protein and polysaccharide are bound together since the protein moiety was not completely removed during the purification process. Also, these results suggest that the antiviral activity of the protein bound polysaccharide is related to the net electrical 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 be enhanced by sulfation or partial digestion.

It is well known that the antiviral activities of polysaccharides

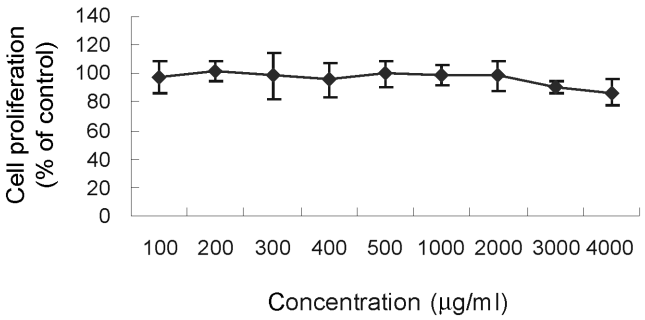

Fig. 4. Effect of GLPG on cell proliferation of Vero cells by trypan blue exclusion method. Vero cells were seeded at a concentration of 2×10^3 cells per well in 96-well plates and incubated at 37°C with GLPG at concentration range from 100 to 4000 μg/ml for 2 or 4 d. The cell untreated with GLPG was used as a control in the experiment. Results shown represent the mean ± S.D. for at least three separate experiments.

are associated with their anionic features, and that they inhibit the early stages of viral infection such as attachment and penetration (Marchetti *et al*., 1994). However, the mechanism underlying the antiviral activity of *G. lucidum* polysaccharide is still unclear. Therefore, our findings present first evidence on its possible mode of the action. Vero cell monolayers in 24-well plates were treated with GLPG before, during, and after virus infection. Based on these three ways of delivering-drugs we found that GLPG can inhibit HSV infection *in vitro*. Moreover, the efficiency of protection against virus infection post-infection was somewhat lower than that achieved pre-incubation, i.e. Fig. 3 indicates that GLPG shows minimal inhibition at lower concentrations when treated after infection, whereas inhibition was evident when GLPG was treated before or during infection. A significant reduction in the viral infection was only found when higher concentrations of GLPG were added after virus infection. It is likely that this is because GLPG inhibits secondary infection by progeny virus rather than inhibiting viral intracellular replication events.

It wondered whether GLPG prevents virus infection by blocking virus adsorption onto host cells, and if so, does it then exert its effect by interacting either with the virus particles or with the host cells. To ascertain the site of interaction, we applied GLPG to Vero cells for 2 h and then removed it before virus infection, or GLPG and virus were mixed and allowed to stand for a short time, and then treated to cells, as described in *Materials and Methods*. Our results showed a strong inhibition of viral infection in both situations (Fig. 3A, B), which could be explained by a strong or irreversible interaction between polysaccharide and the cell membrane, resulting in the blocking of receptors used 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 polysaccharide and the envelope of HSV particles also may be presented. The binding of GLPG to glycoproteins gB, gC and/or gD of the virion interrupts the interaction between the cell receptor and the virus and inhibit viral infection. This implies that the antiviral activity of GLPG is based on an interaction between GLPG and viral glycoproteins and their cell receptors. In other words, GLPG exerts its inhibitory effect by interacting with the positive charges on the virus or on the cell surface, thereby, preventing the virus penetrating the host cells.

In conclusion, GLPG showed strong antiviral activity against HSV-1 and HSV-2 in Vero cells. This result is consistent with other reports (Eo *et al*., 1999; Kim *et al.*, 2000), but they used polysaccharides isolated from the spores or fruiting bodies of *G. lucidum*. The antiviral activity of GLPG may be due its inhibiting HSV attachment to cells, in addition its inhibition of viral penetration would augment its antiviral activity. Show less cytotoxicity and possess good SIs and have potentially use a development bases for antiviral agents. The molecular entity of GLPG is currently in progress. However, the steps affected by GLPG during viral replication have yet to be elucidated.

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