Antitumor and anti-angiogenic activity of *Ganoderma lucidum* polysaccharides peptide

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ABSTRACT

AIM: To investigate the antitumor and anti-angiogenic activity of *Ganoderma lucidum* polysaccharides peptide (GLPP). **METHODS:** Antitumor effect of GLPP was observed in tumor-bearing mice *in vivo*. At the same time, the effects of GLPP on proliferation of tumor cells and human umbilical cord vascular endothelial cell (HUVEC) were detected by MTT assay *in vitro*. Subsequently, spleen lymphocytes proliferation of nude mice was stimulated by LPS or ConA. To investigate the anti-angiogenic effect of GLPP, GLPP 80 μg per disc and GLPP-treated serum 10 μL per disc were added to the chick chorioallantoic membrane (CAM) respectively in *vivo*. **RESULTS:** GLPP 50, 100, and 200 mg/kg inhibited growth of Sarcoma 180 in BALB/c mice markedly by 35.2 %, 45.2 %, and 61.9 %, respectively. GLPP which was directly added to the cultured medium did not inhibit PG cell proliferation *in vitro*; but GLPP-treated serum 50, 100, 200 mg/kg potently inhibited PG cell proliferation by 22.5 %, 26.8 %, and 30.3 %, respectively; and reduced the xenograft (human lung carcinoma cell PG) in BALB/c nude mice greatly *in vivo* by 55.5 %, 46.0 %, and 46.8 %, respectively. Lymphocytes proliferation of nude mice could be stimulated by LPS 5 mg/L but not by ConA 2.5 mg/L, indicating that GLPP could not promote the T lymphocyte proliferation and neutral red phagocytosis of peritoneal macrophages of nude mice. The CAM assay showed that GLPP and GLPP-treated serum had anti-angiogenic effect. GLPP (1, 10, and 100 mg/L) inhibited HUVEC proliferation *in vitro* with the inhibitory rate of 9.4 %, 15.6 %, and 40.4 %, respectively. **CONCLUSION:** GLPP has antitumor and anti-angiogenic activity. The anti-angiogenesis of GLPP may be a new mechanism underlying its anti-tumor effects.

INTRODUCTION

Ganoderma lucidum (Leyss, ex Fr) Karst (*Gl*) has been widely used as a medicine to promote health and longevity in China for thousands of years. The *Gl* and its components were reported to be effective in the treatment of chronic hepatopathy, hypertension, hyperglycemia and neoplasia under modern pharmacological research in recent 30 years[1]. However, the most attractive nature of the *Gl* is its anti-tumor effect[2-8], which was demonstrated to be associated with its polysaccharides fraction[2-6]. A series of experiments have demonstrated that *Ganoderma lucidum* polysaccharides peptide (GLPP) could stimulate host immune functions. Macrophages and lymphocytes could secret anti-tumor cytokines such as TNF-α and interferon-γ (IFN-γ)[7] to inhibit the tumor cells proliferation. So, the antitumor effect of GLPP was supposed to be the result of en-

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hanced immune function.

Several lines of direct evidence showed that angiogenesis was essential for the growth and persistence of solid tumors and their metastases[9-11]. In this study, we investigated the influence of GLPP on tumor and angiogenesis, and found anti-angiogenesis may be a new mechanism of anti-tumor effect of GLPP.

MATERIALS AND METHODS

Animals Inbred male 6-8-week-old (body weight 18-22 g) BALB/c (H-2 d) mice (Grade II, Certificate No scxk11-00-0004) and BALB/c nude mice (Grade III, Certificate No scxk11-00-0010) were purchased from the Department of Experimental Animals, Health Science Center, Peking University, Beijing, China. All procedures were in accordance to the Institute Ethical Committee for Experimental Use of Animals.

Drugs GLPP were isolated from boiling water extract of the fruiting body of *Ganoderma lucidum* (Leyss ex Fr) Karst (*Gl*), followed by ethanol precipitation, dialysis and protein depletion using Sevag method. It was a kind of polysaccharides peptide with M_r 512 500, and the ratio of polysaccharides of peptides is 94.8 %:5.2 %. The polysaccharides were consisted of *D*-rhamnose, *D*-xylose, *D*-fructose, *D*-galactose, and *D*-glucose with molar ratio of 0.549:3.614:3.167:0.556:6.89 and linked together by β-glycosidic linkages. It is a hazel-colored and water soluble powder, kindly provided by Fuzhou Institute of Green Valley Bio-Pharm Technology.

Antitumor experiment in tumor-bearing mice Murine Sarcoma 180 (S180) was purchased from Beijing Tumor Institute and maintained by weekly transplantation of the tumor cells into the peritoneal cavity of mouse. Human lung carcinoma cell line (PG) was purchased from Department of Pathology, Peking University Health Center. PG cells were cultured with 10 % FCS RPMI-1640 in a humidified environment at 37 ºC, 5 % CO_2. Tumor cells (Sacoma 180 with 2×10^6/mouse or PG cells with 2×10^6/nude mouse) were injected into the axillary fossa of the mice right foreleg. The mice were divided into 4 groups randomly. Different doses of GLPP 50, 100, and 200 mg/kg were given intragastrically once a day. The BALB/c mice implanted sarcoma 180 were killed 10 d later and the BALB/c nude mice implanted PG cell were killed 33 d later and the tumors were cut and weighted. At the same time, blood samples were collected from the orbital vein and the serum (GLPP-treated serum) was separated and filtered, then stocked at -70 ºC.

PG cells proliferation assay PG cells were washed with PBS and were dispersed in 0.125 % trypsin-0.02 % edetic acid solution. A cell suspension (2×10^7 cells/L) was made with 10 % FCS RPMI-1640, planted onto 96-well culture plates (0.1 mL/well), and incubated at 37 ºC, 5 % CO_2 for 24 h. The media was replaced with 0.1 mL of RPMI-1640 +5 % FCS and the GLPP 0, 0.1, 1, 10, 100 mg/L or 1:2 diluted GLPP-treated serum 0, 50, 100, 200 mg/kg was applied, respectively. After 48 h, cells proliferation was estimated based on the cellular reduction of tetrazolium salt MTT[12] by a Microplate reader (BIO-RAD, Model 550), using a test wavelength of 540 nm.

Preparation of spleen lymphocytes of BALB/c nude mice Mice were killed and the spleens were chopped with two slides and filtered over a fine nylon mesh. Cells were washed three times in Hanks' balanced salt solution. Cells were finally suspended in 10 % FCS RPMI-1640 supplemented with benzylpenicillin 100 kU/L, streptomycin 100 mg/L. Microplate wells received spleen cell suspension (2×10^6/well) added with ConA 2.5 mg/L or LPS 5 mg/L, and incubated at 37 ºC, 5 % CO_2 for 72 h. Cell proliferation was estimated based on the method of MTT.

Neutral red phagocytosis assay of peritoneal macrophages Macrophages were obtained from the peritoneal exudates[13]. Mice peritoneal exudates cells (PECs) were harvested by peritoneal lavage using cold Hanks' solution. PECs were washed twice and resuspended in a RPMI-1640 medium containing 10 % FCS. Peritoneal macrophages were further isolated from the PECs by incubating the PECs (2×10^5/well) in a 96-well plate at 37 ºC, 5 % CO_2 for 2 h in a humidified atmosphere to allow peritoneal macrophages for adherence. The supernatants were discarded and 100 μL 0.075 % neutral red were added and incubated for another 1 h. Cells were then washed with PBS for three times and incubated with cell lysis buffer (1 mol/L acetic acid: ethanol=1:1) overnight. The result was recorded with a spectro-photometer, using a test wavelength of 540 nm.

Chick chorioallantoic membrane (CAM) assay[14-16] Three-day-old fertilized white Leghorn eggs were cracked, and embryos with intact yolks were placed in petri dishes. After 3 d of incubation (37 ºC and 3 % CO_2), a Glass Microfibre filters disc (Whatman) containing GLPP-treated serum or GLPP was applied to

the CAM of individual embryos. After 48 h of incubation, embryos and CAMs were observed with a stereomicroscope. The positive anti-angiogenic effect was scored when microvessels around the disc were obviously reduced after sample application or an obvious blockage of CAM vessel development was observed.

Human umbilical cord vascular endothelial cell (HUVEC) culture and proliferation assay HUVEC was isolated as the method of Jaffe *et al*[17]. The HUVEC detached by 0.1 % type I collagenase (Gibico) were resuspended in complete DMEM which contained 20 % new born calf serum (BCS), endothelial cell growth supplement (ECGS) 50 mg/L, heparin sulfate 40 kU/L, insulin 0.4 kU/L, streptomycin sulfate 100 mg/L, benzylpenicillin 100 kU/L. The cell suspension was then seeded on gelatin-coated 6-well culture plates (Costa, America) at a density of 4×10^7 cells/L and incubated under 5 % CO_2 at 37 ºC. Cultures were fed with fresh medium every 48 h. The typical cobblestone morphology was observed using an inverted microscope (OLYMPUS). For the proliferation assay, HUVEC were dispersed in 0.05 % trypsin-0.02 % edetic acid solution. Cell suspension (4×10^7 cells/L) was made with DMEM+20 % BCS, planted onto gelatinized 96-well culture plates (0.1 mL/well), and incubated at 37 ºC, 5 % CO_2 for 24 h. The media was replaced with 0.1 mL of DMEM+5 % BCS and GLPP 1, 10, and 100 mg/L was added, respectively, and incubated at 37 ºC, 5 % CO_2 for 48 h. Cell proliferation was estimated based on the method of MTT.

Statistical analysis Statisitical significance was determined by one-way ANOVA employing the computer SPSS statistic package. $P<0.05$ was considered significant.

RESULTS

Antitumor effect of GLPP on locally implanted sarcoma 180 in BALB/c mice *in vivo* GLPP 50, 100, 200 mg/kg inhibited growth of Sarcoma 180 by 35.2 %, 45.2 %, and 61.9 %, respectively (Tab 1).

Effect of GLPP on proliferation of PG cells *in vitro* GLPP did not inhibit PG cell proliferation *in vitro* when was added directly to the cultured medium; but GLPP (50, 100, and 200 mg/kg)-treated serum markedly inhibited PG cell proliferation by 22.5 %, 26.8 %, and 30.3 %, respectively (Tab 2).

Antitumor effect of GLPP on locally implanted PG human lung carcinoma cell in BALB/c nude mice *in vivo* GLPP 50, 100, and 200 mg/kg inhibited the xenograft (human lung carcinoma cell PG) in BALB/c nude mice markedly *in vivo* with inhibitory rate of 55.5 %, 46.0 %, 46.8 %, respectively (Fig 1, Tab 3).

Tab 1. The inhibition of Sarcoma 180 primary tumors in BALB/c mice by GLPP 50, 100, 200 mg/kg ig for 10 d *in vivo*. Tumor weight index means the ratio of tumor weight to body weight (g/g). *n*=10. Mean±SD. $^cP<0.01$ *vs* NS.

Groups	Dose/ mg·kg^{-1}	Body weight/g	Tumor weight/g	Tumor weight index/%	Inhibitory rate/%
NS	-	16.8±2.2	1.40±0.28	8.4±2.0	
GLPP	50	16.6±2.0	0.91±0.39^c	5.5±2.4^c	35.20
	100	16.1±2.4	0.77±0.29^c	4.6±1.8^c	45.25
	200	16.4±1.9	0.53±0.20^c	3.2±1.0^c	61.88

Tab 2. Effect of GLPP and GLPP-treated serum on proliferation of PG cells *in vitro*. *n*=6. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* NS-treated serum.

Groups	Dose/mg·L^{-1}	$OD_{540\ nm}$
RPMI-1640	-	0.123±0.009
GLPP	0.1	0.113±0.015
	1.0	0.120±0.014
	10.0	0.122±0.014
	100.0	0.119±0.008
	Dose/mg·kg^{-1}	$OD_{540\ nm}$
NS-treated serum	-	0.142±0.024
GLPP-treated serum	50.0	0.110±0.013^b
	100.0	0.104±0.015^b
	200.0	0.099±0.021^c

Lymphocyte proliferation assay Lymphocytes of nude mice treated with or without GLPP could be stimulated by LPS 5 mg/L but not by ConA 2.5 mg/L (Fig 2).

Neutral red phagocytosis assay of peritoneal macrophages GLPP 50, 100, and 200 mg/kg on nude mice did not have influence on neutral red phagocytosis (Tab 4).

GLPP inhibits angiogenesis The microvessels around the disc were obviously reduced at 48 h after 80 μg GLPP per disc or 10 μL GLPP (50 mg/kg)-treated serum/disc application indicating there was potent inhi-

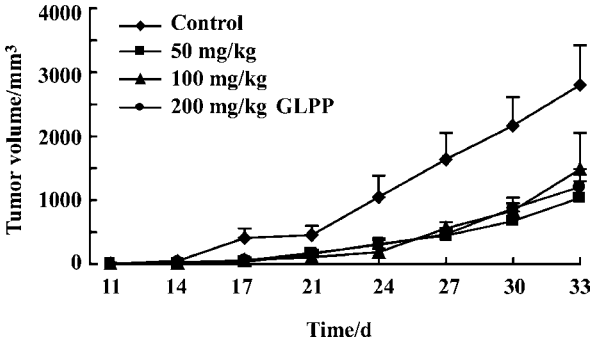

Fig 1. Inhibitory effects of GLPP on PG human lung cacinoma primary tumors of BALB/c nude mice. *n*=5. Mean±SD.

Tab 3. Antitumor effect of GLPP on PG carcinoma xenograft in BALB/c nude mice in *vivo*. *n*=5. Mean±SD. $^cP<0.01$ *vs* NS.

Groups	Dose/ mg·kg^{-1}	Body weight/g	Tumor weight/g	Tumor weight index/%	Inhibitory rate/%
NS	-	24.4±1.7	2.6±0.8	10.9±3.5	
GLPP	50	24.5±1.6	1.2±0.6^c	4.9±2.5^c	55.47
	100	22.7±1.5	1.4±0.7^c	6.3±3.2^c	46.04
	200	22.1±2.5	1.4±0.6^c	6.4±2.9^c	46.79

bition of angiogenesis (Fig 3). There was no evidence of toxicity in any of the chick embryos tested.

HUVEC proliferation assay GLPP 1,10, and 100 mg/L directly inhibited HUVEC cell proliferation *in vitro* with inhibitory rate of 9.4 %, 15.6 %, and 40.4 %, respectively (Fig 4).

DISCUSSION

In the present study, we investigated the antitumor activity of GLPP, a purified polysaccharide isolated from *Ganoderma lucidum*. The results showed that GLPP inhibited the growth of implanted Sarcoma 180 markedly, as the same as prior[5]. On the basis of the preceding *in vivo* results, we added GLPP or GLPP-treated serum directly to the PG Human lung Carcinoma cells. Unexpected results showed that GLPP could not inhibit their proliferation when it was added directly to the tumor cell cultures. While, GLPP-treated serum 50, 100, 200 mg/kg could markedly inhibit PG cells proliferation indicating that GLPP had no direct cell toxicity in tumor cells. And some experiments had proved

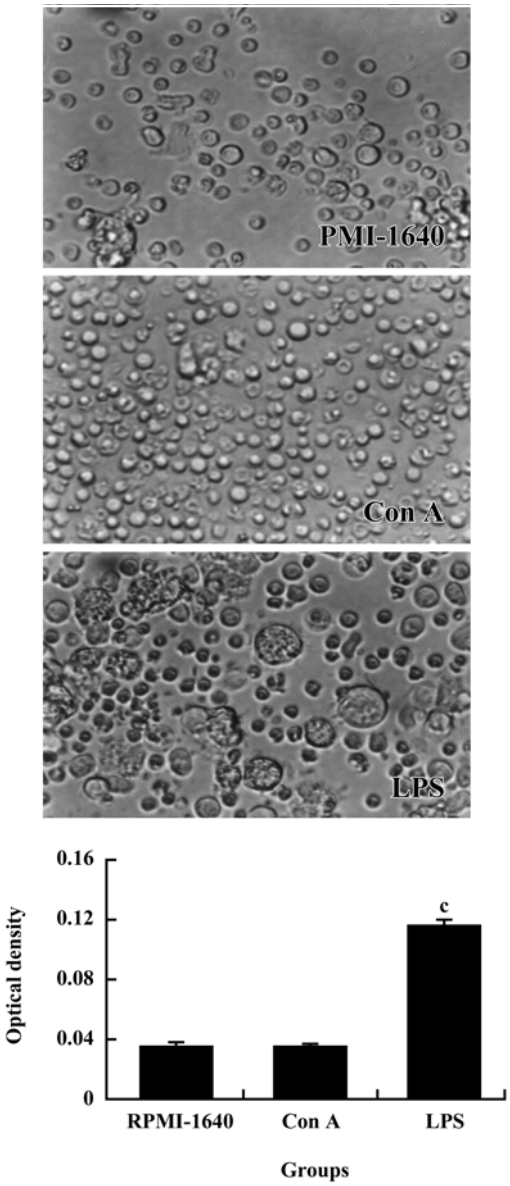

Fig 2. Lymphocyte proliferation assay of the nude mice. (×400). *n*=6. Mean±SD. $^cP<0.01$ *vs* RPMI-1640.

Tab 4. The influence of GLPP on phagocytosis of peritoneal macrophages of the nude mice. *n*=10. Mean±SD.

Groups	Dose/mg·kg^{-1}	$OD_{540\ nm}$
RPMI-1640	-	0.84±0.17
GLPP	50	0.86±0.08
	100	0.86±0.10
	200	0.84±0.10

that GLPP promoted TNF-α and IFN-γ release from immunocytes[4,5,18]. TNF-α and IFN-γ play important roles in suppressing tumor cell growth and inducing

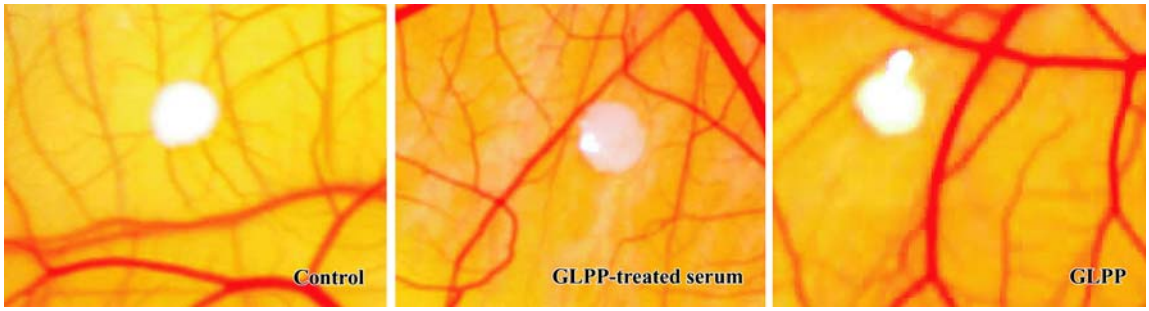

Fig 3. Inhibition of angiogenesis by GLPP 50 mg/kg -treated serum (10 μL per disc) or GLPP 80 μg per disc. *n*=5.

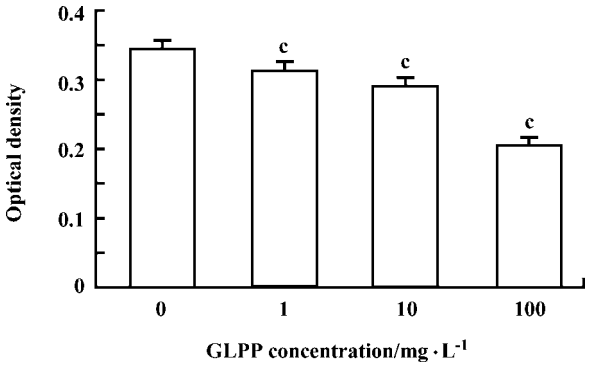

Fig 4. GLPP inhibited HUVEC proliferation in a dose-dependent manner. *n*=6. Mean±SD. $^{c}P<0.01$ *vs* control.

apoptosis of tumor cells. Many scholars had suggested that the anti-tumor effect of GLPP was mainly mediated by the host immunological mechanism. So we wanted to observe whether or not GLPP still have anti-tumor effect on immune-default nude mice.

We observed that at GLPP 50, 100, 200 mg/kg also inhibited the xenograft (human lung carcinoma cell PG) in BALB/c nude mice markedly *in vivo*. The lymphocytes of nude mice could be stimulated by LPS but not by Con A suggesting GLPP could not promote the T lymphocyte proliferation of nude mice. GLPP had no effects on neutral red phagocytosis of peritoneal macrophages in nude mice.

The above results demonstrated that the cell-mediated immunity was default in nude mice, but GLPP still has high anti-tumor activity on the mice, which indicated that other anti-tumor pathway was involved in anti-tumor effects of GLPP.

Tumor growth and metastasis is angiogenesis-dependent. To stimulate angiogenesis, tumors upregulate the production of a variety of angiogenic factors, including fibroblast growth factors (aFGF and bFGF) and vascular endothelial cell growth factor/vascular permeability factor (VEGF/VPF)[19]. Many malignant tumors, however, also generate inhibitors of angiogenesis[20], including angiostatin[21] and thrombospondin[22]. It is becoming clear that the angiogenic phenotype is the result of a net balance between these positive and negative regulators of neovascularization[21-23]. Anti-angiogenesis might represent an important mechanism underlying anti-tumor activity.

To test the ability of GLPP to inhibit angiogenesis *in vivo*, we used the chick chorioallantoic membrance (CAM) assay. GLPP or GLPP-treated serum potently inhibit angiogenesis. To determine whether or not GLPP had effect on the endothelial cell proliferation, HUVEC proliferation was measured. The results showed that GLPP directly inhibited HUVEC cell proliferation *in vitro* in accordance with our primary results that GLPP induced endothelial cell apoptosis.

There are two classes of angiogenesis inhibitors – 'direct' and 'indirect'. Direct angiogenesis inhibitors prevent vascular endothelial cells from proliferating, migrating or avoiding cell in response to a spectrum of pro-angiogenic proteins[24]. Indirect angiogenesis inhibitors generally prevent the expression of or block the activity of a tumor protein that activates angiogenesis, or block the expression of its receptor on endothelial cells. Many of these tumor-cell proteins are the products of oncogenes that drive that angiogenic switch[25,26].

Cytokines IFN-γ, IL-12, and IP-10 (interferon-inducible 10) could inhibit angiogenesis *in vivo* or *in vitro*[27-29]. IL-12 and IFN-γ also regulated the expression of tumor VEGF[30]. GLPP improved host immune functions and augmented cytokines production such as interferon-γ (IFN-γ) and IL-12[31]. So the angiogenic activity of GLPP may be dependent on both pathways: direct and indirect.

In conclusion, anti-angiogenesis of GLPP may be one of the mechanisms in its anti-tumor effects.

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