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Ganoderma lucidum polysaccharides peptide inhibits the growth of vascular endothelial cell and the induction of VEGF in human lung cancer cell

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Abstract

Ganoderma lucidum Polysaccharide Peptide (*Gl*-PP) has shown some effects as anti-tumors in mice and potential anti-angiogenesis. In this study, we elucidated the possible mechanism of *Gl*-PP action on anti-angiogenesis of tumor. Our research indicated that the proliferation of HUVECs was inhibited by *Gl*-PP in a dose-dependent fashion, but not because of cytotoxicity. Flow cytometric studies revealed that *Gl*-PP treatment of HUVECs could induce cell apoptosis directly. Moreover, addition of *Gl*-PP also led to a reduction of Bcl-2 anti-apoptotic protein expression and an increase of Bax pro-apoptotic protein expression of HUVECs. Therefore, inducing cell apoptosis by *Gl*-PP might be the mechanism of inhibiting HUVEC proliferation. Human lung carcinoma cells PG when exposed to high dose of *Gl*-PP in hypoxia for 18 h resulted in a decrease in the secreted VEGF. Taken together, these findings support the hypothesis that the key attribute of the anti-angiogenic potential of *Gl*-PP is that it may directly inhibit vascular endothelial cell proliferation or indirectly decrease growth factor expression of tumor cells.
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Keywords: *Ganoderma lucidum* polysaccharide peptide (*Gl*-PP); Anti-angiogenesis; Human umbilical cord vascular endothelial cell (HUVEC); Cell apoptosis; VEGF

Introduction

Angiogenesis, the formation of new blood vessels from preexisting ones, is necessary for the growth, progression and metastasis of solid tumors (Folkman, 1971, 1990; Hanahan and Folkman, 1996). In turn, tumor cells can secrete a variety of angiogenic factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which can stimulate angiogenesis as well (Hanahan and Folkman, 1996; Kerbel, 1991; Kerbel et al., 1998; Rak et al., 2002).

Ganoderma lucidum (Leyss, ex Fr.) Karst (*Gl*) has been widely used as medicine to promote health and longevity in China for thousands of years. The most attractive property of *Gl* is its anti-tumor effect (Xia et al., 1989; Liey et al., 1992; Wang et al., 1997; Zhang and Lin, 1999; Hu and Lin, 1999; Sliva et al., 2002; Liu et al., 2002; Lin et al., 2003a; Chen et al., 2004; Jiang et al., 2004; Gao et al., 2005), which has been demonstrated to be mainly associated with its polysaccharides

fraction by mediating immune system mechanisms (Xia et al., 1989; Liey et al., 1992; Wang et al., 1997; Zhang and Lin, 1999; Hu and Lin, 1999; Sliva et al., 2002; Chen et al., 2004). *Gl*-PP has also been shown to possess anti-angiogenic property by our research group, and anti-angiogenesis might be a new mechanism for anti-tumor effect of *Gl*-PP (Cao and Lin, 2004). However, the mechanism of *Gl*-PP on anti-angiogenesis remains to be determined.

Since vascular endothelial cell plays a key role in angiogenesis, we have chosen it for studying the anti-angiogenic mechanisms of *G. lucidum* Polysaccharides Peptide (*Gl*-PP) in vitro. Results from our laboratory showed that *Gl*-PP could inhibit the proliferation of vascular endothelial cells, but without cytotoxicity. Since the presence of *Gl*-PP in vascular endothelial cell culture affects cell proliferation, we want to know whether the vascular endothelial cells are undergoing programmed cell death. Our studies show that *Gl*-PP-treated HUVECs shifted in annexin V-FITC/PI-labeling increase by flow cytometry after about 48 h. In addition, increasing evidence suggests that Bcl-2 family proteins play a central role in cell apoptosis. Bcl-2 and Bax are the two prototype members of this large family of proteins, Western blotting

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revealed a reduction in Bcl-2 anti-apoptotic protein expression and an increase of Bax pro-apoptotic protein expression in *Gl*-PP-treated HUVECs. All of this suggests that *Gl*-PP can induce cell apoptosis of HUVEC.

On the other hand, *Gl*-PP has no distinct effect on tumor cell proliferation (Wang et al., 1997; Zhang and Lin, 1999; Sliva et al., 2002; Cao and Lin, 2004), demonstrated by the experiment in which we investigated the inhibitory effect of *Gl*-PP on the secretion of a primary angiogenic cytokine VEGF by human lung carcinoma cells.

Together, these results suggest that *Gl*-PP has an anti-angiogenic effect on tumor, which takes effect by directly inducing vascular endothelial cell apoptosis or indirectly inhibiting growth factors secretion and expression in tumor cells.

Materials and methods

Gl-PP extracted from **G. lucidum**

G. lucidum Polysaccharides (*Gl*-PP) were isolated by extracting the fruiting body of *G. lucidum* (Leyss. ex Fr.) Karst (*Gl*) with boiling water. The content of *Gl*-PP in the *G. lucidum* is 2.33%. It is a polysaccharide peptide with a molecular weight of 512,500, and the ratio of polysaccharides to peptide is 94.84%:5.16%. The polysaccharides consist of D-rhamnose, D-xylose, D-fructose, D-galactose and D-glucose linked together by β-glycosidic linkages with molar ratios of 0.549:3.614:3.167:0.556:6.89 (Lin et al., 2003b). It is a hazel-colored water soluble powder, kindly provided by Fuzhou Institute of Green Valley Bio-Pharm Technology.

Human umbilical cord vascular endothelial cell (HUVEC) culture and identify

Human umbilical cords were obtained from Beijing Woman and child health care hospital, with permission from the Ethical Society. HUVEC was isolated using the method of Jaffe et al. (1973). The HUVECs detached by 0.1% type I collagenase (Gibco) were resuspended in complete DMEM with 20% new born calf serum (BCS), 50 μg ml^{-1} ECGS (Endothelial Cell Growth Supplement), 40 u ml^{-1} heparin sulfate, 0.4 u ml^{-1}

Insulin, 100 μg ml^{-1} streptomycin sulfate, and 100 U ml^{-1} penicillin. The cell suspension was then seeded on gelatin-coated culture plates (6-well culture plates, Costa, America) at a density of 40,000 cells ml^{-1} and incubated under 5% CO_2 at 37 °C. Cultures were fed with fresh medium every 48 h. The typical cobblestone morphology was observed using an inverted microscope (OLYMPUS). Immunofluorescence staining of human VIII factor (specially expressed in vascular endothelial cell) was visualized using Laser confocus scanning microscopy.

HUVEC proliferation assay

HUVEC were harvested using 0.05% trypsin−0.02% EDTA solution. Cells were suspended (40,000 cells ml^{-1}) in DMEM with 20% BCS, plated onto gelatinized 96-well culture plates (0.1 ml/well), and incubated at 37 °C, 5% CO_2 for 24 h. The media were replaced with 0.1 ml of DMEM with 5% BCS and incubated for 24, 48 and 72 h, or with the addition of (1, 10, 100 μg ml^{-1} *Gl*-PP) incubated for 48 h at 37 °C, 5% CO_2. Cell proliferation was determined using MTT assay (Carmichael et al., 1987).

Cytotoxicity assays

The cytotoxicity of *Gl*-PP was determined by the lactate dehydrogenase (LDH) assay which measures the activity of LDH released into the medium from dead cells as a result of cytotoxicity. Cell-free culture supernatants from *Gl*-PP (0, 10, 100 μg ml^{-1} *Gl*-PP) treated HUVECs were collected and then measured using LDH assay.

Flow cytometry of HUVEC stained with Annexin V-FITC/PI

Annexin V, a calcium-dependent phospholipids-binding protein with a high affinity for phosphatidylserine, was used to detect cell apoptosis. 200,000 cells were plated onto gelatinized culture flask in DMEM containing 20% BSC, and incubated at 37 °C, 5% CO_2 for 24 h. The media was replaced with 1 ml of DMEM with 5% BCS and the 1, 10, 100 μg ml^{-1} *Gl*-PP, and incubated at 37 °C, 5% CO_2 for 48 h. Cells were harvested, washed with PBS and resuspended in
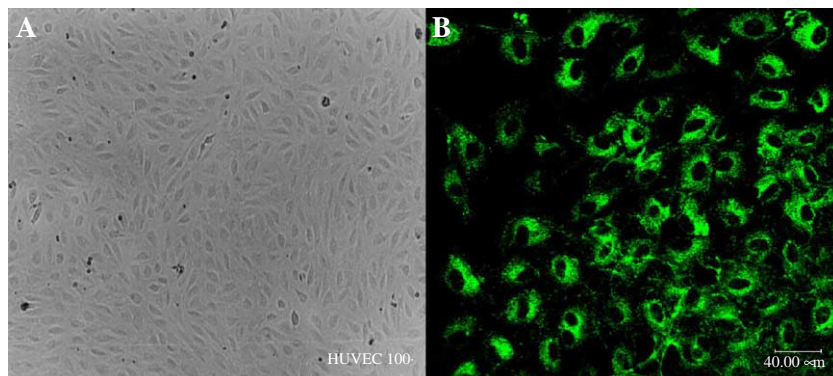

Fig. 1. (A) Morphology of cultured HUVEC. (B) Immunofluorescence staining of human VIII factor (specially expressed in vascular endothelial cell) in HUVEC.

binding buffer (10 mM HEPES/NaOH, pH7.4, 140 mM NaCl, 2.5 mM CaCl$_2$). 10 µl of Annexin V-FITC and 5 µl of PI were added to each sample and the cells were incubated in the dark for 15 min, then resuspended in 300 µl of binding buffer before flow cytometric analysis. In each sample, a minimum of 10,000 cells were counted and stored in list mode. Data analysis was performed using standard Cell Quest software (Becton-Dickinson).

Western blot analysis

HUVEC treated with *Gl*-PP for 48 h were harvested and washed three times with PBS, and resuspended in 50 µl lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl$_2$, 1 mM EDTA, 1% Triton-100, 0.1% SDS, 1 mM PMSF). After centrifugation, the supernatant was collected and the protein contents were determined using Bradford reagent. Electrophoresis was performed on 12% SDS-PAGE, and with a loading of 60 µg protein in each lane. After transferring to a nitrocellulose membrane, membranes were subsequently preblocked in TBS containing 5% nonfat milk power and then incubated with Rabbit polyclonal anti-Bcl-2 antibody, or mouse monoclonal anti-Bax antibody 1 : 100 (Santa Cruz Biotechnology, Inc). The bands were visualized using a secondary HRP-coupled anti-rabbit or anti-mouse (1 : 2000) antibodies (Santa Cruz Biotechnology, Inc) and ECL-detection system (Santa Cruz Biotechnology, Inc).

VEGF secretion in cancer cell

PG lung carcinoma cells were grown in 24 well plates in complete medium and incubated at 37 °C, 5% CO$_2$ for 24 h. The medium was replaced with RPMI-1640 with 5% FCS and the 0, 2, 20, 200 µg ml^{-1} *Gl*-PP, and incubated at 37 °C, 95% N$_2$/5% CO$_2$ for 18 h in hypoxia. Conditioned media were analyzed for VEGF protein content by an ELISA kit as per manufacturer's instructions (Jingmei Biotech).

Fig. 3. Inhibition of HUVEC proliferation by *Gl*-PP in vitro. *Gl*-PP was applied to HUVEC in a 48 h proliferation assay. Proliferation ratio (%) indicates the cell proliferation rate in different dose *Gl*-PP medium compared with DMEM when cell numbers are considered as 100%. Each bar represents the mean ± SD. $n = 6$, **$P < 0.01$ vs DMEM.

Statistical analysis

Statisitical significance was determined by one-way ANOVA using the computer SPSS (10.0) statistic package. *P* values below 0.05 were considered significant.

Results

HUVEC culture and identification

Cells were verified as endothelial by morphology and the presence of human VIII factor (also called Von Willebrand factor). Typical cobblestone morphology of cultured HUVEC is observed under microscopy (Fig. 1A), and human VIII factor was positive in culture HUVEC using immunofluorescence staining (Fig. 1B).

HUVEC growth curve

The third passage HUVECs were incubated in DMEM with 5% BCS for 24, 48 and 72 h, the cell proliferation result is shown in Fig. 2.

Effect of Gl-PP on the proliferation of HUVECs

Cells were incubated with different concentrations of *Gl*-PP (1, 10, 100 µg ml^{-1}) for 48 h. As shown in Fig. 3, *Gl*-PP at 1,
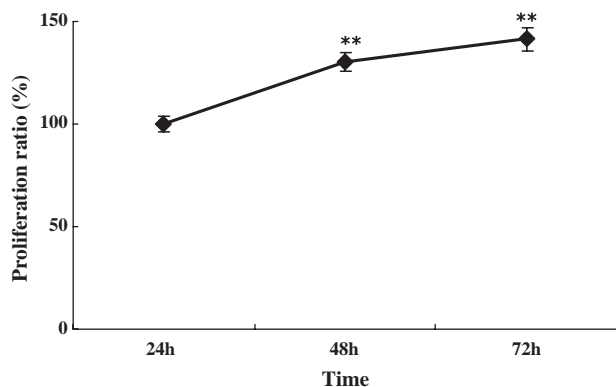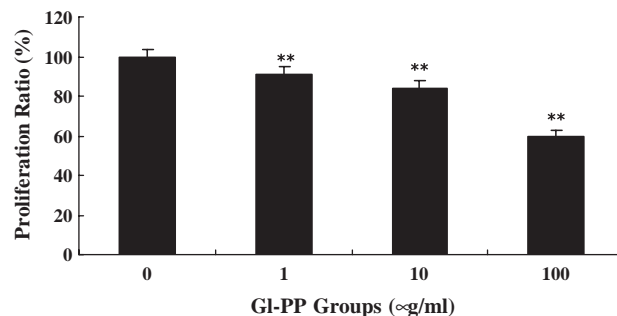

Fig. 2. The growth curve of HUVECs in DMEM with 5% BCS. Proliferation ratio (%) indicates the cell proliferation rate at other times compared with at 24 h when cell numbers are considered as 100%. $n = 4$, results are shown as mean ± SD, **$P < 0.01$ vs 24 h.
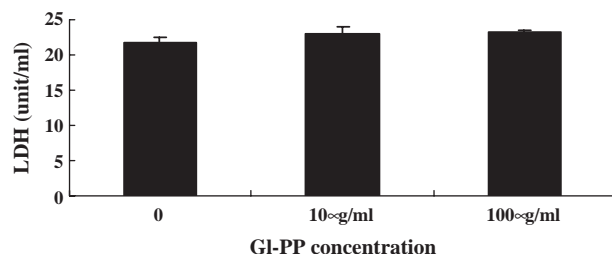

Fig. 4. LDH activity of released HUVEC treated with *Gl*-PP for 48 h. Each bar represents the mean ± SD, $n = 4$.
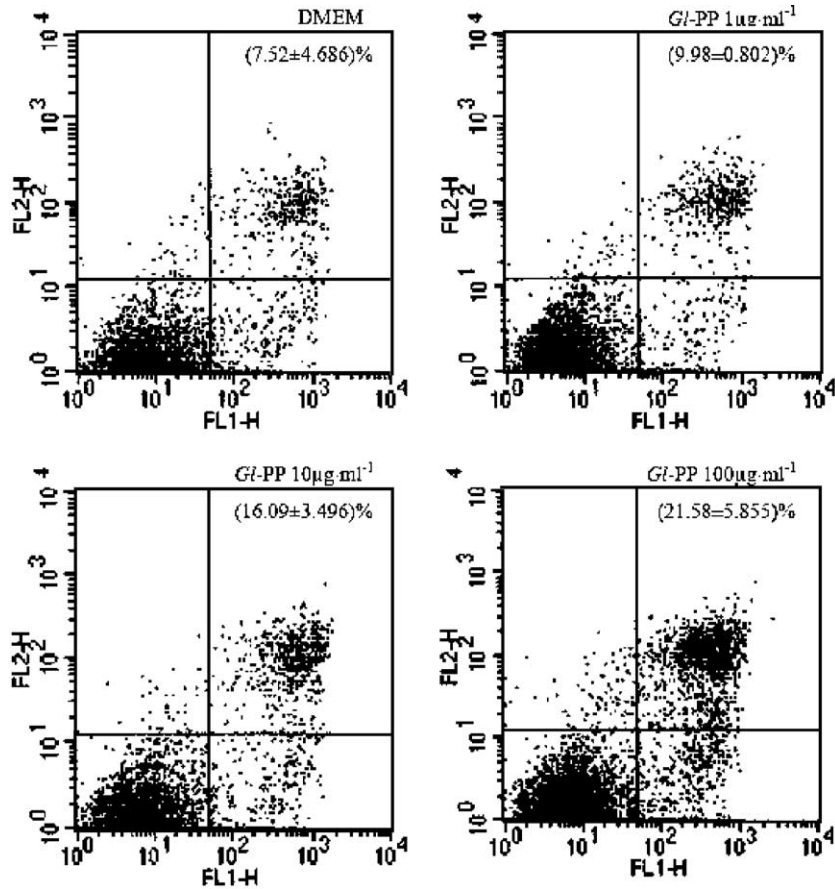
Fig. 5. HUVECs apoptosis induced by *Gl*-PP. Flow cytometry was carried out to quantify the percentage of HUVECs, after undergoing apoptosis treated with *Gl*-PP (1, 10, 100 μg ml^{-1}) for 48 h, DMEM were used as control. Cells were stained with Annexin V-FITC/PI, gating was performed to analyze Annexin V-FITC/PI positive. Density plots were produced and analyzed using the Cell Quest software package of a FACScan flow cytometer. FL1-H: FITC conjugated Annexin V; FL2-H: PI.

10, 100 μg ml^{-1} inhibited the proliferation of HUVECs in a dose-dependent fashion.

Influence of Gl-PP on LDH release from HUVEC

The cytotoxic potential of *Gl*-PP was determined by measuring the activity of LDH released into the medium from dead cells as a result of cytotoxicity. Cell-free culture supernatants from *Gl*-PP (0, 10, 100 μg ml^{-1}) treated HUVECs for 48 h were measured, and no significant difference was observed among them about the activity of LDH (Fig. 4).

HUVECs apoptosis induced by Gl-PP

Fig. 5 shows annexin V-FITC staining in conjunction with PI staining in HUVECs treated for 48 h with different concentrations of *Gl*-PP. *Gl*-PP at 10, 100 μg ml^{-1} showed a distinct shift in annexin fluorescence intensity. The mean fluorescence intensity is significantly different between control and *Gl*-PP (*$P < 0.05$, **$P < 0.01$ vs DMEM).

Gl-PP regulation of Bcl-2 protein level in HUVEC

Being the apoptosis inhibitor Bcl-2 protein was highly expressed in HUVEC. Two concentrations of *Gl*-PP (10, 100

μg ml^{-1}) had potential effects in decreasing the expression of apoptosis inhibiting protein Bcl-2 compared with DMEM control, 100 μg ml^{-1} *Gl*-PP decreased the Bcl-2 protein levels significantly ($P < 0.05$, Fig. 6).
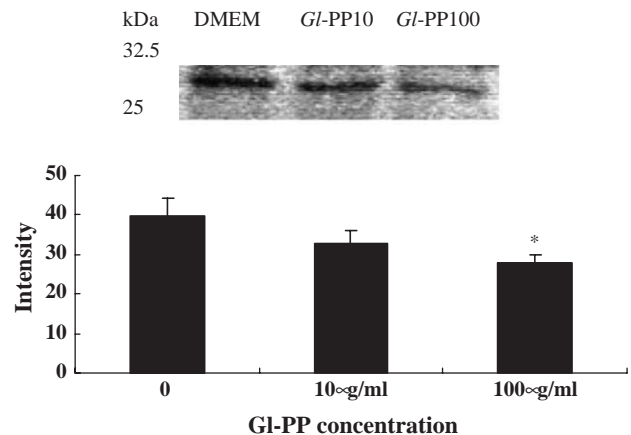

Fig. 6. Protein expression of Bcl-2 in HUVEC treated with *Gl*-PP for 48 h. The third passage HUVEC were incubated with *Gl*-PP at a concentration of 0, 10, 100 μg ml^{-1} for 48 h. 5% DMEM medium were used as control. Total protein was extracted and Bcl-2 expression was analyzed by Western blot. Molecular weight of Bcl-2 is 28 kDa. Each bar represents the mean±SD. $n = 3$, *$P < 0.05$ vs DMEM.
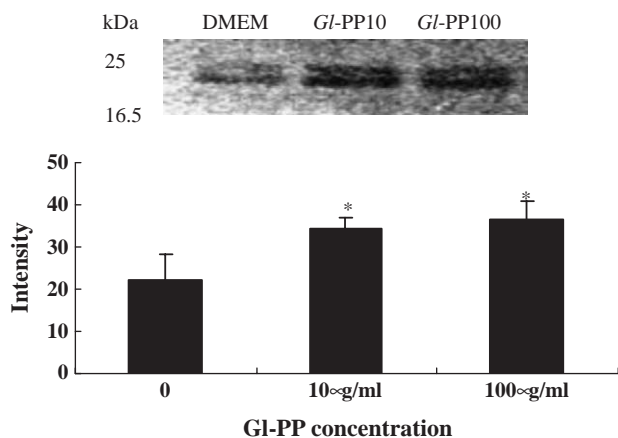
Fig. 7. Bax expression in HUVEC treated with *Gl*-PP for 48 h. The third passage HUVEC were incubated with *Gl*-PP at a concentration of 10, 100 μg ml^{-1} for 48 h, 5% DMEM medium were used as control. Total protein was extracted. And Bax expression of Bax was analyzed using Western blot. Molecular weight of Bax is 23 kDa. Each bar represents the mean±SD. $n=3$, *$P<0.05$ vs DMEM.

Gl-PP regulation of Bax protein level in HUVEC

Bax is the apoptosis promoter. Two concentrations of *Gl*-PP (10, 100 μg ml^{-1}) increased the expression of apoptosis inducing protein Bax compared with DMEM control (Fig. 7).

VEGF secretion by cancer cells

PG human lung carcinoma cells secrete VEGF in hypoxia. Different concentrations of *Gl*-PP were used to treat PG human lung carcinoma cells for 18 h in hypoxia, and 200 μg ml^{-1} *Gl*-PP decreased the secreted VEGF content significantly (Table 1).

Discussion

Many researchers have shown that *Gl* possesses anti-tumor activity. Possible mechanisms include enhancing immune function (Xia et al., 1989; Zhang and Lin, 1999; Hu and Lin, 1999; Sliva et al., 2002), induction of differentiation (Liey et al., 1992), etc. Recent studies revealed that *Gl* also had a potential effect of anti-angiogenesis which might be one of the important mechanisms on anti-tumor (Miura et al., 2002; Kimura et al., 2002; Cao and Lin, 2004).

Angiogenesis depends on several aspects such that the endothelial cells must proliferate to provide the necessary number of cells for the growing vessels, and the cells need to be capable of migration, etc. Due to the key role vascular endothelial cell plays in angiogenesis, we decided to explore the effect of *Gl*-PP on HUVEC.

HUVEC proliferation assay results showed that *Gl*-PP directly inhibited HUVEC cell proliferation in vitro. However, the mechanism of *Gl*-PP inhibitory effect on HUVECs is unclear, which could be the cytotoxicity effect? The cytotoxic potential was determined by measuring the activity of LDH released into the medium from dead cells (Sepp et al., 1996;

Repetto et al., 2001). The results indicated that there was no significant difference in LDH activity between *Gl*-PP treated cells and control.

Because the presence of *Gl*-PP in HUVECs culture affects proliferation, we asked whether the endothelial cells are undergoing programmed cell death. After initiation of apoptosis, most cell types translocate the membrane phospholipids (PS) from the inner surface of the plasma membrane to the outside (Koopman et al., 1994; Zhang et al., 1997; Van-Engeland et al., 1998). PS can be detected by staining with an FITC conjugate of annexin V, 38-kDa protein that binds PS. Since HUVEC exposure time in *Gl*-PP was long (48 h), in addition to positive membrane staining with annexin V, most of the cells were also positive for annexin V and PI (Fig. 5), which indicated a more advanced stage of apoptosis. In addition, recent advances in the study of signaling pathways involved in apoptotic cell death have begun to elucidate the relationship between caspases, Bcl-2 family members, and mitochondria. Increasing evidence suggests that the Bcl-2 family proteins play a central role in regulating cell death. Bcl-2 and Bax are the two main members of this large family of proteins. Bcl-2 can prevent programmed cell death in response to numerous stimuli (Cho and Kim, 1998; Allen et al., 1998). Conversely, pro-apoptotic proteins such as Bax can accelerate cell apoptosis, and, in some cases, they are sufficient to cause apoptosis independent of additional signals (Allen et al., 1998). We investigated the whole cell extract of *Gl*-PP-treated HUVECs and the control, the results showed that *Gl*-PP reduced Bcl-2 expression and increased Bax expression in HUVEC. These findings suggest that *Gl*-PP induces apoptosis of HUVECs, which could be the cause for inhibiting endothelial cell proliferation.

There are two classes of angiogenesis inhibitors — 'direct' and 'indirect'. Direct angiogenesis inhibitors prevent vascular endothelial cells from proliferating, migrating or responding to a spectrum of pro-angiogenic proteins (Kerbel, 1991). Indirect angiogenesis inhibitors generally prevent the expression of or block the activity of a protein that activates angiogenesis, or block the expression of its receptor on endothelial cells. Many of these tumor-related proteins are the products of oncogenes that drive and activate the angiogenic switch (Kerbel et al., 1998; Rak et al., 2002).

Research has shown that VEGF is expressed in many types of solid tumors (Ramakrishnan et al., 2000; Karth et al., 2000; Wong et al., 2003; Koukourakis et al., 2004), generally mediated by transcriptional activator known as hypoxia inducible factor 1 (HIF-1) in carcinoma (Forsythe et al.,

Table 1
Effect of *Gl*-PP on VEGF expression in culture supernatants of PG cell in hypoxia

Gl-PP dose (μg ml^{-1})	VEGF (pg/ml) of PG in hypoxia
0	258.68±2.348
20	256.37±3.653
200	239.99±2.935**

$n=3$, mean±SD; **$P<0.01$ vs control.

1996; Karth et al., 2000; Laughner et al., 2001; Wong et al., 2003). In hypoxia, VEGF expression could be induced. VEGF plays a key role in angiogenesis progression (Brown et al., 1997; Veikkola et al., 1999; Zimmermann et al., 2001). Our studies suggested that *Gl*-PP not only showed inhibitory effect on vascular cell proliferation, but also exerted an inhibitory action on the secretion of VEGF by human lung carcinoma cells in hypoxia (Table 1).

In summary, the anti-angiogenic activity of *Gl*-PP is achieved through directly inhibiting vascular endothelial cells proliferation or indirectly decreasing growth factor secretion of tumor cells.

Acknowledgments

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