Polysaccharides isolated from *Phellinus gilvus* inhibit melanoma growth in mice

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Abstract

There is no information about the effect of polysaccharides from fungus, *Phellinus gilvus* (PG) on melanoma. The effect of PG on the proliferation and apoptosis of the B16F10 melanoma cell line was determined by a sulforhodamine B (SRB) and a sandwich enzyme-linked immunosorbent assay. The in vivo effect of PG on B16F10 melanoma cells allografted in athymic nude mice was investigated. PG decreased cell proliferation and increased cell apoptosis in a dose dependent manner in vitro. Also, PG significantly inhibits melanoma growth in mice. The PG anti-tumor effect in vivo was associated with a significant increase in the melanoma apoptosis rate. These findings support PG as a therapeutic agent against melanoma.

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1. Introduction

Cancer is one of the leading causes of death in the world despite newly developed tools for treatment and diagnosis. Among them, melanoma is a malignant neoplasm of melanocytes most frequently arising from the skin and the incidence and mortality rates have increased at annual rates of 2–3% for the last 30 years [1,2].

Relapse after curative surgical treatment of melanoma remains a significant clinical challenge [3,4] and trials of post-surgical adjuvant therapy have proved largely unsuccessful with the majority inducing severe side effects at therapeutically effective doses. In the present study, therefore, we have investigated the ability of a naturally-occurring product to inhibit melanoma growth with the view to developing new anti-tumor substances with low toxic potential.

In recent studies, several natural products have been investigated for their anti-tumor activity in vitro and in vivo [5–8]. Among these products, polysaccharides isolated from Basidiomycete mushrooms represent an unlimited source for anti-tumor activity [9].
Some of these polysaccharides, such as Lentinan (Lentinus edodes), Schizophyllan (Schizophyllum commune), and Krestin (Coriolus versicolor) are now used as adjuvant in the treatment of cancer disease [10–12].

PG used in this study is a mushroom belonging to the Hymenochaetaceae basidiomycetes and it is a new genus developed by agriculturists in 2003. PG has advantages over the other Phellinus mushrooms in that it has a very short growth period (3 months) making it cheaper to produce. In addition, our recent studies have demonstrated significant anti-inflammatory properties for PG, including inhibition of pulmonary inflammation [13], prevention of intraperitoneal adhesion under infectious circumstances [14], and promotion of dermal wound healing [15]. Thus, we predict that medicinal application of PG has much advantage in medical cost-cutting as well as benefits to adjuvant therapy in future.

We have also recently demonstrated that polysaccharides isolated from PG inhibit growth of gastric tumor in vivo (unpublished data) and proliferation of the sarcoma 180 tumor cell in vitro [16]. However, there is no information about the anti-tumor effect of PG on melanoma in vitro and in vivo. Here, we demonstrate that PG profoundly inhibits melanoma in vitro and in vivo and that this effect is associated with the induction of apoptosis.

2. Materials and methods

2.1. Drugs

The fruiting body of PG was kindly provided by Gyeongbuk Agricultural Technology Administration (Daegu, Korea). A seed culture was grown in a 250 ml flask containing 50 ml of PMP medium (2.4% potato/dextrose broth plus 1% malt extract, 0.1% peptone) at 28 °C on a rotary incubator at 150 rpm for 4 days. To obtain fruiting body of PG, a culture was grown in an oak sawdust block for 90 days. The yield of fruiting bodies was 97 g dried weight per block. It was extracted under optimal conditions of water extraction for maximal anti-tumor activity, distilled water (1:25) at 100 °C for 10 h (unpublished data). The recovery procedure for polysaccharides from water extract of PG followed an established method in our previous study [14,15]. Concentration of polysaccharides was determined by measuring total sugar by the anthrone method [17] with glucose as the standard material. Adriamycin (AM) was purchased from Sigma Chemical Co. (St Louis, MO). AM was prepared by dissolving 0.85% NaCl. All materials were stored at 4 °C until used.

2.2. Cell lines

The aggressively growing B16F10 murine melanoma cells were purchased from Korea Cell Line Bank (KCLB®, Korea). The cells were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of streptomycin, and 100 U/ml of penicillin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and performed using trypsin–EDTA to detach the cells from their culture flasks once or twice weekly (all from Life Technologies, Inc., Grand Island, NY).

2.3. Cell proliferation assay

Cell proliferation activity of PG or AM against B16F10 murine melanoma cells was evaluated by SRB assay [18]. The B16F10 (1 × 10⁵ cells/well) were plated in four cycles of triplicates in 96-well plates and incubated for 24 h in a DMEM medium. The tumor cells were treated with 18.75–300 μg/ml of PG, 0.006–60 μg/ml of AM, or no additions (control) and further incubated for 48 h. After incubation, 50% trichloroacetic acid (TCA) was added and fixation was allowed to proceed for 2 h at 4 °C. The TCA-fixed cells were stained for 30 min with SRB. After staining, Tris base (10 mM, pH 10.5) was added (all from Sigma Chemical Co.). The optical density was measured at 490 nm. Under each condition, the absorbance of each represented the cell proliferation activity (the absorbance of cells treated with PG or AM divided by the absorbance of the untreated cells).

2.4. Cell apoptosis assay

TMK-1 cells were grown, harvested, and replated as described above. Cell apoptosis was determined by a sandwich enzyme-linked immunosorbent assay
(Cell Death Detection ELISAplus; Boehringer Mannheim, IND) according to the manufacturer’s instructions.

2.5. Animals

Sixty female athymic nude mice at 7–8 weeks of age were purchased from Charles River Korea (Orient, Korea) and acclimatized under the controlled conditions for 1 week before the experiment. The animals were fed with commercial rat feed from Orient Inc., Korea. Food and water were provided ad libitum to the animals. The Guidelines for Animal Care and Use of Kyungpook National University approved the housing, care and use of animals, as well as procedures to minimize discomfort.

2.6. Tumor cell inoculation and administration of drug

B16F10 murine melanoma cells were harvested by trypsinization, centrifuged, resuspended in phosphate buffered saline (PBS) at a density of $1 \times 10^6$ cells/ml, and kept on ice.

Thirty mice were inoculated subcutaneously on the right flank with a 0.1 ml mixture ($10^6$ cells/100 ml PBS) of murine melanoma cells by using a 27-gauge needle. The drug treatment started 2 days after B16F10 inoculation. The mice received 0.3 ml intraperitoneal daily injections of PG (100 mg/kg per day, $n=10$, PG group), AM (0.1 mg/kg per day, $n=10$, AM0.1 group) or 0.85% NaCl ($n=10$, control group) from day 2 to day 10 after tumor cell inoculation. The number of surviving animals was examined every day.

Body weights of all animals were measured every 2 days during treatment period to detect life-threatening toxicity by drugs.

2.7. Immunohistochemistry of proliferating cell

Immunohistochemical staining for proliferating cell was performed on formalin-fixed, paraffin-embedded sections (4 μm) of melanoma tumors with the anti-bromodesoxyuridin-peroxidase (Anti-BrdU-POD, Roche Ltd, Mannheim, Germany). Sections were deparaffinized by xylene and rehydrated through a graded series of ethanol and PBS. The sections were refixed with ethanol/HCl (70%/2.3 M) for 10 min. After washing slides by PBS containing 10% FBS, the sections were stained by the diaminobenzidine (DAB) components using a DAB substrate kit (Vector Laboratories, Inc., CA) for 10 min. Using this procedure, proliferating cells are stained brown. Negative controls were run by replacing the DAB substrate solution with PBS.

2.8. Colorimetric TUNEL assay

Formalin-fixed paraffin-embedded sections (4 μm) of the melanoma solid tumors were subjected to non-radioactive system for apoptotic cells with the DeadEnd™ Colorimetric TUNEL System (Promega Corp., Madison, WI). Sections were deparaffinized by xylene and rehydrated through a graded series of ethanol and PBS. The sections were pretreated with proteinase K (20 μg/ml) for 10 min, rinsed with PBS, and refixed in 10% formalin in PBS for 5 min. After washing 10% formalin by PBS, the sections were incubated in terminal deoxynucleotidyl transferase (TdT) reaction mix for 60 min and rinsed with 0.3% hydrogen peroxide for 5 min to block endogenous peroxidase activity. Cell permeabilization was performed by
incubating the sections in streptavidin HRP diluted in PBS, which is detected using the DAB components. Using this procedure, apoptotic nuclei are stained dark brown. Omission of the TdT enzyme during processing was used as a negative control. Apoptosis was detectable by morphological findings, chromatin condensation, nuclear DNA fragmentation, or apoptotic bodies with a dark-brown microscope.

2.9. Statistical analysis

Values are expressed as mean±SD. Analysis of changes in tumor volume and weight among the control, PG, AM1-treated groups of mice was performed using analysis of variance followed by multiple comparisons and Fisher’s LSD test using the SAS statistical package (release 8.1; SAS Institute Inc., Cary, North Carolina, USA). Results were considered significant for \( P<0.05 \). The \( P \) values listed in the legends are for the overall analysis of variance.

3. Results

3.1. PG inhibits melanoma tumor growth in vitro

We conducted dose–response studies on proliferation and apoptosis of B16F10 murine melanoma cells in vitro to determine the effects of PG or AM on melanoma tumor growth. The cell proliferation activity was determined by the colorimetric SRB assay. The colorimetric assay determined the number of viable cells proliferating. PG inhibited the proliferation of B16F10 cells in a dose-dependent manner \( (R^2=0.99, P=0.0001) \) and AM also inhibited the proliferation of the tumor cells in a dose-dependent manner \( (R^2=0.91, P<0.0001) \). The minimum concentration of PG and AM that significantly induced the apoptosis of B16F10 melanoma cells after 48 h of incubation was 75 and 0.06 \( \mu g/ml \) \( (P<0.0001, \text{Fig. 1c and d}. \)

3.2. PG inhibits melanoma solid tumor growth in vivo

Athymic nude mice allografted with B16F10 cells were divided into control, PG, and AM1 treated groups. Tumor growth was monitored daily from day 10 to day 16 after tumor cell inoculation. Tumor in the control group grew rapidly, reaching an average volume of 1697.2±321.9 mm\(^3\) by day 16 after the inoculation of B10F10 melanoma cells. In contrast, the inhibition of tumor growth on mice that were administered PG or AM1 intraperitoneally was profound, with tumor volume remaining at an average of 801.3±290.8 or 678.9±280.8 mm\(^3\) \( (\text{Fig. 2a–c, } P=0.0258) \). There were no differences in tumor volumes between PG and AM1 groups. At 16 days after the inoculation of B16F10 cells, the tumors of all animals were removed carefully and the weights were recorded. Average of tumor weights in the control group were 0.99±0.3 g. Conversely, average tumor weights of mice treated with PG or AM1 were significantly reduced compared to that in control group \( (P=0.0128) \). Average weights of mice treated with PG or AM1 were 0.54±0.31 or 0.53±0.37 (Fig. 2d). These results indicated that PG has as significant an inhibitory effect as AM1 on melanoma tumor growth in nude mice.

3.3. PG extends survival of melanoma tumor bearing mice

Athymic nude mice allografted with B16F10 cells were divided into control, PG, and AM0.1 treated groups. Mice received drugs for 9 days from day 2 to day 10 after tumor cell inoculation. The effects of PG or AM0.1 on the survival rate of mice are shown in Fig. 3. The survival rate of mice treated with PG or AM0.1 was prolonged compared with that in control group. However, all mice treated with PG or AM0.1 died at day 21 after tumor cell inoculation (Fig. 3).
3.4. Body weight changes during treatment period

In the melanoma solid tumor experiment, neither life threatening toxicity nor a loss of body weight was observed in the experimental animals during the treatment with PG or AM1. In the survival experiment, although weight loss was initially observed in mice treated with PG on 2 day after beginning of treatment, the mice gradually recovered body weight by 8 day (Fig. 4a and b). In the control group, the mice gained weight abnormally from the end of treatment to death (data not shown). This may be attributable to enormous growth of melanoma in abdomen. These results indicate that in athymic nude mice chemoprevention therapy for melanoma with PG promotes survival and is associated with very low clinical toxicity.

3.5. PG promotes melanoma apoptosis in vivo

Immunostained sections from the control-, PG-, and AM1-treated groups of animals were analyzed for differences in proliferation and apoptosis to determine whether inhibition of tumor growth in vivo by PG and AM1 resulted from the inhibition of proliferation or the stimulation of apoptosis.
Antiproliferative effects of PG on melanoma tumors were determined by immunolocalization of Anti-
BrdU-POD from formalin-fixed, paraffin-embedded sections. There was a marginal difference in stain-
ing for the proliferation marker, BrdU, in melanoma tumors obtained from PG-treated athymic
nude mice compared with those from the control mice (28.4 ± 9.5 vs 50.6 ± 8.7%, P = 0.0601, 
Fig. 5a). AM1-treated mice (35.9 ± 7.9%, P = 0.0601) also showed marginal decrease in prolif-
eration compared with control. Apoptotic effects of PG on melanoma tumors were determined by in
situ cell death detection enzyme-linked colorimetric assay on formalin-fixed, paraffin embedded sec-
tions. Apoptosis was detectable by morphological findings, chromatin condensation, nuclear DNA
fragmentation, or apoptotic bodies. Melanoma tumors from PG-treated mice demonstrated a
statistically significant increase in apoptosis compared with those from the control (48.6 ± 5.3 vs
21.6 ± 7.7%, P = 0.0001). AM1-treated mice (43.5 ± 5.9%, P = 0.0001) also showed significant
increase in apoptosis compared with control. There was no significant difference in apoptosis between
PG and AM1 (Fig. 5b). These data suggested that the PG achieves anti-tumorigenic effects in vivo via induction of tumor cell apoptosis.

4. Discussion

World-wide, there are approximately 220 known species of *Phellinus* mushroom and it found mainly in tropical areas of America and Africa [19] with varying medicinal effects [20–26]. Here, we showed that polysaccharides isolated from a kind of *Phellinus*, PG suppressed the growth of B16F10 melanoma cells and prolonged survival rate in nude mice compared with controls. This is the first report of PG inhibiting melanoma growth in vivo and in vitro and suggests a potential therapeutic role for PG as an adjuvant for the treatment of melanoma.

In modern medicine, natural products like PG for the prevention or treatment of tumor are attractive chemoprevention agents because of their very low clinical toxicity compared with chemical anti-tumor drugs [27,28]. Indeed, recently, we have demonstrated the safety of a single orally-administered dose of PG in rats [29]. Furthermore, in this study, the absence of body weight changes during treatment provides supportive evidence for the low clinical toxicity of PG.

Previous studies, by others and ourselves, have shown that intraperitoneal injection of polysaccharides isolated from *Phellinus* mushroom was effective in inhibiting tumor growth and metastasis [30], pulmonary inflammation [13], and oxidant and hepatotoxic activity [23]. We also observed that the intraperitoneal route of PG administration was effective in inhibiting gastric tumor growth (unpublished data). Therefore, the intraperitoneal route was chosen similarly here in order to determine whether PG would inhibit melanoma growth in vivo.
Because the processes of proliferation generally modulate in vivo tumor growth, we investigated the modulation of this process by PG in our tumor model. Despite the significant antiproliferative effect of PG in vitro, we only observed a marginal difference in the antiproliferative effect of PG against melanoma cells in vivo. The discrepancy between the in vitro and in vivo effects of PG on the proliferation of melanoma cells is intriguing. The reason for this discrepancy may be because of the sensitivity of the assay. It is also possible that the effect that was observed (PG 28.4 ± 9.5% vs control 50.6 ± 8.7%, \( P = 0.0601 \), Fig. 5a), although not statistically significant, is biologically significant.

Recent study suggests that in melanoma, the mechanism of activation involves the triggering of apoptosis and angiogenesis [31,32]. Previous work has shown that Phellinus mushrooms inhibit tumor growth by a variety of mechanisms including activating macrophages through protein tyrosine kinase and protein kinase C [33], induction of anticarcinogenic phase II enzymes [25], inactivation of ERK1/2 and p38 MAP kinases [21] and stimulation of humoral and cell mediated immunity [34]. However, the role of Phellinus mushroom in inhibiting tumor growth by induction of apoptosis has not previously been demonstrated. In our model, we have demonstrated for the first time that the anti-tumorigenic mechanism of PG in vivo and in vitro most likely results from a significant induction of apoptosis. Our study supports the process of apoptosis as an important mechanism of PG anti-tumor effect. The implication of this observation is that PG may be used in synergy with other antiproliferative agents against melanoma. It is likely the mechanism of PG anti-tumor action involves multiple processes.

AM has been used for more than 30 years in treating a variety of malignancies [35]. A number of mechanisms have been postulated including intercalation into DNA, damage to DNA via free radical formation or interference with DNA unwinding [36]. Skladanowski and Konopa [37] and Zaleskis et al. [38] reported that AM induces apoptosis by induction of DNA fragmentation and cell shrinkage in tumor cells. In our study, AM1 treatment of melanoma tumors also demonstrated a significant increase in apoptosis in accordance with the results from Skladanowski and Konopa [37].

In summary, we have demonstrated for the first time that polysaccharides isolated from PG is capable of inhibiting melanoma growth in vivo. The mechanism of PG’s anti-tumor effects involved the promotion of cellular apoptosis. Further studies regarding the mechanism of anti-tumor effects of PG in vivo will assist our understanding of the potential for Phellinus mushrooms in clinical approaches to melanoma prevention and treatment. We are exploring other processes such as inhibition of angiogenesis by PG in mechanism of tumor.
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