The Antitumor Activity of *Ganoderma lucidum* (Curt.:Fr.) P. Karst. (Ling Zhi) (Aphyllophoromycetideae) Polysaccharides Is Related to Tumor Necrosis Factor-α and Interferon-γ

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**ABSTRACT:** In the present study, the antitumor activity of GL-B, a polysaccharide isolated from *Ganoderma lucidum* (Curt.:Fr.) P. Karst. and its mechanism were studied in vivo and in vitro. The results were as follows: (1) GL-B 50, 100, 200 μg ml⁻¹ inhibited the growth of implanted Sarcoma 180 in vivo significantly and dose dependently. (2) GL-B directly added to the culture medium neither induced HL-60 apoptosis nor restrained its proliferation in vitro. (3) The macrophage or T lymphocyte culture medium treated with GL-B (GL-B-M-CM or GL-B-T-CM) 50, 100, and 200 μg ml⁻¹ significantly induced HL-60 apoptosis and inhibited its proliferation. GL-B significantly increased tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) release in dose-dependent and time-dependent instances. (4) As untreated macrophages and T lymphocytes produced little or no TNF-α and IFN-γ, and macrophage culture medium with normal saline (N-M-CM) or T lymphocyte culture medium with normal saline (N-T-CM) did not inhibit HL-60 proliferation or induce its apoptosis, it seemed that the antitumor activity of GL-B was related to apoptosis induced by TNF-α-release from macrophages and IFN-γ-release from T lymphocytes.

**KEY WORDS:** Apoptosis, *Ganoderma lucidum*, HL-60, interferon-γ, macrophage, polysaccharides, proliferation, Sarcoma 180, T lymphocyte, tumor necrosis factor-α.

**ABBREVIATIONS**

- Con A: concanavalin A
- CY: cyclophosphamide
- ELISA: enzyme-linked immunoabsorbent assay
- FCS: fetal calf serum
- IFN-γ: interferon-γ
- LPS: lipopolysaccharide
- NBS: newborn bovine serum
- PEC: peritoneal exudate cells
- PI: propidium iodide
- TNF-α: tumor necrosis factor-α
- TNF-β: tumor necrosis factor-β

Many investigators have demonstrated that the polysaccharides isolated from *Ganoderma lucidum* could significantly inhibit the growth of implanted Sarcoma 180 and Lewis lung carcinoma in animal models in vitro (Sasaki, 1971; Miyazaki and Nishiyama, 1981; Sone and Okuda, 1985; Maziyama et al., 1989). Although its antitumor activity is beyond doubt, the mechanisms remain unclear. Some scholars have suggested that its antitumor activity may be mediated to
some extent by the activation of host immune functions. However, such a hypothesis requires substantial evidence. In this article, we describe our investigation of the antitumor activity of GL-B, a mixture of partially purified polysaccharides isolated from G. lucidum, at a cellular and molecular level both in vivo and in vitro.

MATERIALS AND METHODS

Animals

Inbred male and female 1–2-month-old (body weight 18–25g) BALB/c mice were purchased from the Department of Experimental Animals, Beijing Medical University.

Drug

*Ganoderma lucidum* polysaccharide (GL-B) consists of seven fractions of polysaccharide isolated from *G. lucidum*. It is a yellowish and water-soluble powder with molecular weights of 7,000 to 9,000 provided by the Department of Phytochemistry, College of Pharmacy, Beijing Medical University (Li and He, 1991).

Cell Lines

HL-60 and Sarcoma 180 were obtained from Beijing Tumor Institute. L929 cells were provided by the Department of Immunology, Beijing Medical University. RPMI 1640 powder was from Gibco BRL. MTT, lipopolysaccharide (LPS), and concanavalin A (Con A) were from Sigma.

Preparation of T Lymphocytes and Peritoneal Macrophages

Mice were killed and the spleens were chopped with two slides and filtered over a fine nylon mesh. Cells were washed three times in Hank’s balanced salt solution containing 5% heat-inactivated newborn bovine serum (NBS). Cells were finally suspended in RPMI-1640 supplemented with 100 IU ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 1 mmol l⁻¹ sodium pyruvate, 2 mmol l⁻¹ L-glutamine, 3.4 × 10⁻³ mmol l⁻¹ 2-mercaptoethanol, and 10% NBS. Rat peritoneal exudate cells (PECs) were harvested by peritoneal lavage using cold Hank’s solution containing 5% fetal calf serum (FCS). PECs were washed twice and resuspended in an RPMI 1640 medium containing 10% FCS. Peritoneal macrophages were further isolated from the PECs by incubating the PECs in a 24-well plate at 37°C in a humidified atmosphere for 2–4 h to allow for peritoneal macrophage adherence. After this time, the nonadherent cells were removed by washing three times with warm RPMI 1640 medium. More than 95% of the adherent cell population was macrophages, as determined by morphology and esterase staining.

Conditioned Media

To investigate the effect of GL-B on stimulating cytokine production by T lymphocytes and macrophages, and the effect of GL-B conditioned media with T lymphocytes and macrophages on proliferation and apoptosis of tumor cells, a pure population of macrophages or T lymphocytes was incubated separately in an RPMI 1640 medium containing 10% NBS with or without various concentrations of GL-B at 37°C for 12–72. The conditioned media, which were called macrophage culture medium with GL-B (GL-B-M-CM) and T lymphocyte culture medium with GL-B (GL-B-T-CM), were then collected, filtered, and stored at −70°C, respectively, for use.

Assay of Cytokines

Conditioned media from GL-B stimulated macrophages or T lymphocytes were assayed for activity of tumor necrosis factor (TNF-α) and interferon-γ (IFN-γ). TNF-α was assayed by bioassay methods using L929 cells and IFN-γ by solid-phase enzyme-linked immunoabsorbent assay (ELISA) as described by the available kit.

Treatment of HL-60 Cells

HL-60 cells, maintained in an RPMI 1640 medium containing 10% FCS, were cultured at an initial concentration of 1×10⁵ ml⁻¹ in the presence or absence of 20% (vol/vol) of GL-B stimulating conditioned media or normal media. For detecting the direct effect of GL-B on HL-60
growth, the culture was treated with GL-B at different concentrations. Cultures were then incubated at 37°C in a humidified atmosphere for 3 days.

Antitumor Experiment in Tumor-Bearing Mice

Sarcoma 180 cells were injected subdermally into the axillary fossa of the right foreleg. The mice were divided into several groups randomly. Different doses of drugs were administered by stomach tube, once a day. The mice were killed 10 days later and the tumors were cut and weighed.

Analysis of Apoptosis

HL-60 cells were separated from the medium by centrifugation and washed with phosphate-buffered saline (PBS) after incubation. Hypodiploid DNA was analyzed using the method of propidium iodide (PI) labeling and flow cytometry. After washing, samples were resuspended and placed in the dark at 4°C. PI fluorescence of individual nuclei was analyzed using a FACSscan flow cytometer (Becton Dickinson).

Determination of Cells Proliferation

MTT (20 μl, 5 mg l⁻¹ every well) was added to the cell culture 4 h before the end of incubation. After incubation, gave away supernatant, and 150 μl of isopropanol was added and OD was assessed using the Enzyme Labeling Instrument (Bio-Rad).

Statistical Analysis

Results were expressed as X ± SD and analyzed by t-test to compare the difference between the groups with the Statistica software for Windows® (4.5, Statsoft Inc., 1993).

RESULTS

Antitumor Effect of GL-B on Locally Implanted Sarcoma 180 In Vivo

Table 1 shows the antitumor effect of GL-B on Sarcoma 180 in BALB/c mice in vivo. GL-B 50, 100, and 200-mg kg⁻¹ inhibited the growth of Sarcoma 180 in a dose-dependent instance. The inhibitory rates are 27.70%, 55.83%, and 66.70%, respectively. The inhibitory rate of the GL-B group treated with cyclophosphamide (CY) was higher than that of the GL-B or the CY group. Their inhibitory rates were 80.98%, 45.45%, and 75.95%, respectively (Table 2).

Effect of GL-B on Proliferation of HL-60 and Sarcoma 180 Cells In Vitro

On the basis of the above results, we further added GL-B directly to the in vitro HL-60 and Sarcoma 180 culture media. We unexpectedly found that GL-B had no effect on proliferation of either HL-60 or Sarcoma 180 cells (Tables 3 and 4). Table 3 shows that GL-B 400 μg ml⁻¹ slightly stimulated HL-60 cell proliferation.

| TABLE 1 | Antitumor Effect of GL-B on Sarcoma 180 in BALB/c Mice (n = 10, X ± SD) |
|------------------|------------------|------------------|------------------|------------------|
| Dose (mg·kg⁻¹) | Body weight (g) | Tumor weight (mg) | Inhibitory rate (%) |
| Groups          | Origin | | difference | | |
| NS              | 23.61 ± 1.40 | 10.32 ± 2.60 | 2.02 ± 0.16
| GL-B            | 24.05 ± 1.75 | 7.57 ± 1.82 | 1.46 ± 0.61 | 27.70 |
| 50              | 23.90 ± 1.62 | 6.33 ± 1.77** | 0.89 ± 0.45** | 55.83 |
| 100             | 23.52 ± 1.48 | 6.46 ± 1.46** | 0.67 ± 0.47** | 66.70 |
| 200             | 23.84 ± 1.43 | 5.84 ± 1.68** | 0.44 ± 0.54** | 78.02 |
| CY              | 20.20 ± 1.10 | 5.84 ± 1.68** | 0.44 ± 0.54** | 78.02 |

*p < 0.05 vs. NS; **p < 0.01 vs. NS
TABLE 2

Antitumor Effect of GL-B on Sarcoma 180 in BALB/c Mice (n = 10, \( \bar{x} \pm SD \))

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg( \times )kg(^{-1} ))</th>
<th>Body weight (g)</th>
<th>Tumor weight (mg)</th>
<th>Inhibitory rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Origin difference</td>
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</tr>
<tr>
<td>NS</td>
<td></td>
<td>23.14 ± 1.62</td>
<td>9.58 ± 2.11</td>
<td>1.87 ± 0.78</td>
</tr>
<tr>
<td>GL-B</td>
<td>100</td>
<td>22.78 ± 1.14</td>
<td>7.22 ± 1.73</td>
<td>1.02 ± 0.51 ( \Delta )</td>
</tr>
<tr>
<td>CY</td>
<td>20</td>
<td>22.87 ± 1.55</td>
<td>6.65 ± 1.42</td>
<td>0.45 ± 0.48 ( \Delta )</td>
</tr>
<tr>
<td>GL-B + CY</td>
<td>100+20</td>
<td>23.35 ± 1.34</td>
<td>6.71 ± 1.36</td>
<td>0.36 ± 0.17 ( ** )</td>
</tr>
</tbody>
</table>

\( ^{*}p < 0.05 \) vs. NS; \( **p < 0.01 \) vs. NS

\( \Delta p < 0.05 \) vs. GB + CY; \( \Delta \Delta p < 0.01 \) vs. GL-B + CY

Effect of GL-B on Inducing HL-60 Cells Apoptosis In Vitro

Table 5 indicates GL-B 50, 100, 200 \( \mu \)g ml\(^{-1} \) cannot induce HL-60 cell apoptosis; the proportions of apoptic cells were 0.87 ± 0.24%, 0.65 ± 0.31%, 0.63 ± 0.45%, respectively. The positive control drug Etopside Vp-16 significantly induced HL-60 cell apoptosis; the apoptic cells was 67.70 ± 4.04%.

Effects of GL-B-M-CM and GL-B-T-CM on Proliferation of HL-60 Cells In Vitro

Tables 6 and 8 show that every concentration of GL-B-M-CM and GL-B-T-CM significantly inhibited proliferation of HL-60 cells in vitro. Compared with RPMI 1640 and N-M-CM or N-T-CM groups, \( p < 0.05 \) or 0.01.

Effect of GL-B-M-CM and GL-B-T-CM on Inducing Apoptosis of HL-60 Cells In Vitro

Table 8 shows that 50, 100, and 200 \( \mu \)g ml\(^{-1} \) of GL-B-M-CM significantly induced HL-60 cells apoptosis; the proportions of apoptic cells were 18.81 ± 0.93%, 20.98 ± 1.57%, 23.00 ± 0.56%, respectively, compared with the N-M-CM group, 7.44 ± 1.07%, \( p < 0.01 \).

Table 9 shows that 50, 100, and 200 \( \mu \)g ml\(^{-1} \) of GL-B-T-CM significantly induced HL-60 cell apoptosis; the proportions of apoptic cells were 19.39 ± 1.13%, 21.94 ± 0.84%, 22.85 ± 1.49%, respectively, compared with the N-T-CM group, 7.75 ± 1.14%, \( p < 0.01 \).
TABLE 6
Effect of GL-B-M-CM on Proliferation of HL-60 Cells In Vitro (n = 8, X ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration mg·ml⁻¹</th>
<th>OD 570 nm</th>
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</thead>
<tbody>
<tr>
<td>RPMI 1640</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-M-CM</td>
<td></td>
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<tr>
<td>GL-B-M-CM</td>
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</table>

TABLE 7
Effect of GL-B-T-CM on Proliferation of HL-60 Cells In Vitro (n = 8, X ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration mg·ml⁻¹</th>
<th>OD 570 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640</td>
<td></td>
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<tr>
<td>N-T-CM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GL-B-T-CM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vp-16</td>
<td>80</td>
<td>0.208 ± 0.116 ΔΔ</td>
</tr>
</tbody>
</table>

TABLE 8
Effect of GL-B-M-CM on Apoptosis of HL-60 Cells In Vitro (n = 3, X ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration mg·ml⁻¹</th>
<th>Apoptotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640</td>
<td></td>
<td>1.29 ± 0.46</td>
</tr>
<tr>
<td>N-M-CM</td>
<td></td>
<td>7.44 ± 1.07</td>
</tr>
<tr>
<td>GL-B-M-CM</td>
<td>50</td>
<td>18.81 ± 0.93 ΔΔ</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>20.98 ± 1.57 ΔΔ</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>23.00 ± 0.56 ΔΔ</td>
</tr>
<tr>
<td>Vp-16</td>
<td>80</td>
<td>71.45 ± 2.43 ΔΔ</td>
</tr>
</tbody>
</table>

TABLE 9
Effect of GL-B-T-CM on Apoptosis of HL-60 Cells In Vitro (n = 3, X ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration mg·ml⁻¹</th>
<th>Apoptotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 1640</td>
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<tr>
<td>N-T-CM</td>
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<tr>
<td>GL-B-T-CM</td>
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</table>

FIGURE 1. Effect of GL-B on apoptosis of HL-60 cells incubated for 72 h in vitro.
(A) Control; (B) GL-B 50 µg·ml⁻¹; (C) GL-B 100 µg·ml⁻¹; (D) GL-B 200 µg·ml⁻¹; (E) Vp-16 80 µg·ml⁻¹.

FIGURE 2. Effect of GL-B-M-CM on apoptosis of HL-60 cells incubated for 72 h in vitro.
(A) Control; (B) GL-B 50 µg·ml⁻¹; (C) GL-B 100 µg·ml⁻¹; (D) GL-B 200 µg·ml⁻¹; (E) Vp-16 80 µg·ml⁻¹.
level during 24–48 h, and subsided after 72 h. The TNF-α level in the supernatant of 12.5–400 μg·ml⁻¹ GL-B cultured with macrophages rose during 24 h as the dose increased (Fig. 5).

**Effect of GL-B on IFN-γ Production of T Lymphocytes**

Figure 6 shows that the IFN-γ level in the supernatant of 200 μg·ml⁻¹ GL-B cultured with T lymphocytes rose in 24 h and subsided after 48 h. The IFN-γ level in the supernatant of 12.5–200 μg·ml⁻¹ GL-B cultured with T lymphocytes rose during 24 h as the dose was increased to 400 μg·ml⁻¹. At that level it started to subside (Fig. 7).

**DISCUSSION**

In the present study, we investigated the antitumor activity of GL-B, a purified polysaccharide isolated from *Ganoderma lucidum*. The results showed that GL-B inhibited the growth of implanted Sarcoma 180 significantly and in a dose-dependent, combination immunotherapy, GL-B with a chemotherapy such as cyclophosphamide achieved the best effect. On the basis of the preceding in vivo results, we added GL-B directly to...
FIGURE 5. Effect of different doses of GL-B on TNF-α induction in the supernatant of murine peritoneal macrophages cultured with LPS after 24 h incubation (n = 4, x ± SD).

The in vitro cultures of Sarcoma 180 and HL-60 cells. Unexpected results showed that directly adding GL-B to the in vitro tumor cell cultures cannot inhibit their proliferation, even at the very high concentration of 400 μg·ml⁻¹. GL-B also has no effect on inducing tumor cell apoptosis measured by using a FACScan flow cytometer. It seems that GL-B alone has no direct effect on proliferation of tumor cells.

In a further study, GL-B was first added to the culture medium of macrophages and T lymphocytes, then the conditioned GL-B-M-CM and GL-

FIGURE 6. Effect of 200 μg·ml⁻¹ GL-B in IFN-γ induction in the supernatant of murine spleen cells cultured with ConA after 3–72 h of incubation.
B-T-CM media were added to the in vitro HL-60 culture medium as a drug. The results showed that both GL-B-M-CM and GL-B-T-CM significantly inhibited proliferation of HL-60; the FACScan flow cytometer also found that both of them significantly induced HL-60 cell apoptosis.

To reveal further the mechanism of the antitumor effect of GL-B-M-CM and GL-B-T-CM, the level of TNF-α in GL-B-M-CM and IFN-γ in GL-B-T-CM were assayed by bioassay and ELISA, respectively. TNF-α and IFN-γ were known to play important roles in suppressing tumor cell growth and inducing apoptosis of many different kinds of tumor cells (Thomson, 1991; Malorni and Rainaldi, 1996). Many experiments showed that TNF-α and IFN-γ cooperated with each other in inducing tumor cell apoptosis (Volm and Mattern, 1995; Rawadi et al., 1996; Sveinbjörnsson and Rushfeldt, 1997). Our experimental results showed that the levels of TNF-α in GL-B-M-CM and IFN-γ in GL-B-T-CM rose significantly in a dose-dependent mode. Moreover, our results showed that there was a positive correlation between the level of TNF-α in GL-B-M-CM and IFN-γ in GL-B-T-CM and the antitumor effect of GL-B-M-CM and GL-B-T-CM.

In the present study, we investigated the antitumor activity and mechanism of GL-B. The results were as follows: first, GL-B did inhibit the growth of implanted Sarcoma 180 in vivo significantly and dose-dependently. Second, GL-B directly added to a culture medium neither induced HL-60 apoptosis nor restrained its proliferation in vitro. Third, the macrophage or T lymphocyte culture medium treated with GL-B significantly induced HL-60 apoptosis and inhibited its proliferation. GL-B significantly increased TNF-α and IFN-γ release dose- and time-dependently. TNF-α and IFN-γ were known to play important roles in suppressing tumor cell growth and inducing apoptosis of tumor cells. Finally, as untreated macrophages and T lymphocytes produced little or no TNF-α and IFN-γ, and N-M-CM or N-T-CM did not inhibit HL-60 proliferation or induce its apoptosis, it seemed that the antitumor activity of GL-B was related to promoting TNF-α release from macrophages and IFN-γ release from T lymphocytes.

REFERENCES


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