Regulatory effect of *Ganoderma lucidum* polysaccharides on cytotoxic T-lymphocytes induced by dendritic cells *in vitro*[1]

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KEY WORDS *Ganoderma lucidum;* polysaccharides; dendritic cells; cytotoxic T-lymphocytes; serine endopeptidases

ABSTRACT

AIM: To study the regulatory effects of *Ganoderma lucidum* polysaccharides (*Gl*-PS) on cytotoxicity and mechanism of specific cytotoxic T-lymphocytes (CTL) induced by dendritic cells (DC) *in vitro* during the stage of antigen presentation. **METHODS:** Cultured murine bone marrow-derived DC were pulsed with P815 tumor cell lysates and co-incubated with or without various concentrations of *Gl*-PS (0.8, 3.2, or 12.8 mg/L) at the same time. P815 specific CTL were induced by spleen lymphocytes stimulated with mature DC. Non-adherent cells and culture supernatants were harvested on d 5 for analysis of specific cytotoxicity with lactate dehydrogenase (LDH) activity assay, mRNA expression of IFNγ, granzyme B with RT-PCR assay, and protein expression of IFNγ, granzyme B with ELISA or Western blot assay, respectively. **RESULTS**: Three concentrations of *Gl*-PS promoted LDH activities released into culture supernatants ($P<0.01$). It also increased mRNA expression of IFNγ in CTL (*Gl*-PS 12.8 mg/L *vs* RPMI medium 1640, $P<0.05$) and granzyme B in CTL ($P<0.01$). Protein production of IFNγ in culture supernatants ($P<0.05$) and protein expression of granzyme B in CTL (*Gl*-PS 12.8 mg/L *vs* RPMI medium 1640, $P<0.05$) were also augmented by *Gl*-PS. **CONCLUSION:** *Gl*-PS is shown to promote the cytotoxicity of specific CTL induced by DC which were pulsed with P815 tumor antigen during the stage of antigen presentation, and the mechanism of cytotoxicity is believed to be going through IFNγ and granzyme B pathways.

INTRODUCTION

Ganoderma lucidum (*Gl*) is effective in inhibiting tumor growth in animal experiments[1] and used as adjuvant of antitumor therapy in clinic[2-3]. Combination of *Ganoderma lucidum* polysaccharides (*Gl*-PS), one of the main efficacious ingredients of *Gl*, with antitumor drugs such as mitomycin or etoposide could antagonize the inhibitory effects of these drugs on mixed lymphocyte culture (MLC) [4]. The tumor inhibitory effects of *Gl*-PS have been deeply investigated[3, 5-9] and believed to be through immune mechanisms, such as cytokines secretion, induction of tumor cell apoptosis, *et al*.

Dendritic cells (DC), a kind of important professional antigen-presenting cells (APC), are pivotal for the initiation of primary immune response of both helper and cytotoxic T-lymphocytes[10-13]. Our previous study has demonstrated that *Gl*-PS could promote not only the maturation of cultured murine bone marrow-derived DC *in vitro*, but also the immune response initiation in-

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Received 2002-03-11 Accepted 2002-09-27

duced by DC[14]. Since DC play important roles in cytotoxic T-lymphocytes (CTL) induction and anti-tumor immunity[15,16], in the present study, we established the culture of specific CTL induced by DC which pulsed with P815 cell lysates, investigated whether *Gl*-PS had regulatory effects on specific cytotoxicity of CTL during the stage of antigen presentation, and further explored the mechanisms of CTL cytotoxicity.

MATERIALS AND METHODS

Animal Male or female C57BL/6j (H-2^b) mice (Grade II, Certificate No 01-3046) and BALB/c (H-2^d) mice (Grade II, Certificate No 01-3044) were purchased from the Department of Experimental Animal, Health Science Center, Peking University, Beijing, China. Mice were used at 6-8 weeks old.

Drugs *Gl*-PS was isolated from boiling water extract of wood-cultured *Gl*, followed by ethanol precipitation, dialysis, and protein depletion using Sevag method. It was a kind of polysaccharides peptide with molecular weight of 584 900. *Gl*-PS was a kind of hazel powder and dissolved in serum free RPMI medium 1640 (Gibco BRL), then filtered through a 0.22-μm filter and stored at 4 °C. They were further diluted to indicated concentrations (0.8, 3.2, or 12.8 mg/L) prior to each assay.

Preparation of bone marrow-derived DC and DC pulsed with P815 tumor antigen P815 was a kind of mastocytoma (H-2^d) and was maintained *in vitro* in a complete medium (RPMI medium 1640 supplied with 10 % FCS). Cell lysates were prepared from freshly cultured cells[17]. P815 cells were counted and suspended in serum-free RPMI medium 1640 at 1×10^{10} cells/L. These cells were disrupted by four cycles of freeze/thaw (-20 °C/37 °C water bath). The lysed cells were centrifuged at 600×*g* for 5 min, and the supernatants were collected and centrifuged at 13 000×*g* for 60 min. The final supernatants were collected and sterilized by passing through 0.22-μm filter and stored at -2 °C. DC (derived from C57BL/6j) were cultured and detected as described previously[14], except that on d 5, non-adherent cells were incubated at a concentration of 3×10^6 cells/L, pulsed with 1×10^9 cells/L P815 cell lysates, and treated with indicated concentration of *Gl*-PS at 37 °C for 18 h. Serum-free RPMI medium 1640 was used as control.

Induction of specific CTL[18] P815 specific CTL were induced by DC pulsed with P815 tumor antigen. Mature DC was pretreated with 25 mg/L mitomycin. Mononuclear lymphocytes were isolated from mouse splenocytes by Ficoll-Urografin density gradient. BALB/c mononuclear lymphocytes (1×10^7 cells/well) were cultured with DC (1×10^4 cells/well) in 24-well flat bottom tissue culture plates (2 mL/well) and incubated at 37 °C, 5 % CO_2 for 5 d. Non-adherent cells and culture supernatants were harvested for assay.

Lactate dehydrogenase (LDH) activity assay P815 cells (2×10^8 cells/L) were incubated with 1×10^9 cells/L CTL (effector cells:target cells=5:1) for 24 h. The activities of LDH released into culture supernatants were measured with LDH-cytotoxic test kit (Beijing Chem). LDH activity was determined with a standard curve.

RNA extraction and semi quantitative RT-PCR CTL were collected and washed once with diethylpyrocarbonate (DEPC)-treated PBS, and the total RNA were extracted with TRIzol reagent (Gibco, BRL) according to the manufacturer's protocol. RNA precipitates were dissolved in DEPC-treated double distilled water containing RNasin (R) ribonuclease inhibitor (Promega). The concentration of RNA was determined spectrophotometrically (DU640, BECKMAN). The mRNA expressions of IFNγ, granzyme B in CTL were performed by reverse transcription polymerase chain reaction (RT-PCR) with access RT-PCR system (Promega) according to the manufacturer's protocol. To ensure that equal amounts of starting material were used in each RT-PCR reaction, RNA was reverse transcripted and amplified with β-actin-specific primers. The cycle number of PCR amplification was 28, which was chosen to ensure that amplification of all specific cDNA products was exponential. Specific primer sequences of IFNγ[19], granzyme B[20], or β-actin×[19] were as follows and the size of production was 376, 330, or 478 bp, respectively: IFNγ sense primer: 5'-CACGGCACAGTCATTGA-AAGCCTA-3'; IFNγ antisense primer: 5'-TGAGGCTGGATTCCGGCAACA-GCT-3'; Granzyme B sense primer: 5'-CCCAGGCGCA-ATGTCAAT-3'; Granzyme B antisense primer: 5'-CCAGGATAAGAAACTCGA-3'; β-actin sense primer: 5'-AGGGAAATCGTGGGT-GACATCAAA-3'; β-actin antisense primer: 5'-ACTCATCGTACTCC-TGCTTGC-TGA-3'. All of the products were electrophoresed on 2 % agarose gel and stained with ethidium bromide. The expression intensities of two coamplified bands were quantified with Gel Doc 2000 system and Quantity One software (BIO-RAD), and expressed as a ratio (IFNγ

vs β-actin, granzyme B *vs* β-actin).

Cytokine assay The protein levels of mouse IFNγ in culture supernatants of CTL were determined with a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Jingmei Biotech) according to the manufacturer's protocol. Microtiter plates were coated with specific antibodies to capture IFNγ in cell culture supernatants. A second layer antibody was then added. Cytokine concentrations were determined with a standard curve derived from known amounts of IFNγ using absorbance reading at 450 nm on a spectrophotometer (BIO-RAD). The minimum detection level of IFNγ was 28 ng/L.

SDS-polyacrylamide electrophoresis and Western blot The protein levels of mouse granzyme B expressed in CTL were determined by Western blot. The total protein of CTL was extracted with TRIzol reagent (Gibco, BRL) according to the manufacturer's protocol. Protein contents were determined with Bradford reagent. Equal amounts of protein (30 μg) were subject to 12 % SDS-PAGE and transferred to nitrocellulose membrane (Amersham UK). The membranes were subsequently preblocked in TBS containing 5 % nonfat milk power and then incubated with goat polyclonal anti-granzyme B antibody (N-19) (Santa Cruz Biotechnology, Inc) at dilution of 1:100 followed by peroxidase-conjugated rabbit anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, Inc). The antigen-antibody complex was visualized with Western blotting luminol reagent (ECL, Santa Cruz Biotechnology, Inc). The bands were quantified with Gel Doc 2000 system and Quantity One software (BIO-RAD).

Statistical analysis Data were expressed as mean±SD. Statistical significance was determined by one-way ANOVA followed by least-significant difference (LSD). $P<0.05$ was considered significant.

RESULTS

Regulation of *Gl*-PS on CTL cytotoxicity LDH activities released into the culture supernatants of CTL and P815 co-culture were augmented when P815 specific CTL were induced by DC treated with *Gl*-PS (0.8, 3.2, or 12.8 mg/L) during the stage of P815 lysates pulsing. Significant difference was observed between each concentration of *Gl*-PS and RPMI medium 1640 control ($P<0.01$, Tab 1).

Regulation of *Gl*-PS on mRNA expression of IFNg in CTL mRNA of IFNγ were highly expressed in P815 specific CTL induced by DC treated with or without *Gl*-PS during the stage of P815 lysates pulsing (Fig 1). Three concentrations of *Gl*-PS (0.8, 3.2, or 12.8 mg/L) increased mRNA expression ratios of IFNγ (IFNγ *vs* β-actin) compared with RPMI medium 1640 control. Significant difference was observed between RPMI medium 1640 control and *Gl*-PS 12.8 mg/L ($P<0.05$, Tab 2).

Tab 1. LDH activity in culture supernatants of specific CTL and P815 co-culture. Specific CTL were induced by *Gl*-PS-treated DC pulsed with P815 cell lysates. *n*=3. Mean ±SD. $^cP<0.01$ *vs* RPMI medium 1640.

Group	Concentration/ mg·L^{-1}	10^{-3}×LDH activity/U·L^{-1}
RPMI medium 1640	-	1.63±0.12
Gl-PS	0.8	2.04±0.17^c
	3.2	2.61±0.06^c
	12.8	2.41±0.11^c

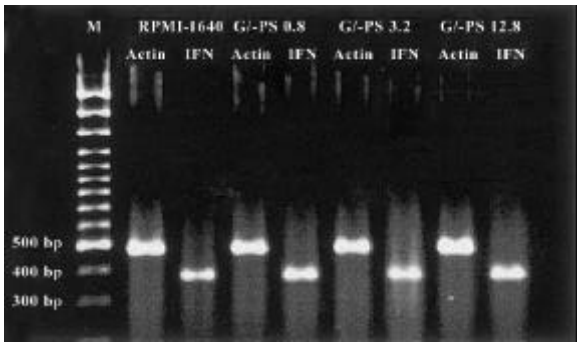

Fig 1. mRNA expression of IFNg and b-actin in CTL induced by *Gl*-PS-treated DC pulsed with P815 cell lysates. M: Marker; RPMI-1640: RPMI medium 1640; Actin: b-actin; IFN: IFNg.

Regulation of *Gl*-PS on protein production of IFNg in CTL The protein productions of IFNγ in culture supernatants of CTL were augmented when CTL were induced by DC treated with *Gl*-PS (0.8, 3.2, or 12.8 mg/L) during the stage of antigen presentation. Significant difference was observed between each concentration of *Gl*-PS and RPMI medium 1640 control ($P<0.05$, Tab 3).

Regulation of *Gl*-PS on mRNA expression of granzyme B in CTL mRNA of granzyme B were highly expressed in P815 specific CTL induced by DC treated with or without *Gl*-PS during the stage of P815 lysates

Tab 2. mRNA expression of IFNg in CTL induced by *Gl*-PS-treated DC pulsed with P815 cell lysates. The intensity ratio was expressed as ratio of IFNg *vs* b-actin. *n*=3. Mean±SD. [a]*P*>0.05, [b]*P*<0.05 *vs* RPMI medium 1640.

Group	Concentration/ mg·L^{-1}	Intensity ratio/%
RPMI medium 1640	-	77±7
Gl-PS	0.8	86±9[a]
	3.2	85±8[a]
	12.8	88±8[b]

Tab 3. Production of IFNg in culture supernatants of CTL induced by *Gl*-PS-treated DC pulsed with P815 cell lysates. *n*=3. Mean±SD. [b]*P*<0.05, [c]*P*<0.01, *vs* RPMI medium 1640.

Group	Concentration/ mg·L^{-1}	Content of IFNγ/ μg·L^{-1}
RPMI medium 1640	-	2.6±0.4
Gl-PS	0.8	4.7±0.3[b]
	3.2	6.4±1.1[c]
	12.8	6.2±0.4[c]

pulsing (Fig 2). It also showed that three concentrations of *Gl*-PS (0.8, 3.2, or 12.8 mg/L) could increase mRNA expression ratios of granzyme B (granzyme B *vs* β-actin) when it was compared with RPMI medium 1640 control. Significant difference was observed between each concentration of *Gl*-PS and RPMI medium 1640 control (P<0.01, Tab 4).

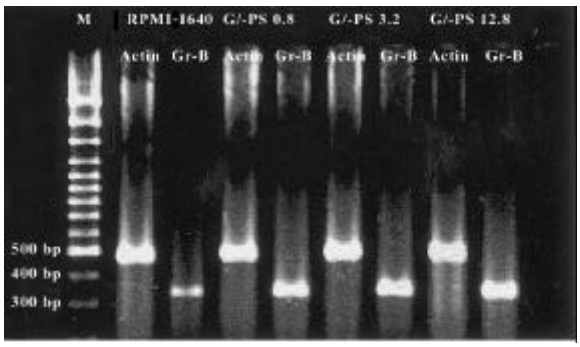

Fig 2. mRNA expression of granzyme B and b-actin in CTL induced by *Gl*-PS-treated DC pulsed with P815 cell lysates. M: Marker; RPMI-1640: RPMI medium 1640; Actin: b-actin; Gr-B: Granzyme B.

Tab 4. mRNA expression of granzyme B in CTL induced by *Gl*-PS-treated DC pulsed with P815 cell lysates. The intensity ratio was expressed as ratio of granzyme B *vs* b-actin. *n*=3. Mean±SD. [c]*P*<0.01 *vs* RPMI medium 1640.

Group	Concentration/ mg·L^{-1}	Intensity ratio/%
RPMI medium 1640	-	55±8
Gl-PS	0.8	98±10[c]
	3.2	90±16[c]
	12.8	91±18[c]

Regulation of *Gl*-PS on protein production of granzyme B in CTL Protein of granzyme B was highly expressed in P815 specific CTL induced by DC treated with or without *Gl*-PS during the stage of P815 lysates pulsing. The expression of granzyme B of RPMI medium 1640 was regarded as 100 % (Fig 3). It also showed that the highest concentration of *Gl*-PS (12.8 mg/L) could significantly increase the protein expression of granzyme B when it was compared with RPMI medium 1640 control (P<0.05, Tab 5).

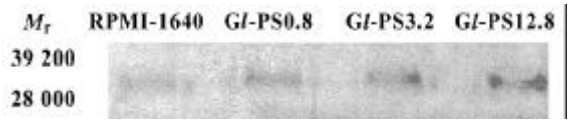

Fig 3. Protein expression of granzyme B in CTL induced by *Gl*-PS-treated DC pulsed with P815 cell lysates. RPMI-1640: RPMI medium 1640.

Tab 5. Production of granzyme B in CTL induced by *Gl*-PS-treated DC pulsed with P815 cell lysates. *n*=3. Mean±SD. [b]*P*<0.05 *vs* RPMI medium 1640.

Group	Concentration/ mg·L^{-1}	Intensity ratio/%
RPMI medium 1640	-	100±0
Gl-PS	0.8	100±1
	3.2	105±19
	12.8	125±12[b]

DISCUSSION

The immunotherapies of tumor have developed from antibodies or cytokines to generation of effector cells, most of which were "tumor vaccine", but they only gained limited success[21]. The main reason was that tumor has successfully avoided the immune response through various mechanisms, such as limited availability of tumor-associated antigens, tumor local micro-milieu which could inhibit immune cells, tolerance, *et al*[22,23]. Fortunately, recent insights into the role of DC as the most important APC in initiating or priming immune responses of both helper and cytotoxic lymphocytes provide the basis for more effective immunotherapy of tumor.

The primary function of DC includes activation and differentiation of naive T-lymphocytes[10-13]. Our previous study showed that *Gl*-PS could promote the proliferation of one-way MLC induced by DC, indicating that the immunomodulatory effects of *Gl*-PS maybe have relationship to DC[14]. Another function of DC is modulating immunity against pathogens including malignant cells[10,16,21]. The tumor inhibitory activity of *Gl*-PS was via immune system[7,8], and we investigated whether or not *Gl*-PS could regulate the antitumor activity during antigen presenting stage.

T-lymphocyte response to tumor cells will require initial antigen presentation by professional APC such as DC. Administration of DC pulsed with tumor antigen can lead to not only tumor specific immune responses, but also antitumor activity. Immature DC has a high capability for antigen capture and processing. DC was used as tumor vaccine by pulsing with tumor antigen, and was effective in presenting tumor antigen, activating CTL to recognize and kill tumor cells[24]. This DC vaccine has special meaning for tumor escaping from immunity. LDH activity assay is a kind of method to detect the cytotoxicity. Our study demonstrated that *Gl*-PS could promote the specific cytotoxic effects of CTL induced by DC pulsed with P815 lysates during the stage of antigen presentation, which may be effective in eliminating tumor escaping from immunity. The clinical trail of DC reinfusion in tumor treatment showed that repeat reinfusion with low doses of DC enhanced antitumor immunity, while over-dose of DC inhibited protective antitumor immunity. The DC concentration in our study was low, and treatment with *Gl*-PS exhibited up-regulation of CTL activity, which may be useful in decreasing the number of DC and reducing the side effects of DC vaccine. Moreover, in clinic, most of the circulating DC and DC residing in or near malignant tissues were defective in their ability to differentiate into mature DC[25], one of the reasons was that they might be defective in their capacity of taking up, processing, and presenting tumor-associated antigens. And the effects of *Gl*-PS in promoting antigen presenting would be helpful in restoring the function of DC in body. Another reason was that tumor local micromilieu such as secretion of IL-10 that inhibiting DC differentiation, maturation, and migration. Our previous study showed that *Gl*-PS promoted the maturation of DC, up-regulated the co-expression of I-A/I-E and CD11c on DC surface, mRNA expression and protein secretion of IL-12 p40 unit[14], which could antagonize the effects of inhibitory factors of DC.

The cytotoxicities of CTL are conducted by several mechanisms[26-28], one is cytokines such as IFNγ, and another is tumor cells lysis pathway through perforin-granzyme and death signal through *Fas-FasL*. In general, *Fas-FasL* is important in maintenance of body stabilization, while perforin-granzyme pathway is more important in pathologic situation. Granzyme B is a kind of lymphocyte serine proteases located in cytoplasmic granules of CTL and NK cells, which is synthesized as inactive proenzymes, and stored within cytolytic granules and released during degranulation. A new perspective suggests that granzyme B enters cells *via* receptor-mediated endocytosis, after that it can mediate apoptotic signals and induce rapid fragmentation of DNA of target cells *via* activation of two complementary pathways, a cytosolic pathway involving proapoptotic caspase family, and a nuclear pathway probably involving a cell cycle regulating protein and/or kinase Cdc2. It has been reported that *Gl*-PS increased IFNγ production[8], and our results confirmed such discovery. *Gl*-PS could increase the mRNA transcription and protein secretion of IFNγ by specific CTL induced by DC pulsed with tumor antigen during the stage of antigen presentation, which might be one of the mechanisms of antitumor activity through immunity. And we also found that *Gl*-PS could increase the mRNA and protein expression of granzyme B, which may be another cytotoxic mechanism involved in *Gl*-PS. The mRNA and protein expression of IFNγ did not correspond with each other. Neither did granzyme B. Since the mRNA expression is prior to protein expression, which may be one reason of discordance. Cytokines secretion played key roles in the early stage of pathogens invasion, while apoptosis induced by CTL was

more important in late stage[29], which may be another reason of expression discordance.

However, our observation was obtained through *in vitro* culture system, and need further investigation of *in vivo* experience to observe the regulatory effects of *Gl*-PS on specific CTL induced by DC during the stage of antigen presentation.

ACKNOWLEDGMENTS *Gl*-PS was kindly provided by Prof LIN Shu-Qian of Fuzhou Institute of Green Valley Bio-Pharm Technology.

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