Activation of Macrophages by Polysaccharide–protein Complex from *Lycium barbarum* L.

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Macrophages play crucial roles in innate immunity. This paper reports that a polysaccharide–protein complex isolated from *Lycium barbarum* (LBP) is able to activate macrophages. LBP was isolated from *Lycium barbarum* fruit and separated to five homogenous fractions, designated LBPF1, LBPF2, LBPF3, LBPF4 and LBPF5. It was found that LBP (50 mg/kg, i.p.) markedly upregulated the expressions of CD40, CD80, CD86 and MHC class II molecules on peritoneal macrophages. In *in vitro* studies showed that LBP and LBPF1-5 activated transcription factors NF-κB and AP-1 by RAW264.7 macrophage cells, induced TNF-α, IL-1β, IL-12p40 mRNA expression, and enhanced TNF-α production in a dose-dependent manner. Furthermore, LBP (50 mg/kg, i.p.) significantly enhanced macrophage endocytic and phagocytic capacities *in vivo*. These results indicate that LBP enhances innate immunity by activating macrophages. The mechanism may be through activation of transcription factors NF-κB and AP-1 to induce TNF-α production and upregulation of MHC class II costimulatory molecules. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: *Lycium barbarum* polysaccharide–protein complex; macrophage; NF-κB; AP-1; TNF-α; endocytosis; phagocytosis.

INTRODUCTION

Biological response modifiers (BRMs) are substances that stimulate the body’s immune response to infection and disease. BRMs can be cytokines produced endogenously by mammalian immune cells or derivatives of microorganisms, fungi, algae, lichens and photosynthetic plants (Leung et al., 2006; Schepetkin and Quinn, 2006). In recent decades, polysaccharides isolated from botanical sources have extraordinarily attracted a great deal of attention in the biomedical area because of their broad spectrum of therapeutic properties and relatively low toxicity (Schepetkin and Quinn, 2006). One of the pharmacological mechanisms of these botanical polysaccharides is thought to influence the innate immunity through activating macrophages (Omardsdottir et al., 2005; Schepetkin et al., 2005).

*Lycium barbarum* (*L. barbarum*) is an important Chinese medicine used to treat and prevent diseases such as insomnia, liver dysfunction, diabetes, visual degeneration and cancer. It has been recognized that the bioactive components of *L. barbarum* are polysaccharide–protein complex (LBP), which is a type of β-glycan possessing a core backbone of (1→6)-β-galactosyl residues, about half of which are substituted at C-3 by galactosyl or arabinosyl groups (Peng and Tian, 2001, Fig. 1). The carbohydrate is linked O-glycosidically to serine/threonine residues of the protein part (Qin et al., 2001). LBP generally consists of six monosaccharides (galactose, glucose, rhamnose, arabinose, mannose and xylose) and 18 amino acids (Huang et al., 1998; Gan et al., 2003, 2004). In structure–function attributes, β-glycan structure is thought to contribute to the biological function (Tzianabos, 2000). Previous studies have shown that LBP can enhance the immune function (Gan et al., 2003, 2004), protect liver damage (Ha et al., 2005), lower the blood glucose level (Luo et al., 2004), reduce the side effects of chemotherapy and radiotherapy (Gong et al., 2004, 2005) and act against cancer (Gan et al., 2004; Zhang et al., 2005; Chao et al., 2006). It is speculated that one of the mechanisms by which LBP enhances innate immunity may be through induction of the innate immune response by activating macrophages.

Macrophages play a major role in host defense against infection. Macrophages express a broad range of pattern recognition receptors (PRRs) to bind the conserved structures of pathogens, ingest bond microbes into vesicles, and produce reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (mainly nitric oxide) to destroy microbes (Aderem and Underhill, 1999; Taylor et al., 2005). Activated macrophages also secrete cytokines TNF and IL-1, and chemokines to induce inflammatory reactions to microbes (Pylkkänen et al., 2004). In addition, macrophages can present antigen to T cells and produce IL-12 to coordinate innate and adaptive immune responses (Watford et al., 2003). Furthermore, macrophages are involved in tissue remodeling after infections and injury, clearance of apoptotic cells and hematopoiesis (Tsirigiani et al., 2006; Krysko et al., 2006). (2009)
en et al., 2006). The present study investigated the mechanism of macrophage activation induced by LBP and compared the immunostimulatory activities of its different fractions.

MATERIALS AND METHODS

Animal and cell line. Female BALB/c mice, 6 weeks old, were obtained from the Singapore Laboratory Animal Centre. All procedures were approved by the Institutional Animal Care and Use Committee, National University of Singapore. The RAW264.7 murine macrophage cell line was ordered from the American Type Culture Collection (ATCC, TIB-71). The cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C in a 5% CO₂ humidified incubator.

Reagents. Ovalbumin (OVA), lipopolysaccharide (LPS, L3755, Escherichia coli serotype 026:B6) E-TOXATE® kit, FITC-dextran and FITC-Staphylococcus aureus were purchased from Sigma (St Louis, MO). The protein assay kit was from Bio-Rad (Hercules, CA). The mouse B cell isolation kit, lipofectamine transfection reagents assay kit was from Bio-Rad (Hercules, CA). The mouse IgM/kappa monoclonal antibody was from Sigma (St Louis, MO). 3H-thymidine was from GE Healthcare (Buckinghamshire, UK). Luciferase assay reagents were from Promega (Madison, WI). 3H-thymidine was from GE Healthcare (Buckinghamshire, UK).

LBP preparation and treatment. L. barbarum fruit was purchased from Eu Yan Sang Chinese medicine store (Singapore). The fruit was imported from Zhongning county, Ningxia province China. Voucher herbarium specimens are deposited at the WHO Immunology Centre, National University of Singapore. LBP was isolated from L. barbarum fruit as described previously (Chen et al., 2008). Briefly, L. barbarum dried fruit was extracted with water and the water extract was precipitated with ethanol. Free proteins were removed with Sevag reagent (CHCl₃:BuOH = 4:1). Crude LBP was obtained by dialysis and lyophilization. The LBP was then separated by DEAE-cellulose ion exchange chromatography (successively eluted with water, followed by 0.05 M, 0.1 M, 0.2 M and 0.5 M NaCl) and further purified by size exclusion chromatography (eluted with water) using FPLC. Five homogenous fractions, designated as LBPF1, LBPF2, LBPF3, LBPF4 and LBPF5 were obtained (Chen et al., 2008). The molecular weights of LBPF1, LBPF2, LBPF3 and LBPF4 were 150 kDa, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Chen et al., 2008). The molecular weight of LBPF5 was 293 kDa, as determined by size exclusion chromatography (Chen et al., 2008). The carbohydrate contents were 48.2%, 30.5%, 34.5%, 20.3% and 23.5%, respectively, as determined by phenol–sulfuric acid assay (Chen et al., 2008). The protein contents were 1.2%, 4.8%, 4.1%, 13.7% and 17.3%, respectively, as measured by the Bradford method using a protein assay kit (Chen et al., 2008). The LBP and LBPF1-5 were dissolved in PBS or normal saline (for in vivo experiment), filtered through a 0.22 μm filter and stored at 4 °C. The RAW264.7 cells were stimulated with LBP or LBPF1-5 at various concentrations and time points at 37 °C in a 5% CO₂ humidified incubator.

Flow cytometric analysis. Cells were washed with cold wash buffer (PBS/0.1% NaCl/1% FBS). 10⁶ cells were stained with 0.5 μg of APC-conjugated anti-mouse CD11b (Rat IgG2b, APC, M1/70), anti-mouse CD40 (rat IgG2a, FITC, 3/23), anti-mouse CD80 (American hamster IgG2, FITC, 16-10A1), anti-mouse CD86 (anti-IgG2a, FITC, GL1), anti-mouse I-A/I-E (rat IgG2a, FITC, 2G9), isotype controls American hamster IgG1 (APC, G235-2356), American hamster IgG2 (FITC, B81-3) and rat IgG2a (FITC, R35-95) were from BD Biosciences (San Diego, CA). Luciferase assay reagents were from Promega (Madison, WI). 3H-thymidine was from GE Healthcare (Buckinghamshire, UK).

Luciferase assay. The RAW264.7 cells were transiently transfected with NFAT-luc, AP-1-luc or NF-κB plasmid DNA using lipofectamine transfection reagent according to the manufacturer’s protocol. The transfected cells were incubated for 48 h. 1 × 10⁵ cells were stimulated with LBP or LBPF1-5 100 μg/mL for 6 h. PMA (20 ng/μL) plus ionomycin (0.5 μg/mL) was a positive control.
At the end of stimulation, the cells were harvested and lysed with 20 μL of lysis buffer (25 mM Tris-phosphate, 8 mM MgCl₂, 2 mM DTT, 1% Triton X-100, and 10% glycerol, 2 mM 1,2-diaminocyclohexan-N,N,N,N-tetraacetic acid). The lysate was mixed with 100 μL of luciferase assay reagent. Luciferase activity was measured by a luminometer (Biotrace). The background obtained with the lysis buffer was subtracted in each experimental value, and the specific transactivation was expressed as the fold induction over untreated cells.

**Real-time RT-PCR.** Total RNA was extracted from LBP- or LBPF1-5-treated RAW264.7 cells using Qiagen RNeasy mini kit and reverse transcribed to cDNA using the Invitrogen SuperScrip™ first-strand synthesis system. The PCR primers and fluorogenic probes for the target genes (TNF-α, IL-1β and IL-12p40) and endogenous control (β-actin) were purchased as TaqMan® Gene Expression Assays. The PCR volume was 20 μL, composed of 1 μL of 20× mixed primers and probe, 10 μL of 2× TaqMan® Fast Universal PCR Master Mix and 9 μL of cDNA template (500 ng diluted in RNase-free water). PCR was performed in an optical 96-well reaction plate on the ABI 7500 Fast Real-time PCR System. Each sample was run in triplicate. The thermal cycle conditions were 20 s hold at 95 °C, followed by 50 cycles of 1 s at 95 °C (denature) and 20 s at 60 °C (annealing/extension). The relative quantification of the target gene expression was calculated by comparative CT (ΔΔCT) method using the SDS 1.3.1 software. Values reported have a 95% confidence interval (CI) as determined by the software.

**ELISA.** TNF-α, IL-1β and IL-12p40 and p70 were quantified by sandwich ELISA using BD Biosciences OptiEIA™ set according to the manufacturer’s instructions. The absorbance was measured at 450 nm with a reference wavelength of 570 nm using a spectrometer (Tecan Sunrise).

**Endocytosis and phagocytosis assay.** Mice were injected i.p. with 1 mL of FITC-dextran (1 mg/mL, endocytosis assay) or 1 mL of FITC-<i>Staphylococcus aureus</i> (1 mg/mL, phagocytosis assay) 30 min prior to killing. Peritoneal cells were harvested, washed and resuspended in DMEM medium supplemented with 10% FBS. The cells were applied to 24-well tissue culture plates with a microscope glass cover slip in the bottom at 37 °C in a 5% CO₂ humidified incubator for 6 h. The cover slip with adherent cells was picked up and mounted with DABCO–glycerol media. The cells were observed and photographed under a fluorescent microscope (Olympus BX-60, magnification, ×40).

**Test of LPS contamination.** LPS contamination was tested by <i>Limulus</i> amebocytes lysate (LAL) assay and B cell proliferation assay. The LAL assay was performed using an E-TOXATE® kit according to the manufacturer’s instruction. In brief, 100 μL of samples (10 mg/mL), standards or endotoxin-free water (negative control) was mixed with 100 μL of LAL for 1 h at 37 °C and observed for gelation. B cell proliferation was performed using negatively selected B cells from BALB/c mouse spleen. 2 × 10⁵ B cells were stimulated with LPS (1 μg/mL), or LBP (100 μg/mL), or LBPF1-5 (100 μg/mL) at 37 °C in a 5% CO₂ humidified incubator for 72 h. The cells were pulsed with ³H-thymidine (0.5 μCi/well) for the last 18 h. The amount of ³H-thymidine incorporated into the cells was measured using a β-scintillation counter (Packard TopCount).

**Statistical analysis.** Data are presented as mean ± SD, except for the relative quantification of cytokine mRNA, which was presented as 95% of CI. Each experiment was repeated at least three times. Differences were analysed for significance using the Student’s unpaired, two-tailed t-test by the SPSS 13.0 software. A value of <i>p</i> < 0.05 was used as the threshold for significance.

**RESULTS**

**Effects of LBP on the expression of CD40, CD80, CD86 and MHC class II molecules on macrophages**

Macrophages are types of antigen presenting cells which enhance their antigen presenting ability by upregulating the expression of MHC class II molecules and costimulators such as CD40, CD80 and CD86 (Hancock et al., 1996). To investigate whether LBP upregulates the expression of such molecules on macrophages, LBP i.p. was injected i.p. into mice and the peritoneal macrophages were harvested 7 days later. Expression of such molecules was analysed by flow cytometry. As shown in Fig. 2, 3.9% of peritoneal cells in the mice injected with saline expressed CD40. In contrast, the expression was increased to 74.1% in the LBP-treated mice. Similarly, the expression of CD80, CD86 and MHC class II after LBP treatment were upregulated from 18.3%, 14.5% and 64.5% to 94.1%, 90.4% and 96.9%, respectively.

**Effects of LBP and LBPF1-5 on activation of transcription factors**

Transcription factors are critical for macrophage activation. Transcriptional signaling is necessary for inducible expression of a suite of genes required to initiate inflammation and eliminate pathogens (Guha and Mackman, 2001). Therefore, the study investigated whether LBP activates three key transcription factors, including NFAT, AP-1 and NF-κB. The RAW264.7 cells were transfected with the three corresponding plasmids containing the luciferase reporter gene, then the transfected cells were stimulated with LBP and LBPF1-5, and the luciferase activity was measured by luciferase assay. As shown in Fig. 3, LBP and LBPF1-5 significantly activated AP-1 and NF-κB (p < 0.01–0.05, compared with medium). The luciferase activities were increased 1.5- to 2.5-fold by LBP or LBPF1-5 stimulation. In contrast, NAFT was completely suppressed.

**LBP and LBPF1-5 induce TNF-α, IL-1-β and IL-12p40 mRNA expression**

As activation of transcription factors initiates gene transcription, the study next investigated whether LBP induces TNF-α, IL-1β and IL-12p40 mRNA expression. After RAW264.7 cells were treated with LBP or LBPF1-5...
for 48 h, TNF-α, IL-1β and IL-12p40 mRNA expression were increased strikingly, of which TNF-α mRNA was increased 1.5- to 2.7-fold (Fig. 4A), IL-1β mRNA was increased 1600- to 4700-fold (Fig. 4B), and IL-12p40 was increased 1 360 000- to 6 810 000-fold (Fig. 4C). The extremely high levels of relative expression of IL-12p40 and IL-1β mRNA after LBP stimulation were because they were expressed at a very low level in the untreated cells (Ct values were 50 and 35, respectively, data not shown).

**LBP and LBPF1-5 enhance TNF-α production**

As LBP induces TNF-α, IL-1β and IL-12p40 mRNA expression, it may induce their protein production. To answer this question, RAW264.7 cells were stimulated with LBP or LBPF1-5 at various concentrations, including 1, 10, 100 and 500 μg/mL for 48 h. As shown in Fig. 5, LBP and LBPF-5 induced TNF-α production in a dose-dependent manner. LBPF3 appeared to be the most potent. The effects were visible at 1 μg/mL and 100 μg/mL of LBP and LBPF1-5 induced 7.6–19.3 ng/mL of TNF-α. In contrast, RAW264.7 cells did not produce any IL-1β and IL-12p40 and p70 detectable by ELISA after LBP and LBPF1-5 stimulation.

**LBP enhances endocytosis and phagocytosis in vivo**

Endocytosis and phagocytosis are the main functions of macrophages. Macrophages engulf large molecules by endocytosis while destroying microbes by phagocytosis. Animal models were set up to investigate whether LBP enhances macrophage functions. Mice were injected i.p. with LBP daily for 7 days and 30 min prior to killing were injected i.p. with FITC-dextran (endocytosis...
LBP and LBPF1-5 are free of LPS contamination

The LAL results showed that the quantity of endotoxin in crude LBP and LBPF1-5 was less than 0.015 EU/mg (negative). Our previous work has demonstrated that LBP does not stimulate B cell proliferation. Therefore, the study also tested whether LBP was contaminated by LPS during preparation by B cell proliferation assay. Unlike LPS, LBP and LBPF1-5 did not stimulate B cell proliferation (Fig. 7).

DISCUSSION

*L. barbarum* is believed to possess multiple health benefits. This study demonstrated that one of the pharmacological mechanisms of LBP is through enhancement of innate immunity by activating macrophages. In detail, LBP induced the expression of CD40, CD80, CD86 and MHC class II molecules on mouse peritoneal macrophages. LBP activated transcription factors NF-κB and AP-1, induced TNF-α, IL-1β and IL-12p40 mRNA expression, and enhanced TNF-α production by RAW264.7 macrophage cells. All its five fractions were active. LBPF3 appeared to be the most potent in activation of transcription factors and induction of cytokine production. Peritoneal macrophages from LBP-treated mice were very active in endocytosis and phagocytosis. The data indicate that the mechanism of macrophage activation by LBP may be through activation of transcription factors NF-κB and AP-1 to induce TNF-α production and upregulation of MHC class II costimulatory molecules.

LBP and LBPF1-5 significantly activated transcription factors AP-1 and NF-κB using the RAW264.7 macrophage cell line. In contrast, NFAT was completely inhibited. The reason that NFAT induction was suppressed after LBP treatment is not known, probably because it mainly participates in the regulation of T-cell function and development (Macian, 2005). Unlike NFAT, NF-κB plays crucial roles in macrophage activation. NF-κB induction is essential for the expression of a wide variety of immune response genes, including proinflammatory cytokines, chemokines and adhesion molecules (Beinke and Ley, 2004). AP-1 proteins have been implicated in invasive cell growth and matrix metalloprotease production and in cell line models, and have been suggested to mediate the induction of inflammatory genes such as TNF (Hu et al., 2007).
Figure 6. LBP enhances endocytosis and phagocytosis in vivo. BALB/c mice were injected with LBP (50 mg/kg) i.p. daily. Saline was control. Mice were killed 7 days later. 30 min prior to killing, mice were injected i.p. with 1 mL of FITC-dextran (1 mg/mL) (A, B) or 1 mL of FITC-Staphylococcus aureus (1 mg/mL) (C, D). Peritoneal cells were harvested, washed and incubated in 24-well tissue culture plates with a microscope glass cover slip in the bottom for 6 h. The cover slip with adherent cells were observed and photographed under a fluorescent microscope (Olympus BX-60, magnification, ×40). (A) Saline + dextran. (B) LBP + dextran. (C) Saline + Staphylococcus aureus. (D) LBP + Staphylococcus aureus.

As expected, the induction of NF-κB and AP-1 triggered TNF-α, IL-1β and IL-12p40 mRNA expression. However, only TNF-α production was detectable by ELISA, and RWA264.7 cells constitutively secreted a substantial level of TNF-α. The lack of IL-1β and IL-12p40 and p70 production could be because their absolute mRNA expression was low. IL-12, secreted from dendritic cells (DCs) and macrophages, is a crucial cytokine that can direct Th1 cell differentiation. Nevertheless, it was found that LBP induced IL-12p40 and p70 production from DCs, and T cells stimulated with LBP-treated DCs produced a higher level of IFN-γ, indicating that LBP can induce Th1 response (manu-

by the LAL assay. The result showed that the samples were not contaminated with LPS, as was further supported by the fact that LBP and its five fractions did not stimulate B cell proliferation, whereas LPS did. This result demonstrated that macrophage activation is by LBP, but not by LPS.

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Activation of T lymphocytes by polysaccharide–protein complex from Lycium barbarum L.

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ABSTRACT

T lymphocytes play central roles in adaptive immunity. Lycium barbarum L. (L. barbarum), also known as wolfberry, is a Chinese herbal medicine with various biological activities, such as enhancing immunity, protecting liver damage, and reducing the side effects of chemotherapy and radiotherapy. Here, we report that polysaccharide–protein complex from L. barbarum (LBP) is able to activate T cells. LBP was isolated from L. barbarum and separated to five homogenous fractions, designated LBPF1, LBPF2, LBPF3, LBPF4, and LBPF5. We found that LBP, LBPF4, and LBPF5 significantly stimulated mouse splenocyte proliferation. The proliferation proved to be of T cells, but not B cells. Cell cycle profile analysis indicated that LBP, LBPF4, and LBPF5 markedly reduced sub-G1 cells. LBP, LBPF4, and LBPF5 could activate transcription factors NFAT and AP-1, prompt CD25 expression, and induce IL-2 and IFN-γ gene transcription and protein secretion. LBP (i.p. or p.o.) significantly induced T cell proliferation. Our results suggest that activation of T lymphocytes by LBP may contribute to one of its immuno-enhancement functions.

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1. Introduction

Lycium barbarum L. (L. barbarum), commonly known as wolfberry, is a well-known Chinese herbal medicine as well as tonic which has been used in East Asia for thousands of years to treat and prevent diseases such as insomnia, liver dysfunction, diabetes, visual degeneration, and cancer. People in such areas believe wolfberries have multiple health benefits and use them to make tea, soup, stew and wine or chew them like raisins. The bioactive components of L. barbarum fruit have been mainly attributed to its polysaccharide–protein complex (LBP), which contains several fractions separated by ion exchange chromatography and size exclusion chromatography [1–3]. LBP fractions generally consist of 6 monosaccharides (galactose, glucose, rhamnose, arabinose, mannose, and xylose) and 18 amino acids [1–3]. Their structures are (β-glycan possessing a backbone of (1→6)-β-galactosyl residues, about half of which are substituted at C-3 by galactosyl or arabinosyl groups [4]. The carbohydrate is linked O-glycosidically to serine/threonine residues of the protein part [5]. The β-glycan structural aspect of a polysaccharide is thought to correlate with the biological function [6]. Previous studies have shown that LBP can enhance the immune function [2,3], protect liver damage [7], lower blood glucose level [8], reduce the side effects of chemotherapy and radiotherapy [9,10], and act against cancer [3,11,12]. While LBP was found to induce lymphocyte proliferation and cytokine production [2,3], its mechanism on activation of T cells and the immunostimulatory effect of different fractions have not been reported yet.

T cells are thymus-derived lymphocytes and play a central role in generation and regulation of immune response to protein antigens. Mature T cells leaving the thymus are either CD4+ or CD8+ and express CD3 and T cell receptor (TCR) molecules. Based on the lymphokines they produce, CD4+ T-helper (Th) cells are divided into Th1 and Th2 subsets. Th1 cells produce IFN-γ to promote cell-mediated immunity; Th2 cells secrete IL-4 to promote humoral immunity [13]. CD8+ cytotoxic T lymphocytes (CTLs) are a major effector for protection against cancer as well as many infectious diseases [14]. T cell activation is composed of a cascade of events, including TCR/CD3 recognition of peptide-major histocompatibility complex (MHC) I or II, formation of immunological synapse, and triggering of multiple signaling pathways. Activated T cells then become effector cells to perform functional responses such as cytokine secretion, proliferation, and differentiation [13,14]. In this present study, we investigated the mechanism of T cell activation induced by LBP and compared the immunostimulatory activities of different fractions.

2. Material and methods

2.1. Animals and cell line

Female C57BL/6 and BALB/c mice, 6–week old, were obtained from the Singapore Laboratory Animal Centre. All animals were housed at...
2.4. Proliferation assay

Sevag reagent (CHCl₃: ethanol. The precipitate was dissolved in water. One under reduced pressure and precipitated with 5 volumes of absolute

in a 5% CO₂ humidified incubator at 37 °C in a 5% CO₂ humidified incubator for 72 h and were pulsed with ³H-thymidine (0.5 μCi/well) for the last 18 h. The cells were harvested on glass fiber filters using a Filtermate cell harvester (Packard). The amount of ³H-thymidine incorporated into cells was measured using a β-scintillation counter (TopCount, Packard). The results are expressed as stimulation index (SI), as calculated by dividing cpm of stimulated cells with cpm of unstimulated cells.

2.5. Protease digestion

Protein contained in LBP was destroyed by incubation with protease. Briefly, 20 mg of crude LBP, LBPF4, or LBPF5 was dissolved in 10 ml of 0.2% SDS/10 mM EDTA solution. Protease was added to 1 mg/ml. The reaction mixture was incubated in a water bath at 37 °C overnight, followed by dialysis against water for 3 days and lyophilization.

2.6. Cell cycle profile analysis

LBP- or LBPF1-5-treated mouse splenocytes were harvested, washed, and fixed in 1 ml of 70% ethanol at 4 °C overnight. The cells were resuspended in 1 ml of staining solution (100 μg/ml RNase, 40 μg/ml PI, and 0.1% Triton X-100 in PBS) for 30 min at room temperature. Cell cycle profile was analyzed by flow cytometry (Beckman Coulter).

2.7. Flow cytometric analysis of CD25

LBP- or LBPF1-5–treated mouse splenocytes were harvested and treated with 50 μl of monoclonal rat anti–mouse CD25 (20 μg/ml) at 4 °C for 40 min, washed, and followed by 100 μl of FITC–conjugated goat anti–rat IgG (10 μg/ml) and incubated at 4 °C for 40 min in the dark. After incubation, the cells were washed and resuspended in 0.5 ml of PBS/0.2% paraformaldehyde. CD25 expression was quantified by flow cytometry (Dako).

2.8. Quantitative real-time reverse transcription PCR

Total RNA was extracted from LBP- or LBPF1–treated mouse splenocytes using Qiagen RNeasy mini kit and reverse transcribed to cDNA using Invitrogen SuperScript™ first-strand synthesis system. PCR primers and fluorogenic probes for all of the target genes (IL-2, IL-4, TNF-α, IFN-γ) and endogenous control (β-actin) were purchased as TaqMan® Gene Expression Assays. PCR volume was 20 μl of composed of 1 μl of 20× mixed primers and probe, 10 μl of 2×TaqMan® Fast Universal PCR Master Mix, and 9 μl of cDNA template (500 ng diluted in RNase-free water). PCR was performed in optical 96-well reaction plate on the ABI 7500 Fast Real-time PCR System. Each sample was run in triplicate. The thermal cycle conditions were 20-second hold at 95 °C, followed by 50 cycles of 1 s at 95 °C (denaturation) and 20 s at 60 °C (annealing/extension).

The relative quantification (RQ) of the target gene expression was calculated by comparative C_T (ΔΔC_T) method using the SDS 1.3.1 software. In this method, the threshold cycle (C_T) is the fractional cycle number at which the fluorescence passes the threshold, which level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The amount of target gene expressed is normalized to an endogenous reference (R-
actin) and is relative to a calibrator (negative control, i.e. untreated cells). The target CT and endogenous reference CT is calculated for each sample. The CT of the endogenous reference is then subtracted from the CT of the target gene. This value is known as ΔCT. The ΔCT of each sample is then subtracted from the ΔCT of the calibrator, and this value is known as ΔΔCT.

2.9. ELISA

Cytokines, including IL-2, IL-4, IFN-γ, TNF-α in the cell culture supernatant, were quantified by sandwich ELISA using BD Biosciences OptiEIA™ set according to the manufacturer’s instruction. Briefly, Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 100 µl of purified anti-mouse IL-2, IL-4, IFN-γ, or TNF-α mAbs. The plates were blocked with PBS/10% FBS and incubated with 100 µl of supernatant from cell culture or serially diluted cytokine standards. 100 µl of Working Detector (biotinylated anti-mouse IL-2, IL-4, IFN-γ, or TNF-α mAb + streptavidin-HRP conjugate) was added for 1 h at room temperature followed by addition of 100 µl of one step substrate reagent for 30 min. Color development was stopped by 50 µl of stop solution. The absorbance value was measured at 450 nm with a reference of 570 nm using a spectrometer (Tecan Sunrise).

2.10. Luciferase assay

Jurkat cells were transiently transfected with NFAT-luc, AP-1-luc, or NF-κB plasmid using lipofectamine transfection reagent according to the manufacturer’s instruction. The transfected cells were incubated for 48 h. 1×10^5 cells were stimulated with 100 µg/ml of LBP or LBPF1-5 for 6 h. PMA (20 ng/ml) and ionomycin (0.5 µg/ml) were positive controls [18]. At the end of stimulation, cells were harvested and lysed with 20 µl of lysis buffer (25 mM Trisphosphate, 8 mM MgCl2, 2 mM DTT, 1% Triton X-100, 10% glycerol, and 2 mM 1,2-diaminocyclohexane-N,N,N,N'-tetraacetic acid). The lysate was mixed with 100 µl of luciferase assay reagent. Luciferase activity was measured by a luminometer (Biotrace). The background obtained with the lysis buffer was subtracted in each experimental value, and the specific trans-activation was expressed as the fold induction over untreated cells.

2.11. In vivo activation of T cells by LBP

LBP was given to BALB/c mice daily either i.p. at 0.5, 1.5, 5, 15, or 50 mg/kg or p.o. at 5, 15, or 50 mg/kg. Each dosage consisted of 4 mice. Normal saline was negative control. After seven days, mice were sacrificed.
sacrificed and spleen cells were harvested. Splenocyte proliferation was determined by 3H-thymidine incorporation assay in the presence or absence of Con A (1 µg/ml).

2.12. Statistical analysis

Data are presented as mean±S.D., except for the RQ of cytokine mRNA, which was presented as 95% of confidence interval. Each experiment was repeated at least three times. Differences were analyzed for significance using the Student's unpaired, two-tailed t-test by the SPSS 13.0 software. A p value less than 0.05 was used as the threshold for significance.

3. Results

3.1. Characteristics of LBP

Crude LBP was separated by ion exchange chromatography on a DEAE-cellulose column, which was successively eluted with H2O, 0.05 M, 0.1 M, 0.25 M, and 0.5 M NaCl. Five intermediate fractions, designated LBP1–5, were obtained (Fig. 1A). All of them peaked at A490 and A280, indicating they contained both polysaccharide and protein. LBP1–5 were further purified by size exclusion chromatography on a Sephacryl S-300 column. As shown in Fig. 1B–F, five fractions, designated LBPF1–5, were obtained. All fractions showed single peak at A490 and A280, indicating they were homogenous molecules. The molecular masses of LBPF1–4 were around 150 kDa determined by SDS-PAGE. The molecular mass of LBPF5 was 290 kDa determined by gel filtration. The carbohydrate contents of LBPF1–5 were 48.2%, 30.5%, 34.5%, 20.3%, and 23.5%, respectively, as measured by the phenol-sulfuric acid assay using glucose as standard. The protein contents were 1.2%, 4.8%, 4.1%, 13.7%, and 17.3%, respectively, as measured by the Bradford method.

3.2. Effects of LBP on splenocyte, T and B cell proliferation

To screen the immunomodulatory activities of LBP and its active fractions, mouse splenocytes were stimulated with LBP or LBPF1–5 at serial concentrations for 72 h. Cell proliferation was measured by 3H-thymidine uptake assay. As shown in Fig. 2A, crude LBP, LBPF4, and LBPF5 induced splenocyte proliferation in a dose-dependent manner. At 300 µg/ml, LBP significantly enhanced splenocyte proliferation by about 4.5-fold. In contrast, LBPF1, LBPF2, and LBPF3 did not stimulate splenocyte proliferation. The proliferative effects of LBPF4 and LBPF5 were not higher than that of crude LBP. As splenocytes contain both T and B cells, to study which subset that LBP stimulates, CD3+ T cells and CD19+ B cells were isolated from splenocytes by negative selection. More than 85% of remaining cells were T or B cells, as determined by flow cytometry (data not shown). The purified T or B cells were stimulated with 100 µg/ml of LBP, LBPF4, or LBPF5 for 72 h and cell proliferation was measured. The result showed that LBP, LBPF4, and LBPF5 activated T cells, but not B cells (Fig. 2B). As positive controls, Con A (3 µg/ml) stimulated T cell proliferation by 80-fold, while LPS (5 µg/ml) stimulated B cell proliferation by 13-fold (data not shown). To further investigate whether the protein part of LBP contributes to such effects, LBP, LBPF4, and LBPF5 were digested with protease to destroy protein. The lymphocyte proliferative activities of LBP, LBPF4, and LBPF5 were significantly reduced after treatment with protease (Fig. 2C).

3.3. Effects of LBP on cell cycle progression

As LBP could stimulate T cell proliferation, it might promote cells to enter S and G2/M phase of the cell cycle. To test this hypothesis, mouse splenocytes were stained with PI after LBP stimulation and the cell cycle profile was analyzed by flow cytometry. The result showed that the percentage of cells in S and G2/M phases did not increase after treatment with LBP or LBPF1–5 (Fig. 3). However, crude LBP, LBPF4, and LBPF5 could reduce the percentage of apoptotic cells. Up to 67% of untreated mouse splenocytes underwent apoptosis after in vitro culture for 48 h. In contrast, the percentage of apoptotic cells was lowered to 38%, 47%, and 44% in the presence of crude LBP, LBPF4, and LBPF5, respectively; whereas LBPF1, LBPF2, and LBPF3 did not have such an effect.

3.4. Activation of CD25 by LBP

To investigate whether LBP enhances the expression of T cell activation marker CD25, mouse splenocytes were stimulated with 100 µg/ml of LBP or LBPF1–5 for 48 h. CD25 expression was...
Fig. 4. Effects of LBP and LBPF1–5 on CD25 expression. 2×10⁶ mouse splenocytes were stimulated with 100 µg/ml of LBP or LBPF1–5 for 48 h. Con A (3 µg/ml) was positive control. CD25 expression was determined by flow cytometry. Results are representative of three independent experiments.
determined by flow cytometry. Fig. 4 shows that 4.3% of resting splenocytes expressed CD25. After stimulation with crude LBP, LBPF4, and LBPF5, CD25 expression was increased to 12.5%, 18.7%, and 10.9% of the cells, respectively; whereas LBPF1, LBPF2, and LBPF3 failed to enhance CD25 expression.

3.5. Induction of cytokine mRNA expression by LBP

To investigate whether crude LBP and its five fractions induce cytokine gene expression, mouse splenocytes were stimulated with 100 µg/ml of crude LBP, or LBPF1–5 for 48 h. Cytokine gene expression, including IL-2, IL-4, IFN-γ, and TNF-α, were quantified by real-time PCR. As shown in Fig. 5A, IL-2 mRNA expression was enhanced 2-, 9-, and 1.5-fold after simulation with LBP, LBPF4, and LBPF5, respectively. IL-4 mRNA expression was enhanced 5-, 4-, and 24-fold after simulation with LBP, LBPF4, and LBPF5, respectively (Fig. 5B). IFN-γ mRNA expression was enhanced 200,000-, 120,000-, and 1700-fold after simulation with LBP, LBPF4, and LBPF5, respectively (Fig. 5C). It is noted that the high level of IFN-γ mRNA relative expression after simulation with LBP, LBPF4, and LBPF5 was due to its extremely low expression in the untreated cells. TNF-α mRNA expression was enhanced 6-, 5-, and 2-fold after stimulation with LBP, LBPF4, and LBPF5, respectively (Fig. 5D). In contrast, LBPF1, LBPF2, and LBPF3 did not induce the mRNA expression of these cytokine genes. As positive

Fig. 5. Relative quantification of cytokine mRNA upon treatment of LBP or LBPF1–5. 2×10⁶ mouse splenocytes were stimulated with 100 µg/ml of LBP, or LBPF1–5 for 48 h. Con A (3 µg/ml) was positive control. RNA was extracted and reverse transcribed to cDNA. Gene expression was measured by real-time RT-PCR. The relative mRNA expression was normalized to the endogenous control gene β-actin and calibrated by untreated cells. Results are represented as 95% confidence interval (CI) of triplicate. *95% CI; **99% CI; ***99.9% CI, compared to untreated cells. (A) IL-2. (B) IL-4. (C) IFN-γ. (D) TNF.

Fig. 6. Dose–response and kinetics of cytokine production upon treatment with LBP or LBPF1–5. 2×10⁶ mouse splenocytes were stimulated with 1, 3, 10, 30, 100 µg/ml of LBP, or LBPF1–5 for 48 h in the dose–response assays, or with 100 µg/ml of LBP, or LBPF1–5 for 6, 12, 24, 48, 72 h in the kinetic response assays. Cytokines secreted into the culture supernatant were measured by ELISA. Values are represented as mean±S.D. of four replicates. *P<0.05; **P<0.01; ***P<0.001, compared to untreated cells. (A) Dose–response of IL-2 production. (B) Kinetics of IL-2 production. (C) Dose–response of IFN-γ production. (D) Kinetics of IFN-γ production.
control, Con A induced IL-2, IL-4, IFN-γ, and TNF-α mRNA expression 21-, 636-, 188,000-, and 8-fold, respectively.

3.6. Induction of cytokine production by LBP

As LBP induces cytokine gene transcription, it may induce cytokine production. To address this question, mouse splenocytes were treated with LBP or LBPF1-5 at serial concentrations and different time points. Secreted cytokines, including IL-2, IL-4, IFN-γ, and TNF-α, were measured by ELISA. LBP, LBPF4, and LBPF5 significantly induced IL-2 and IFN-γ production both in a dose-dependent and time-dependent manner, while LBPF1, LBPF2, and LBPF3 did not have such functions (Fig. 6). 30 µg/ml to 100 µg/ml of crude LBP, LBPF4, or LBPF5 significantly induced IL-2 and IFN-γ production (Fig. 6, A and C, P<0.001–0.01). Both IL-2 and IFN-γ production peaked at 48 h after stimulation (Fig. 6, B and D). This was consistent with the proliferation results. However, IL-4 and TNF-α were undetectable by ELISA after stimulation with LBP, LBPF4, and LBPF5.

3.7. Activation of NFAT and AP-1, but not NF-κB by LBP

Transcription factors NFAT, AP-1, and NF-κB play important roles in T cell activation. Once activated, they can enter the nucleus and bind the promoter or enhancer of cytokine genes to trigger the mRNA transcription. To investigate whether induction of cytokine mRNA expression and cytokine production by LBP is due to activation of transcription factors, Jurkat T cells were transiently transfected with a luciferase reporter plasmid NFAT-luc, AP-1-luc, or NF-κB-luc. The transfected cells were then stimulated with 100 µg/ml of LBP or LBPF1-5 for 6 h. Luciferase activity was measured by luciferase assay. Crude LBP, LBPF4 and LBPF5 activated NFAT 4- to 6-fold, and AP-1 2- to 3-fold compared to medium control (Fig. 7). In contrast, NF-κB was not activated by LBP. As positive control, PMA in combination with ionomycin activated NFAT and AP-1 50- and 80-fold, respectively (data not shown).

3.8. Activation of T lymphocytes in vivo by LBP

To verify whether LBP activates T cells in vivo, we first injected LBP i.p. to BALB/c mice. Splenocytes were harvested and cell proliferation was measured in the presence or absence of Con A. We found that splenocytes from LBP-injected mice had stronger proliferative activity than those from saline-injected mice after culture in vitro for 72 h in the presence or absence of Con A (Fig. 8, A and B). The effect was visible at a low dose of 0.5 mg/kg of LBP and was significantly different at 15 and 50 mg/kg of LBP. Similar effects were found when LBP was given p.o. to mice (Fig. 8, C and D). The results were consistent with the in vitro data.

4. Discussion

In this present study, we isolated LBP from L. barbarum fruit and purified it to five homogenous fractions: LBPF1, LBPF2, LBPF3, LBPF4, and LBPF5. We found that crude LBP, LBPF4, and LBPF5 could induce T cell proliferation, activate transcription factors NFAT and AP-1, prompt CD25 expression, and induce cytokines IL-2 and IFN-γ.
production. Our results indicate that the mechanisms of T cell activation by LBP may involve activation of CD25 via activation of transcription factors NFAT and AP-1. We are the first to compare the efficacy of different LBP fractions in T cell activation. Although LBP fractions are [α–glycans [1,4,5], which may correlate with their biological function (6), only LBPF4 and LBPF5 are able to activate T cells, whereas LBPF1, LBPF2, and LBPF3 could not. This could be because LBPF4 and LBPF5 contain more protein than LBPF1, LBPF2, and LBPF3, as further supported by the finding that the T cell–stimulatory effect of LBPF4 and LBPF5 were significantly compromised when the proteins were digested (Fig. 2C). We speculate the protein part of LBP may play important role in activation of T cells, though we were not able to precisely correlate the exact structure with its T cell–stimulatory activity. Nevertheless, activation of dendritic cells and macrophages by LBP is a different profile. All fractions are active, but LBPF4 and LBPF5 are less potent than LBPF1, LBPF2, and LBPF3 (manuscript in preparation). This may be because they contain less amount of carbohydrate that may play key roles in binding the corresponding receptors on DCs and macrophages.

We also found that materials with higher purity such as LBPF4 and LBPF5 did not display markedly higher immunostimulating activity than the crude parental LBP. Initially we doubted that crude LBP may contain other active components but these may have been removed during the process of ion exchange chromatography and size exclusion chromatography. To answer this question, we collected the eluted solution besides LBPF1-5 outside the peak range of A490 and A280, where the suspicious active components may exist. However, the collected material failed to stimulate mouse splenocyte proliferation. We also mixed the stuff with LBPF4 or LBPF5 in splenocyte proliferation assay. But still, no enhanced cell proliferation was observed compared to LBPF4 or LBPF5 alone. Therefore, the components able to activate T cells are LBPF4 and LBPF5 only. From our data, LBP, unlike Con A or PHA, is a weak T-cell stimulus. It induces T cell proliferation 3– to 4-fold only (Fig. 2). This may be the reason that the homogenous LBPF4 and LBPF5 do not have higher immunostimulatory activity than crude LB. On the other hand, this may be good for the host to enhance immunity, as the strong T-cell stimulants, such as Con A and PHA, may cause inflammation and are harmful.

We are the first to systematically elucidate the profile of LBP-induced cytokine production from T cells. T cells secrete a number of cytokines, such as IL-2, IL-4, IFN–γ, and TNF. IL-2 is the essential cytokine for T lymphocyte growth. IL-2–induced proliferation occurs via pro-proliferative signals through the proto-oncogenes c-myc and c-fos, in combination with anti-apoptotic signals through Bcl-2 family members [19]. IFN–γ promotes the differentiation of TH1 cells and therefore a predominantly cell-based immune response. It functions primarily on macrophages to enhance antimicrobial properties [20]. IL-4 is crucial for the differentiation of naïve Th cells into the Th2 cells that promote humoral immunity and provide protection against intestinal helmintes. It also has a central role in the pathogenesis of allergic inflammation [21]. Upon stimulation with antigen, CD+ T cells can differentiate in a highly polarized manner into Th1 or Th2 subsets [22]. ELISA and real-time PCR data showed that crude LBP, LBPF4, and LBPF5 induced T cells to produce IL-2 and IFN–γ at both mRNA and protein levels. However, IL-4 and TNF–α were undetectable by ELISA, although their genes were transcribed after stimulation with LBP, LBPF4 and LBPF5. This could be because IL-4 mRNA expression was low and the protein amount translated from mRNA might not be enough to be measured by ELISA, or the IL-4 secreted in the supernatant had been fully utilized. The reason for the absence of TNF–α secretion could be that it is mainly produced by macrophages, with T cells producing only some. There may not be sufficient macrophages to secrete TNF–α measurable by ELISA in the stimulated 2×10^6 mouse splenocytes. The induction of IFN–γ production by LBP indicates it may prime Th1 response. In the previous phases I clinical trial investigating the effect of L. barbarum on Epstein-Barr virus (EBV) inhibition, we found that L. barbarum could reduce serum EBV DNA copies in subjects with elevated serum IgA anti-EBV (data not shown). The mechanism could be because LBP helps to activate CTLs to kill EBV-infected cells.

Cytokine gene transcription is initiated with the participation of transcription factors, of which NFAT, AP-1, and NF-κB are three key factors. NFAT was initially identified as an inducible nuclear factor that could bind the IL-2 promoter in activated T cells [23]. AP-1 proteins are the main transcriptional partners of NFAT during T-cell activation [24,25]. NF-κB is triggered by pro-inflammatory stimuli and genotoxic stress, including the following: cytokines, such as TNF and IL-1; bacterial-cell wall-components, such as LPS; viruses; and DNA-damaging agents [26]. Our data showed that LBP, LBPF4, and LBPF5 could activate NFAT and AP-1 transcription factors using Jurkat T cells, but not NF-κB. This is understandable as activation of NF-κB needs TNF and IL-1β, both of which are mainly produced by macrophages.

In summary, the results presented in this study suggest that LBP enhances immunity by activating T cells. The active fractions appear to be LBPF4 and LBPF5. Crude LBP, LBPF4 and LBPF5 could activate transcription factors NFAT and AP-1, induce IL-2 and IFN–γ gene transcription and protein production, and stimulate T cells to produce Th1 cytokines. We conclude that the activation of T lymphocytes by LBP may contribute to one of its known immuno-enhancement functions.

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References


Reversal of Apoptotic Resistance by *Lycium barbarum* Glycopeptide 3 in Aged T Cells

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**Objective** To study whether *Lycium barbarum* glycopeptide 3 (LBGP3) affects T cell apoptosis in aged mice. **Methods** LBGP3 was purified with DEAE cellulose and Sephadex columns. Apoptotic “sub-G1 peak” was detected by flow cytometry and DNA ladder was resolved by agarose gel electrophoresis. Levels of IFN-γ and IL-10 were measured with specific kits and mRNA expression was detected by RT-PCR. Apoptosis-related proteins of FLIP, FasL, and Bcl-2 were determined by Western blotting. **Results** LBGP3 was purified from Fructus Lycii water extracts and identified as a 41 kD glycopeptide. Treatment with 200 μg/mL LBGP3 increased the apoptotic rate of T cells from aged mice and showed a similar DNA ladder pattern to that in young T cells. The reversal of apoptotic resistance was involved in down-regulating the expression of Bcl-2 and FLIP, and up-regulating the expression of FasL. **Conclusion** *Lycium barbarum* glycopeptide 3 reverses apoptotic resistance of aged T cells by modulating the expression of apoptosis-related molecules.

**Key words**: *Lycium barbarum* glycopeptide 3; Aged T cells; Cytokines; Apoptosis; Senescence

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**INTRODUCTION**

Apoptosis plays an important role in maintaining the integrity and homeostasis of metazoan. In immune system, development and activity of T cells are closely correlated with cellular apoptosis, through which the aged, dysfunctional T cells are selectively destroyed and prevented from accumulating in the body[1]. However, apoptotic resistance occurs in the aging processes. In an IL-2 and ConA-induced apoptosis model, for instance, its mechanism in aged T cells is closely associated with the functional regulation of apoptosis proteins, such as FasL, FLIP, survivin, and T cell type transition from Th1 to Th2[2-3]. Up to the present, there are only a few studies about how to regulate apoptosis by drugs in aged T cells.

*Lycium barbarum* polysaccharides, the main active components of *Fructus lycii*, have been proved to enhance the response of SAMP aged mouse T splenic cells to SRBC, in which concentration-dependent increase in free calcium ion was detected in splenic cells and celiac macrophages[4]. *Lycium barbarum* glycopeptide 3 (LBGP3), one of the polysaccharides, plays a critical role in the above-mentioned response. It was reported that LBGP3 enhances the proliferation of splenic cells in aged mice[5] and activates the function of monocytes by increasing the expressions of IL-2 and TNF-α mRNA and protein levels in a dose-dependent manner. However, little is known about the effect of LBGP3 on aged T cells from the viewpoint of apoptosis.

In the present research, the effect of LBGP3 on apoptosis of T cells in aged mice was examined and the expression of certain apoptosis-related proteins was detected. The results show that LBGP3 could efficiently reverse apoptotic resistance of T cells in aged mice and is thus a promising drug to modulate the aging process.

**MATERIALS AND METHODS**

**Animals**

C57BL/6J mice were purchased from Laboratory Animal Center, Chinese Academy of Medical Sciences. The 2-month old young mice and 26-month old mice were used in the experiment. The mice were sacrificed at the end of treatment. Their tissues and
organs were immediately collected and stored at 
-70°C for experiments.

Reagents

Rabbit anti-FasL polyclonal antibody, rabbit 
anti-Bcl-2 polyclonal antibody, and rabbit anti-actin 
polyclonal antibody were purchased from Santa Cruz. 
Rabbit anti-FLIP polyclonal antibody and rabbit 
anti-Bax polyclonal antibody were bought from 
NeoMarkers. CellTiter 96 aqueous non-radioactive 
and SV total RNA isolation system were purchased 
from Promega. Cell proliferation assay and 
Superscript™ one-step RT-PCR were bought from 
Gibco BRL. IFN-γ ELISA kit and IL-10 
irradiation-immune kit were from Beijing Jingmei Co. 
Ltd. DEAE-cellulose, Sephadex G-75, and 
CM-Sephadex C-50 were from Pharmacia. ConA and 
IL-2 were from Sigma.

Purification of LBGP3

Lycium (~500 g) was minced and soaked in a 
three-fold volume of distilled water for 24 h at room 
temperature and filtered, aqueous solution was 
collected. The pellets were again soaked in a 1.5-fold 
volume of H₂O for additional 6 h, and the filtered 
solution was kept. The pooled mixture from this 
procedure was further concentrated by rotary 
evaporation at room temperature. After centrifugation 
at 3000 rpm for 5 minutes, the supernatant was 
deposited with a 4-fold volume of absolute ethanol. 
The deposition was dissolved with H₂O, and repeatedly 
extracted seven times with a 1/5-fold volume of 
savage agent (CHCL₃:CH₃CH₂CH₂OH = 4:1). 
Solid crude LBP was made by dialyzing the final 
aqueous mixture for 48 h, and vacuum-dried at 4°C. 
LBP was purified by column chromatography with 
DEAE-cellulose (0.15 mol/L NaHCO₃ as an elution 
buffer), and the third peak component (designated as 
LBGP3) was collected, and further purified by 
Sephadex G-75 (washed with 0.15 mol/L NaCl) and 
CM-Sephadex C-50 (washed with 0.25 mol/L 
phosphate buffer). The purity of LBGP3 was 
determined by HPLC Shimadzu LC-10A (column: 
TSK-2005SW), using 50 mmol/L NaH₂PO₄-Na₂HPO₄ 
(pH 7.0) as an elution buffer at the flow rate of 1 mL/min 
(monitored at the wavelength 280 nm).

Determination of Molecular Weight

SDS-PAGE electrophoresis (12%) was performed to 
measure the LBGP3 molecular weight following 
standard electrophoresis protocol. The gel was 
stained with Comassie brilliant blue R250. The 
protein standard markers used included rabbit 
phosphorylase (97.4 kDa), bovine serum albumin 
(66.2 kDa), rabbit actin (43.0 kDa), bovine carboxylase 
(31.0 kDa), trypsin inhibitor (20.1 kDa), and egg white 
bacteriolysin (14.4 kDa). The measured Rfs of the 
proteins were used to plot a standard curve with 
which the LBGP3 molecular weight was calculated.

Isolation and Stimulation of T Cells

The animals were sacrificed with their spleens 
removed aseptically and rinsed with RPMI-1640 
medium. Single cell suspension was prepared by 
rubbing the tissue against sterile stainless steel wire 
meshes (100 μm) in 10 mL PBS and filtered through 
an aseptic fiberglass. Erythrocytes were lysed in 5 
ml Tris-NH₄Cl lysis buffer. The cell suspension was 
centrifuged at 800 rpm, and the pellets were 
resuspended in 2 mL RPMI-1640 medium. The cells 
were loaded onto a nylon fiberglass column and 
icubated at 37°C for 45 min. After the column was 
washed with RPMI-1640 medium, T cell fractions 
were collected and counted. The purified T cells were 
stimulated with ConA (6 μg/mL) and IL-2 (10 ng/mL) 
for 18 h to induce apoptosis. The cells were washed 
with RPMI-1640 medium, and cultured in the same 
medium at 37°C in an atmosphere containing 5% 
CO₂.

Determination of Cell Proliferation

Proliferation of T cells was detected by CellTiter 
96 aqueous non-radioactive cell proliferation assay. 
The cells were exposed to LBGP3 at 50, 100, 200, 
500, and 1000 μg/mL before 20 μL/well MTS/PMS 
mixture solution was added to the wells. The cells 
were incubated for an additional 4 h at 37°C in an 
atmosphere containing 5% CO₂ and read at OD₄₉₀.

Analysis of T Cell Apoptosis by Flow Cytometry

The purified aged T cells at 2×10⁶/mL were 
divided into 2 groups. One group was treated with 
ConA/IL-2, the other group with ConA/IL-2 plus 200 
μg/mL LBGP3. After cultured for 0, 6, 24 h, the cells 
were washed 2 times with cold phosphate buffer and 
fixed for 24 h in 70% ethanol. The cells were washed 
thrice with cold phosphate buffer, incubated with 100 
μL/mL RNase for 30 min at 37°C and stained with 50 
μg/mL PI for 30 min at 4°C. After filtration through 
nylon membrane meshes, cell apoptosis was analyzed 
with a flow cytometer FACS420. The data were 
analyzed using Cellplus2.0 software and the ratio of 
apoptosis was calculated.

DNA Extract and Agarose Gel Electrophoresis

T (2×10⁶) cells were plated in a 6-well plate and 
divided into 3 groups: young cell group, control 
group, and LBGP3 treatment group. After stimulated...
with IL-2/ConA for 18 h, the cells were collected by centrifugation at 800×g for 5 min, 100 μL lytic buffer containing 100 μg/mL RNase was then added to the cell pellets. After incubated at 37℃ for 0.5 h, 100 μL lytic buffer containing 100 μg/mL protease K was added to the solution and incubated at 50℃ for an additional 2.5 h. The same volume of phenol: chloroform: isopropanol (25:24:1) was used to extract protein. The aqueous phase was transferred to a new tube and 1/10 volume of 3 mol/L sodium citrate (pH 5.2) was added. DNA was precipitated with a 2.5-fold volume of cold ethanol in dry ice for 30 min. After centrifugation, DNA was washed once with 70% ethanol, air-dried, and redissolved in 20 μL TE (pH 8.0), 10 μg DNA sample was loaded onto the 1.8% agarose gel and run at 30 volt for 2.5 h in TAE buffer and stained in 1% EB TAE buffer. The result was recorded and analyzed with the UVP gel imaging system.

Influence of LBGP3 on Expression of IFN-γ and IL-10 in Aged T Cells

T cells (1×10^6) were plated in a 6-well plate and divided into 3 groups: young cell group, control group, and LBGP3 treatment group. After treated for indicated times, culture medium was collected for measuring the concentration of IL-10 with liquid scintillation counting[7]. Reagent PR was added and mixed, the admixture was allowed to stand for 20 min at room temperature. IL-10 marked with I 125 and antibodies. After centrifugation at 3500 rpm for 25 min, radioactivity of the pellet was measured with an automatic γ-arithmometer. Referring to the standard curve, the concentration of IL-10 was then measured.

The concentration of IFN-γ was measured by double antibody sandwich ELISA[8-9]. The test samples were added to a 96-well plate, in which mouse anti-IFN monoclonal antibody was embodied. After incubated for 120 min at room temperature, the plate was washed four times and 100 μL well working reagent was added, the plate was then sealed and incubated for 60 min at room temperature. After washed four times, developing and ending were carried out consequently. The value of ODb10 was read and the concentration of IFN-γ was measured based on the standard curve.

Influence of LBGP3 on Expression of IL-10 and IFN-γ mRNA Detected by RT-PCR

After treated with LBGP3, aged T cells were washed twice with PBS. The total RNA was purified with an extraction kit (Promega), following its standard protocol. The extracted RNA was stored at -70℃.

The Sibaiheng Primer 5.0 software was used to design the primers of IL-10 and IFN-γ and β-actin housekeeping gene as an internal control.

IL-10 (496 bp):
- sense 5’AACACACAGGCTTGACTTGAG3’
- antisense 5’GTCCCACACATCCTGACTACT3’.
IFN-γ (256 bp):
- sense 5’ACTTTGTGGAGGCAAGTAGGAAG3’
- antisense 5’ATGTTCCAGGACAGGTGTGTG3’.
β-Actin (733 bp):
- sense 5’GAACCCTAAGGCACACTGAA3’
- antisense 5’CTGCTGGAAAGTGACAGTGAG3’.

Superscript™ one-step RT-PCR kit was employed to analyze the expression of the above cytokines.

Western Blotting

After treated with IL-2/ConA, young and aged T cells were washed twice with cold PBS. The cells were lysed in a lysis buffer (Tris 50 mmol/L, NaCl 150 mmol/L, Na3HPO4 0.02%, SDS 0.1%, NP-40 1%, PMSE 1 mmol/L, leupeptin 10 μg/mL, pepstatin A 1 μg/mL, DTT 1 mmol/L, glycerol 10%, pH 8.0). The protein concentration was measured with a Bio-Rad protein assay kit. SDS-PAGE was performed with a 12% gel and 40 μg per sample was loaded onto the gel. A PVDF membrane was transferred for 2 h at a constant current of 0.8 mA/cm² gel area. The first-antibody (1:200) and the second-antibody (1:1000 horseradish peroxidase) were used. After an illuminant substrate was added, UV gel imaging was performed to obtain the results.

Data Analysis

Data from the various groups were compared by Student’s t-test. In each case, P<0.05 was considered statistically significant. All data listed in the figures or the tables were expressed as (T±s).

RESULTS

Purification of LBGP3

LBGP3 was analyzed by HPLC with a TSK-2000SW column, and the retention time of LBGP3 was 10.282 min, indicating that the purification procedure yielded a high purity of LBGP3. LBGP3 was further analyzed by SDS-PAGE, and a single band was observed (data not shown). Based on the flow rates of protein standard markers with the bromophenol blue front as 1.0, the calculated molecular weight of LBGP3 was 41 kD.

Effective Concentration of LBGP3 for T Cells

When aged T cells were exposed to different concentrations of LBGP3, the proliferation of T cells was more effective at 200 μg/mL LBGP3 (Fig. 1),
which was used in the subsequent experiments.

**FIG. 1.** Effect of LBGP3 on the proliferation of aged T cells. The cell growth was determined by CellTiter 96 aqueous non-radioactive cell proliferation assay.

**Effect of LBGP3 on Apoptotic Rate of Aged T Cells**

In the classical apoptotic process, after fixation and incubation in PBS, aggregated chromatin partially flows out from the cells and forms the sub-G1 peak, named “apoptotic peak”, which can be detected by flow cytometry[10]. When the aged cells were cultured for 6 and 24 h with LBGP3, the apoptotic cells constituted 29.8%±4.9% and 63.1%±8.5%, respectively, higher than those in the control groups (23.5%±3.8% and 42.0%±6.7%, respectively), similar to those in the young mice group (32.6%±4.1% and 67.2%±9.6%, respectively, n=3, P<0.05), suggesting that LBGP3 could recover the apoptotic rate of T cells from the spleen of aged mice up to that in the young group (Fig. 2).

**FIG. 2.** Percentage of splenic T cell apoptosis after treatment with 200 µg/mL LBGP3 in aged T cells determined by flow cytometry.

**Agarose Gel Electrophoresis**

The DNA ladder pattern of aged mouse T cells occurred less than that of the young mouse T cells[11]. A similar DNA pattern generated after incubation of the aged T cells with LBGP3, consistent with the results detected by flow cytometry (Fig. 3).

**FIG. 3.** DNA ladder pattern of the aged mouse splenic T cells after exposure to LBGP3. The cells were stimulated with IL-2/ConA for 18 h. Ten µg of DNA was loaded into a 2% agarose gel and run at 30 voltage for 2 h, and stained with 2% ethidium bromide. 1: young T cells; 2: aged T cells; 3: aged T cells plus LBGP3; M: DNA markers.

**Effect of LBGP3 on Eexpression of IFN-γ and IL-10 in Aged T Cells**

T cells could be divided into two kinds according to the cytokines they produce. Th1 cells could secret IL-2, INF-γ, and TNF-α, whereas Th2 cells could generate IL-4, IL-5, and IL-10. The transition from Th1 to Th2 occurs from middle to old age and Th2 cells are resistant to apoptosis induced by CD95. Hence, we determined the expression of INF-γ and IL-10 to indicate the transition of T cells. The increased INF-γ expression and decreased IL-10 expression were shown after exposure to LBGP3 treatment (Fig. 4). The changes in INF-γ and IL-10 expression were consistent to those in mRNA expression (Fig. 5).

**Effect of LBGP3 on Expression of Apoptosis-related Proteins**

A number of signal molecules are involved in apoptosis, such as FasL, FLIP, Bax, and Bcl-2[12-15]. In old T cells, lower expression of FasL and higher expression of Bcl-2 resulted in apoptotic resistance in...
FIG. 4. Changes of IFN-γ (A) and IL-10 (B) levels in splenic aged T cells after treatment with LBGP3. *P<0.05, **P<0.01 vs control.

FIG. 5. Changes of IFN-γ (A) and IL-10 (B) mRNA expression in aged splenic T cells after treatment with LBGP3 for 18 h. The expression levels were detected by RT-PCR with the Actin expression as an internal control. The figures were representatives of two independent experiments.

contrast to the young T cells (Fig. 6, middle lanes). Increased FasL expression and decreased Bcl-2 expression in aged T cell were observed after treatment with LBGP3, suggesting that the sensitivity of aged T cells to apoptosis was associated with the apoptosis-related molecules. However, the change in Bax expression was not obvious.

FIG. 6. Changes in expression of apoptosis-related proteins after exposure to LBGP3. The expressions of FasL and FLIP proteins (A), Bcl-2 and Bax proteins (B) were determined by Western blotting after treatment of aged T cells with LBGP3 for 18 h. 1: young T cells; 2: aged T cells; 3: aged T cells plus LBGP3.

DISCUSSION

Senescence in immune system is a progressive and irreversible physiological decline, leading to infection, malignancy, and abnormal immune diseases. Studies showed that apoptosis is correlated
with the occurrence of these diseases, and stimulation with antigens can induce apoptosis to destroy the useless cells[16], especially apoptosis of lymphocytes. Our previous study showed that the expressions of IL-2R in aged T cells as well as FasL and NF-kB in young T cells are decreased, though the expression of FLIP in aged T cells is higher than that in the young T cells, suggesting that signal molecules play an important role in apoptosis[17-19]. The present study showed that increased Bcl-2 expression and decreased FasL could lead to apoptotic resistance in aged T cells. Our previous study has proved that the apoptotic rate of T cells in aged mice is lower than that of young T cells. The apoptosis resistance in aged T cells may be related to the transition of T cells. Our previous study showed transformation from type I to type II T cells in aged mice. In this study, we have proved that expression of IFN-γ and IL-10 in T cells of aged mice is changed after treatment with LBGP3.

As a Chinese traditional medicine, *Lycium barbarum* has been used to delay aging in Asia for thousands of years. However, little is known about its biological activities. It was reported that *Lycium barbarum* can regulate apoptosis[19]. We purified *Lycium barbarum* glycoprotein 3 by 3-step chromatography and showed that it could regulate the apoptosis of aged T cells. The results obtained by flow cytometry and agarose gel electrophoresis indicate that the apoptosis rate of aged and young T cells after exposure to LBGP3 was similar, suggesting that the apoptosis resistance of aged T cells can be reversed by treatment with LBGP3. DEcreased Bcl-2 expression and increased FasL expression are correlated with apoptosis resistance and action of LBGP3. The exact molecular targets of LBGP3 on apoptosis pathways are under investigation in our laboratory.

REFERENCES


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Lycium barbarum polysaccharides regulate phenotypic and functional maturation of murine dendritic cells

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Abstract

Lycium barbarum polysaccharides (LBPs) have been known to have a variety of immunomodulatory functions including activation of T cells, B cells and NK cells. Dendritic cells (DC) are potent antigen-presenting cells that play pivotal roles in the initiation of the primary immune response. However, little is known about the immunomodulatory effects of LBPs on murine bone marrow derived dendritic cells (BMDC). In the present study, the effects of LBPs on the phenotypic and functional maturation of murine BMDC were investigated in vitro. Compared to the BMDC that were only subjected to treatment with RPMI1640, the co-expression of I-A/I-E, CD11c and secretion of IL-12 p40 by BMDC stimulated with LBPs (100 \( \mu \)g/ml) were increased. In addition, the endocytosis of FITC-dextran by LBPs-treated BMDC (100 \( \mu \)g/ml) was impaired, whereas the activation of proliferation of allogenic lymphocytes by BMDC was enhanced. Our results strongly suggest that LBPs are capable of promoting both the phenotypic and functional maturation of murine BMDC in vitro.

Keywords: Lycium barbarum polysaccharides (LBPs); Dendritic cells (DC); Mixed lymphocyte reaction (MLR)

1. Introduction

Lycium barbarum, a well-known Chinese traditional medicine and also an edible food, plays multiple roles in pharmacological and biological functions including anti-aging activity, hypoglycemic and hypolipidemic effects, strengthening yang and improving eyesight. It has been reported that polysaccharides purified from Lycium barbarum are especially effective in modulating immune functions and inhibiting tumor growth (Gong et al., 2005; Gan et al., 2004; Cao et al., 1994). The immuno-modulation effects of LBPs are extensive, including increasing macrophage phagocytosis, the form of antibody secreted by spleen cells, spleen lymphocyte proliferation and CTL activity. Recently, the antitumor effects of LBPs have been investigated and are believed to be going through immune mechanisms (Gan et al., 2003, 2004).

DC, one of the most potent antigen-presenting cells (APC), are important for the initiation of primary immune response of both helper and cytotoxic T lymphocytes (Banchereau et al., 2000; Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2001; Liu, 2001). After antigen capture, the DC precursors migrate to T cell regions of draining lymph nodes where they mature into functional DC. The functional DC further stimulate naive T cells by triggering the signaling pathway involving both major histocompatibility complex (MHC) molecules presenting antigen-peptides and co-stimulatory molecules (Austyn et al., 1988; Rock, 1996). The initial contact between DC and resting T cells may be mediated by a transient, high affinity interaction between DC-SIGN on the DC and the adhesion molecule ICAM-3 on the T cells (Geijtenbeek et al., 2000), followed by interactions through other adhesion molecules and their corresponding ligands (ICAM-1/LFA-1,
LFA-1/CD2). Following TCR engagement, an intimate interaction often referred to as the immunological synapse evolves, where multiple interactions between co-stimulatory molecules on DC and their ligands on T cells result in final DC maturation and T cell activation (Banchereau et al., 2000; Lanzavecchia and Sallusto, 2000). There are no reports about LBPs inducing maturation of murine BMDC. Therefore, LBPs regulating phenotypic and functional maturation of murine BMDC were investigated in this study.

2. Materials and methods

2.1. Source of mice

Male or female C57BL/6J (H-2b) and BALB/c (H-2b) mice were purchased from the Department of Experimental Animal, College of Medicine, Zhejiang University, Hangzhou, China. Mice were used at 4–6 weeks of age.

2.2. Source of drugs

LBPs were purchased from Pharmagenesis Beijing Office. The percentage of polysaccharide of LBPs was about 84.32% according to the phenol-sulfuric acid colorimetric method. The molecular weights of LBPs were estimated to be 31,000 through high performance gel filtration chromatography. The LBPs mainly consist of mannose, glucose, galactose, arabinose, rhamnose, and xylose.

2.3. Generation of bone marrow-derived murine myeloid DC

DC were prepared as described previously with minor modifications (Inaba et al., 1992). Briefly, bone marrow cells were flushed from the femur and tibiae of C57BL/6J mice and depleted of RBC by hypotonic lysis using Tris-NH4Cl. Cells with a starting number of 2 × 10^5 cells per ml were cultured in RPMI 1640 in six-well flat bottom plates (Orange Scientific) at 37°C, 5% CO2, supplemented with 10% fetal calf serum (FCS), 2 mmol/l L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 30 ng/ml rmGM-CSF (Peprotech) and 20 ng/ml rmIL-4 (Peprotech). On day 3, the old medium was replaced with fresh medium. On day 5, cells were purified by MACS columns (Miltenyi Biotech). CD11c^+ DC were acquired and divided into 3 groups. In parallel, 3 groups of DC were incubated at a concentration of 1 × 10^5/ml with either 100 μg/ml LBPs, serum-free RPMI media 1640 or 100 ng/ml LPS (serotype 0111:B4; Sigma). The serum-free RPMI media 1640 and 100 ng/ml LPS groups were used as controls. On day 7, cells and culture supernatants were collected for further experiments and analysis.

2.4. Flow cytometric analysis

Cell surface expression of I-A/I-E or CD11c was determined by immunofluorescence staining. On day 7, cells were harvested, washed twice with PBS, and resuspended in washing buffer (PBS containing 2% FCS and 0.1% sodium azide). Cells were first blocked with 20% mixed mouse and rat serum for 15 min at 4°C, and then stained with PE conjugated anti-mouse I-A/I-E antibody (BD Pharmingen) and FITC conjugated anti-mouse CD11c antibody (BD Pharmingen) for 30 min at 4°C in the dark. Lastly the antibody-treated cells were washed twice with washing buffer. Cell surface co-expression of I-A/I-E and CD11c was detected by flow cytometry (Becton Dickinson). All the obtained data were analyzed by the CellQuest software package.

2.5. Cytokine assay

On day 7, DC culture supernatants were collected and the concentration of mouse IL-12 p40 unit was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Biosource) according to the manufacturer’s instructions. Cytokine concentrations were determined according to absorbance readings at 450 nm on a universal microplate reader (Bio-Tek Instruments).

2.6. Endocytosis is assay

In order to analyze the endocytic capacity of DC on day 7, 1 × 10^5 cells were incubated at 37°C for 1 h with 1 mg/ml FITC-dextran (Sigma). In the parallel experiment 1 × 10^5 cells were incubated at 4°C for 1 h. After incubation, the cells were washed twice with cold HBSS. The cells were analyzed on a flow cytometer.

2.7. Mixed lymphocyte reaction (MLR) induced by DC

Responder mononuclear lymphocytes from H-2^b^ BALB/c’s splenocytes were isolated by Ficoll-Urografin density gradient. On day 7, mature DC were harvested as inducers, treated with 25 μg/ml mitomycin C (AppliChem) for 45 min, then 5 × 10^5 cells were added to allogeneic lymphocytes (1 × 10^6 cells per well) in flat-bottom 96-well tissue culture plates for 120 h. Cell proliferation was estimated according to the cellular reduction of tetrazolium salt MTT (Sangon) by the mitochondrial dehydrogenase of viable cells into a blue formazan product that was measured spectrophotometrically.

2.8. Statistics

The results were expressed as means ± SD of the indicated number of experiments. The statistical significance was estimated using a Student’s t-test for unpaired observations. A p value of <0.05 was considered to be significant.

3. Results

3.1. LBPs up-regulate the co-expression of I-A/I-E and CD11c on DC surface

LBPs (100 μg/ml) or LPS (100 ng/ml) treated DC showed an increased co-expression of I-A/I-E and CD11c on DC surface, and the double positive cells ratio were (41.42 ± 1.43)% or (54.13 ± 3.99)%, respectively, whereas that of RPMI 1640 was (35.68 ± 2.40)%. The difference was significant by paired t test analysis: n = 4, P < 0.01. One groups’ figure of flow cytometric analysis is shown (Fig. 1).

3.2. LBPs increase IL-12 p40 production of DC

LBPs (100 μg/ml) or LPS (100 ng/ml) treated DC showed an increased production of IL-12 p40 in culture supernatants of DC, and the production was 94.1 ± 12.2 pg/ml, 263.6 ± 13.8 pg/ml, respectively, compared with 57.3 ± 17.2 pg/ml of RPMI 1640. A significant difference was observed between RPMI 1640 control and 100 μg/ml LBPs group or LPS group (Fig. 2). The difference was significant by paired t test analysis: n = 4, P < 0.01.

3.3. LBPs inhibit the FITC-dextran uptake by DC

Mannose-receptor-mediated endocytosis was analyzed by the uptake of FITC-dextran. In DC treated by LBPs (100 μg/ml) or LPS (100 ng/ml), the percentage (30.96 ± 3.30 and 19.66 ± 2.01, respectively) of FITC-stained cells had a significantly lower uptake of FITC-dextran, compared with RPMI 1640 (68.52 ± 1.66)%. Parallel experiments were also
performed at 4°C (15.93 ± 1.02), FITC-dextran was internalized significantly less than DC at 37°C. The difference was significant by paired t test analysis: n = 4, P < 0.01). One groups’ figure of flow cytometric analysis is shown (Fig. 3).

3.4. LBPs facilitate the allostimulatory capacity of DC

The effects of LBPs on MLR induced by DC are illustrated in Fig. 4. LBPs (100 μg/ml)-treated DC or LPS (100 ng/ml)-treated DC stimulated proliferative responses more effectively than RPMI 1640-treated DC, and the proliferation ratio of the lymphocytes was (129.9 ± 12.48)% or (153.8 ± 14.15)%, respectively, compared with (100 ± 9.12)% of RPMI 1640 (Fig. 2). The difference was significant by paired t test analysis: n = 5, P < 0.05). (RPMI 1640 control served as 100%).

4. Discussion

It has been well-known for decades that LBPs are biologically active components of Lycium barbarum with potential pharmacological and biological functions. This is the first report that we are aware of showing the effects of LBPs on the generation and maturation of murine BMDC. In this study, we have shown that LBPs can induce maturation of murine BMDC and ready them for T cell-mediated immune responses. LBPs significantly increase the production of bioactive IL-12 p70 in murine BMDC in the presence of GM-CSF/IL-4.
Also, we investigated the capacity of LBPs-treated DC to internalize FITC-dextran via mannose receptor-mediated endocytosis. Both mechanisms are complex, energy-dependant processes that require the coordinated action of the actin cytoskeleton and are characteristic and distinctive properties of immature vs. mature DC (Sallusto et al., 1995; Garrett and Mellmann, 1999). These data further support the view that exposure to LBPs promotes the generation of functionally active, mature DC.

It is well-known that tumors are sources of biological substances and release certain immunosuppressive factors to evade the immune surveillance system of the host (Kanto et al., 2001). Through analysis of tumor specimens obtained from patients, a reduced number of DC infiltrated into tumors correlated with a poor prognosis for patients (Zeid and Muller, 1993; Tsuge et al., 2000). Also, the phenotypes and function of DC could be altered in tumors. The reduction of expression of co-stimulatory molecules, defective cytokine production, and full allostimulatory activity could be found in DC infiltrated into tumor tissue, which implies that tumor-derived factors can impede DC maturation (Gabrilovich et al., 1997; Troy et al., 1998). These effects appear to be maturation-dependent, acting only on DC precursors and not mature DC. Therefore, it may be better to use mature DC for clinical applications.

LBPs as a traditional Chinese herb have been extensively used in improving the immune activity of patients treated by chemical or radiation therapy. According to the results of this study, LBPs enhance the phenotypic and functional maturation of DC. Therefore, LBPs may increase the antitumor effects of DC-based vaccine therapy. Now we are investigating the antitumor effects of a combination therapy through an LBPs- and DC-based vaccine in a tumor-bearing mouse model.

Acknowledgments

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Immunomodulation and antitumor activity by a polysaccharide–protein complex from *Lycium barbarum*

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Abstract

The modulation of a polysaccharide–protein complex from *Lycium barbarum* (LBP\textsubscript{3p}) on the immune system in S180-bearing mice was investigated. The mice inoculated with S180 cell suspension were treated p.o. with LBP\textsubscript{3p} (5, 10 and 20 mg/kg) for 10 days. The effects of LBP\textsubscript{3p} on transplantable tumors and macrophage phagocytosis, quantitative hemolysis of sheep red blood cells (QHS), lymphocyte proliferation, the activity of cytotoxic T lymphocyte (CTL), interleukin-2 (IL-2) gene expression and lipid peroxidation were studied. LBP\textsubscript{3p} could significantly inhibit the growth of transplantable sarcoma S180 and increase macrophage phagocytosis, the form of antibody secreted by spleen cells, spleen lymphocyte proliferation, CTL activity, IL-2 mRNA expression level and reduce the lipid peroxidation in S180-bearing mice. The effect is not dose-dependent in a linear fashion. A total of 10 mg/kg dose is more effective than 5 and 20 mg/kg doses. This suggests that LBP\textsubscript{3p} at 10 mg/kg has a highly significant effect on tumor weight and improves the immune system.

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Keywords: *Lycium barbarum*; Polysaccharide–protein complex; Transplantable tumor; Immune system

1. Introduction

During the past three decades, many polysaccharides and polysaccharide–protein complexes have been isolated from mushrooms, fungi, yeasts, algae, lichens and plants. The biological activities of these polysaccharides have attracted more attention recently in the biochemical and medical areas because of their immunomodulatory and antitumor effects [1]. The search for novel polysaccharides with antitumor properties stems from the basic shortcomings of cancer chemotherapy and radiotherapy. In particular, the great majority of chemical compounds, which have been identified as cytotoxic to cancer cells, are also toxic to normal cells [2]. Hence, the discovery and identification of new safe drugs, without severe side effects, has become an important goal of research in the biomedical sciences. The enhancement or potentiation of host defense mechanisms has been recognized as a possible means of inhibiting tumor growth without harming the host.
*Lycium barbarum* plays multiple roles in pharmacological and biological functions as a well-known Chinese traditional medicine and also a kind of food. *L. barbarum* polysaccharide–protein complex (LBP) is its important bioactive component. It was reported that the crude LBP could significantly suppress the growth of malignant tumor in vivo [3,4]. However, the antitumor effect of LBP is speculative because of the unclear purity of the crude LBP and the mechanism of antitumor action of LBP is still not fully understood. In the present work, the immunomodulatory effect of LBP3p, the third fraction of LBP, on S180-bearing mice was investigated and the lipid peroxidation was also discussed.

2. Materials and methods

2.1. Preparation of LBP3p

*L. barbarum* was collected in Zhongning, Ningxia, China and identified by Prof. Qiong Luo, Medical School of Wuhan University, Wuhan, China. The voucher specimen of this plant (FST 505) is deposited in the Department of Food Science and Technology, Huazhong Agricultural University, Wuhan, China. A total of 100 g of dried powdered *L. barbarum* were first extracted with acetone/petroleum (1:1, 300 ml/C2H3) to remove pigments. The dried residual plant material was then extracted with 80% ethanol (300 ml/C2H3) to remove oligosaccharides. The pellets were extracted with distilled water (300 ml) at 80 °C every 2 h for four times, and the extracts were pooled and concentrated. The crude polysaccharide–protein complex (LBP) was precipitated by five volumes of ethanol. After centrifugation, the precipitate (3.35 g) was redissolved in distilled water (30 ml) and applied to DEAE-cellulose (OH⁻) anion exchange chromatography column (column size: 2.6 × 40 cm, 30 g). The column was eluted with H₂O (480 ml), 0.05 M NaCl (500 ml), 0.10 M NaCl (500 ml) and 0.25 M NaCl (500 ml). A neutral and three acidic fractions were obtained, LBP₁ (0.535 g), LBP₂ (0.511 g), LBP₃ (0.659 g) and LBP₄ (1.333 g), respectively. Each fraction was dialyzed against distilled water for 3 days and then purified on Sephadex G200 column (column size: 2.6 × 60 cm, 10 g) with distilled water (200 ml) as the eluant. A purified fraction from LBP₃ (LBP₃p) was obtained after lyophilization with a yield of 0.297 g. It was identified to be homogeneous by SDS-PAGE [5], which showed a single band after staining with Ag [6] and periodic acid-schiff [7]. Chemical composition of LBP₃p was 63.56% neutral sugars, 24.80% acidic sugars and 7.63% proteins as determined by phenol–H₂SO₄ [8], carbazole [9] and Lowry method [10], respectively. It contained six monosaccharides: galactose, glucose, rhamnose, arabinose, mannose, xylose and the molar ratio was 1:2.12:1.25:1.10:1.95:1.76, respectively, by gas chromatography [11]. The molecular weight of LBP₃p was 1.57 × 10⁵ by laser light scattering [12].

2.2. Experimental animals

Male Kunming mice (18 ± 2) g were provided by the Animal Center, Institute of Health and Epidemic Prevention, Wuhan, China. The mice were housed under normal laboratory conditions (21 ± 2 °C, 12/12-h light–dark cycle) with free access to standard rodent chow and water. Under sterile condition, 0.2 ml of S180 cell suspension (sterile normal saline 1:3 dilution) was inoculated subcutaneously to mouse armoit. The mice inoculated were divided into five groups: S180-bearing control group, 20 mg/kg cyclophosphamide (CTX) and 5, 10, 20 mg/kg LBP₃p treatment groups. A normal control group was also used in this experiment. CTX and LBP₃p were administered p.o. for 10 days once daily. Normal control and S180-bearing control groups received the same volume of normal saline. On day 11, all animals were executed. The mice, thymus and tumors were weighed, and a count on tumor inhibition rate was made. Thymus index was expressed as the thymus weight relative as body weight.

2.3. Macrophage phagocytosis assay

The mice were injected i.p. with 1.0-ml 6% starch broth 3 days prior to sacrifice. On day 11, the mice were injected i.p. with 1.0-ml 1% cock red blood cells (CRBC). After 30 min, the macrophages were collected, fixed with methanol and stained with Giemsa. The number of CRBC ingested by 100 macrophages were counted in an optical microscope and expressed as phagocytosis index (PI). Besides, the percentages of macrophages that phagocytosed CRBC were deter-
mined and expressed as phagocytic efficiency index (PEI).

2.4. Quantitative hemolysis of sheep red blood cells (QHS) assay

QHS assay was performed using the methods of Simpson and Gozzo [13] with some modifications. In brief, 0.2 ml of 20% sheep red blood cells (SRBC) prepared in normal saline was injected to animals, i.p., 4 days prior to the assay. On day 4 following immunization, the spleens were removed and single cell suspensions of $10^6$ cells/ml were prepared in PBS. A total of 1.0 ml of 0.4% SRBC and 1.0 ml of 10% guinea pig serum were mixed with 1.0 ml of cell suspension and incubated for 1 h at 37 °C. After centrifugation at 3000 rpm for 3 min, the absorbance of the supernatant was measured at 413 nm using spectrophotometer (Shimadzu UV-1201).

2.5. Lymphocyte proliferation assay

Spleens were aseptically removed from sacrificed mice with scissors and forceps in cold phosphate-buffered saline (PBS) and gently homogenized with a loose teflon pestle. $5 \times 10^3$ cells were cultured in RPMI-1640 medium supplemented with 10% newborn bovine serum (NBS) at 37 °C in an atmosphere of 5% CO₂ in the presence of concanavalin A (ConA, 5 mg/l). After treatment for 72 h, 10 µl MTT (5 g/l) was added to every well and the plate was incubated for another 4 h. The plate was then centrifuged at 2000 rpm for 10 min and the supernatants were discarded. A total of 150 µl dimethyl sulfoxide was added to each well. The plate was then shaken until crystals were dissolved. The absorbance $A_{570}$ was detected on the ELX800 Microplate Reader (Bio-TEK, USA) [14].

2.6. Assays of cytotoxic T lymphocyte (CTL)

The CTL activity from spleen was studied using a Cytotox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega). S180 cells were used as target cells and seeded in 96-well U-bottom culture plates at $5 \times 10^4$ cells/well in RPMI-1640. Spleen cells were added at $5 \times 10^5$ cells/well. The plates were centrifuged at 2000 rpm for 4 min to facilitate cell to cell contacts and then they were incubated for 4 h at 37 °C. Lactate dehydrogenase (LDH) activity was measured in 50 µl well of the supernatants by addition of the enzyme substrate and absorbance recording at 490 nm. Three kinds of control measurements were performed: a target spontaneous release, a target maximum release and an effector spontaneous release. To determine the percentage of target cells killed, the following equation was used: % lysis=$(E-ES-TS)/(M-ES) \times 100$; where $E$=mean of absorbances in the presence of effector cells, ES=mean of absorbances of effector cells incubated alone, TS=mean of the absorbances of target cells incubated with medium alone, M=mean of maximum absorbances after incubating target cells with lysis solution.

2.7. Interleukin-2 (IL-2) gene expression

Mice were sacrificed and their spleen were removed immediately, frozen in liquid nitrogen and then stored at $-80$ °C. Total RNA was extracted with TRIZol reagent (Gibco BRL) according to the manufacture’s instruction and resuspended in 100 µl RNase-free water. Total RNA was quantified by using a spectrophotometer at 260 nm and the purity was assessed by determining the ratio of $A_{260}/A_{280}$ (nm). Complementary DNA (cDNA) was prepared by incubation RNA with Molony murine leukemia virus (M-MLV) reverse transcriptase (200 units, Promega), 10 mM dNTP (Roche) and Oligo (dT)₁₅ (200 ng, Promega) at 37 °C for 60 min in 25 µl Tris–HCl buffer (50 mM, pH 8.3). Following inactivation of the enzyme by incubation at 95 °C for 5 min, the cDNAs were amplified in a polymerase chain reaction (PCR) with the following primer sets: 5'-TGCAGCTCGCATCTTGTTCA-3' and 5'-ACTCTCTACTATCGGAAGA-3' for IL-2 (the product size 468 bp); 5'-TGCGTCAGAAGGACTCCTATG-3' and 5'-TCTTCTCGATACTCAGGGAC-3' for $\beta$-actin as an internal control (product size: 591 bp). These sets were designed from the published nucleic acid sequences available from GenBank databases. PCR amplification was carried out with a reaction mixture composed of primers, dNTP and 1 unit Taq DNA polymerase (Promega). After heating samples at 95 °C for 5 min, 30 cycles of PCR were performed consisting of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and extension at 72
For 1 min with a final extension at 72°C for 5 min. An aliquot of PCR products was separated on a 1.0% agarose gel containing ethidium bromide and measured semiquantitatively using a Bio-Rad Multi-Analyst System. To compare the relative mRNA expression level from each of the samples, the value is presented as the ratio of the IL-2 band intensity of RT-PCR product over the corresponding h-actin RT-PCR product. The PCR products were sequenced to confirm the specificity of the oligonucleotide primers.

2.8. Lipid peroxidation assay

Ten percent homogenates of liver, spleen and serum were prepared in cold normal saline. After centrifugation at 3000 rpm at 4°C for 20 min, 1.0-ml 15% trichloroacetic acid (TCA) and 1.0-ml 6.7% thiobarbituric acid (TBA) were mixed with 1.0 ml of the supernatant and then boiled for 15 min. The contents were centrifuged at 3000 rpm for 10 min and the absorbance was measured at 532 nm. The content of protein was determined using Bradford’s method. The concentration of TBARS was calculated using an extinction coefficient of 155 mM⁻¹ cm⁻¹ [15].

2.9. Statistical analysis

Results were expressed as mean ± standard deviation (S.D.). The statistical significance of difference between groups was evaluated by analysis of vari-

Table 1
Effects of LBP₃p on thymus indexes tumor weights in S180-bearing mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (mg/kg)</th>
<th>Thymus index (mg/g)</th>
<th>Tumor weight (g)</th>
<th>Inhibitory rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>–</td>
<td>3.42 ± 0.67***</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S180 control</td>
<td>–</td>
<td>2.01 ± 0.70</td>
<td>1.48 ± 0.32</td>
<td>–</td>
</tr>
<tr>
<td>CTX</td>
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<td>2.23 ± 0.37</td>
<td>0.77 ± 0.30***</td>
<td>47.77</td>
</tr>
<tr>
<td>LBP₃p</td>
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<td>2.55 ± 0.15*</td>
<td>1.07 ± 0.39*</td>
<td>28.14</td>
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<tr>
<td></td>
<td>10</td>
<td>2.80 ± 0.34**</td>
<td>0.84 ± 0.44***</td>
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<td></td>
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<td>2.45 ± 0.30</td>
<td>1.31 ± 0.12*</td>
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</table>

S180-bearing mice were administered p.o. with CTX 20 mg/kg and LBP₃p, 5, 10, 20 mg/kg for 10 days once daily. Normal control and S180-bearing control groups received the same volume of normal saline. Thymus index, tumor weight and the inhibitory rate were determined on day 11. Values are mean ± S.D. of 10 mice.

* Significantly different from S180 control group at p<0.05.
** Significantly different from S180 control group at p<0.01.
*** Significantly different from S180 control group at p<0.001.

Table 2
Effects of LBP₃p on macrophage phagocytosis and the form of antibody secreted by spleen cells in S180-bearing mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (mg/kg)</th>
<th>PEI (%)</th>
<th>PI</th>
<th>A₄₁₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>–</td>
<td>37.40 ± 3.58***</td>
<td>1.15 ± 0.09***</td>
<td>0.447 ± 0.012**</td>
</tr>
<tr>
<td>S180 control</td>
<td>–</td>
<td>28.40 ± 3.64</td>
<td>0.76 ± 0.05</td>
<td>0.340 ± 0.028</td>
</tr>
<tr>
<td>CTX</td>
<td>20</td>
<td>30.80 ± 2.28</td>
<td>0.81 ± 0.07</td>
<td>0.356 ± 0.021</td>
</tr>
<tr>
<td>LBP₃p</td>
<td>5</td>
<td>33.20 ± 3.03*</td>
<td>0.90 ± 0.05</td>
<td>0.390 ± 0.016**</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>35.00 ± 3.67**</td>
<td>1.07 ± 0.07***</td>
<td>0.424 ± 0.014**</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>31.60 ± 2.07</td>
<td>0.86 ± 0.09</td>
<td>0.381 ± 0.015*</td>
</tr>
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</table>

S180-bearing mice were administered p.o. with CTX 20 mg/kg and LBP₃p, 5, 10, 20 mg/kg for 10 days once daily. Normal control and S180-bearing control groups received the same volume of normal saline. The phagocytosis index (PI), phagocytic efficiency index (PEI) and quantitative hemolysis of sheep red blood cells (QHS) were determined according to Section 2. Values are mean ± S.D. of 10 mice.

* Significantly different from S180 control group at p<0.05.
** Significantly different from S180 control group at p<0.01.
*** Significantly different from S180 control group at p<0.001.
ance, followed by Student’s $t$-test. A significant difference was set at $p<0.05$.

3. Results

3.1. Effects of LBP$_{3p}$ on thymus indexes and tumor weights in S180-bearing mice

As shown in Table 1, 5 and 10 mg/kg LBP$_{3p}$ caused a significant increase in the thymus index compared with S180 control group. No significant increase was observed in CTX-treated animals. LBP$_{3p}$ could significantly inhibit the growth of mouse transplantable sarcoma S180. The inhibitory rate was 28.14%, 43.05% and 11.88%, respectively, when the concentration of LBP$_{3p}$ was 5, 10 and 20 mg/kg. The inhibitory effect of LBP$_{3p}$ 10 mg/kg was the strongest and close to that of CTX 20 mg/kg, a positive control.

3.2. Effects of LBP$_{3p}$ on macrophage phagocytosis and humoral immunity in S180-bearing mice

A significant reduction in macrophage phagocytosis and QHS assay was induced in S180-bearing mice. LBP$_{3p}$ could markedly increase macrophage phagocytosis and the antibody secreted by spleen cells in

<table>
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<th>Table 3</th>
<th>Effects of LBP$_{3p}$ on spleen lymphocyte proliferation and CTL activity in S180-bearing mice</th>
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<tr>
<td>Group</td>
<td>Concentration (mg/kg)</td>
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<tr>
<td>Normal</td>
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<td>control</td>
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<td>S180</td>
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S180-bearing mice were administered p.o. with CTX 20 mg/kg and LBP$_{3p}$ 5, 10, 20 mg/kg for 10 days once daily. Normal control and S180-bearing control groups received the same volume of normal saline. The lymphocyte proliferation and CTL activity were determined according to Section 2. Values are mean ± S.D of 10 mice.

*Significantly different from S180 control group at $p<0.05$.
**Significantly different from S180 control group at $p<0.001$.

S180-bearing mice (Table 2). The effect of LBP$_{3p}$ 10 mg/kg was the best and it could restore macrophage phagocytosis and humoral immunity in S180-bearing mice closely to those of normal control.

3.3. Effects of LBP$_{3p}$ on cellular immunity in S180-bearing mice

Spleen lymphocyte proliferation and CTL activity were significantly decreased in S180-bearing mice. LBP$_{3p}$ could remarkably increase spleen lymphocyte proliferation and CTL activity in S180-bearing mice (Table 3). LBP$_{3p}$ 10 mg/kg could regulate cellular immunity in S180-bearing mice nearly to that of normal control.
3.4. Effects of LBP3p on IL-2 gene expression in S180-bearing mice

Effect of LBP3p on IL-2 mRNA level in murine spleen was determined by a semi-quantitative RT-PCR. As seen in Fig. 1, the expression level of IL-2 was significantly decreased in S180-bearing mice compared with that in normal control. LBP3p markedly augmented IL-2 gene expression in S180-bearing mice. LBP3p 5, 10 and 20 mg/kg increased IL-2 mRNA level by 3.29-, 4.80- and 2.23-fold, respectively.

3.5. Effects of LBP3p on lipid peroxidation in S180-bearing mice

The effects of LBP3p on lipid peroxidation in S180-bearing mice are shown in Table 4. The results showed that MDA contents were increased in liver, spleen and serum of S180-bearing mice and LBP3p could significantly inhibit the lipid peroxidation. LBP3p 10 mg/kg could decrease the lipid peroxidation in S180-bearing mice nearly to that of normal control.

4. Discussion

The relation between the occurrence, growth and decline of tumor and immune states is the essential problem of tumor immunology. The discovery and identification of new antitumor drugs, which can potentiate the immune function has become an important goal of research in immunopharmacology and oncotherapy. This study demonstrates the favourable antitumor effect of LBP3p, a polysaccharide–protein complex from *L. barbarum* and its immunomodulative activity. LBP3p could significantly inhibit the growth of mouse transplanted sarcoma S180 after treatment for 10 days and the inhibitory effect of LBP3p 10 mg/kg was close to that of CTX 20 mg/kg, a positive control.

Protective immunity against tumor is composed of both humoral and cellular immunity. The humoral defence via antibody response is mediated by B cells and other immune cells involved in antigen processing and immunization. The antigen–antibody complex can counteract toxin and defend the infection induced by pathogen. Cell-mediated immune defense was mediated specifically by T cells including cytotoxic T cells. T cells can kill tumors and produce many lymphocyte factors consisting of macrophage mobile factor, lymphotoxin, transfer factor and interferon, which can enhance macrophage phagocytosis and the capacity of killing target cells [16]. In this experiment, we found that LBP3p could significantly increase macrophage phagocytosis, the antibody secreted by spleen cells, spleen lymphocyte proliferation, CTL activity in S180-bearing mice that suggested that LBP3p could enhance the humoral immunity and cell-mediated immunity in S180-bearing mice. LBP3p has potent tumor therapeutic activity by improving the immune system.

Since cytokines play a prominent role in the development of immune response, we also investigated the effect of LBP3p on the production of IL-2 in S180-bearing mice. IL-2 stimulates the proliferation of CTL, helper T lymphocytes, natural killer (NK) cells, lym-
phokine activated killer (LAK) cells and macrophages, all of which can participate in immunological antitumor mechanisms [17]. IL-2 mRNA level was found to be augmented in LBP$_{3p}$-treated S180 murine spleen in the present study, which implied that IL-2 may be involved in the early phase of immune response and induces CTL production. The increase in IL-2 also may explain the antitumorigenic properties of LBP$_{3p}$.

The function of immune cells largely relies on the structures and functions of their membranes [18]. Membrane phospholipid is the main object attacked by free radical. The more the degree of unsaturation of membrane phospholipid, the more the lipid peroxidation and the damage to cell membranes. The damages to the structure and function of the membranes of immune cells induced by lipid peroxidation have an adverse effect on the immune function. The present study showed that LBP$_{3p}$ significantly decreased the lipid peroxidation of immune cells in S180-bearing mice, demonstrating indirectly that LBP$_{3p}$ could potentiate the immune function in S180-bearing mice to some extent.

In this experiment, the inhibitory effects of LBP$_{3p}$ on tumor and its immunomodulative activity were not in a dose-dependent manner. The effect of LBP$_{3p}$ 10 mg/kg was the best, LBP$_{3p}$ 5 mg/kg taking second place. It was reported that the key to regulate the immune function by polysaccharide was the level of state in body, not the dose. The immune level can be regulated to the normal by polysaccharide in autoimmune process and no effect was evident at higher doses above the limit. It was achieved by integral harmony function in which network of immune-neuro-endocrine interactions was the priority [19].

In conclusion, the antitumor and immunostimulatory activities of LBP$_{3p}$ have been demonstrated in mice. Further studies on the mechanism by which LBP$_{3p}$ induces these effects and additional clinical usefulness in therapies of cancer are needed.

References


A polysaccharide-protein complex from *Lycium barbarum* upregulates cytokine expression in human peripheral blood mononuclear cells

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Abstract

The production of cytokine is a key event in the initiation and regulation of an immune response. Many compounds are now used routinely to modulate cytokine production, and therefore the immune response, in a wide range of diseases, such as cancer. Interleukin-2 and tumor necrosis factor-\(\alpha\) are two important cytokines in antitumor immunity. In this study, the effects of *Lycium barbarum* polysaccharide-protein complex (LBP3p) on the expression of interleukin-2 and tumor necrosis factor-\(\alpha\) in human peripheral blood mononuclear cells were investigated by reverse transcription polymerase chain reaction (RT-PCR) and bioassay. Administration of LBP3p increased the expression of interleukin-2 and tumor necrosis factor-\(\alpha\) at both mRNA and protein levels in a dose-dependent manner. The results suggest that LBP3p may induce immune responses and possess potential therapeutic efficacy in cancer.

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Keywords: *Lycium barbarum*; Polysaccharide-protein complex; Cytokine; Gene expression; Bioassay

1. Introduction

During the past three decades, many polysaccharides and polysaccharide-protein complexes have been isolated from mushrooms, fungi, yeasts, algae, lichens and plants. The biological activities of these polysaccharides have attracted more attention recently in biochemical and medical fields because of their immunomodulatory and antitumor effects (Ooi and Liu, 2000). The search for novel polysaccharides with antitumor properties stems from the basic shortcomings of cancer chemotherapy and radiotherapy. In particular, the great majority of chemical compounds, which have been shown to be cytotoxic to cancer cells, are also toxic to normal cells (Kim et al., 1996). Hence, the discovery and identification of new safe drugs, without severe side effects, has become an important goal of research in the biomedical sciences. The enhancement or potentiation of host defense mechanisms has been recognized as a possible means of inhibiting tumor growth without harming the host.

*Lycium barbarum* plays multiple roles in pharmacological and biological functions as a well-known Chinese traditional medicine and also a kind of food. *L. barbarum* polysaccharide-protein complex (LBP) is its important bioactive component. Recently, our group demonstrated that LBP3p, the third fraction of LBP, could significantly suppress the growth of S180 solid tumor in vivo and restore the immune status of S180-bearing rats, as witnessed by macrophage phagocytosis, the form of antibody secreted by spleen cells, spleen lymphocyte proliferation and the activity of cytotoxic T lymphocytes (CTL) (Gan and Zhang, in press). However, there is no clear understanding of the molecular or cellular basis of immunostimulation by LBP3p. Cytokines are mediators of all aspects of immunoregulation, so the capacity to induce or enhance cytokine production could be a major mechanism by which LBP3p exerts immunomodulatory effects. In the present work, the expression of interleukin-2 and tumor necrosis factor-\(\alpha\) was investigated by using a semiquantitative reverse transcription and polymerase chain reaction (RT-PCR) technique for the quantification of cytokine mRNA and bioassay for the quantification of secreted cytokines after addition of LBP3p to human peripheral blood mononuclear cells.
2. Materials and methods

2.1. Preparation of LBP3p

LBP$_{3p}$, the third fraction of LBP, was extracted with hot water from L. barbarum (planted in Zhongning, Ningxia, China) and then isolated by anionic exchange chromatography and gel filtration chromatography (He and Zhang, 1995). It was identified as a polysaccharide-protein complex by ultraviolet, infrared spectroscopy and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Laemmli, 1970) and was stained with Ag (Wray et al., 2001). It was shown to be homogeneous by Sephadex G-200 gel filtration chromatography and SDS-polyacrylamide gel electrophoresis (Pinilla and Luu, 1999). It contained 1:2.12:1.10:1.95:1.76, respectively, by gas chromatography (Laemmli, 1970) and was stained with Ag (Wray et al., 2001). As a positive control, 10 mg/l phytohemagglutinin was added to the cultures.

2.2. Cell isolation and culture

Thirty milliliters of venous blood were drawn into plastic syringes containing 0.2% EDTA from healthy volunteers, who had abstained from any drugs for at least 2 weeks before sampling. Human peripheral blood mononuclear cells were separated on a gradient of Ficoll with a density of 1.077 g/l (Luttmann et al., 1996). After three washings with phosphate-buffered saline, the cells were resuspended in 100 µl RNase-free water. Total RNA was extracted using TRIzol reagent (Gibco BRL) according to the manufacturer’s instructions and resuspended in 100 µl RNase-free water. Total RNA was quantified by using a spectrophotometer at 260 nm and purity was assessed by determining the ratio of $A_{260}/A_{280}$ nm. All samples had ratios above 1.75.

2.3. RNA extraction

After cultivation of human peripheral blood mononuclear cells, total RNA was extracted using TRizol reagent (Gibco BRL) according to the manufacturer’s instructions and resuspended in 100 µl RNase-free water. Total RNA was quantified by using a spectrophotometer at 260 nm and purity was assessed by determining the ratio of $A_{260}/A_{280}$ nm. All samples had ratios above 1.75.

2.4. RT-PCR of interleukin-2 and tumor necrosis factor-α mRNA

Complementary DNA (cDNA) of human peripheral blood mononuclear cells was prepared by incubating the RNA with Molony murine leukemia virus reverse transcriptase (200 units, Promega), 10 mM dNTP (Roche) and random primers (200 ng, Promega) at 37 °C for 60 min in 25 µl Tris–HCl buffer (50 mM, pH 8.3). Following inactivation of the enzyme by incubation at 95 °C for 5 min, the cDNAs were amplified in a PCR with the following primer sets: 5'-ACTCACCAGGTGCTCCTAT-3' and 5'-AGACTTGTCTACCTAATGGA-3' for interleukin-2 (the product size 266 bp), 5'-TCTCGAACCCTGGGTACAA-3' and 5'-ACCGCAACTCTGACTCCTAT-3' for tumor necrosis factor-α (the product size 123 bp). To control equal template concentrations and amplification efficiency, a cDNA sequence of β-actin was amplified using the following primers: 5'-GCATGGAGTCTCTGGCAT-3' and 5'-CTAGAAGCGATTGGCGTGG-3' (the product size 320 bp). These sets were designed from the published nucleic acid sequences available from GeneBank databases. PCR amplification was carried out with a reaction mixture composed of primers, dNTP and 1 unit Taq DNA polymerase (Promega). After samples were heated at 95 °C for 5 min, 30 cycles of PCR were performed consisting of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 1 min with a final extension at 72 °C for 5 min. An aliquot of PCR products was separated on a 1% agarose gel containing ethidium bromide and measured semiquantitatively using a Bio-Rad Multi-Analyt System. To compare the relative level of mRNA expression in each of the samples, the value is presented as the ratio of the band intensity of the cytokine RT-PCR product to the corresponding β-actin RT-PCR product. Calibration studies were carried out with various numbers of PCR cycles, from 20 to 40, to ensure that the amount of PCR product for each cytokine and β-actin was not on the plateau of amplification (data not shown). The PCR products were sequenced to confirm the specificity of the oligonucleotide primers. To exclude genomic DNA contamination, total RNA was amplified by PCR without reverse transcription.

2.5. Bioassay of interleukin-2

The activity of interleukin-2 in supernatants of human peripheral blood mononuclear cells was assayed using CTLL-2 cells bioassay (Tada et al., 1986). Briefly, the CTLL-2 cells were maintained on 96-well plastic plates at a concentration of 4 × 10$^{3}$/well in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated newborn bovine serum in the presence of 5% CO$_2$ at 37 °C. Then 10 µl of the supernatant of human peripheral blood mononuclear cells was added to each well. After a 24-h incubation, 10 µl 3-[4, 5-dimethylthiazol-2-yl]-2, 5-
diphenyl tetrazolium bromide (MTT) (5 g/l) was added and the incubation was continued for another 4 h. The formazan crystals were dissolved in 150 μl dimethyl sulfoxide and the absorbance was read at 570 nm on an ELX800 Microplate Reader (Bio-TEK, USA). Different dilutions of standard interleukin-2 were used to calibrate the assay.

2.6. Bioassay of tumor necrosis factor-α

The activity of tumor necrosis factor-α in the culture supernatants of human peripheral blood mononuclear cells was assayed using the L929 cell bioassay (Green et al., 1984). Briefly, L929 cells were maintained on 96-well plastic plates at a concentration of 4 × 10⁴/well. Then 10 μl of the supernatant of human peripheral blood mononuclear cells was added to each well. After a 24-h incubation, 10 μl MTT (5 g/l) was added and the incubation was continued for another 4 h. Dimethyl sulfoxide (150 μl) was used to dissolve the formazan crystals and the absorbance was read at 570 nm. Different dilutions of standard tumor necrosis factor were used to calibrate the assay.

2.7. Statistical analysis

Each experiment was performed at least four times. Results are expressed as the means ± S.D. Statistical analysis was done using an unpaired two-tailed Student’s t-test. P-values < 0.05 were considered significant.

3. Results

3.1. Kinetics of cytokine gene expression and cytokine secretion in LBP3p-stimulated human peripheral blood mononuclear cells

As the production of a cytokine is highly dependent on the time after stimulation, the time kinetic profile of interleukin-2 and tumor necrosis factor-α mRNA expression and their secretion induced by LBP3p were determined by a semiquantitative RT-PCR and bioassay in human peripheral blood mononuclear cells (Fig. 1). LBP3p significantly enhanced interleukin-2 mRNA expression at 8 h, peaking at 12 h and returning to baseline levels at 15 h. At 24 h, a marked decrease was observed. The kinetic curve of interleukin-2 secretion was associated with that of mRNA expression. With respect to tumor necrosis factor-α mRNA expression, human peripheral blood mononuclear cells exposed to LBP3p showed a significant increase as early as 2 h after treatment. The greatest increase was observed at 4 h after treatment, returning to baseline levels at 8 h, being undetectable at 24 h. For tumor necrosis factor-α secretion, a significant increase was observed in supernatants at 4 h after treatment and production peaked at 8 h.

3.2. Effects of LBP3p on interleukin-2 and tumor necrosis factor-α mRNA levels and their activities in human peripheral blood mononuclear cells

As a significant difference in the time kinetic profiles of interleukin-2 and tumor necrosis factor-α was observed in human peripheral blood mononuclear cells after LBP3p administration, we determined the effects of LBP3p on interleukin-2 and tumor necrosis factor-α mRNA levels and their activities when their production peaked. After treatment of human peripheral blood mononuclear cells with LBP3p, for 12 h, interleukin-2 mRNA level and its activity were significantly increased compared with those of the negative control, which contained only the cells. Treatment with LBP3p 5, 10, 20 and 40 mg/l increased interleukin-2 mRNA by 1.8-, 3.9-, 7.0- and 7.4-fold, respectively (Fig. 2B). The activity of interleukin-2 was increased by...
4.3-, 7.7-, 14.2- and 16.0-fold, respectively, as compared to that of the negative control (Fig. 2C).

After treatment with LBP3p for 4 h, tumor necrosis factor-α mRNA level was significantly increased in human peripheral blood mononuclear cells. Treatment with LBP3p 5, 10, 20 and 40 mg/l increased tumor necrosis factor-α mRNA level by 2.4-, 3.9-, 6.1- and 15.4-fold, respectively (Fig. 2B). The activity of tumor necrosis factor-α after treatment with LBP3p 5, 10, 20 and 40 mg/l for 8 h was increased by 7.1-, 9.1-, 13.6- and 15.2-fold, respectively, as compared to that of the negative control (Fig. 2C).

4. Discussion

The use of cytokines has a long history in immunotherapy, with interferon-α being the first cytokine used in tumor immunotherapy in 1957. Cytokines can regulate the immune response and are secreted by immune effector cells as well as a large variety of other cells, including tumor cells. Several cytokines are capable of mediating tumor regression in some malignancies. Interleukin-2 and tumor necrosis factor-α are two of the most extensively studied cytokines for tumor immunotherapy purposes. Interleukin-2 stimulates the proliferation of cytotoxic T lymphocytes, helper T lymphocytes, natural killer cells, lymphokine-activated killer cells and macrophages, all of which can participate in immunological antitumor mechanisms (Mizuno et al., 2000). Tumor necrosis factor-α is a peptide, which plays a pivotal role in host defense (Beutler, 1995). It may act on monocytes and macrophages in an autocrine manner to enhance various functions, such as
cytotoxicity to tumor cells, and to induce the expression of a number of other immunoregulatory and inflammatory mediators (Liu et al., 1996). However, because of the short half-lives of interleukin-2 and tumor necrosis factor-α in serum, systemic administration of high doses of interleukin-2 and tumor necrosis factor-α is needed, resulting in severe side effects, such as vascular leak syndrome, edema, anemia, fever and hypotension (Villikka and Pyrhonen, 1996). One strategy to reduce the incidence and/or severity of cytokine therapy is induction therapy. The present study provides evidence for the first time that severity of cytokine therapy is induction therapy. The immunomodulating and antitumor activity of LBP3p may be at least partly related to the level of expression of interleukin-2 and tumor necrosis factor-α.

The kinetics of expression is different for each cytokine and is dependent on the type of cytokine and the cell. It is generally accepted that protein synthesis, the production of cytokines and their gene expression are differently regulated (Liu et al., 1999). In the present study, the greatest increase in both gene expression and the secretion of interleukin-2 was at 12 h, while tumor necrosis factor-α gene expression and secretion peaked at 4 and 8 h in human peripheral blood mononuclear cells after LBP3p administration. It seemed that the change in tumor necrosis factor-α expression occurred earlier than that of interleukin-2. The expression of interleukin-2 remained high for a long time, whereas that of tumor necrosis factor-α remained high for only a short time. Therefore, this sort of kinetic study may provide a possible means for improving the immunomodulating and antitumor action of LBP3p, although the kinetics of cytokine expression are quite complicated.

Many mushroom polysaccharides and polysaccharide-protein complexes have been considered as antitumor and immunomodulating agents. Some researchers showed that the antitumor action of mushroom polysaccharide complexes, such as PSK, a protein-bound polysaccharide extracted from the mycelia of Coriolus versicolor (Hirose et al., 1990) and the polysaccharice-protein complex (PSPC) isolated from the culture filtrate of Tricholoma lobayense (Liu et al., 1996), was due to the potentiation of the host immune system through the regulation of cytokines in the cytokine network. It has been reported that PSK induces the expressions of genes for IL-1α, IL-1β, IL-6, IL-8, TNF-α, etc. in human peripheral blood mononuclear cells in vitro. The effect of LBP3p on cytokine expression is similar to that of mushroom polysaccharide-protein complexes to some extent.

In conclusion, our results demonstrated that LBP3p, a polysaccharide-protein complex isolated from L. barbarum, could significantly upregulate the expression of interleukin-2 and tumor necrosis factor-α at both mRNA and protein levels in a dose-dependent manner in human peripheral blood mononuclear cells. In order to investigate the overall antitumor effect of LBP3p, study of the in vivo induction of gene expression and the production of immunomodulating cytokines in the tumor-bearing mice is in progress.

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References


A Randomized, Double-Blind, Placebo-Controlled, Clinical Study of the General Effects of a Standardized Lycium barbarum (Goji) Juice, GoChi™

HARUNOBU AMAGASE, Ph.D., 1 and DWIGHT M. NANCE, Ph.D. 2

ABSTRACT

Background: This randomized, double-blind, placebo-controlled clinical trial is the first study reported from outside China that has examined the general effects of the orally consumed goji berry, Lycium barbarum, as a standardized juice (GoChi™; FreeLife International LLC, Phoenix, AZ) to healthy adults for 14 days.

Methods: Based upon the medicinal properties of Lycium barbarum in traditional Asian medicine, we examined by questionnaire subjective ratings (0–5) of general feelings of well-being, neurologic/psychologic traits, gastrointestinal, musculoskeletal, and cardiovascular complaints as well as any adverse effects. Also, measures of body weight, body–mass index, blood pressure, pulse rate, and visual acuity were assessed before and after consuming 120 mL of GoChi/day or placebo control solution. Data were statistically analyzed for changes between day 1 and day 15.

Results: Significant differences between day 1 and day 15 were found in the GoChi group (N/H11549 16) in increased ratings for energy level, athletic performance, quality of sleep, ease of awakening, ability to focus on activities, mental acuity, calmness, and feelings of health, contentment, and happiness. GoChi also significantly reduced fatigue and stress, and improved regularity of gastrointestinal function. In contrast, the placebo group (N/H11549 18) showed only two significant changes (heartburn and happiness). No significant changes in musculoskeletal or cardiovascular complaints were observed in either group. All parametric data (body weight, etc.) were not significantly different between groups or between day 1 and day 15 for either group.

Conclusions: These results clearly indicate that daily consumption of GoChi for 14 days increases subjective feelings of general well-being, and improves neurologic/psychologic performance and gastrointestinal functions. The data strongly suggest that further research is indicated to confirm and extend knowledge of the potential effects of Lycium barbarum upon human health.

INTRODUCTION

Lycium barbarum (goji) is in the family Solanaceae and its ripe fruit has been used in Asian countries, such as China, Korea, and Japan, for more than 2500 years as a traditional herbal medicine and functional food for its benefits to anti-aging, vision, kidney, and liver functions.1–4 In his Compendium of Medicine, Li Shi-zen named L. barbarum as a top-grade medicinal material that can nourish the liver and kidney, supplement energy, and improve eyesight.1-3

Shennong’s Classic of Materia Medica (Shennong Bencaojing) also mentioned that “long term use of goji can contribute to agility and longevity.” Ni Zhu-Mo, the renowned Chinese herbalist, also said in his Ben Cao Hui Yan (Convergent Speech on the Materia Medica) that “Goji can supplement energy, blood, adjust...
midity, and enjoys ten magic functions.34

O-Ser glycopeptide structure.50 The main chains of the gly-turonic acid, and 18 amino acids, and they share a Glycan- monosaccharides (Ara, Rha, Xyl, Man, Gal, and Glc), galac-they differ somewhat in composition, the LBP contain six found to be complex glycopeptides consisting of acidic het-

matography.10,38,50,51 Their structural composition has been

terminally termed group of unique, water-soluble glycoconjugates—collec-tive names of biological activities, in-

barbarum barbarum barbarum barbarum, recent studies indicate that extracts from bar-

Among the chemical constituents of L. barbarum fruit,

Among the chemical constituents of L. barbarum fruit,

L. barbarum L. barbarum L. barbarum L. barbarum, there are various small molecules such as
differentiation, and therefore, a high content of polysaccharides with proven pharmacologic activities is considered to be an indicator for

tory activities are found in Traditional Chinese Medicine,
ying liquid chromatography with the glucan analysis

ASA07120) was supplied by FreeLife International LLC, in

Subjects, 18 years old and older, were recruited for the
tal complaints, cardiovascular effects (blood pressure and

additional effects of L. barbarum L. barbarum L. barbarum L. barbarum. To begin to address this, in

AMAGASE AND NANCE
were under anticoagulant therapy with Coumadin® (Bristol-Myers Squibb Company, Cincinatti OH, generic, warfarin), fruit juices, were pregnant or breast feeding, were being treated for any immune, liver, or kidney-related conditions, or had any acute or chronic medical or psychiatric condition. Subjects were excluded from the study if they were healthy. Subjects were excluded from the study if they could not be healthy. Subjects were excluded from the study if they could not be healthy.

Myers Squibb Company, Cincinatti OH, generic, warfarin), fruit juices, were pregnant or breast feeding, were being treated for any immune, liver, or kidney-related conditions, or had any acute or chronic medical or psychiatric condition. Subjects were excluded from the study if they were healthy. Subjects were excluded from the study if they could not be healthy. Subjects were excluded from the study if they could not be healthy.

Myers Squibb Company, Cincinatti OH, generic, warfarin), fruit juices, were pregnant or breast feeding, were being treated for any immune, liver, or kidney-related conditions, or had any acute or chronic medical or psychiatric condition. Subjects were excluded from the study if they were healthy. Subjects were excluded from the study if they could not be healthy. Subjects were excluded from the study if they could not be healthy.
tolic and diastolic), and pulse rate were measured. The visual test indicators included reports of any eye fatigue, and a vision test was performed. Subjects also completed the 48-item clinical survey/questionnaire, which included any adverse effects. On both test days, subjects were asked to skip breakfast prior to completing the test protocol. The study was noninvasive, and subjective results were collected with the graded scores through questionnaire sheets and interviews. Quantitative data collected included body weight, height, BMI, body fat, total water content (Tanita BF-679W; Tokyo, Japan), pulse rate, and blood pressure (Omron HEM-637; Vernon Hills, IL). The vision tests included conscious eye-fatigue and distance vision, as checked by Graham Field #1264, 10-feet chart.

Statistical analysis
Dietary background data were analyzed with the non-parametric Mann–Whitney $U$ test (placebo vs. GoChi). All parametric data (body weights, BMI, etc.) were analyzed by $t$-test for independent and for dependent groups. Descriptive statistics were calculated for placebo and GoChi for all dependent measures and summarized as means and standard errors. For all clinical symptom questions under all categories (well-being, gastrointestinal, etc.), each question was graded and the scores were analyzed for changes between day 1 and day 15 with the nonparametric Wilcoxon matched-pairs tests. Differences were considered significant at $p/\alpha < 0.05$.

RESULTS
Parametric data
All parametric data, such as body weights, BMI, etc., were analyzed by $t$-test for independent and for dependent groups, and there were no significant changes in any of these dependent measures between day 1 and day 15 for either group or any group differences, as shown in Tables 2 and 3.

Clinical symptoms
Significant differences ($p/\alpha < 0.05$) between day 1 and day 15 were found for the GoChi group for multiple questions in several categories as shown in Table 4, Table 5, Table 6 and Figure 1. GoChi significantly increased athletic performance, quality of sleep, focus on activities, calmness, and

| TABLE 2. PARAMETRIC DATA (BODY WEIGHT, ETC.) MEANS/SEM ON DAY 1 AND DAY 15a |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Variable                      | Placebo (N=18)  | Day 1           | SEM             | Day 15          | SEM             | GoChi (N=16)    | Day 1           | SEM             |
| Body weight (kg)              | 76.2 ± 4.09     | 76.2 ± 4.13     |                 |                 |                 | 88.7 ± 17.36    | 88.7 ± 17.51    |                 |
| Height (cm)                   | 167.4 ± 2.72    | 169.3 ± 2.79    |                 |                 |                 | 171.2 ± 11.53   | 171.9 ± 11.85   |                 |
| BMI (kg/m²)                   | 27.0 ± 0.98     | 26.4 ± 0.96     |                 |                 |                 | 30.4 ± 4.15     | 29.9 ± 4.05     |                 |
| Body fat (%)                  | 32.9 ± 2.19     | 31.9 ± 2.35     |                 |                 |                 | 33.1 ± 9.29     | 31.5 ± 9.97     |                 |
| Water content (%)             | 47.2 ± 1.41     | 47.7 ± 1.50     |                 |                 |                 | 47.3 ± 5.98     | 48.0 ± 6.36     |                 |
| Systolic blood pressure (mm Hg)| 119.2 ± 3.20   | 115.9 ± 2.06    |                 |                 |                 | 125.2 ± 13.56   | 124.0 ± 8.72    |                 |
| Diastolic blood pressure (mm Hg)| 78.6 ± 2.26   | 74.8 ± 2.60     |                 |                 |                 | 80.6 ± 9.60     | 78.8 ± 11.07    |                 |
| Pulse                        | 76.3 ± 2.84     | 71.7 ± 1.99     |                 |                 |                 | 75.7 ± 12.04    | 70.6 ± 8.44     |                 |
| Left eye (/20)                | 43.0 ± 8.28     | 40.1 ± 7.98     |                 |                 |                 | 44.7 ± 35.14    | 41.5 ± 33.87    |                 |
| Right eye (/20)               | 43.3 ± 6.76     | 35.0 ± 5.31     |                 |                 |                 | 44.0 ± 28.68    | 38.5 ± 22.53    |                 |

aNo statistical difference has been detected between these days. bFreeLife International LLC, Phoenix, AZ.

BMI, body–mass index.
The GoChi group, with a tendency to increased energy level and mental acuity, and better ability to focus on their activities. More than 80% of people who consumed GoChi had easier awakening, improved mental acuity, and increased athletic performance.

GoChi group also showed a significant reduction in diastolic blood pressure.

In contrast to the GoChi group, the placebo group (N/H11005) showed only two statistically significant changes during 14 days of consuming placebo. No significant changes were found for any variable (Tables 4 and 5).

Several subjects who were consuming GoChi reported less fatigue, more energy during the day, and increased athletic performance. Around 50% of people who took GoChi had less interest in sweets and ate less. Several subjects in the GoChi group also showed a significant reduction in diastolic blood pressure (Table 5).

In the psychologic and neurologic area, the extract from *L. barbarum* has been shown to have effects on the brain and neuronal function. Experimentally, LBP protects neurons against toxicity of fibrillar Aβ and caspase-3 activity. It has neuroprotective effects against threitol (DTT) by lowering the DTT-induced LDH release and caspase-3 activity. It has neuroprotective effects against cytotoxic actions of dithiothreitol (DTT) by lowering the DTT-induced LDH release and caspase-3 activity. It has neuroprotective effects against cytotoxic actions of dithiothreitol (DTT) by lowering the DTT-induced LDH release and caspase-3 activity.

In terms of gastrointestinal symptoms, the GoChi group reported more vivid dreams. In the present study, subjects reported an increase in endurance/energy in the daytime and reduced fatigue within 14 days of consuming GoChi. In the present study, subjects reported an increase in endurance/energy in the daytime and reduced fatigue within 14 days of consuming GoChi. In the present study, subjects reported an increase in endurance/energy in the daytime and reduced fatigue within 14 days of consuming GoChi. In the present study, subjects reported an increase in endurance/energy in the daytime and reduced fatigue within 14 days of consuming GoChi.

In the present study, subjects reported an increase in blood urea nitrogen (BUN) after strenuous exercise, and accelerated the clearance of BUN after exercise. Pulse, day 1 76.3 75.7 0.8687

Pulse, day 15 71.7 70.6 0.7113

DBP, day 1 78.6 80.6 0.5533

DBP, day 15 74.8 78.8 0.3226

SBP, day 1 119.2 125.2 0.2801

SBP, day 15 115.9 124.0 0.0528

Water content, day 1 47.2 47.3 0.9792

Water content, day 15 47.7 48.0 0.8902

Fat, day 1 33.0 33.1 0.9555

Fat, day 15 31.9 31.5 0.9003

BMI, day 1 27.0 30.4 0.1463

BMI, day 15 26.4 29.9 0.1209

Height, day 1 167.4 171.2 0.3608

Height, day 15 169.3 171.9 0.5191

Body wt, day 1 76.2 88.7 0.1106

Body wt, day 15 76.2 88.7 0.1139

A purified LBP was shown to increase adaptability to an exercise load, to enhance resistance to fatigue, and to accelerate the elimination of lactic acid in mice. The study period. In the psychologic and neurologic area, the extract from *L. barbarum* has been shown to have effects on the brain and neuronal function. Experimentally, LBP protects neurons against toxicity of fibrillar Aβ and caspase-3 activity. It has neuroprotective effects against threitol (DTT) by lowering the DTT-induced LDH release and caspase-3 activity. It has neuroprotective effects against cytotoxic actions of dithiothreitol (DTT) by lowering the DTT-induced LDH release and caspase-3 activity.
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<th>GoChi</th>
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<td>16</td>
</tr>
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<td><strong>Energy in a day, day 15</strong></td>
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<td><strong>Focus on activities, day 1</strong></td>
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<td><strong>Focus on activities, day 15</strong></td>
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<td><strong>Feel healthy, day 15</strong></td>
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<tr>
<td><strong>Feel happy, day 15</strong></td>
<td>18</td>
<td>16</td>
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</table>

Bold indicates significant day 1 versus day 15 effect by nonparametric Wilcoxon matched pairs test.
which are associated with age-related neurodegenerative diseases. A /H9252H9252 peptides induce a rapid activation of c-Jun N-terminal kinase (JNK) by phosphorylation. Pretreatment with an aqueous extract of L. barbarum markedly reduced the phosphorylation of JNK-1 [Thr183/Tyr185] and its substrates c-Jun-I [Ser 73] and c-Jun-II [Ser 63].8 These studies support the anti-aging properties of L. barbarum claimed by traditional Asian medicine, and may be related to the psychologic and neurologic effects found in the present study.

LBP facilitated the recovery of peripheral red blood cells and platelets after mitomycin C–induced myelosuppression in mice.9 These effects on blood cells may be related to the subjective reports of increased endurance and decreased fatigue with use of GoChi.

Because L. barbarum is reported in several animal studies to be neuroprotective against the loss of retinal ganglion cells in glaucoma12 or age-related macular degeneration, 18 we tried to detect any changes in the visual functions in our subjects. However, the present study with healthy volunteers did not show any differences.

LBP is reported to enhance food conversion rate and the content of zinc and iron in female weanling mice, and to reduce their body weight after 21 days of consumption.11 This shows that LBP can modulate metabolism in vivo and may correspond with the present study's result regarding the effects of GoChi on subjective assessments of physical performance and gastrointestinal function. In addition, LBP may be processed as prebiotics in the gastrointestinal tract, helping to increase regularity by improving quality and quantity of gut microflora.

There are several experimental reports showing an antidiabetic effect of L. barbarum because it is well known in traditional Chinese herbal medicine for diabetes. Animal studies have shown that oral intake of LBP can restore abnormal oxidative indices to near-normal levels in blood, liver, and kidney in rats made diabetic by intraperitoneal injection.

### TABLE 5. DESCRIPTIVE STATISTICS: MEANS/ERRORS, GASTROINTESTINAL, DIARRHEA, AND MUSCULOSKELETAL/CARDIOVASCULAR QUESTIONS

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Mean ± SEM</th>
<th>N</th>
<th>Mean ± SEM</th>
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</thead>
<tbody>
<tr>
<td>Body weight, day 1</td>
<td>17</td>
<td>1.29 ± 0.427</td>
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<td>1.69 ± 0.481</td>
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<td>Body weight, day 15</td>
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<td>1.06 ± 0.277</td>
<td>16</td>
<td>1.31 ± 0.405</td>
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<td>Constipation, day 1</td>
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<td>0.72 ± 0.253</td>
<td>16</td>
<td>0.63 ± 0.315</td>
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<tr>
<td>Diarrhea, day 1</td>
<td>18</td>
<td>0.22 ± 0.129</td>
<td>16</td>
<td>0.81 ± 0.356</td>
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<tr>
<td>Diarrhea, day 15</td>
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<td>0.44 ± 0.217</td>
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<td>0.94 ± 0.433</td>
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<td>Regularity, day 1</td>
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<td>1.28 ± 0.378</td>
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<tr>
<td>Regularity, day 15</td>
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<td>1.22 ± 0.358</td>
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<td>0.69 ± 0.299</td>
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<td>Stomach discomfort, day 1</td>
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<td>0.83 ± 0.318</td>
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<td>1.69 ± 0.416</td>
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a FreeLife International LLC, Phoenix, AZ.

Bold indicates significant difference day 1 versus day 15 by nonparametric Wilcoxon matched-pairs test. A/C, air conditioner.
Injection of streptozotocin. LBP reduces blood glucose levels, oxidative stress, and DNA damage in rats with non-insulin-dependent diabetes mellitus (NIDDM), decreases malondialdehyde and nitric oxide in serum of fasting rats, and elevates serum levels of superoxide dismutase. Furthermore, LBP reduced cellular DNA damage in peripheral lymphocytes of NIDDM rats. These experimental results suggest that L. barbarum and LBP may be of benefit in the treatment of diabetes and possibly other metabolic diseases.

Further studies including blood analysis on biochemical markers will clarify the detailed mechanisms of these actions of GoChi found in the present human clinical study.

**CONCLUSIONS**

The results shown in this randomized, placebo-controlled, double-blind clinical study clearly indicate that daily consumption of GoChi for 14 days increases subjective feelings of general well-being, neurologic/psychologic traits, and gastrointestinal functions. These are consistent with traditional uses.

**ACKNOWLEDGMENTS**

The authors thank Kathleen Fry, M.D., former President of the American Holistic Medical Association, for her supervision of this clinical trial. George Y.C. Wong, Ph.D. at Strang Cancer Research Laboratory, Cornell University Medical College is also thanked for his critical review of the research questionnaire. All sources of financial support come from FreeLife International. Principal investigator/corresponding author and study subjects are employees of FreeLife International and the coauthor is a member of FreeLife's Independent Scientific Advisory Board.

**FIG. 1.** Changes in clinical scores between days 1 and 15 for GoChi™ group (solid bars) and placebo group (open bars). Categories of questions are shown below graph.

**TABLE 6. SUMMARY OF STATISTICAL ANALYSIS; SIGNIFICANT DIFFERENCES BETWEEN DAY 1 AND DAY 15 FOR PLACEBO AND GOCHI™ a FOR THE MULTIPLE QUESTIONS ARE AS FOLLOWS**

<table>
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<td>GoChi Fatigue</td>
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<td>Athletic performance</td>
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<td>Stress</td>
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<td>7.0</td>
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<td>Sleep</td>
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AMAGASE AND NANCE
Research Report

Characterizing the neuroprotective effects of alkaline extract of *Lycium barbarum* on β-amyloid peptide neurotoxicity

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ARTICLE INFO

Abstract

Lycium barbarum is an oriental medicinal herb that has long been used for its anti-aging and cell-protective properties. Previous studies have shown that aqueous extracts from *L. barbarum* exhibit neuroprotection via inhibiting pro-apoptotic signaling pathways. Other active components can also be accomplished by novel alkaline extraction method, which may give different profiles of water-soluble components. We hypothesize that another active component obtained by alkaline extraction method exerts different biological mechanisms to protect neurons. In this study, we aim to examine the neuroprotective effects from the alkaline extract of *L. barbarum*, namely LBB, to attenuate β-amyloid (Aβ) peptide neurotoxicity. Primary cortical neurons were exposed to Aβ-peptides inducing apoptosis and neuronal cell death. Pretreatment of LBB significantly reduced the level of lactate dehydrogenase (LDH) release and the activity of caspase-3 triggered by Aβ. "Wash-out" procedures did not reduce its neuroprotective effects, suggesting that LBB may not bind directly to Aβ. We have further isolated three subfractions from LBB, namely LBB-0, LBB-I and LBB-II. LBB-I and LBB-II showed differential neuroprotective effects. Western blot analysis demonstrated that LBB-I and LBB-II markedly enhanced the phosphorylation of Akt. Taken together, our results suggested that the glycoconjugate isolated from novel alkaline extraction method can open up a new avenue for drug discovery in neurodegenerative diseases.

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Keywords:
Lycium barbarum
Neuroprotection
β-Amyloid
Alkaline extraction
1. Introduction

Alzheimer’s disease (AD) is one of the most common causes of dementia, affecting more than 4.5 million people in the US. With the rapid expansion of aged population, it is estimated that the number of AD patients will increase by almost 3-fold in 2050 (Hebert et al., 2003). It is likely to be an important public issue that brings great financial burden to our society. The presence of senile plaques is one of the pathological hallmarks in AD (Mattson, 2004; Selkoe, 2001), in which aggregations of beta-amyloid peptide (Aβ-peptide) can be identified. It has been shown that soluble, oligomeric or fibrillar forms of Aβ exhibit certain extent of neurotoxicity. Previous experiments have demonstrated that Aβ-peptides are toxic to cultured neurons (Busciglio et al., 1992; Estus et al., 1997; Grace et al., 2002). Evidence from in vivo experiments also showed that Aβ is one of the pathological factors leading to neuronal loss, tau phosphorylation and activation of microglia (Geula et al., 1998; Maurice et al., 1996). The mechanisms of Aβ to impose toxicity on neurons have been studied extensively. It has been suggested that the activation of caspase (Harada and Sugimoto, 1999; Troy et al., 2000), stress kinases, and induction of oxidative stress (Folin et al., 2006) are involved in the apoptotic processes.

Although many studies had been directed to AD treatment, there is still yet promising intervention for curing the disease. The cholinesterase inhibitor is the most commonly used class of drug that had been approved by the U.S. Food and Drug Administration (FDA) for the treatment of AD (Doody, 2003; Giacobini, 2000). Neuroprotection is the attempt to preserve normal cellular interaction in the brain and minimize loss of neuronal functions in pathological conditions. Currently, much attention has been focused on the potential of using natural herbs as neuroprotective agent.

The fruits of Lycium barbarum, also called Fructus lycii or Gouqizi, have long been used in oriental medicine for the nourishment of the kidney, liver and eyes. Recent research has documented its anti-aging properties. There are many studies showing its beneficial effects on age-related conditions such as reduction of free radicals in our body (Li et al., 2007). The most common components of L. barbarum found in the aqueous extract of the fruit are polysaccharides and polysaccharide proteins. For instance, the polysaccharides of L. barbarum protect rat testes from heat-induced damage and protects mouse testicular cells from H2O2-induced DNA damage (Luo et al., 2006). Its beneficial effects can be found in irradiation, chemotherapy, or mitomycin C-induced mice myelosuppression by promoting the recovery of peripheral white blood cell and red blood cell count in these mice models (Gong et al., 2005; Hai-Yang et al., 2004). Apart from the above protective effects, extracts of L. barbarum are also a potential agent for treatment of diabetes since its polysaccharides can reduce blood glucose, triglyceride and total cholesterol levels in diabetic or hyperlipidemic rabbits (Luo et al., 2004). Polysaccharides from L. barbarum also demonstrate their hypoglycemic effects in a model of non-insulin-dependent diabetes mellitus (NIDDM) rats (Zhao et al., 2005b).

Since L. barbarum has protective functions on different cell types and can ameliorate an age-related disease (Chan et al., 2007), this laboratory was the first to carry out studies to investigate its neuroprotective properties from a water extract of the fruit. Our data suggested that the water-soluble extracts can protect cultured neurons from β-amyloid-induced neurotoxicity by suppression of the c-Jun N-terminal (JNK) signaling pathway (Yu et al., 2005). Its aqueous extract can also protect neurons from dithiothreitol (DTT)-induced endoplasmic reticulum reducing stress (Yu et al., 2006a). In spite of the fact that aqueous extracts elicit neuroprotection, there are other active components that make L. barbarum to be known as anti-aging herbal medicine. We hypothesize that other active components can be found by novel alkaline extraction method. This provides a novel way in drug discovery of neuroprotective agent. In the present study, we aim to characterize any potential neuroprotective effects of the alkaline extract of

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![Fig. 1](image_url) - Protective effects of the alkaline extract LBB on Aβ-peptide-triggered neurotoxicity. Rat primary cortical neurons were pretreated with different dosages of LBB for 1 h, neurons were exposed to Aβ(25-35) peptide (25 μM) for 24 h. (a) The level of general cell death was assayed by LDH assay which detected the release of LDH in culture medium. (b) Caspase-3 like activity was determined by the colorimetric caspase-3 like assay. DEVD cleavage was expressed as fold of control, which was calculated as: [s.a. of Aβ-treated/LBB-treated/s.a. of control]. Results were expressed as mean ± SE from at least 3 independent experiments. Data were analyzed by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test. *p<0.001 relative to control. **p<0.001 relative to cultures treated with Aβ only.
L. barbarum. The results show that the alkaline fractions from L. barbarum may have protective effects on Aβ-peptide-induced neurotoxicity and more research is required to explore their possibility as neuroprotective agent for AD.

2. Results

2.1. Alkaline extract LBB protects neurons from Aβ25-35 peptide-triggered toxicity

Fig. 1a shows the fold of control for the release of LDH in various treatment groups. Cultured neurons have normal turnover with baseline of LDH of 19.8 ± 0.03% of total lysis. The exposure of neurons to Aβ-peptide resulted in a 1.6 ± 0.02-fold increase in the release of LDH when compared to the control cultures. Pretreatment of LBB at 0.1, 1, 10, 100 and 500 μg/ml for 1 h significantly reduced Aβ-triggered LDH release. LBB at 500 μg/ml almost completely attenuated Aβ-peptide toxicity. Treatment with LBB per se in all our tested dosages did not cause significant increase in LDH release.

Since Aβ-peptide can trigger its neurotoxicity through the activation of caspase-3 and hence apoptosis, we had carried out colorimetric caspase-3-like activity assay in different treatment groups. In our control group, the specific activity of caspase-3 was 0.33 ± 0.02 pmol/min/μg. Exposure of neurons to Aβ25-35 peptide significantly increased the activity of caspase-3 to 2.06 ± 0.1-fold. Fig. 1b illustrates that pretreatment of LBB at 0.1, 1, 10 and 500 μg/ml significantly attenuated the activity of caspase-3 triggered by Aβ-peptide. However, 100 μg/ml of LBB did not show significant neuroprotective effects. The results suggest a biphasic neuroprotective effect to attenuate caspase-3 activity, implying that perhaps two active components exist in LBB. The presence of extract in all
tested dosages did not significantly alter basal activity of caspase-3. The result was further confirmed by using Western blot analysis, in which the level of cleaved caspase-3 in LBB pretreatment groups (10 and 500 μg/ml) was much lower than that without LBB pretreatment.

Aside from the biological assays, LBB also preserved the morphology of neurons. Representative images showing the protective effects of different dosages of LBB against 24 h of exposure to Aβ-peptide are depicted in Fig. 2. Under a phase-contrast microscope, the cell bodies of cultured neurons in control group were round and dark with a network of processes visible throughout the culture plates (Fig. 2a). After the exposure to Aβ-peptide, cultured neurons were damaged as reflected by the disappearance of normal cell bodies and the presence of fragmented neuritis (Fig. 2b). In the groups pretreated with LBB, the morphology of neurons was better preserved with the presence of normal cell bodies and intact processes (Figs. 2d, f). As shown in Figs. 1a and b, both 10 and 500 μg/ml exhibit neuroprotection. Therefore, they can both preserve intact morphology of neurons.

2.2. Effects of wash-out procedures and acidification of LBB on its neuroprotective properties

It is believed that certain drugs exert its effects by interacting with its target molecules; and hence preventing its subsequent interaction with cell membrane or receptors. In order to investigate if LBB would also exert its neuroprotective effects through a similar mechanism, the wash-out procedures were carried out. During this experiment, LBB extract was allowed to be cultured with neurons for 1 h and then were washed.

**Fig. 3** – Effects of (a) wash-out and (b) acidification procedures on the neuroprotective activities of LBB. (a) In order to find out if LBB binds directly to Aβ-peptide to elicit its cytoprotective effects, ‘wash-out’ experiment was performed. Neurons were pretreated with LBB for 1 h. Afterwards, LBB-containing medium was removed and neurons were then washed with PBS prior to exposure to Aβ-peptide. The release of LDH was measured by LDH assay. Results were expressed as fold of control. (b) LBB was acidified by dissolving it in medium containing concentrated HCl (1 M), followed by incubation at 80 °C with shaking (40 rpm) for 18 h. The solution was neutralized with medium containing NaOH (1 M). Neurons were treated with the ‘acidified LBB’ for 1 h prior to incubation with Aβ25-35 for 24 h. DEVD-cleavage activity was measured by colorimetric caspase-3 like assay. Results were expressed as fold of control. Results were expressed as mean ± SE from at least 3 independent experiments. Data were analyzed by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test. *p<0.001 relative to control. #p<0.001 relative to cultures treated with Aβ only.

**Fig. 4** – LBB attenuated Aβ1-42 triggered neuronal cell death. Neurons were pretreated with LBB (10, 100, 500 μg/ml) for 1 h, then exposed to Aβ1-42 (25 μM) for 24 h. They were then fixed with methanol:acetone (1:1) for 20 min and stained with DAPI (1 μg/ml in PBS) for 10 min. Apoptotic bodies were counted in five different fields per treatment in triplicate under a fluorescent microscope. Percentage of apoptotic cells was calculated as: (no. of apoptotic neurons/ no. of total neurons) × 100%. Results were expressed as mean ± SE from at least 3 independent experiments. Data were analyzed by one-way ANOVA for multiple comparison and Student–Newman–Keuls test as post hoc test. *p<0.001 relative to control. #p<0.001 relative to cultures treated with Aβ only.

Apart from the biological assays, LBB also preserved the morphology of neurons. Representative images showing the protective effects of different dosages of LBB against 24 h of exposure to Aβ-peptide are depicted in Fig. 2. Under a phase-contrast microscope, the cell bodies of cultured neurons in control group were round and dark with a network of process visible throughout the culture plates (Fig. 2a). After the exposure to Aβ-peptide, cultured neurons were damaged as reflected by the disappearance of normal cell bodies and the presence of fragmented neuritis (Fig. 2b). In the groups pretreated with LBB, the morphology of neurons was better preserved with the presence of normal cell bodies and intact processes (Figs. 2d, f). As shown in Figs. 1a and b, both 10 and 500 μg/ml exhibit neuroprotection. Therefore, they can both preserve intact morphology of neurons.
away by PBS before the addition of Aβ-peptide. The release of LDH from cultures after wash-out procedures is shown in Fig. 3a. The results showed that LBB elicited neuroprotection even though it has been washed out. The neuroprotection of wash-out group was similar to that of non-wash-out group. The results suggest that it is less likely for the extract to bind directly to Aβ-peptide in preventing its interaction to cell membrane.

The acidification procedures were performed to confirm that glycoconjugates but not other components are responsible for its neuroprotective effects. After the procedures, the activity of caspase-3 did not reduce in any LBB pretreatment group (Fig. 3b). During the acidification process, most polysaccharides and proteins were destroyed. Hence, our results confirmed that the glycoconjugate of LBB is the major components accounted for its neuroprotective properties.

2.3. Protective effects of LBB on Aβ1–42 peptide-triggered toxicity

We used full-length Aβ1–42 peptide as another toxin model to reconfirm the protective effects of LBB against Aβ-peptides. DAPI binds strongly to DNA and therefore allows the
visualization of condensed and fragmented nucleus, if any, during apoptosis. Apoptotic and normal cells were identified under a fluorescent microscope after DAPI staining. The results are shown in Fig. 4. A 20.9±3.3% of the total neurons in the control group were found to be apoptotic. When neurons were exposed to Aβ-peptide for 24 h, the number of apoptotic body increased to 34.3±2.1%. Pretreatment with 10, 100 or 500 μg/ml LBB for an hour significantly reduced the percentage of apoptotic neurons. The result is consistent to those shown in the first part, further confirmed the results that the alkaline extract is neuroprotective against neurotoxicity of Aβ-peptides.

2.4. Effects of LBB subfractions on Aβ25–35 peptide-treated neurons

A typical dose-dependent response of the alkaline extract on Aβ-peptide-exposed neurons was not observed in Figs. 1a and b. This suggests that LBB composes of a number of components which account for its protective function. In order to have a better understanding on the extract, LBB was further purified into three subfractions, -0, -I and -II. We tested their effects on culture neurons exposed to Aβ-peptide. LBB-0 did not exert neuroprotective effects against Aβ-peptide toxicity (data not shown). The effects of LBB-I and -II pretreatment on the release of LDH and the activity of caspase-3 are shown in Fig. 5. In the control group, the specific activity of caspase-3 was 0.34±0.03 pmol/min/μg of protein. Exposure of neurons to Aβ-peptide significantly increased the activity of caspase-3 to 2.4±0.2-fold. Statistical analysis shows that LBB-II has higher confidence to significantly reduce the level of LDH as well as the activity of caspase-3 (p<0.001) than that of LBB-I (p<0.05).

We also examined the possible neuroprotective properties of the subfractions. Aβ-peptide was at first added into the culture for 1 h. Afterwards, LBB-I or -II was added and co-incubated with neurons for another 23 h. The effects of post-treatment of the subfractions are shown in Fig. 6. Both LBB-I and -II in our tested dosages could significantly reduce the level of LDH. However, only 100 μg/ml LBB-II significantly attenuated Aβ-peptide-triggered activity of caspase-3 (p<0.05), LBB-B1 at 10 μg/ml even enhanced the activity of caspase-3. The results further confirm the data shown in Fig. 5 by showing that LBB-II has higher neuroprotective potential than that of LBB-I.

2.5. The alkaline extracts and its subfractions enhanced the phosphorylation of Akt

Western blot analysis was carried out to investigate the possible mechanisms of the neuroprotective effects of LBB, LBB-I and LBB-II. The results are shown in Fig. 7. Since several studies have shown that the activation of Akt pathway can protect neurons from Aβ-induced apoptosis (Wei et al., 2002; Martin et al., 2001), we investigated if our extracts could cause alteration in Akt phosphorylation. According to previous study, exposure of neurons to Aβ-peptide led to the phosphorylation of Akt starting from 15 min and gradually decreased after 12 to 24 h (Lesne et al., 2005). In addition, we have received comments from previous published results showing an increase of Akt phosphorylation 24 h after treatment by another Chinese medicine, Nerium indicum (Yu et al., 2004). Therefore, we have chosen 6 h to examine the phosphorylation of Akt. As shown in the figure, treatment of Aβ25–35 peptide for 6 h reduced the level of pAkt (Ser 473). Pretreatment of neurons with 10, 100 or 500 μg/ml LBB markedly enhanced the phosphorylation of Akt. For LBB-I, only the dosage 100 μg/ml enhanced the phosphorylation of
Akt. For LBB-II, the level of phosphorylation of Akt increased with dosages of LBB dependently in the treatment groups. Our results in Western blot analysis matched with those in Figs. 5 and 6, which may explain why neuroprotective effect of LBB-II is better than LBB-I. Neurotoxicity of Aβ25-35 peptide involved the phosphorylation of JNK-1. However, treatment of LBB did not attenuate JNK phosphorylation, suggesting that the neuroprotective effects of LBB-I and -II did not mediate by inhibiting JNK signaling pathway. Taken together, the activation of the Akt signaling pathway can partly explain why the extracts can protect the neurons from Aβ-peptide-induced neurotoxicity.

3. Discussion

The present study shows that the alkaline extract from L. barbarum, namely LBB, and its subfractions prevent Aβ-peptide-induced neurotoxicity in cortical cultured neurons. It has been well documented that aqueous extraction is the most commonly used method for the isolation of bioactive glycoconjugates from medicinal herbs including L. barbarum. Due to the lower water solubility and gelatinous properties, the alkaline extracts were often disregarded. There are few studies demonstrating the use and effectiveness of alkaline extraction method. Up to now, none of this method has explored the use of alkaline extract as neuroprotective agents. Hence, our results suggest that alkaline extraction method can be used as an alternative route in search for new protective agent in neurodegenerative diseases.

Polysaccharides are macromolecules that often appear in many different forms and in different locations in plants (Paulsen and Barsett, 2005). They are structurally a heterogeneous group of compounds which are neutral or acidic and may consist of only one type of monosaccharide or two and could contain up to ten different types of sugars. Polysaccharides may be in repeating units and can be linear or branched and substituted with different organic groups such as sulphated and carboxylic groups. The biological active polysaccharides isolated from natural sources are often charged and found to be pectic type polymer, e.g., arabinins, arabinogalactans and rhhamnogalacturonans. These compounds encompass many biological activities, including anti-cancer (Lavi et al., 2006; Seo et al., 2005; Wang et al., 2006) and anti-inflammation (Carvalho et al., 2006; Pinello et al., 2006). There are increasing lines of evidences showing the neuroprotective, cognitive-enhancing or anti-aging potential of glycoconjugates. For instance, Verbena officinalis Linn. polysaccharides can protect neurons from Aβ-peptide-induced neurotoxicity in vitro (Lai et al., 2006); the polysaccharide separated from flowers of N. indicum has demonstrated its neuroprotective effects in an AD cell culture model (Yu et al., 2004, this issue). Apart from Aβ toxicity, glycoconjugates also exert protection against other toxins. For example, the glycoconjugates isolated from Cordyceps sinensis can attenuate H2O2-induced injury in PC12 cells (Li et al., 2003), and the acylated oligosaccharides from Polygala tenuifolia can protect rats in potassium cyanide-induced anoxia animal model for cerebrovascular disease (Ikeya et al., 2004).

Since glycoconjugates from medicinal herbs are potential candidates for the treatment of various diseases, several studies have been carried out to investigate the isolation, purification and structures of these biological active macromolecules. Direct aqueous extractions followed by gel column chromatography remain the most popular method used for the isolation of glycoconjugates (Ding et al., 2003; Lavi et al., 2006; Wang et al., 2002; Yeung et al., 2006; Zhao et al., 2005a,b). Up to date, there are only few reports discussing the use of alkaline reagents for the extraction of polysaccharides and polysaccharide proteins. It has been reported that rats with carrageenan-induced edema and scalded edematous hyper-algesia showed a reduced inflammatory response when they were treated with a branched (1→3)-α-D-glucan isolated from the alkaline (sodium hydroxide) extract of Dictyophora indusiata Fisch. The studies find that there are conformational difference of glucan between its neutral and highly alkaline soluble form (Hara et al., 1982). Another example is glucan isolated from the alkaline extract of the fruiting bodies of Amanita muscaria, which shows significant anti-tumor activities in mice implanted with Sarcoma 180 tumor. The researchers find that the molecular weight of this glucan changes in alkaline and neutral solutions (Kiho et al., 1992). It has been shown that the glucan isolated from the alkaline extract of the fruiting bodies of Hericium erinaceus can process immunomodulation activity (Dong et al., 2006). A recent study also finds that the alkali-soluble glycoconjugates of Rhizoclonium riparium alga have immunomodulation properties in which they can induce gene expression for interleukin-1β (IL-1β) in a murine-derived macrophage cell line (Hsu et al., 2006). Together with our results, it is suggested that the alkaline extracts can be a useful isolation method to obtain nature glycoconjugates with biological activities.

Our data have just suggested that the alkaline soluble glycoconjugates of L. barbarum could attenuate Aβ-peptide-induced neurotoxicity. The crude extract LBB significantly reduced the activity of caspase-3 triggered by Aβ. Interesting, this protective effect was not dose-dependent, with 100 µg/ml LBB impose little neuroprotection. This biphasic effect suggested that there might be two active components in LBB. Two subfractions with different chemical profiles were produced after further processing of the crude LBB extract. We found that LBB-II has a higher content of galacturonic acid, which may explain why LBB-II has better neuroprotective effects.

Perhaps, one may question the delivery of polysaccharides into the brain. Heparin is proteoglycan associated with various kinds of biological activities in the brain. Recent studies have shown that low molecular weight heparin derivatives exert protective effects against Aβ-peptide cytotoxicity and reduce Aβ-peptide-induced inflammatory response in vitro (Bergamaschini et al., 2002). This compound can pass through the blood-brain barrier (BBB) (Leveugle et al., 1998; Ma et al., 2002) and attenuate Aβ-peptide-induced abnormal tau formation in hippocampal neurons (Dudas et al., 2002). Since heparin has a polysaccharide backbone and has the potential to pass through BBB to elicit its protective effects, it demonstrates the possibility that other neuroprotective polysaccharides might also process similar properties. At present, although it is still unknown whether polysaccharides from LBB could pass through BBB, such opportunity to cross BBB cannot be excluded and further investigation is deserved to confirm this issue.
Table 1 - Glycosyl-residue composition of the alkaline extract isolated from L. barbarum

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* %Dry weight basis.  
* Mol%.

APT<sub>25-35</sub> peptide was used as one of the toxin models in this study. The short APT<sub>1-42</sub> peptide is the core fragment of the full-length APT<sub>1-42</sub> peptide, yet it is highly toxic (Harada and Sugimoto, 1999; Pike et al., 1993, 1995; Yankner et al., 1990). Although it cannot be found naturally in AD brains, studies including ours have shown that the short APT<sub>25-35</sub> peptide can trigger neurotoxicity similar to those caused by the native full-length APT<sub>1-42</sub> peptide (Harada and Sugimoto, 1999; Pike et al., 1993, 1995; Yankner et al., 1990). Injection of APT<sub>25-35</sub> peptide into hippocampus of rats cause significant neuronal loss and memory deficits (Stepanichev et al., 2004, 2005). The short APT<sub>25-35</sub> peptide contains methionine residue 35, which can also be found in APT<sub>1-42</sub> peptide and is important for its neurotoxic properties (Butterfield and Bush, 2004; Varadarajan et al., 1999).

Previously, many studies demonstrated that the activation of survival pathways might safeguard neurons from harmful conditions. The Akt survival pathway is one of the pathways in neuroprotection (Chong et al., 2005; Ding et al., 2006; Zhao et al., 2005a). Its anti-apoptotic effects have been demonstrated in many in vitro studies (Datta et al., 1999). Apart from this, its activation is in response with neurotrophic factors such as insulin-like growth factor I (Willame-Morawek et al., 2005) and nerve growth factor (Saito et al., 2004). Akt activation therefore plays an important role in neuroprotection. It has been shown that APT<sub>1-42</sub>-peptide could downregulate Akt expression (Magrane et al., 2005; Nakagami, 2004). There are several lines of evidence suggesting that some neuroprotective medicinal herbs, such as Stephania longa (Zhang et al., 2005) and N. indicum (Yu et al., 2004), extracts protect neurons through stimulating the Akt survival pathway. Here, we report that the alkaline extract from L. barbarum might also protect cultured neurons from APT<sub>1-42</sub>-peptide toxicity through the stimulation of the same survival pathway. However, in our previous study, we found that an aqueous extract of L. barbarum (LBA) protected neurons from APT toxicity through inhibition of the JNK-c-jun signaling pathway (Yu et al., 2005). In fact, our data show that LBB could not alter the phosphorylation of JNK. This suggests that the polysaccharides isolation from the alkaline extracts, resulting in its differential modulation on neurons. The chemical composition of LBB was further investigated (Table 1). We found that LBB showed major difference in rhamnose, xylose and galacturonic acid content. Comparing the LBB to LBA extracts, LBB exhibits neuroprotective effects ranging from 0.1 to 500 μg/ml, while that for LBA ranged from 0.1 to 100 μg/ml.

Taken together, the alkaline extract from L. barbarum exhibits neuroprotective effects against extracellular Aβ peptide-induced apoptosis. LBB may elicit its neuroprotective effects partly via stimulation of the Akt signaling pathway, which is different to that of LBA. The differences in composition and biological activities between the alkaline and aqueous extracts suggest that alkaline extraction method may impose structure modification on the glycoconjugate. In conclusion, alkaline extraction method can be a new direction for the search of potential drug leads in neurodegenerative diseases.

4. Experimental procedures

4.1. Materials

L. barbarum L. was purchased from Ning Xia Huizu Autonomous Region, People’s Republic of China. Glycosyl composition analysis by gas chromatography-mass spectrometry following by methylation was performed by the Complx Carbohydrate Research Center [University of Georgia, Athens, GA; supported in part by the Department of Energy-funded Center for Plant and Microbial Complex Carbohydrates (DF-FG09-93ER-20097)]. The amino acid composition analysis was determined by post-column ninhydrin detection on a Beckman amino acid analyzer Instruments (Models 6300) by the Scientific Research Consortium, Inc., USA.

Materials used for neuronal cell culture were purchased from Gibco-BRL (Burlington, Ontario, Canada). Other chemicals were obtained from the following companies: 4′-diamidino-2-phenylindole (DAPI), Aβ-peptides (both 1–42 and 25–35 fragments), protease inhibitor cocktail, phosphatase inhibitor cocktail and anti-β-actin monoclonal antibody were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Caspase-3 substrate (Ac-DEVE-pNA) was purchased from Calbiochem, Inc. (La Jolla, CA, USA). LDH cytotoxicity assay kit from Roche Diagnostics (Mannheim, Germany). Rabbit polyclonal antibodies for Akt (Ser 473), cleaved caspase-3 and JNK detection were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit antibodies were from DAKO (Glostrup, Denmark). PVDF membrane was from Bio-Rad (Richmond, CA, USA). Biomax X-ray film was from Kodak (Tokyo, Japan). Enhanced chemiluminescence (ECL) detection kit was from Amersham (Buckinghamshire, UK). Re-Blot Western blot recycling kit was purchased from Chemicon (Temecula, CA, USA).

4.2. Preparation and isolation of Lycium barbarum extracts (LBB, LBB-O, LBB-I and LBB-II)

The dried fruits of L. barbarum (400 g) were crushed into small pieces and were defatted by refluxing with 95% ethanol, and the residue was extracted successively with boiling distilled water (3 h) for three times. The water-insoluble residues were then extracted twice with 1 M NaOH at 4 °C (each for 4 h). The filtrate from the alkaline extraction was neutralized with 3 M
HCl at room temperature, dialyzed (molecular cut-off size 3000–5000 Da, Spectrum), contracted and centrifuged. Addition of 95% EtOH (3 Vol) to the supernatant yielded a water-soluble crude polysaccharide, LBB (4.4 g, 1.1%), as the precipitate. LBB was redissolved in water (1%) and was fractionated on a diethylaminoethylcellulose (DEAE)-Sepharose Fast flow column (Cl−, 60×5 cm, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) by sequential elution with water and followed by 0–0.8 M gradient of NaCl. The fractions were collected according to the sugar profile as detected by phenol–H₂SO₄ method, concentrated, neutralized with acetic acid, dialyzed (molecular cut-off size 3000–5000 Da, Spectrum) and freeze dried. LBB-0 (0.56 g, 12.7%), LBB-I (0.43 g, 9.8%) and LBB-II (0.36 g, 8.2%) were obtained from H₂O, 0.2 M and 0.4 M, respectively. No carbohydrate was detected in the 0.8 M NaCl eluate. All crude and purified extracts have been examined for endotoxin (Sigma) and were proved to be endotoxin free.

4.3. Primary neuronal cultures

Primary cortical neurons were prepared from embryonic day 17 Sprague–Dawley rats (Laboratory Animal Unit, The University of Hong Kong) according to our previously published methods (Chang et al., 2002; Lin et al., 2004, 2006; Suen et al., 2003a,b; Yu et al., 2004, 2005, 2006a,b). In brief, cerebral cortices were mechanically dissociated in phosphate-buffered saline (PBS) with glucose (18 mM). Cells were seeded onto 6-well or 4-well plates or (2×10⁶ cells/well or 0.4×10⁶ cells/well respectively) pre-coated with poly-L-lysine (25 μg/ml). The culture medium consisted of minimum essential medium (MEM) supplemented with 5% heat-inactivated fetal bovine serum, glucose (18 mM), L-glutamine (2 mM), insulin (5 μg/ml), progesterone (0.02 μM), putrescine (100 μM), selenium (30 μM), penicillin (50 U/ml) and streptomycin (50 μg/ml). Neurons were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and were cultured for 7 days prior to treatments.

4.4. Treatment of neurons

In order to find out whether LBB and its subfractions, LBB-0, LBB-I and LBB-II, exerted neuroprotective effects, neurons were pretreated with different dosages of the extracts for 1 h, and then exposed to Aβ (25 μM) for 24 h. The Aβ-peptides were incubated with autoclaved Milli-Q water at 37 °C for a day prior to use. The preparative method gave a mixture of fibrillar and oligomeric forms of Aβ-peptide. As the toxicity of Aβ can be exerted by fibrillar and oligomeric forms, treatment of Aβ mixture can simulate the real situation in Alzheimer’s disease patients. For post-treatment, neurons were exposed to Aβ (25 μM) for 1 h and the alkaline extracts were added into the cultures for co-incubation of another 23 h. For ‘wash-out’ experiment, neurons were pretreated with LBB for 1 h. Afterwards, LBB-containing medium was removed and neurons were then washed with PBS prior to exposure to Aβ-peptide. Acidification procedure was carried out to destroy the protein and carbohydrate prior to treatment. LBB was dissolved in medium containing concentrated HCl (1 M), followed by incubation at 80 °C with shaking (40 rpm) for 18 h. Medium containing NaOH (1 M) was added to neutralize the medium.

Neurons were treated with the ‘acidified LBB’ for 1 h prior to incubation with Aβ for 24 h.

4.5. Measurement of general cytotoxicity

In order to determine the level of general cytotoxicity, cultured medium was collected for LDH activity assay. The assay was conducted as described elsewhere (Lai et al., 2006; Suen et al., 2003a; Yu et al., 2005, 2006a,b). Briefly, the culture medium was incubated with the assay buffer for 30 min in dark. Release of LDH was determined by measuring the absorbance at 492 nm. Results were expressed as the fold of control (fold of control was calculated as follow: absorbance of Aβ-treated/LBB-treated/absorbance of control).

4.6. Caspase-3-like activity assay

Caspase-3-like activity was determined by using the colorimetric caspase-3-like assay as described elsewhere (Suen et al., 2003a; Fang et al., 2005; Yu et al., 2004, 2005, 2006a,b, this issue). Neurons were scratched and lysed in lysis buffer containing DTT (5 mM), EDTA (0.1 mM), HEPES (50 mM, pH 7.4) and Triton-X (0.2%). Fifty micrograms of protein from each sample was incubated with caspase-3 substrate (Ac-DEVD-pNA) for 2 h at 37 °C. During the reaction, a yellow product (pNA) was cleaved from the substrate (i.e., DEVD cleavage) and its absorbance at 405 nm was determined for caspase-3-like activity. The values of specific activity (s.a., unit=pmol/min/μg) were calculated. Results were expressed as the fold of control.

4.7. DAPI staining

In order to reconfirm the neuroprotective properties of LBB, it is essential to demonstrate if it can protect neurons form the full-length Aβ₁–₄² peptide toxicity, which also triggers neuronal apoptosis. It is also important to show results other than caspase-3 biochemical assay. Therefore, we employed DNA staining by DAPI fluorescence probe by counting apoptotic bodies. Apoptotic bodies were recognized by DNA fragmentation and condensation as described previously (Chang et al., 2002; Suen et al., 2003a; Lai et al., 2006; Yu et al., 2004, 2005, 2006a,b, this issue). Neurons were pretreated with different dosages of LBB for 1 h, and then exposed to Aβ₁–₄² (25 μM) for 24 h. After treatment, neurons were fixed with methanol: acetone (1:1) for 20 min. DAPI (1 μg/ml in PBS) was added to the cells for 10 min, followed by washing with PBS for 3 times. Five different fields were counted per treatment in triplicate under a fluorescent microscope. A total number of about 200 neurons were counted in each field. Results were expressed as the percentage of apoptotic cells.

4.8. Western blot analysis

To examine the level of phosphorylated form of Akt, neurons after treatment were harvested and lysed in ice-cold lysis buffer containing Tris–HCl (10 mM, pH 7.4), NaCl (1 mM), Na₂PO₄ (20 mM), Na₃VO₄ (2 mM), Triton-X-100 (1%), glycerol (10%), deoxycholate (0.5%), SDS (0.1%), phenylmethysulfonyl fluoride (PMSF, 1 mM), protease inhibitor cocktail and phosphatase inhibitor cocktail. The lysate was homogenized and then centrifuged at 20,000×g for 30 min at 4 °C to obtain
the protein content. Equal amounts of protein were separated on 12.5% SDS-polyacrylamide gels. Proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membranes, and the membranes were blocked with 5% non-fat milk in TBST (TBS containing 0.1% Tween-20), followed by immunostaining with primary antibodies. Anti-phosphorylated Akt, anti-non-phosphorylated Akt, anti-cleaved caspase-3, anti-phosphorylated JNK, anti-non-phosphorylated JNK were used at 1:1000 dilution for 2 h at room temperature. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at 1:2000 dilution for 1 h at room temperature. Bands were developed on Biomax X-ray films using the ECL detection kit. The membranes were then stripped with the use of Re-Blot Western blot recycling kit and re-probed with monoclonal anti-β-actin antibody at 1: 5000 dilution, and goat anti-mouse HRP (1:2000) secondary antibody.

4.9. Statistical analysis

All values obtained were expressed as means±standard error (SE) from at least 3 independent experiments. The significance of differences among different groups was determined by one-way ANOVA, followed by Student–Newman–Keuls as post hoc test.

Acknowledgments

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Lycium barbarum polysaccharides: Protective effects against heat-induced damage of rat testes and H2O2-induced DNA damage in mouse testicular cells and beneficial effect on sexual behavior and reproductive function of hemicastrated rats

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Abstract

Lycium barbarum, a famous Chinese medicinal herb, has a long history of use as a traditional remedy for male infertility. Polysaccharides are the most important functional constituent in L. barbarum fruits. We systematically investigated the effect of L. barbarum polysaccharides (LBP) on rat testis damage induced by a physical factor (43 °C heat exposure), on DNA damage of mouse testicular cells induced by a chemical factor (H2O2), and on sexual behavior and reproductive function of hemicastrated male rats. The results showed that LBP provided a protective effect against the testicular tissue damage induced by heat exposure. When compared with negative control, a suitable concentration of LBP significantly increased testis and epididymis weights, improved superoxide dismutase (SOD) activity, and raised sexual hormone levels in the damaged rat testes. LBP had a dose-dependent protective effect against DNA oxidative damage of mouse testicular cells induced by H2O2. LBP improved the copulatory performance and reproductive function of hemicastrated male rats, such as shortened penis erection latency and mount latency, regulated secretion of sexual hormones and increased hormone levels, raised accessory sexual organ weights, and improved sperm quantity and quality. The present findings support the folk reputation of L. barbarum fruits as an aphrodisiac and fertility-facilitating agent, and provide scientific evidence for a basis for the extensive use of L. barbarum fruits as a traditional remedy for male infertility in China. © 2006 Elsevier Inc. All rights reserved.

Keywords: Lycium barbarum; Polysaccharides; Reproductive function; Fertility; Infertility; DNA damage; Sexual hormones; Sexual behavior; Testis; Rats; Mice; Hemicastrated rats; Traditional Chinese medicine

Introduction

Lycium barbarum L. belongs to the plant family Solanaceae. Red-colored fruits of L. barbarum have been used as a traditional Chinese herbal medicine for thousands of years (Gao et al., 2000). The earliest known Chinese medicinal monograph documented medicinal use of L. barbarum around 2300 years ago. L. barbarum fruits have a large variety of biological activities and pharmacological functions and play an important role in preventing and treating various chronic diseases, such as diabetes, hyperlipidemia, cancer, hepatitis, hypo-immunity function, thrombosis, and male infertility (Gao et al., 2000; Li, 2001). L. barbarum polysaccharides (LBP) isolated from the red-colored fruits are the most important functional factor (Qi et al., 2001; Peng et al., 2001b; Wang et al., 2002a; Gan et al., 2003, 2004; Zhang et al., 2005). Five L. barbarum polysaccharides (LbGp1–LbGp5) were separated and structurally elucidated (Peng et al., 2001a,b; Peng and Tian, 2001).

There have been some reports on the antifertility effects and fertility-promoting or aphrodisiac effects of medicinal plant extracts (Arletti et al., 1999; Mazaro et al., 2002; Carro-Juárez et al., 2004), but few researchers have reported on the effects of plant polysaccharides on male reproductive function. The pro-
fertility effect of *L. barbarum* fruits was first described in the sixteenth century by the great Chinese herbalist Li Shizhen, and nowadays *L. barbarum* fruits are included in most fertility-promoting Chinese remedies (Wang et al., 2002b). However, the male fertility-promoting mechanism of action of *L. barbarum* fruits remains largely unclear. In recent decades, some Chinese clinical studies have shown that *L. barbarum* fruits cause a significant improvement in the quality of sperm (Yin and Guo, 1993; Li, 2001). Wang et al. (2002b) reported that LBP could inhibit time- and hyperthermia-induced structural damage in murine seminiferous epithelium, and delay apoptosis in this system, under both normothermic and hyperthermic culture conditions. Although many important findings about biological activities and pharmacological functions of LBP have been obtained, there has been little information concerning the effect of LBP on reproductive system and function.

For the last decade, we have been conducting systematic studies on the biological activities and functions of crude or pure LBP from *L. barbarum* fruits, such as hypoglycemic, hypolipidemic, antifatigue, antioxidant and immune activity effects (Luo et al., 1997, 1999a,b, 2004). The objectives of this study are: (1) to evaluate protective effects of LBP against damage by a physical factor (heat exposure) in rat testes; (2) to assess protective effects of LBP against DNA damage induced by a chemical factor (H₂O₂) in mouse testicular cells; and (3) to investigate stimulating effects of LBP on sexual behavior and reproductive function of hemicastrated male rats.

**Materials and methods**

**Plant materials and chemicals/reagents**

Dried fruits of *L. barbarum* were purchased in a local market. They were from Ningxia, a well-known production area for *L. barbarum* in China. Lauryl sarcosine, hydrogen peroxide (H₂O₂), Na₂EDTA, and ethidium bromide (EtBr) were purchased from Sigma Chemical Co. (St. Louis, MO) and Dulbecco's modified Eagle's medium (DMEM), low melting point agarose, and normal melting point agarose were from GIBCO Co. (Grand Island, NY). Superoxide dismutase (SOD) and malondialdehyde (MDA) kits were from Jiancheng Bioengineering Institute (Nanjing, China). Testosterone (T) reagent kits, luteinizing hormone (LH) radioimmunoassay kits, follicle stimulating hormone (FSH) and estradiol (E₂) radioimmunoassay kits were obtained from Jiuding Ltd. Co. (Tianjin, China). Testosterone propionate, progesterone, and estradiol benzoate were from Jinyao Amino Acids Ltd. Co. (Tianjin, China). Chloroform, methanol, ethanol, and propanone were from Shanghai Reagents Co. (Shanghai, China). Other chemicals and reagents used were obtained from Sigma. All chemicals and reagents were analytical grade.

**Experimental animals**

Thirty-six adult male Wistar rats weighing from 180 to 220 g were used for assessing protective effect of LBP on reproductive system (tissues) damaged by physical factors (heat exposure), and 6 adult male Kong Ming mice (20±2 g) for investigating effect of LBP on DNA damage induced by chemical factors (H₂O₂). Ninety-two adult Wistar rats (46 male and 46 female) weighing from 180 to 220 g were used for examining the sexual behavior and reproductive function. They were housed in groups of two in rectangular cages (40 × 25 × 20 cm) with wire mesh lids under standardized animal room conditions (12 hr light/dark photoperiod at 23 °C and ~60% humidity). Food in pellets and tap water were available ad libitum. Animal experiments were performed according to the guidelines for the care and use of laboratory animals established by Wuhan University (Wuhan, China), which are in accordance with the Declaration of Helsinki of the World Medical Association (Wang et al., 2002b).

**Preparation of L. barbarum polysaccharides (LBP)**

*L. barbarum* fruits were dried at 60 °C and ground to fine powder. The ground powder samples were refluxed to remove lipids with chloroform:methanol solvent (2:1) (v/v). After filtering, the residues were air-dried, and then refluxed again with 80% ethanol at 80 °C to remove oligosaccharides. The residues were extracted four times in boiled water and filtered. The combined filtrates were concentrated by a rotavapor at 60 °C, and then precipitated using 95% ethanol, 100% ethanol and acetone, respectively. After filtering and centrifuging, the precipitate was collected and vacuum-dried. The dried *L. barbarum* polysaccharides (LBP) obtained were stored in a refrigerator till further use.

**Assessment of protective effect of LBP on rat testes damaged by a physical factor (heat exposure)**

The treatment of heat exposure followed the method of Miura et al. (2002) with minor modifications. The tested rats were anesthetized with sodium pentobarbital, and then their four legs were tied on vertical holders. Their scrotum containing the testes were submerged in warm water (43 °C) for 15 min. Thirty-six male rats were randomly assigned to one of six treatment groups (n = 6 for each group), including four experimental groups with four different concentrations of LBP (10, 50, 100, and 200 mg/kg per day) (43 °C heat exposure) and two control groups, i.e., a negative control group (0.9% saline) (43 °C heat exposure), and a normal control group (0.9% saline) without heat exposure. The rats in the experimental groups were administered through gastric gavage with four different concentrations of LBP dissolved in 0.9% normal saline one time per day for 14 consecutive days, while two control groups received the same volume of 0.9% normal saline without LBP every day. After 15 days, the rats of the negative control and four experimental groups were exposed to heat. Twenty-four hours after heat exposure, blood samples were drawn from the femoral artery of rats for measuring sexual hormones (i.e., T, LH, and FSH) in plasma, and then all tested rats (including normal control group) were sacrificed. The testes and epididymides were removed and weighed (mg/100 g body weight).
The testicular tissues were taken, treated and fixed in 10% polyformaldehyde and paraffin wax, and sliced in 4 μm sections and stained with hematoxylin and eosin (H&E) for morphological observation using an optical microscope at a 100× magnification. Protein content of the testicular tissues was determined by the Lowry method (Lowry et al., 1951). MDA levels and SOD activity of the testicular tissues were examined following the kit instructions. MDA assay was measured by the thiobarbituric acid (TBA) colorimetric method (Ohkawa et al., 1979). SOD assay was based on the ability to inhibit oxidation of oxyamine by the xanthine–xanthine oxidase system. MDA and SOD values were calculated using the optical density (OD) values observed (532 nm for MDA and 550 nm for SOD) and protein content, and expressed as nmol/mg protein and U/mg protein, respectively. The levels of serum T, LH, and FSH were determined with traditional radioimmunoassay (RIA) methods and followed the procedure instructions of the corresponding kits.

Assessment of protective effect of LBP against DNA damage induced by a chemical factor (H2O2) in mouse testicular cells

Male mice were sacrificed by cervical dislocation. Separation and preparation of mouse testicular cells followed the method described by Zhang et al. (2001). The isolated testicular cells (10⁶–10⁷ cells/mL) were randomly divided into three sample groups. All cell samples (1 mL testicular cell suspension + 2 mL DMEM culture solution) were incubated at 37°C. The cells of the LBP+H2O2 group were treated with four different concentrations of LBP (50, 100, 200, and 400 μg/mL, respectively), and 1 h later 30 μL of 100 μmol/L H2O2 was added and incubated for 25 min. The cells of the normal control group were treated with 30 μL of double distilled water. The cells of the negative control group were treated with 30 μL of 100 μmol/L H2O2. DNA damage in mouse testicular cells was estimated using the comet assay as reported by Singh et al. (1988) with minor modification, i.e., single cell gel electrophoresis (SCGE) which was performed under alkaline conditions following the procedure of Zhang et al. (2001). Three slides were prepared for each treatment. Two hundred cells per slide were randomly counted for tail frequency and 25 cells measured for tail length. Comets were observed using an Olympus BX51 fluorescence microscope attached to a solid-state camera (Olympus, Japan). Tail frequency (%) (the percentage of the cells with tail) and tail length (μm) (=maximum total length between comet head and tail−head diameter) were used to assess the DNA damage levels.

Investigation of the stimulating effect of LBP on sexual behavior and reproductive function of hemicastrated rats

The female rats were ovariecotomized under anesthesia, and after 2 weeks brought into estrous by subcutaneous injection of estradiol benzoate (200 μg/kg) 48 h before testing, and by subcutaneous injection of progesterone (2 mg/kg) 4 h before testing. They were screened with non-experimental sexually experienced males and only those exhibiting good sexual receptivity (solicitation behavior and lordosis in response to mounting) and no rejection behavior, were used (Arletti et al., 1999). Forty-six screened female rats were used for the mating experiment in this study.

Forty-six sexually vigorous male rats (ejaculation latency shorter than 15 min in at least the last three sessions) were also screened for the mating experiment. They were randomly divided into experimental group (n = 12), positive control group (n = 12), negative control group (n = 12), and normal control group (n = 10). Except for the rats of normal control group, the rats were anesthetized through intraperitoneal injection of 3% sodium pentobarbital (30 mg/kg), and castrated/orchiectomized via a midline scrotal incision (2 cm) to allow unilateral (right) testis removal. Three days after the surgery, the male rats of the positive control group got a subcutaneous injection of testosterone propionate (2 mg/kg). The male rats of the experimental group received 10 mg/kg of LBP (the dose used was confirmed to be more effective in our preliminary experiment) dissolved in 0.9% normal saline, whereas the male rats of negative control and normal control groups received 10 mg/kg of 0.9% normal saline per day through gastric gavage for 21 consecutive days.

Sexual behavior parameters observed in this study included erection latency, mount latency, and percentage of mount. Fourteen days after the start of gastric gavage, erection latency of the tested male rats was recorded using a BL–420E biological function system (electronic stimulating instrument) (Taimong Co., Chengdu, China). Erection latency was time (in seconds) from first stimulus to penis erection. Twenty-one days after the start of gastric gavage, major sexual behavior parameters of the tested male rats were investigated in a sound-proof dark room under a dim red light. After a 10-min adaptation period in a plastic observation cage, a stimulus-receptive female was introduced to the male by dropping it gently into the cage. The following parameters were recorded within 15 min: mount and intromission latency, time from introduction of the female to the occurrence of the first mount or intromission; ejaculation latency, time from the first intromission to ejaculation; percentage of mount (mounting rats). Tests were terminated immediately after the first postejaculatory intromission; or if intromission did not occur within 15 min of the introduction of the female, or if ejaculation latency exceeded 30 min (Arletti et al., 1999).

Twenty-two days after the start of gastric gavage, blood samples were drawn from the femoral artery of the tested rats for determining serum sexual hormones (T, LH, FSH, and E2) (see method described above), and then all tested rats were sacrificed by cervical dislocation. The left testes and epididymides of the tested rats were removed to carefully separate and weigh foreskin gland, seminal vesicle-prostate gland, and laevoatar ani muscle (LAM) and weights are expressed as milligram per 100 gram body weight (mg/100 g). Sperm count and sperm motility from proximal caudal epididymides were investigated. The epididymidal tissues were mashed and homogenized in a 10-mL tube with 6 mL of normal saline and incubated at 37 °C for 10–15 min to make the sperms fully dissociate and to release the sperms. The sperms were counted.
using a haemocytometer under an optical microscope \((\times 200)\) and expressed as sperm number per millilitre \((\text{no.} \times 10^6/\text{mL})\). The sperm motility \(\%\) was assessed by counting at least 200 sperms and calculated by the formula: \((\text{number of motile sperms} \times 100\%) / (\text{total number of motile and immotile sperms})\).

All sample analysis of sperms was conducted immediately after the sperm samples were collected.

**Statistical analysis**

All data are expressed as mean± standard deviation (S.D.). All calculations and statistical analyses were performed with SPSS software for Windows version 10.0 (SPSS Inc., Chicago, IL). The significance of the difference between the means of the control and treated groups was evaluated by Student’s \(t\)-test or \(\chi^2\)-test. Significance was set as \(P<0.05\) or \(P<0.01\).

**Results**

**Protective effect of LBP on rat testes against damage by a physical factor (heat exposure)**

To evaluate effect of heat treatment on rat testis tissues, the scrotum of rats were immersed at 43 °C for 15 min. The weights of testes and epididymis, SOD activity, and serum sexual hormone (T, LH, and FSH) levels in the heat-exposed rats (negative control) were significantly \((P>0.01\) or \(P>0.05\) lower while MDA levels were significantly \((P>0.01\) higher than those of the heat-unexposed rats (normal control) (Table 1). LBP had a protective effect on rat testes against damage by heat exposure. When compared with negative control, LBP treatment at three concentrations \((10, 50, \text{and} 100 \text{ mg/kg·per day})\) significantly \((P>0.01\) or \(P>0.05\) increased testis and epididymis weights in the damaged testes, and all four concentrations \((10, 50, 100, \text{and} 200 \text{ mg/kg·per day})\) of LBP treatments increased SOD activity and reduced MDA levels significantly \((P>0.01\) or \(P>0.05\)). Also, LBP \((10 \text{ mg/kg per day})\) significantly \((P>0.01\) increased T, LH, and FSH levels, in comparison to negative control. Other concentrations \((50, 100, \text{and} 200 \text{ mg/kg per day})\) of LBP also increased T, LH, and FSH levels, but mostly not significantly (Table 1).

Histological examination showed normal morphological features in the testis of normal controls. The seminiferous tubule showed successive stages of transformation of spermatogonum into spermatooza and compact and regular arrangements of cells. Seminiferous epithelium images were made up of spermatogonum, spermatocytes, spermatids, and sperms (Fig. 1A). The testsis of heat-exposed rats (negative control) had significant degenerative changes and destruction (Fig. 1B). Seminiferous tubules became irregular (thin and small). The lumen was filled with cellular debris. Both spermatids and sperms disappeared. Few spermatogonum were observed in certain seminiferous tubules. Inside spermatocytes, chromatin agglutination, unclear nuclear membrane, sparse nucleoplasm, and “vacuoles” in nucleus were observed. This indicated that the testis tissues of negative control were significantly damaged by heat exposure. Administration of various doses of LBP partly restored the morphological structure of seminiferous tubules in the damaged testis. The examined results showed better protective effect on the damaged testis in the LBP \(10 \text{ mg/kg·per day group}\) than other LBP groups \((50, 100, \text{and} 200 \text{ mg/kg·per day})\). The histological results were consistent with the results of the above biochemical assays. To some extent, the morphological structure of seminiferous tubules for the LBP \(10 \text{ mg/kg·per day group}\) (Fig. 1C) was close to that of normal control (Fig. 1A), compared with negative control (Fig. 1B). Most seminiferous tubules had intact structure and spermato-gona arrangements were basically regular.

**LBP protection against DNA damage of mouse testicular cells induced by a chemical factor \((\text{H}_2\text{O}_2)\)**

From comet images by fluorescent microscopy, the undamaged DNA is recognized as a fluorescent core, while the presence of strand breaks in the chain (damaged DNA) causes DNA to migrate and form a tail (“comet”) during the electrophoresis (Singh et al., 1988; Zhang et al., 2001). The bigger and more fluorescent the tail, the greater the DNA damage that has been induced. Microscopic examination did not reveal any DNA damage in normal control (untreated by H\(_2\)O\(_2\)), and the shape of the testicular cells was rounded fluorescent head without a tail (Fig. 2A). Mouse testicular cells in negative control (H\(_2\)O\(_2\)) exhibited obvious breakage of DNA chains induced by H\(_2\)O\(_2\). The damaged DNA fragments formed longer comet tail towards anticathode (Fig. 2B). The results for mouse testicular cells treated with different concentrations of LBP (50,

<table>
<thead>
<tr>
<th>Group</th>
<th>Testes (mg/100 g body weight)</th>
<th>Epididymides (mg/100 g body weight)</th>
<th>SOD (U/mg protein)</th>
<th>MDA (nmol/mg protein)</th>
<th>T (nmol/L)</th>
<th>LH (IU/L)</th>
<th>FSH (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control(\text{a}b)</td>
<td>519±122*</td>
<td>192±33**</td>
<td>302.87±67.72**</td>
<td>0.78±0.49**</td>
<td>24.35±3.43</td>
<td>11.09±0.91</td>
<td>4.19±1.01</td>
</tr>
<tr>
<td>Negative control ((43 \text{ °C}))(\text{a}b)</td>
<td>367±56</td>
<td>132±13</td>
<td>206.30±24.55</td>
<td>2.78±1.26</td>
<td>16.09±4.92</td>
<td>8.71±1.48</td>
<td>2.92±0.59</td>
</tr>
<tr>
<td>10 mg/kg·per day LBP ((43 \text{ °C}))</td>
<td>549±78**</td>
<td>209±24**</td>
<td>387.41±44.38**</td>
<td>1.11±0.44**</td>
<td>27.05±4.17</td>
<td>12.60±1.96</td>
<td>4.71±1.24</td>
</tr>
<tr>
<td>50 mg/kg·per day LBP ((43 \text{ °C}))</td>
<td>526±69**</td>
<td>186±31**</td>
<td>274.41±18.98</td>
<td>1.24±0.50**</td>
<td>19.51±5.65</td>
<td>10.61±1.62</td>
<td>3.94±0.85</td>
</tr>
<tr>
<td>100 mg/kg·per day LBP ((43 \text{ °C}))</td>
<td>493±77*</td>
<td>165±17*</td>
<td>270.08±47.60*</td>
<td>1.39±0.66*</td>
<td>18.41±5.51</td>
<td>10.93±1.84</td>
<td>3.67±1.42</td>
</tr>
<tr>
<td>200 mg/kg·per day LBP ((43 \text{ °C}))</td>
<td>465±86</td>
<td>169±33</td>
<td>272.64±28.22*</td>
<td>1.69±0.90*</td>
<td>17.21±4.61</td>
<td>10.98±1.99</td>
<td>3.41±0.71</td>
</tr>
</tbody>
</table>

\(\text{a} P<0.05\) and \(\text{b} P<0.01\), compared with negative control group. SOD, superoxide dismutase; MDA, malondialdehyde; T, testosterone; LH, luteinizing hormone; and FSH, follicle stimulating hormone.

\(b\) 0.9% normal saline per day through gastric gavage.
100, 200, and 400 μg/mL) for 1 h and then 30 μL of H$_2$O$_2$ (100 μmol/L) for 25 min indicated that DNA damage was significantly attenuated, as compared with negative control. LBP treatment (50 μg/mL) showed that the damaged DNA fragments formed shorter comet tails (Fig. 2C), while cells given LBP treatment at 400 μg/mL exhibited few shorter comet tails (not shown). Mean tail frequency (%) and tail length (μm) showed that, when compared with negative control, pretreatments of LBP (50, 100, 200, and 400 μg/mL) significantly ($P<0.01$) reduced the frequencies of cells with tail and the tail length of the damaged testicular cells (Table 2). The results indicated that LBP had a dose-dependent protective effect on DNA oxidative damage induced by H$_2$O$_2$.

**Stimulating effect of LBP on sexual behavior and reproductive function of hemastrad male rats**

Compared with negative control (10 mg saline/kg per day), both LBP group (10 mg/kg per day) and positive control (TP, testosterone propionate, 2 mg/kg per day) significantly improved the copulatory performance of hemastrad male rats, i.e., significantly ($P<0.01$) shortened penis erection latency and mount latency, and clearly ($P<0.05$) improved the percentage of mounting (Table 3). Moreover, the stimulating effect of LBP and TP (positive control) on sexual behavior of the hemastrad male rats was slightly better than that of normal control rats without castration (10 mg saline/kg per day).
Discussion

*L. barbarum* fruits are well known in traditional Chinese herbal medicine and nowadays are widely used as a popular functional food. LBP, a most important functional component in *L. barbarum* fruits, possesses a large variety of bioactivities, such as antiaging, anticancer, immuno-modulating, hypoglycemic, hypolipidemic, antioxidant, antifatigue, and male fertility-facilitating (Gao et al., 2000; Wang et al., 2002a,b; Gan et al., 2003, 2004; Luo et al., 1999a,b, 2004; Zhang et al., 2005). However, the effect of LBP on male reproductive system and function and the related mechanism of action are not well understood.

SOD is a highly specific scavenging enzyme for superoxide radicals (O$_2^-$) and MDA is a reactive end product of lipid peroxidation. SOD activity and MDA level can reflect the degree of damage of testicular tissues induced by ROS (reactive oxygen species, e.g., O$_2^-$ and OH$^•$). The ROS-induced injury normally causes decrease of SOD activity and increase of MDA level in testicular tissues. It is well known that a lower scrotal temperature is required for normal spermatogenesis in most mammalian species because testicular germ cells are vulnerable to heat stress and undergo apoptosis in response to increased scrotal temperature (Ikeda et al., 1999; Miura et al., 2002). SOD is most sensitive to heat, and heat exposure leads to the decrease of the SOD activity in rat testicular tissues. SOD depletion in spermatozoa is thought to be associated with male infertility. SOD activity in spermatozoa showed a significant correlation to the number of motile spermatozoa and MDA concentration was significantly related to the number of immotile spermatozoa (Kobayashi et al., 1991). This study showed that when negative control was exposed to heat (43 °C, 15 min), the SOD activity significantly decreased and the MDA level significantly increased in the testicular tissues (P<0.01) (Table 1). This indicated that the antioxidant system of the testicular tissues in negative control rats had already been damaged. Excessive free radicals in the testicular tissues resulted in development alteration of testicular reproductive cells and retarded sperm growth. LBP possessed strong antioxidant activity and antiperoxidation effect (Zhang, 1993; Luo et al., 2004). All four LBP treatments in the experimental group significantly

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Erection latency (second)</th>
<th>Mount latency (min)</th>
<th>Percentage of mount (%)</th>
<th>T (nmol/L)</th>
<th>LH (IU/L)</th>
<th>FSH (IU/L)</th>
<th>E$_2$ (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (saline)</td>
<td>10</td>
<td>20.56±4.75**</td>
<td>4.80±1.30**</td>
<td>62.5*</td>
<td>13.87±2.83**</td>
<td>14.57±4.79</td>
<td>5.22±1.68</td>
<td>13.98±3.72**</td>
</tr>
<tr>
<td>Negative control (HC+saline)</td>
<td>12</td>
<td>27.75±6.67</td>
<td>8.14±1.95</td>
<td>58.3</td>
<td>2.16±0.60</td>
<td>15.21±3.33</td>
<td>4.89±1.54</td>
<td>19.27±3.03</td>
</tr>
<tr>
<td>Positive control (HC+TP)</td>
<td>12</td>
<td>16.77±3.30**</td>
<td>4.45±1.57**</td>
<td>84.6*</td>
<td>18.80±4.82**</td>
<td>14.39±2.96</td>
<td>6.66±1.97</td>
<td>13.20±3.30**</td>
</tr>
<tr>
<td>HC+LBP</td>
<td>12</td>
<td>17.23±3.83**</td>
<td>4.64±1.86**</td>
<td>78.6*</td>
<td>6.64±1.64**</td>
<td>14.51±2.54</td>
<td>4.99±1.57</td>
<td>16.41±2.78**</td>
</tr>
</tbody>
</table>

P<0.05 and **P<0.01, compared with negative control. T, testosterone; LH, luteinizing hormone; FSH, follicle stimulating hormone; E$_2$, estradiol.

a Saline, 0.9% normal saline (10 mg/kg per day).

b TP, testosterone propionate (2 mg/kg per day).

c LBP, 10 mg/kg per day.
Table 4
Effect of LBP on weight of accessory sexual organs (reproductive glands) and sperm quantity and quality in hemicastrated (HC) male rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Foreskin gland (mg/100 g)</th>
<th>Seminal vesicle-prostate gland (mg/100 g)</th>
<th>Laevator ani muscle (LAM) (mg/100 g)</th>
<th>Sperm count (no. sperm × 10^9/mL)</th>
<th>Sperm motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (saline)</td>
<td>10</td>
<td>9.55 ± 14.21*</td>
<td>520.51 ± 162.75*</td>
<td>61.46 ± 12.80*</td>
<td>13.15 ± 3.36**</td>
<td>63.00 ± 10.69**</td>
</tr>
<tr>
<td>Negative control (HC + saline)</td>
<td>12</td>
<td>33.96 ± 10.05</td>
<td>349.97 ± 88.55</td>
<td>43.22 ± 14.57</td>
<td>7.47 ± 2.75</td>
<td>45.17 ± 11.59</td>
</tr>
<tr>
<td>Positive control (HC + TP)</td>
<td>12</td>
<td>55.53 ± 17.12**</td>
<td>1032.88 ± 235.16**</td>
<td>81.62 ± 23.94**</td>
<td>13.63 ± 2.29**</td>
<td>64.46 ± 12.67**</td>
</tr>
<tr>
<td>HC + LBP</td>
<td>12</td>
<td>48.83 ± 14.36*</td>
<td>509.33 ± 175.10*</td>
<td>60.65 ± 19.90*</td>
<td>12.56 ± 3.74**</td>
<td>63.36 ± 11.91**</td>
</tr>
</tbody>
</table>

*P < 0.05 and **P < 0.01, compared with negative control.

a Saline, 0.9% normal saline (10 mg/kg·per day).
b TP, testosterone propionate (2 mg/kg·per day).
c LBP, 10 mg/kg·per day.

(P < 0.01 or P < 0.05) increased the SOD activity and decreased the MDA level of rat testicular tissues damaged by heat exposure (Table 1). This indicated that LBP could effectively scavenge free radicals, suppress lipid peroxidation, and alleviate the damage to spermatogenic cells induced by heat exposure. The 10 mg/kg·per day treatment was the most effective among four LBP treatments.

Our results were similar to the findings of Lue et al. (2000) who reported that testis weight and testicular sperm counts of the male rats in the heat exposure group (43 °C, 15 min) were decreased to 65.4% and 28.9% of control levels, respectively. In this study, LBP could clearly increase the weights of reproductive organs (testes and epididymides) in heat-exposed rats (Table 1). Histological examination (Fig. 1) showed that LBP could alleviate the harm to the testicular tissue cells caused by heat exposure and partly restore the morphological structure of seminiferous tubules in the damaged testes. The results also indicated that low concentration of LBP (10 mg/kg·per day) had the best protective effect on rat testes damaged by heat exposure, and followed by 50 and 100 mg/kg·per day. However, 200 mg/kg·per day did not have a significant effect (Table 1).

H₂O₂, a strong oxidant and a common chemical in DNA damage, can easily penetrate cell membrane directly into cell nucleus without enzymatic degradation. H₂O₂, one of the main ROS, has been demonstrated to cause lipid peroxidation and DNA damage in cells (Halliwell and Aruoma, 1991). Also, H₂O₂ reacts with Fe²⁺ or Ca²⁺ to produce the hydroxyl radical (OH•) (e.g., H₂O₂ + Fe²⁺ → OH• + OH⁻ + Fe³⁺). OH• has strong oxidation ability. Exposure of DNA to oxidative stress leads to more than 20 different types of base damage, producing oxidized and ring-fragmented nitrogen bases and causing serious DNA damage (Slupphaug et al., 2003).

LBP had significant protective effects against H₂O₂-induced DNA damage in the mouse testicular cells and acted in a dose-dependent manner (Table 2 and Fig. 2). There were two possible antioxidant pathways: (1) LBP directly removed H₂O₂ or scavenged OH• induced by H₂O₂, suppressed lipid peroxidation of testicular tissues cells, protected cell membrane from oxidative stress, and lowered the DNA damage of the testicular cells to maintain normal structure and functions of the cells; (2) LBP indirectly scavenged the free radicals by activating antioxidant enzyme systems in the testicular tissues to alleviate the DNA damage induced by H₂O₂. In most cells there exist multiple protective mechanisms (antioxidant enzyme systems) designed to prevent ROS-induced injury, such as SOD, glutathione peroxidase (GSH-PX), and catalase (Mennella and Jones, 1980; Sanocka et al., 1997). The present study and a previous study (Wang et al., 2002a,b) indicated that LBP could significantly increase the SOD activity and decrease the MDA level to protect testes and spermatozoa against oxidative damage and lipid peroxidation.

“Function Assessment Procedures and Examination Methods of Health Foods” was published by the Bureau of Health Supervision, Ministry of Health, China (Bureau of Health Supervision, 1998), and reported how to evaluate and judge whether health foods could improve male sexual behavior and function. There are two major assessing experiments, i.e., copulatory experiment and penis erection experiment of castrated animals. If a positive result is obtained in the mating experiment or the erection experiment, the tested health food is considered as confirmed to improve male sexual behavior and function. In this study, a hemicastrated male rat model was established to assess the effect of LBP on male sexual behavior. The results showed that LBP could significantly shorten penis erection latency and mount latency and clearly improve the copulatory performance of hemicastrated male rats, as compared with negative control (Table 3), indicating that LBP can be used as a potential natural aphrodisiac. However, this study did not follow all standard measurements for sexual behavior parameters. Standard measurements for sexual behavior may...
include mount latency, intromission latency, ejaculation latency, postejaculatory interval, and additional parameters (Ågmo, 1997). Further assessment of the effect of LBP on male sexual behavior is warranted in the future.

Under normal physiological conditions, serum T, LH, FSH, and E2 levels in male testes are well balanced (Soderstein et al., 1980; Engelking, 2000). One to three days after castration, major sexual hormone levels decrease and E2 level increases significantly. T and E2 are a pair of opposite hormones and their levels in the testes are highly negatively linearly correlated. One of the main physiological functions of testosterone (T) is to promote spermatogenesis. The decrease of T level is an obstacle to spermatogenesis and reduces sperm number and motility. Our investigation showed that the T level in negative control decreased significantly while the E2 level increased, as compared with normal control (Table 3). When compared with negative control, LBP treatment could significantly ($P<0.01$) increase the T level of hemicastrated male rats. The results suggested that LBP might adjust the secretion of sexual hormones and increase sexual hormone levels in the hemicastrated male rats, although the effect of LBP was inferior to that of testosterone propionate. Additionally, the results of sperm count and motility indicated that LBP treatment could significantly ($P<0.01$) improve sperm quantity and quality in the hemicastrated rats (Table 4). This was consistent with the results of Yin and Guo (1993) who conducted a clinical experiment of L. barbarum fruits on a cohort of 42 patients with low sperm count and/or low sperm motility. The patients were administered 15 g of L. barbarum fruits per day for 4 months. It was found that the sperm quality of 79% patients was significantly improved.

The weights of accessory sexual organs of male animals are usually associated with androgen activity and function. Androgens can stimulate the growth of accessory sexual organs (e.g., foreskin gland, seminal vesicle, and prostate) and increase their weights (Zhang et al., 2002). If certain drugs or natural compounds can increase the weights of accessory sexual organs, they should have similar androgen effect (Cheng et al., 1997). In this study, we determined the weights of foreskin gland, seminal vesicle-prostate, and LAM in LBP treatment, positive control (testosterone propionate), negative control, and normal control (Table 4). Because hemicastration of rats reduced the secretion of sexual hormones, both the T level and the weights of accessory sexual organs in negative control decreased significantly ($P<0.01$), as compared with normal control (without castration). LBP treatment could increase ($P<0.05$) both the T level and the accessory sexual organ weights of hemicastrated rats, as compared with negative control (Tables 3 and 4), and exhibited similar androgen effect. However, the LBP effect was lower than positive control and similar to normal control. This indicated that the adjusting effect and function of LBP did not exceed normal level.

In summary, the present study indicated that LBP showed protective effects against damage to the testicular tissue of male rats induced by a physical factor and on the DNA damage to mouse testicular cells induced by a chemical factor. LBP also improved the sexual behavior and reproductive function of hemicastrated rats. The results effectively support the folk reputation of L. barbarum fruits as sexual stimulants and aphrodisiac for traditional remedy of male infertility in China.

Acknowledgment
This work was supported by a key program of the Bureau of Science and Technology of Hubei, China, and the Food Nutrition and Education Fund of DANONE Institute (China). We are grateful to Dr. Harold Corke (The University of Hong Kong) for his revision and comments on the manuscript.

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Protective Effect of Lycium barbarum on Doxorubicin-induced Cardiotoxicity

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³Institute of Material Medicines, Zhejiang Academy of Medical Sciences, Hangzhou, Zhejiang, China

The objective of this work was to explore the hypothesis that Lycium barbarum (LB) may be protective against doxorubicin (DOX)-induced cardiotoxicity through antioxidant-mediated mechanisms. Male SD rats were treated with distilled water or a water extract of LB (25 mg/kg, p.o.) daily and saline or DOX (5 mg/kg, i.v.) weekly for 3 weeks. Mortality, general condition and body weight were observed during the experiment. DOX-induced cardiotoxicity was assessed by electrocardiograph, heart antioxidant activity, serum levels of creatine kinase (CK) and aspartate aminotransferase (AST) and histopathological change. The DOX group showed higher mortality (38%) and worse physical characterization. Moreover, DOX caused myocardial injury manifested by arrhythmias and conduction abnormalities in ECG (increased QT and ST intervals and ST elevation), a decrease of heart antioxidant activity, an increase of serum CK and AST, as well as myocardial lesions. Pretreatment with LB significantly prevented the loss of myofibrils and improved the heart function of the DOX-treated rats as evidenced from lower mortality (13%), normalization of antioxidant activity and serum AST and CK, as well as improving arrhythmias and conduction abnormalities. These results suggested that LB elicited a typical cardioprotective effect on DOX-related oxidative stress. Furthermore, in vitro cytotoxic study showed the antitumor activity of DOX was not compromised by LB. It is possible that LB could be used as a useful adjunct in combination with DOX chemotherapy.

Keywords: doxorubicin; Lycium barbarum; cardiotoxicity; antioxidant.

INTRODUCTION

Doxorubicin (DOX) is a highly effective chemotherapeutic agent used in the treatment of solid and hematopoietic tumors. However, its application is limited due to its dose-dependent cardiotoxicity (reviewed by Minotti et al., 2004). Recent studies have suggested that DOX-induced cardiotoxicity involves the generation of reactive oxygen species (ROS), including O₂•−, •OH and H₂O₂, which result in cell damage by lipid peroxidation, protein cross-linking and DNA fragmentation (Xu et al., 2001; Wang et al., 2004). ROS are normally eliminated by superoxide dismutase (SOD), catalase, glutathione peroxidase and some antioxidants. Because cardiomyocytes have a relatively lower level of antioxidant enzymes, the heart is more susceptible to oxidative damage (reviewed by Peng et al., 2005).

Moreover, the anticancer effects of DOX do not follow identical mechanisms of ROS. The majority of strategies therefore focus on administering antioxidants that protect the cardiomyocytes against DOX-derived ROS (reviewed by Minotti et al., 2004). Several antioxidants, such as vitamin E and lycopene, show protective effects on DOX-induced myocardial damage without reducing their therapeutic efficacy in animal studies (Karimi et al., 2005; reviewed by Minotti et al., 1999). However, co-administration of DOX with these antioxidants has limited clinical success (reviewed by Ladas et al., 2004; Peng et al., 2005). More and more studies have screened antioxidants from natural medicine with the aim of minimizing ROS-mediated cardiac injury by DOX.

The fruit of Lycium barbarum (LB), a traditional Chinese medicine, is commonly used as an antipyretic, antiinflammation and antisenile agent. The aqueous extract of LB was reported to exhibit a concentration-dependent antioxidant activity, including anti-lipid peroxidation, superoxide anion scavenging and anti-superoxide formation (Wu et al., 2004; Luo et al., 2004). The potential effect of LB as an antioxidant against DOX-induced cardiotoxicity was hypothesized in this study. The cardioprotective effect of LB on DOX-caused oxidative stress was examined by malondialdehyde (MDA), a marker of lipid peroxidation, and SOD levels in the heart. Since electrophysiologic parameters have been suggested as one of the methods to detect cardiotoxicity of DOX by several studies (Dragojevic-Simic et al., 2004; Puri et al., 2005), the study also examined whether pretreatment with LB would improve arrhythmias and conduction abnormalities. Because DOX triggers the disruption of cardiac
myocytes and the release of intracellular creatine kinase (CK) and aspartate aminotransferase (AST) into serum (Deatley et al., 1999), the effects of LB on the change of serum CK and AST caused by DOX were examined. Finally, the protective effect of LB on DOX-induced myocardial injury was explored through histopathological study.

MATERIALS AND METHODS

Chemicals. DOX was obtained from Zhejiang Haizheng Pharmaceutical Co. (Taizhou, China) as a 10 mg/bottle lyophilized powder. It was reconstituted in 20 mL of 0.9% saline for injection. The SOD assay kit and the MDA assay kit were purchased from Nanjing Jiancheng Bio-engineering Institute (Nanjing, China).

Preparation of LB extract. Lycium barbarum was purchased from Tong-Ren-Tang Chinese Pharmacy in Hangzhou, and its identification was authenticated by Hua Liu. A voucher herbarium specimen (0318) was deposited at the Institute of Material Medicines, Zhejiang Academy of Medical Sciences, China. 100 g of LB was boiled with 1 L distilled water for 2 h. The extract was filtered and the residue was re-extracted under the same conditions (Wu et al., 2004). The combined filtrates were evaporated to dryness under vacuum. The yield rate of LB aqueous extracts was nearly 18.1%. Water-extracted LB contained carbohydrate (including flavonoids, betaine and polysaccharides), the mainly bioactive component, was proposed to be responsible for the antioxidant and cardioprotective activity (Luo et al., 2004; Wu et al., 2004).

Experimental protocol. Male Sprague-Dawley rats, 250–300 g weight, were obtained from the Zhejiang Medical Animal Centre, Hangzhou. The rats were housed at a temperature maintained at 20–25 °C and humidity at 50–60% and fed with the standard diet and water throughout the experimental period.

The rats were divided into four groups (n = 8 in each group) under two treatments. Two groups were pre-dosed with distilled water (4 mL/kg) by intragastric irrigation daily for 3 weeks. The other two groups were given orally LB solution (25 mg/kg) daily. The water-treated or LB-treated groups were given an i.v. infusion of the vehicle (0.9% saline, 10 mL/kg) or DOX (5 mg/kg) three times, i.e. at 7, 14 and 21 days. The mortality rate and general appearance of the animals were observed and recorded. All rats were weighed every 3 days during the study period. At 25 days, all the rats were anesthetized for ECG assessment. Blood samples were collected for the evaluation of serum levels of CK and AST. The rats were killed and the hearts were removed for biochemical and histological analyses.

Electrocardiogram. All animals were anesthetized with pentobarbital (30 mg/kg, i.v.). The ECG tracings were recorded by the Medlab system (Nanjing Meiyi Co., China). The rate and rhythm disorders were evaluated by measuring changes in electrocardiography parameters.

Measurements of antioxidant activity. Heart samples (1 g each) were homogenized in 10 mL of PBS at 4 °C with a homogenizer (Khan et al., 2005). Homogenates were then centrifuged at 1500 rpm for 30 min. The levels of SOD and MDA in heart homogenate were evaluated with the SOD assay kit and MDA assay kit, respectively.

Determination of serum levels of AST and CK. AST and CK enzyme activities in the serum were measured using an autoanalyser (Model 7020, Hitachi Medico, Japan).

Histopathological examinations. The tissues were fixed overnight in 10% formalin, dehydrated through a graded ethanol series, and embedded in paraffin. Ultrathin sections were stained with hematoxylin and eosin and observed under a light microscope by a pathologist blinded to the treatments.

Effect of LB on the antitumor activity of DOX in vitro. Human lung carcinoma cells A549 were cultured in Dubecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum. The cells were seeded into a 96-well plate at 5000 cells/well and plus additional LB solution (250 μg/mL). The cultures were maintained at 37 °C humidified with 5% CO2 for 24 h. The cells were exposed to DOX (concentration range 1.7 × 10−4 to 2.2 × 10−8 M) for another 24 h. The control cells were treated similarly, except that the LB or DOX was replaced by DMEM. Afterwards, the cytotoxicity was determined by the crystal violet method (Wattanapitayakul et al., 2005). Briefly, after incubation, the cells were washed with PBS twice, and fixed with 11% glutaral. 0.1% crystal violet solution was used as the stain. After 30 min incubation, the medium was removed and the crystal violet stain in the live cells was solubilized in 10% acetic acid. Subsequently, the absorbance was measured at 595 nm. The EC50 of DOX with or without LB on A549 cells was evaluated.

Statistical analyses. All the results were expressed as mean ± SEM and analysed by one-way ANOVA and Tukey’s HSD test. EC50 values were determined by using the curve fitting program. Basic dose response curves (downhill) of GraphPad Prism 4.03 (San Diego, CA, USA). A probability of error (p < 0.05) was selected as the criterion of statistical significance.

RESULTS

General observations

Table 1 shows several animal characteristics in the experimental treatments. After the third injection, the rats in the two control groups lived normally and no death was observed. However, the number of dead rats was three in the DOX group and one in the LB + DOX group, respectively. All the surviving rats in the DOX and LB + DOX groups appeared weak, with hair erection, a hunched posture and weight loss. DOX also led to a significant decrease (p < 0.05) in heart weight and heart-to-body weight ratio. The DOX-induced decrease in heart weight was attenuated by the pretreatment of

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Table 1. Effect of LB and/or DOX treatment on animal mortality and characteristics

<table>
<thead>
<tr>
<th></th>
<th>Mortality</th>
<th>Ascites (mL)</th>
<th>First BW (g)</th>
<th>Final BW (g)</th>
<th>HW (g)</th>
<th>HW/BW ratio (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0%</td>
<td>0</td>
<td>283.5 ± 6.0</td>
<td>389.4 ± 9.0</td>
<td>1.24</td>
<td>3.19 ± 0.07</td>
</tr>
<tr>
<td>LB</td>
<td>0%</td>
<td>0</td>
<td>282.9 ± 7.5</td>
<td>401.4 ± 4.8</td>
<td>1.28</td>
<td>3.18 ± 0.04</td>
</tr>
<tr>
<td>DOX</td>
<td>38%</td>
<td>8.9 ± 0.8a</td>
<td>283.1 ± 8.8</td>
<td>270.2 ± 3.5a</td>
<td>0.76</td>
<td>2.82 ± 0.05a</td>
</tr>
<tr>
<td>LB + DOX</td>
<td>13%</td>
<td>2.8 ± 0.8ab</td>
<td>286.5 ± 8.2</td>
<td>271.1 ± 9.4a</td>
<td>0.81</td>
<td>2.98 ± 0.04ab</td>
</tr>
</tbody>
</table>

BW, body weight. HW, heart weight. All values are mean ± SEM (n = 5–8 per group).

* Significantly different (p < 0.05) from respective values in the control group.

* Significantly different (p < 0.05) from respective values in the DOX group.

LB, although the difference was not statistically significant (p = 0.08). However, a significant difference (p < 0.05) was found in the heart-to-body weight ratio between the LB + DOX group and the DOX group. Ascites was present in the DOX and LB + DOX groups but not in the control and LB groups. Compared with the rats injected with DOX only, the LB + DOX rats had a significantly lower mean volume of ascites (p < 0.05).

Effect of LB and/or DOX treatment on cardiac function

The ECG tracings were recorded for evaluating the influence of treatment with LB and/or DOX on the heart rate and rhythm. The ECG of the control animals was normal. Dosing LB daily had no significant effect on the ECG of the experiment rats. As shown in Fig. 1, i.v. injection of DOX significantly decreased the mean heart rate, increased ST deviation and T-wave amplitude, and prolonged PR, QT and ST intervals (p < 0.05). Pretreatment with LB effectively improved the DOX-induced bradycardia (Fig. 1A) and significantly helped to reduce the DOX-induced increase in ST (Fig. 1B), PR (Fig. 1C) and QT (Fig. 1D) intervals (p < 0.05). However, the protective effect of LB on the DOX-induced deviation of ST segment and elevation of T-wave was limited (Fig. 1E and 1F).

Effects of LB and/or DOX treatment on heart antioxidant activity

As shown in Table 2, MDA levels of the heart homogenates were similar between the control group and the LB group. In the DOX group, MDA levels increased significantly compared with the control group.

Table 2. Effect of treatment with LB and/or DOX on MDA and SOD of hearts homogenates

<table>
<thead>
<tr>
<th></th>
<th>MDA (nmol/g tissue)</th>
<th>SOD (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.1 ± 2.5</td>
<td>54.7 ± 5.7</td>
</tr>
<tr>
<td>LB</td>
<td>37.9 ± 2.1</td>
<td>47.1 ± 3.6</td>
</tr>
<tr>
<td>DOX</td>
<td>51.0 ± 3.6a</td>
<td>36.6 ± 1.7a</td>
</tr>
<tr>
<td>LB + DOX</td>
<td>39.6 ± 1.5ab</td>
<td>55.8 ± 3.3b</td>
</tr>
</tbody>
</table>

All values are mean ± SEM (n = 5–8 per group).

* Significantly different (p < 0.05) from respective values in the control group.

* Significantly different (p < 0.05) from respective values in the DOX group.

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The DOX-induced increase in heart tissue MDA was significantly attenuated in rats pretreated with LB ($p < 0.05$). The rats in the DOX group showed a significant decrease in SOD levels in heart homogenates, whereas in rats treated with LB, it significantly attenuated the DOX-induced increase in SOD levels.

**Effects of LB and/or DOX treatment on serum biochemistry**

As the main serum biochemical signs of myocardial damage, serum CK and AST of the experimental animals were examined and the results are shown in Table 3. There was no significant difference in either CK or AST levels between the control group and the LB group. Compared with the control group, serum CK and AST levels were increased significantly in the DOX group. LB treatment helped to reduce the levels of CK and AST caused by the DOX injection. However, only AST, but not CK, in the LB + DOX groups was found to be statistically significantly different from the respective values in the DOX group.

**Histopathological changes**

No marked histopathological changes were observed in the cardiac tissues from control (Fig. 2A) and LB groups, whereas DOX caused myocardial damage, characterized by cytoplasmic vacuolation and myofibrillar disarrangement (Fig. 2B). The DOX-induced myocardial damage was attenuated partially by administration of LB (Fig. 2C).

**DISCUSSION**

Myocardial injury caused by oxidative stress, which is generated from DOX disposition, is the main bottleneck for the clinical use of DOX. It is believed that antioxidants could attenuate DOX-induced cardiotoxicity by eliminating free radicals (reviewed by Minotti et al., 2004). Provided with this theory, this study examined the potential protective effect of LB on DOX-induced cardiotoxicity.

In the present study, i.v. injection of DOX (5 mg/kg, weekly for 3 weeks) caused marked myocardial damage in rats. Because of the loss of cardiac myocytes and

---

**Table 3. Effect of treatment with LB and/or doxorubicin DOX on serum levels of CK and AST**

<table>
<thead>
<tr>
<th></th>
<th>CK (nmol/L)</th>
<th>AST (nmol/s/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4914 ± 354</td>
<td>2216 ± 150</td>
</tr>
<tr>
<td>LB</td>
<td>6634 ± 936</td>
<td>2248 ± 159</td>
</tr>
<tr>
<td>DOX</td>
<td>12380 ± 3551</td>
<td>3221 ± 300</td>
</tr>
<tr>
<td>LB + DOX</td>
<td>8201 ± 1132</td>
<td>1642 ± 122</td>
</tr>
</tbody>
</table>

All values are mean ± SEM ($n = 5–8$ per group).
* Significantly different ($p < 0.05$) from respective values in the control group.
* Significantly different ($p < 0.05$) from respective values in the DOX group.

The effect of LB on the cytotoxicity activity of DOX in human lung carcinoma cells was investigated in vitro. As Fig. 3 shows, DOX reduced the cell survival in a sigmoid concentration-response manner with an EC$_{50}$ value of 0.55 μM (95% confidence interval, 0.19 to 1.56 μM). The EC$_{50}$ value for DOX co-administration with LB to induce cell apoptosis was 0.45 μM (95% confidence interval, 0.07 to 2.59 μM), which was close to the cytotoxic activity of DOX. These results suggest the antitumor activity of DOX was not altered by LB.
the attenuation of left ventricular function, the ECG of the rats in the DOX group was characterized by arrhythmias and conduction abnormalities, which embodied a decreased heart rate, increased PR, QT and ST intervals, increased ST deviation, and ST elevation in the DOX group of rats. Similar changes in QT and ST segments have been reported by several other studies (Dragojevic-Simic et al., 2004; Puri et al., 2005). A cumulative dose of 15 mg/kg of DOX still resulted in an increase of heart MDA and a decrease of heart SOD. Consistent with the change of serum biochemistry caused by DOX in other studies, the results confirmed the acute cardiotoxicity of DOX increased serum CK and AST levels (Deatley et al., 1999; Puri et al., 2005). As a type of traditional Chinese herb, Lycium barbarum has usually been used to retard the aging process and to improve health. Moreover, LB is reported to be an effective antioxidant, which possesses anti-lipid peroxidation, superoxide anion scavenging and anti-superoxide formation (Huang et al., 1998; Wu et al., 2004). It has been proposed that LB exhibited antioxidative ability through flavonoids and betaine suppressing the initiation or propagation of the ROS chain reactions (Wu et al., 2004). Administration of the aqueous extract of LB decreased the MDA level in the DOX-treated rats in our study, in agreement with the recent report that the LB extract had an inhibitory effect on FeCl₂-ascorbic acid-induced lipid peroxidation in rat liver homogenates in vitro (Wu et al., 2004). LB did not increase the SOD level in the control rats but inhibited the SOD decrease in the DOX-treated rats. These observations indicated the antioxidant potential of LB and confirmed its beneficial effects. Furthermore, pretreatment with LB markedly ameliorated the DOX-induced histological changes of cardiac myocytes, suggesting the protective role of LB on myocardial damage by DOX. Equally important is the fact that LB was effective in decreasing the levels of serum AST and CK, as well as improving the arrhythmias and conduction abnormalities caused by DOX. Furthermore, the study suggested that LB did not abate the antitumor activity of DOX on carcinoma cells. In addition, Lycium barbarum polysaccharide, one of the main components of the aqueous extract from LB, was found to have anticancer activity by immunological enhancement (Gan et al., 2004; Zhang et al., 2005). Taken together, the results of the present study revealed the protective effect of LB against DOX-induced cardiotoxicity, and this effect appears to be due to the antioxidant effect of LB and may be useful in DOX chemotherapy.

The result of this study indicated that the aqueous extract of LB elicited a typical cardioprotective effect on DOX-related oxidative stress and confirmed the earlier studies showing that induction of oxidative stress and lipid peroxidation are among the basic mechanisms responsible for the cardiotoxicity. The findings of the present study encourage further investigation to yield a conclusive statement that LB is a useful adjunct in combination with DOX chemotherapy.

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**Lycium barbarum** glycoconjugates: effect on human skin and cultured dermal fibroblasts

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**Abstract**

*Lycium barbarum* L. (Solanaceae) glycoconjugates (LbGp) display an interesting array of anti-apoptotic and antioxidant properties, which may be beneficial for human skin. We therefore set out to determine the effects of LbGp in full-thickness human skin, and in dermal fibroblasts. It was found that LbGp decreased the level of MMP (matrix metalloproteinase)-1 significantly, but not that of MMP-3 or -13, in the whole human skin system, without compromising the viability of the skin. Consistently, LbGp inhibited skin expansion under mechanical stress, which in this model depends on the activity of MMP-1. We found that one of *L. barbarum* glycoconjugates, the LbGp5, promoted the survival of human fibroblasts cultured in suboptimal conditions. Furthermore, in the presence of LbGp5, these cultures also contained higher levels of the MMP-1 substrate—collagen type I. Together these results suggest that *L. barbarum* glycoconjugates in general, and LbGp5 in particular, may have important skin-protective properties.

**Keywords:** *Lycium barbarum*; Glycoconjugates; Skin and cultured dermal fibroblasts

**Introduction**

*Lycium barbarum* L. (Solanaceae) is a plant highly valued in traditional Chinese medicine for its broad health benefits (for a review, see Huang, 1999). *L. barbarum* glycoconjugates (LbGp) extracted from its fruits (Fructus Lycii) are believed to be the main active therapeutic components of this plant, due mostly to their impressive antioxidant effects (Zhang, 1993; Zhao et al., 2001; Wang et al., 2002). LbGp are peptidoglycans with a short peptide backbone and a complex, branched glycan moiety.

Recently, we have shown that standardized LbGp not only inhibit lipid peroxidation and cytochrome C oxidation, but also protect seminiferous epithelium from structural damage and apoptosis, in a testicular tissue culture system (Wang et al., 2002). This observation prompted us to investigate whether LbGp have beneficial effects on yet another important type of epithelium and its underlying tissues—the skin. The skin is the organ with the most exposure to external insults, such as free radicals, which can cause premature ageing and neoplasms, in part through the upregulation of matrix metalloproteinases (MMPs; Wlaschek et al., 1995; Krengel et al., 2002). Given the antioxidant and
anticancer (Lu and Cheng, 1991; Cao et al., 1994) properties of LbGp, we hypothesized that LbGp may have a protective effect on the skin. We probed this hypothesis with a model system designed to induce MMP expression in full-thickness human skin through the application of a cyclic mechanical force to the skin. Furthermore, we used human dermal fibroblasts to test the activity of one purified component of LbGp—the LbGp5, which was shown previously to have the strongest antioxidant activity among L. barbarum glycoconjugates (Huang et al., 2001).

Materials and methods

Reagents

All reagents were purchased from Fisher Scientific (Philadelphia, PA) except where indicated.

Isolation of LbGp

LbGp were purified from dried fruits of L. barbarum L. (Solanaceae; harvested in Ningxia province of People’s Republic of China) by hot water extraction, ultrafiltration, ethanol precipitation and DEAE chromatography as described elsewhere (Wang et al., 2002). The yield was 8 mg LbGp/g of dry fruit. LbGp5 was further purified by filtration through a 30 kD cut off centrifugal device (Millipore, Bedford, MA). Flow-through was collected, precipitated with one volume of cold ethanol, lyophilized, redissolved in water and filtered through a 10 kD cut off centrifugal device. The upper chamber material was collected, the volume was adjusted to 10 ml with ultrapure water and refiltered through the 10 kD cut off centrifugal. The upper chamber material was collected and lyophilized (yield: 0.45 mg LbGp5/g of dry fruit). Glycoconjugate concentrations were estimated by a sulfuric acid—phenol method according to Dubois et al. (1956). LbGp5 was visualized on 18% SDS-polyacrylamide gel (BioRad, Hercules, CA) by silver staining according to Shevchenko et al. (1996). Briefly, after electrophoresis, gels were incubated in fixation solution (methanol 50%; acetic acid 5%; water 45% v/v) for 20–30 min, washed in water for 60 min, and incubated in 0.02% sodium thiosulfate for 2 min. Gels were then rinsed, incubated in cold 0.1% silver nitrate at 4°C for 30 min, rinsed again and developed using 0.04% formaldehyde in 2% sodium carbonate.

Enzymatic digestion

LbGp5 (100 μg/ml, pH 5.5) was incubated with 10 μg/ml of β-glucosidase from almonds (Sigma, St. Luis, MO) for 15 min at 37°C. The reaction was terminated by heating at 90°C for 5 min. Heating of mock-digested LbGp5 was performed in parallel and did not affect the activity of LbGp5.

In vitro tissue expansion assay

Full-thickness human skin was obtained under informed consent from patients undergoing abdominoplasty surgery. The skin was transported in oxygenated transport medium (Dulbecco’s Modified Eagles Medium (DMEM) (high glucose) JRH Bioscience, Lenexa, KS)+15 mM HEPES (Sigma, St. Louis, MO)+10,000 U Penicillin/Streptomycin (JRH Bioscience) at 4°C (±1°C), immediately defatted and 1-in diameter pieces punched from the whole tissue. The full-thickness skin punch was placed in growth medium (high glucose DMEM (JRH)+0.15 M HEPES (Sigma)+2 mM Glutamine (BioWittaker, Walkersville, MD)+10,000 U Penicillin/Streptomycin (JRH Bioscience)+4.4 μM Insulin (Boehringer Mannheim, Germany)+0.28 μM Hydrocortisone (Calbiochem, San Diego, CA)+0.3 μM ascorbic acid (Calbiochem) in a custom designed bioreactor. The bioreactor consisted of a sterile growth chamber containing ports for medium addition and removal and gas exchange, nylon cord actuators, and a stepper motor controlled by custom software running under the Windows operating system. The skin was attached radially to 10 actuators using sterile garment tags, which do little damage to the skin. Tension on the skin was set at 100 g per attachment, the device assembled and the skin cycled using a sine wave at 10 cycles/min under an atmosphere of 40% O2, 5% CO2 and 50% N2. Tissue surface area was measured at time 0 and then every 24 h using a digital camera photographed through the clear bioreactor top. Changes in surface area were calculated as a percent change in surface area using a customized digitizing program. To test the effects of LbGp on full-thickness human skin in vitro, varying concentrations from 0 to 1% (w/v) of lyophilized LbGp were dissolved in culture medium prior to addition to the bioreactor. Growth medium and LbGp were replaced daily.

Quantitation of tissue levels of MMP-1 by ELISA

Concentrations of matrix metalloproteinase (MMP)-1 protein were measured in culture medium of whole skin in a quantitative sandwich immunoassay. The assay was performed using an MMP-1 ELISA kit from Calbiochem (# QIA 55) and following the manufacturer instructions. Briefly, 5 ml of media removed from the bioreactor after 48 h incubation with various concentrations of LbGp were centrifuged to remove particulate material, concentrated and assayed immediately. Standards were prepared at the same time as the samples and
assayed in duplicate. One hundred microliters of sample or standard was placed into each well of a 96-well microtiter plate and incubated at room temperature for 2 h. The wells were washed 5 × 1 × wash buffer (sodium phosphate, pH 7.0). One hundred microliters of MMP-1 conjugate was added to each well and incubated for 1 h at room temperature, and washed as above prior to adding 100 μl of color reagent (tetra-methylbenzidine) to each well. After a 30-min incubation in the dark, the reaction was stopped with 2.5 N sulfuric acid. Absorbance was measured in a spectrophotometric plate reader at 490 nm. The concentration of MMP-1 protein in the samples was determined by interpolation from the standard curve. Samples were standardized to total protein in the medium as measured by the Bradford Protein Assay (BioRad, Hercules, CA). Assays for MMP-3 and -13 were performed as described above using kits from Calbiochem (#QIA 73) and Amersham (#RPN2621), respectively.

**Histology**

Skin samples were removed from the bioreactor, washed in PBS and placed in 10% formalin. The tissue was dehydrated, embedded in paraffin, sectioned (5 μm) and stained with Hematoxylin and Eosin (H&E). Slides were examined using a Zeiss axioplan 2 microscope equipped with a Hamamatsu color digital camera.

**Lactate/glucose determination**

Lactate and glucose were determined using a YSI 2700 Select dual channel biochemistry analyzer (YSI, Yellow Springs, OH). Briefly, tissue was removed from the bioreactor, washed in DMEM and placed into 5 ml of DMEM (low glucose) medium at 37 °C. Sixty microliters samples of medium were removed at set intervals over a 3-h period. Samples were either analyzed for glucose and lactate immediately or stored at 4 °C for up to 24 h prior to analysis.

**Fibroblast culture**

Human neonatal dermal fibroblasts (passage 3–25; Cambrex, Walkersville, MD) were cultured at 5% CO₂ in complete FBM medium (FBM medium with FGM2 singlequot supplement, containing insulin, basic fibroblast growth factor and 2% fetal bovine serum; Cambrex). For suboptimal culture conditions this medium was replaced with DMEM without serum (Hyclone, Logan, UT).

For the measurement of type-I collagen and cell proliferation, fibroblasts were seeded in 96-well plates (Corning brand) at 6000 cells/well, and were grown for 3 days to late subconfluence. Cells were rinsed subsequently and incubated in suboptimal conditions with 2% calf serum for three additional days, in the absence or presence of LbGp5. Cultures were observed with a Nikon Eclipse TS100 inverted microscope equipped with a Canon camera. At the end of the experiment, cell media were collected for type I collagen quantification, and cells were counted by the colorimetric method based on sulforhodamine B staining and optical density measurement at 575 nm in a microplate reader 3550-UV (BioRad, Hercules, CA), by standard method (Skehan et al., 1990).

**Type I collagen quantization**

Type-I collagen content in the fibroblast culture media was measured by the sandwich enzyme-linked immunosorbant assay (ELISA), as reported before (Dobak et al., 1994). Briefly, 96-well plates (Immulon 4HBX from Dynex, Chantilly, VA) were coated with goat anti-type I collagen antibody (Southern Biotechnology Associates, Birmingham, AL) overnight at 4 °C, and incubated with conditioned media for 1 h. After rinsing, sandwich was completed by adding biotinylated anti-type I collagen antibody for 1 h, followed by horseradish peroxidase-labeled streptavidin (Southern Biotechnology, Birmingham, AL) for 30 min. The assay was developed with peroxidase substrate 2, 2'-Azino-bis-3-ethylbenziazoline-6-sulfonic acid (ABTS; Rockland Immunochemicals, Gilbertsville, PA) and read at 405 nm. Statistical differences between the means were assessed using Student’s t-test analysis.

**Results**

**Effect of LbGp on matrix metalloproteinases in whole skin**

Because skin cancer and aging are associated with the upregulation of metalloproteinases, we tested the effect of LbGp on the expression of these matrix-digesting enzymes. Whole human skin was cycled at 10 cycles/min under 100 g of force per skin attachment and exposed to increasing concentrations of LbGp in the bioreactor for 48 h; then MMP (matrix metalloproteinases) 1, 3 and 13 in the conditioned medium were quantified by ELISA. Fig. 1A shows that in the presence of LbGp, skin samples contained 3–4 times less MMP-1 than the control samples. In contrast, these glycoconjugates had no effect on the expression of MMP-3 and MMP-13 (Fig. 1B, C). Importantly, this decrease in MMP-1 was not due to an overall decrease of skin viability, as determined by the ratios of lactate/glucose in treated and control skin samples. These ratios were comparable regardless of the presence of LbGp, indicating a constant level of metabolic activity in the
tissue (Fig. 1D). LbGp did not interfere directly with the ELISA and lac/glu assays. Skin viability was confirmed by comparative observations of H&E stained paraffin sections of control skin samples and those incubated with LbGp. There was no observable effect on the epidermis, dermis or adnexal structures (results not shown).

Subsequently, we investigated whether this LbGp-induced selective MMP-1 inhibition has physiological relevance. For this we cycled skin and determined the percent change in surface area over time as described in Materials and Methods. This skin expansion model has been shown to be associated with an increase of MMP-1 expression (Estelles et al., 2000). Fig. 2 shows that LbGp inhibits skin expansion in a dose-dependent manner, by over 60%. This inhibition cannot be explained by a general suppression of skin viability (Fig. 1D), and is consistent with the selective MMP-1-blocking effect of these glycoconjugates (Fig. 1A).

Effect of LbGp5 on dermal fibroblasts at suboptimal culture conditions

LbGp fraction is composed of several glycoconjugates of distinct molecular weights: LbGp2 (68.2 kD; Peng and Tian, 2001); LbGp3 (92.5 kD; Huang et al., 1998), LbGp4 (215 kD; Huang et al., 1999) and LbGp5 (23.7 kD; Peng et al., 2001). Of particular interest is LbGp5, because of its relatively low molecular weight and especially strong antioxidant effect in vitro (Huang et al., 2001). Therefore, we decided to determine whether LbGp5 has an effect on human dermal fibroblasts.

It was found that LbGp5 had no effect on fibroblast growth and morphology at optimal conditions (complete FBM medium; not shown). When cells were grown in suboptimal conditions, however, (DMEM without serum), addition of LbGp5 (10–100 µg/ml) resulted in better overall cell morphology (i.e., less rounded cells) as compared to fibroblasts cultured in the absence of this glycoconjugate (compare Fig. 3B and C). Similarly, suboptimal cultures with LbGp5 had significantly higher cell counts than the same cultures without LbGp (compare Fig. 3E’s first two “LbGp” bars and the “ctr (DMEM)” bar). The morphology and cell numbers of

![Fig. 1. Effect of LbGp on the expression of matrix metalloproteinases in human skin. Full-thickness skin punches (surface area ~6.5 cm²) from abdominoplasty were incubated in a bioreactor with increasing concentrations of LbGp while subjected to cyclic force as described in Materials and Methods. LbGp induce a decrease of MMP-1 level (A), but not of other tested matrix metalloproteinases (MMP-3; Panel B, and MMP-13; Panel C) in full thickness human skin conditioned medium as measured by ELISA (the decrease of MMP-3 at LbGp 1 mg/ml is not statistically relevant). (D): skin viability, as measured by lactate to glucose ratio is not affected by LbGp. Error bars represent standard deviation. Number of tests (n) = 3.](image-url)
cultures with LbGp were comparable to cultures maintained continuously at optimal conditions (compare Fig. 3A and C, and Fig. 3E’s first two “LbGp” bars and the “ctr (FBM)” bar).

We examined also the effect of LbGp5 on type-I collagen output in dermal fibroblast cultures. Type-I collagen is a major substrate of MMP-1 in dermal fibroblasts (Welgus et al., 1981). It is also an important metabolic marker (Weber et al., 1995). In cultures incubated during the last 3 days under suboptimal conditions, the type-I collagen level was 60% lower, as compared with cultures maintained at optimal conditions during the full 6-day period. In contrast, in suboptimal cultures incubated with LbGp5 (100 \( \mu \)g/ml), the type-I collagen level was similar to the optimal culture control (Fig. 3F). The effect of LbGp5 on collagen output by dermal fibroblasts under these conditions was dose dependent (LbGp5 in the medium did not interfere with the ELISA assay; data not shown).

In order to confirm that LbGp5 is indeed responsible for these effects, we subjected it to enzymatic digestion. LbGp5 contains \( \beta \)-linked residues, which makes it sensitive to hydrolysis by \( \beta \)-glucosidase. As shown on Fig. 3(D)–(F), \( \beta \)-glucosidase—digested LbGp5 lost most of its cell-protective and collagen-stimulatory potency. Fig. 4 confirms that \( \beta \)-glucosidase treatment resulted in the degradation of LbGp5.

Fig. 2. Dose-dependent inhibition of skin expansion by LbGp. Full thickness human skin punches (\(~10\,cm^2\)) from abdominoplasty were incubated in a bioreactor and subjected to cyclical force as described in Materials and Methods in the presence of increasing concentrations of LbGp over a 5 day period. Skin expansion (% growth) was determined by measuring the surface area once daily for 5 days and expressed as percentage of the surface of the untreated control on day 0. Error bars represent standard deviation. Number of tests (\( n \)) = 3.

Fig. 3. Protective effect of LbGp5 on subconfluent cultures of human neonatal dermal fibroblasts. A–D: Morphology of cultures incubated at (A) optimal conditions (complete FBM medium) for 6 days; (B) 3 days at optimal conditions following 3 days at suboptimal conditions (DMEM without serum) in the absence of LbGp5; (C) 3 days at optimal conditions following 3 days at suboptimal conditions in the presence of LbGp5 (100 \( \mu \)g/ml); (D) 3 days at optimal conditions following 3 days at suboptimal conditions in the presence of LbGp5 (100 \( \mu \)g/ml) pretreated with \( \beta \)-glucosidase, as described in Materials and Methods (magnification \( \times 40 \)). Arrows indicate rounded cells. E: Quantification of cell numbers at conditions depicted on panels A–D. F: Quantification of type-I collagen at conditions depicted on panels A–D. \( P < 0.05 \).
Discussion

LbGp have been reported to have interesting protective properties on seminiferous epithelium, such as preservation of stratified structure of the cultured seminiferous tubules and inhibition of apoptosis (Wang et al., 2002). These results prompted us to test LbGp for protective effects in the dermis, using the whole human skin expansion model. This unique model was developed by Reconstructive Technologies, Inc. (Palo Alto, CA) to generate skin for burn patients, and has been adapted to study MMP expression. It allows for long-term experiments by maintaining the viability of whole human skin for over a week's time. In this system, LbGp inhibited the expression of MMP-1 selectively, without affecting other tested metalloproteinases. This inhibition was strong and physiologically relevant, because at the same concentrations LbGp blocked skin expansion, that is associated with the increase of MMP-1 expression (Estelles et al., 2000). Thus, LbGp could be of interest in the processes involving excessive expression of MMP-1 in the skin, such as skin aging (Varani et al., 2000; Lahmann et al., 2001) or cancers (Tsukifuki et al., 1999; Ye et al., 2001).

The immunostimulatory (Li et al., 1984; Huang et al., 1998), hepatoprotective (He et al., 1993), anti-ageing (Zhisong, 2000), anticancer (Lu and Cheng, 1991) and antioxidant (Zhang, 1993; Huang et al., 2001) effects of LbGp have been studied extensively. Recent physico-chemical analysis showed that LbGp contains five distinct peptidoglycans labeled LbGp1-5 (Zhao et al., 2001). Out of these glycoconjugates, LbGp5 is particularly interesting due to its relatively small molecular weight and particularly strong antioxidant activity (Huang et al., 2001). We therefore tested LbGp5 on human dermal fibroblasts. Subconfluent to confluent dermal fibroblast cultures can be incubated in optimal (complete FBM) medium conditions for many days, without apparent cell loss. If the same cultures are placed in simple DMEM medium without serum, however, cells tend to round up and detach. LbGp5 had no effect on subconfluent cultures incubated in complete FBM medium, but it protected cells from rounding up and detaching when these cultures were exposed to suboptimal conditions (DMEM without serum). In fact, the morphology of fibroblasts incubated in suboptimal conditions in the presence of LbGp5 was very similar to the morphology of the same cells cultured in optimal conditions. This result is consistent with our previous findings in the cultured seminiferous tubule system, where LbGp showed an antiapoptotic and morphology-protective effect (Wang et al., 2002). The underlying mechanism of action remains to be determined, but it seems that LbGp5 could exert its activity through interaction with a growth factor or adhesion receptor on the cell surface. For example, LbGp5 could facilitate binding of fibroblast growth factor (FGF) to its receptor, as does another proteoglycan, perlecan, (Aviezer et al., 1994). Alternatively, LbGp5 could act as a growth factor on its own, interacting with a sugar-binding site on a receptor. Such sites exist, for example, on the FGF receptor, and their binding by proteoglycan heparin results in signal transduction even in the absence of FGF (Gao and Goldfarb, 1995). LbGp5 could also act, by structural analogy, as an agonist of a glycosylated growth or survival factor, whose active site is composed of both sugar and amino-acid moieties.

Finally, because LbGp5 is such an efficient antioxidant, it may exercise its effect, at least partially, through protecting cells from free radical damage. Such protection has been reported for various glycoconjugates composing the extracellular matrix. In this respect, it is interesting to note that the aging of certain tissues such as cornea and endothelium correlates with the decrease of the most efficient anti-oxidant components of their extracellular matrices (Albertini et al., 2000).

The fact that fibroblasts grown in suboptimal conditions in the presence of LbGp5 secrete normal amounts of type-I collagen, shows that LbGp5 does not merely inhibit cell detachment, but allows cells to maintain their regular metabolic functions. This effect, as well as the protective effect of LbGp5 on cells, can be abrogated by pretreating this glycoconjugate with β-glucosidase. The degradation of LbGp5 by this β-linked glycans hydrolyzing enzyme, as visualized by silver staining, results in the loss of its fibroblast-protective effects, confirming the importance of the glycan moiety for the activity of this L. barbarum peptidoglycan.

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