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Immunomodulatory Effects of *Ganoderma lucidum* (W. Curt.:Fr.) P. Karst. (Aphyllophoromycetideae) on CD4+/CD8+ Tumor Infiltrating Lymphocytes in Breast-Cancer-Bearing Mice

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ABSTRACT: *Ganoderma lucidum* (Basidiomycetes) has been recognized as an immunomodulatory natural product. This study was performed in order to investigate the effects of *G. lucidum* polysaccharide (GL-PS) extract on tumor volume and T(CD4+/CD8+) ratio of tumor infiltrating lymphocytes (TILs) in breast cancer bearing mice. Initially, we performed the DTH (delayed type hypersensitivity) test for obtaining the most effective dose of GL-PS extract on induction of cell-mediated immune response. Then, we evaluated the effect of this dose on tumor volume and CD4+/CD8+ T subpopulations that infiltrated into cancerous breast tissue. Results indicated that GL-PS 100 mg/kg/day could effectively increase DTH response against sRBC in BALB/c mice. Furthermore, intraperitoneal injection of this extract in breast cancer bearing mice could increase T-cell infiltration into the tumor. Also, there was a significant increase in the CD4+/CD8+ ratio in tumor infiltrating lymphocytes as well as a decrease in tumor volume. We concluded that GL-PS can exhibit a potent immunomodulatory effect and may be used for potentiation of the immune system against diseases such as cancer and other conditions in which the immune response has been compromised.

KEY WORDS: *Ganoderma lucidum,* Ling Zhi or Reishi mushroom, delayed-type hypersensitivity, tumor infiltrating lymphocytes TILs, CD+/CD8+ ratio, breast cancer

INTRODUCTION

Ganoderma lucidum (W. Curt.:Fr.) P. Karst. (also known as Ling Zhi or Reishi) is a species of Basidiomycetes that belongs to family Ganodermataceae of Aphyllophoromycetideae (Yang et al., 2000; Wasser, 2005). Its water-soluble polysaccharide extract is widely used in traditional Chinese medicine for the prevention of various kinds of diseases, such as hypertension, bronchitis, arthritis, nephritis, gastric ulcer, tumors, and scleroderma (Arisawa et al., 1986; El-Mekkawy et al., 1998; Wasser and Weis, 1999; Yen and Wu, 1999; Kim et al., 2000; Su et al., 2000; Yang et al.,

ABBREVIATIONS

DTH: delayed type hypersensitivity; **FITC:** fluorescein isothiocyanate; **GLPS:** *Ganoderma lucidum* polysaccharide extract; **HEVs:** high endothelial venules; **TILs:** tumor infiltrating lymphocytes; **sRBC:** sheep red blood cell; **CR3:** complement receptor 3; **PBS:** phosphate buffer saline; **PE:** phycoerythrin; **RPE:** rphycoerythrin

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2000; Wasser, 2005). Also, *G. lucidum* has antifibrotic, cholesterol-lowering, and hypoglycemic effects (Park et al., 1997; Berger et al., 2004; Zhang and Lin, 2004; Wasser, 2005). The exact carbohydrate epitope responsible for the antitumor activity and its receptor have not been identified; however, the receptor CR3 (complement receptor 3) has been shown to bind the β-glucan polysaccharide with undefined side chains (Wang et al., 2002). *Ganoderma lucidum* appears to be very safe since oral administration of its extract does not display any toxicity (Eo et al., 1999a,b) the widespread use of *Ganoderma* spp. as traditional medicine also supports this presumption. In the present study, we purchased crude *G. lucidum* extract from Pharmanex Co. (CA, U.S.A.). According to the works of Wang and coworkers, the composition of this crude extract consists of 84.4% polysaccharides and 15.6% proteins. The active component in this crude extract is its carbohydrate fraction (Wang et al., 2002). In this work, we have investigated the effects of *G. lucidum* polysaccharide (GL-PS) extract on tumor volume and T(CD4+/CD8+) ratio of tumor infiltrating lymphocytes (TILs) in breast cancer bearing mice.

MATERIALS AND METHODS

Animals

Eight- to ten-week-old female inbred normal and breast cancer bearing BALB/c mice were purchased from Pasteur Institute, Tehran, Iran. They were given sterilized water and autoclaved standard mouse chow *ad libitum* throughout the study.

Crude Ganoderma lucidum Extract

Crude *G. lucidum* extract was purchased from Pharmanex Co. (CA, U.S.A.). It was dissolved in PBS (phosphate buffer saline) (pH 7.2-7.4) and then filtered through 0.22 μm mesh for further use.

Delayed-type Hypersensitivity (DTH) test

To evaluate the effects of GL-PS extract on DTH response, several groups of mice were injected intraperitoneally with serial concentrations (50, 100, 150, 200, 300, and 500 mg/kg/day) of the extract. The control group was injected with PBS following the same procedure. After 1 hr, mice were primed with 1×10^8 sRBC injected subcutaneously in the back. On day seven, the sensitized animals were challenged with 1×10^8 sRBC injected subcutaneously on the left hind footpad. The increase in the foot pad thickness was measured after 24, 48, and 72 hr by Mauser dial caliper (Germany), and the results expressed as the percentage increase in the foot pad thickness. The results were calculated according to the following formula: *Left foot pad challenged with sRBC – right foot pad challenged with saline × 100/right footpad challenged with saline* (Ebtekar and Hassan, 1993).

Tumor Transplantation and Evaluation of tumor Volume

Tumor tissue was separated from breast cancer bearing mouse and then transplanted subcutaneously to syngenic BALB/c mice. After developing the tumor, mice were divided in two groups. According to the results obtained from DTH test, the GL-PS optimum dose was selected. The first group was inoculated with 100 mg/kg/day GL-PS extract intraperitoneally for seven days and the second group was inoculated with PBS with the same procedure daily. At this stage, tumor volume was also evaluated in both groups three times before injection of GLPS on the first day, three days later, and on the last day before killing. The following formula $V = 1/2 \times LW2$, where V is the volume, L is the length, and W is the width, was used (Singh et al., 1996). After day seven, the animals were killed and the solid tumors were cut into small pieces with forceps and scalpel. The pieces were rinsed twice with phosphate buffer saline (PBS). The suspensions were passed through 150 μm stainless steel mesh and then the cells were washed twice and labeled with monoclonal antibodies.

Immunofluorescent Staining for Evaluation of Intratumor T-cell Subpopulations

For staining of the cells obtained from tumor tissues, fluorescent anti-CD4 and anti-CD8 antibodies (Serotec Co., Germany) were used. We established the reference immunophenotypic pattern using standard procedures. In this study, 100 µl of intratumor cells were treated as follows.

According to company protocol, each sample was immunostained with 10 µl mAbs [anti-CD4 conjugated with fluorescein isothiocyanate (FITC) and anti-CD8 conjucated with rphycoerythrin (RPE)] in flow-cytometry tubes. Samples were then kept in 4°C and in the dark for 30 min. Cell samples were counted on a Becton Dickinson Coulter flow cytometer with serial filter configuration. The analysis was focused on the lymphoid area according to the forward and side scatters. Double-stained cells were analyzed using Coulter software (Lal et al., 1988)

Statistical Analysis

All experiments were carried out in duplicate or triplicate. We used one-way ANOVA and Duncan test to determine which dosage had maximum effect on DTH response. Also, we used the T-test for comparison of tumor volume in the experimental and control groups. In all cases, p values below 0.05 were considered significant.

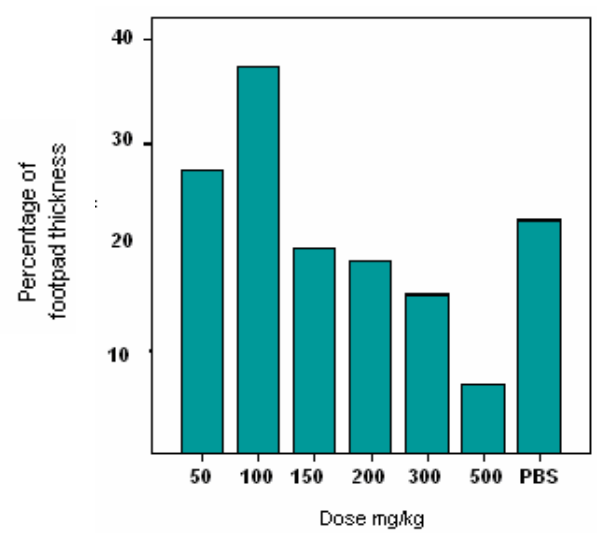

FIGURE 1. Effect of various concentrations of GLPS on DTH response after 24 hr.

RESULTS

Effect of GL-PS on DTH Response

In order to assess the effect of GL-PS on DTH response, the protocol mentioned above was performed. The results obtained by injection of various concentrations of GL-PS (50, 100, 150, 200, 300, and 500 mg/kg/day) were analyzed by Duncan test. The results indicated that GLPS at doses of 50 and 100 mg/kg/day had maximum enhancing effect on the DTH response ($p < 0.05$) after 24hr, and the 100 mg/kg/day dose had the strongest effect on the increase of DTH response ($p < 0.05$); whereas at doses of 300 and 500 mg/kg/day, GL-PS had a suppressive effect on DTH response, and GL-PS at doses of 150 and 200 mg/kg/day had no effect on DTH response when compared with the control group ($p > 0.05$) (see Fig. 1).

Effect of GL-PS on tumor Volume and Modulation of tumor Infiltrating Lymphocytes

The results obtained from tumor volume assay indicated that GL-PS extract caused a significant ($p < 0.05$) decrease in tumor volume in comparison to the control group (see Fig. 2).

Results from evaluation of the lymphocyte subpopulation following intraperitoneal inoculation of GL-PS extract indicated that this extract, at the dosage of 100 mg/kg/day, caused a significant ($p < 0.05$) increase in the CD4+ TILs and also in-

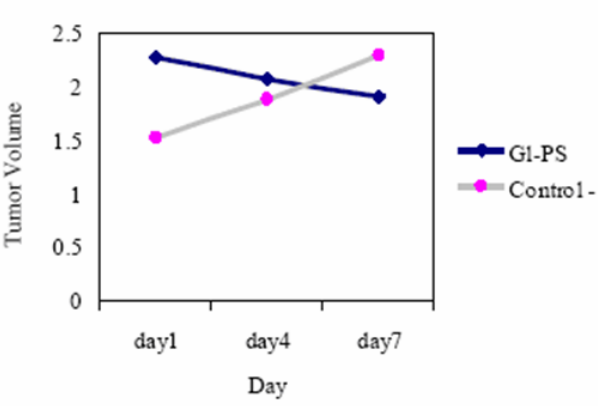

FIGURE 2. Tumor volume at three different days in the experimental group and control group.

creased the ratio of CD4+/CD8+ as compared to the control group (see Fig. 3).

DISCUSSION

Ganoderma lucidum, a medicinal mushroom, has been widely used to promote health and longevity in China and other East Asian countries. The fruit bodies, cultured mycelia, and spores of *G. lucidum* were reported to be effective in treatment of chronic hepatopathy, hypertension, and neophasia (Bao et al., 2002). This fungus has attracted considerable attention because its polysaccharide extracts have been shown to demonstrate antitumor activity (Miyazaki and Nishijima, 1981; Wang et al., 1997). Studies provide evidence indicating that β-D-glucan from medicinal mushrooms may induce biological response by binding to membrane complement receptor type three (CR3, aMb2 integrin, or CD11b/CD18) on the immune effector cells. The discovery of the specific receptor through which these compounds exert their effects may open a new field for further research.

In this study, we investigated the effect of GL-PS extract on tumor volume and T(CD4+/CD8+) ratio of tumor infiltrating lymphocytes in breast cancer bearing mice. Since the DTH test is a reliable marker of cellular immune response (Black, 1999; Descotes, 1999), we initially performed the DTH test to obtain the most effective dose of GL-PS extract. We used this simple test as a primary screening method to obtain a dose of GL-PS extract with the strongest stimulatory effect on cellular immune response. Then, based on the DTH test, the breast cancer-bearing mice were injected with the same dose and route.

This study indicates that GL-PS extract at 100 mg/kg/day has strong immunomodulatory properties, specifically augmenting the cellular branch of immune response. These findings correlate with many of the therapeutic effects reported for GL-PS. In most of the conditions indicated, a dominant cellular response could provide protection against disease progression. Also, according to the results obtained from the DTH test, GL-PS extract can suppress DTH at dosages of 300 and 500 mg/kg/day. While toxicity can be ruled out as a possible reason, the immunosuppressive mechanisms involved need to be further studied for the GL-PS extract at doses higher than 300 mg/kg.

Our findings from intraperitoneal injection of 100 mg/kg/day GL-PS showed that the GL-PS decreased tumor growth in breast cancer bearing mice. The mechanism of inhibition of tumor growth needs to be further studied. Also, intraperitoneal inoculation of GL-PS extract could cause a significant increase in the infiltration of CD4+ subpopulation of T lymphocytes. Today, the essential role of TILs in antitumor immune response is well identified. It has been shown that in tumor progression cases, T-cell infiltration into the tumor has decreased (Bernengo et al., 1983; Muhonen et al., 1994). Studies on cancer therapy using TILs proliferated *in vitro* and injected *ex vivo* indicate

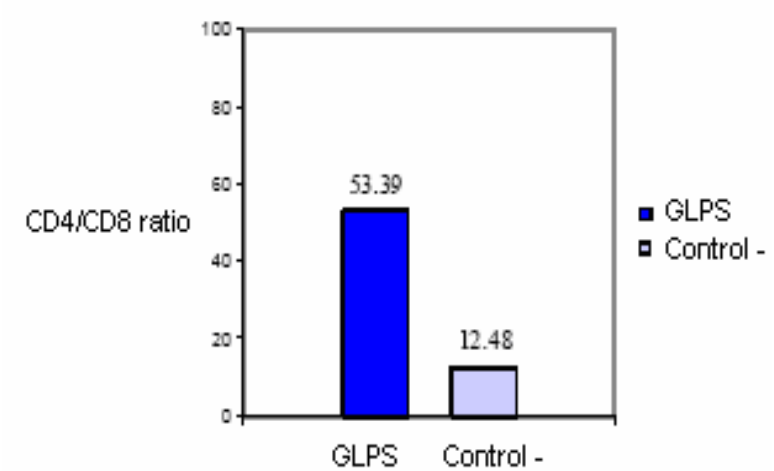

FIGURE 3. The T(CD4+/CD8+) ratio of TILs in the experimental group and control group.

that the injection of these TILs can help tumor treatment (Devita, 1998).

Our findings showed that GL-PS extract can highly increase infiltration of CD4+ T cells, thus, the administration of this extract can be considered as a method for T-cell recruitment into the tumor. This method has another advantage: we do not encounter the lymphocyte homing problem. In the case of an *in vitro* expansion, after *ex vivo* injection, a large number of injected lymphocytes cannot recognize tumor HEVs and therefore probably cannot enter tumor tissue. Further studies should be conducted to elucidate the underlying mechanism. As shown in Fig. 3, GL-PS extract had a stronger stimulatory effect on CD4+ TILs than CD8+ TILs, and thus significantly increased the T(CD4+/CD8+) ratio. In human tumors, there are some reports indicating that the increase in this ratio is related to a better prognosis, but in the current literature on this subject, there is no report to correlate this ratio to tumor prognosis in murine models. According to recent technical advances, it is now relatively easy to propagate a subset of CD4+ T cells from mice bearing aggressive tumors, which possess greater therapeutic potency than CD8+ T cells previously cultured from such animals. This subset of CD4+ T cells are notable for their low L-selectin (CD62 L) expression, and their striking ability to traffic rapidly into the tumors at diverse anatomic locations, including the pulmonary system, skin, and brain (Kjaergaad and Shu, 1999).

In other studies, it has become increasingly apparent that subpopulations of T lymphocytes present in tumor bearing mice possess an extraordinary ability to induce tumor rejection when adoptively transferred to syngenic mice bearing the same tumor (Kagamu and Shu, 1998). Naturally sensitized antitumor cells can be detected in mice bearing highly aggressive, weakly immunogenic tumors. Adoptive immunotherapy with such CD4+ T cells is typically effective only against immunogenic tumors (Kagamu and Shu, 1998). Rejection proceeds regardless of whether the tumor cells detectably express class II molecules or not (Cohen et al., 1994; Greenberg et al., 1998).

The GL-PS extract mediated immunopotentiation of DTH response and increased infiltration of T cell subpopulations in tumors are strong indications of the therapeutic potential of *Ganoderma lucidum* for cancer patients. The controlled manipulation of the immune response by natural pharmacological means is a highly sought goal of clinicians due to favorable therapeutic results and less side effects and toxicity.

Further research on the effect of GL-PS extracts on cytokine patterns, immune cell subtypes, and intracellular signaling events is necessary to shed light on the exact mechanisms and scope of the effect of this compound. Our study indicates that this medicinal mushroom may be administered for the prophylaxis and treatment of disease in immunocompromised patients.

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