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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-α are two important cytokines in antitumor immunity. In this study, the effects of *Lycium barbarum* polysaccharide-protein complex (LBP_{3p}) on the expression of interleukin-2 and tumor necrosis factor-α in human peripheral blood mononuclear cells were investigated by reverse transcription polymerase chain reaction (RT-PCR) and bioassay. Administration of LBP_{3p} increased the expression of interleukin-2 and tumor necrosis factor-α at both mRNA and protein levels in a dose-dependent manner. The results suggest that LBP_{3p} may induce immune responses and possess potential therapeutic efficacy in cancer.

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 LBP_{3p}, 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 LBP_{3p}. Cytokines are mediators of all aspects of immunoregulation, so the capacity to induce or enhance cytokine production could be a major mechanism by which LBP_{3p} exerts immunomodulatory effects. In the present work, the expression of interleukin-2 and tumor necrosis factor-α 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 LBP_{3p} to human peripheral blood mononuclear cells.

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2. Materials and methods

2.1. Preparation of LBP_{3p}

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., 1981) and periodic acid-schiff (Mcmanus, 1946). It was shown to be homogeneous by Sephadex G-200 gel filtration chromatography and SDS-polyacrylamide gel electrophoresis. The molecular weight of LBP_{3p} was 1.57×10^5 by laser light scattering (Bettelheim et al., 1962). The chemical composition of LBP_{3p} was 63.56% neutral sugars, 24.80% acidic sugars and 7.63% proteins, as determined by phenol–H_2SO_4 (Dubois et al., 1956), carbazole (Chaplin, 1986) and Lowry (Lowry et al., 1951) methods, respectively. It consisted of six monosaccharides: galaclose, glucose, rhamnose, arabinose, mannose, xylose; and the molar ratio was 1:2.12:1.25:1.10:1.95:1.76, respectively, by gas chromatrography (Pinilla and Luu, 1999). It contained β-D-glucose linkage (infrared spectroscopy: 890 cm^{-1}) and the linkgage between glycan and protein was through glycan-*O*-serine by β-elimination method (Downs and Pigman, 1976).

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 RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated newborn bovine serum in the presence of 5% CO_2 at 37 °C. The appropriate concentrations of LBP_{3p} were added to the culture medium and the cells and supernatants were collected 2, 4, 8, 12, 15 and 24 h after stimulation. As a positive control, 10 mg/l phytohemagglutinin was used as a mitogen in the same way (Zvetkova et al., 2001).

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′-ACTCACCAGGATGCTCTCTAT-3′ and 5′-AGACTTGTCTACCTAATGGA-3′ for interleukin-2 (the product size 266 bp), 5′-TCTCGAACCCCGAGTGACAA-3′ and 5′-ACCGCACCTCGACTCTCTAT-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′-GCATGGAGTCCTGTGGCAT-3′ and 5′-CTAGAAGCATTTGCGGTGG-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-Analyst 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 4×10^4/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 LBP_{3p}-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 LBP_{3p} were determined by a semiquantitative RT-PCR and bioassay in human peripheral blood mononuclear cells (Fig. 1). LBP_{3p} 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 LBP_{3p} 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.

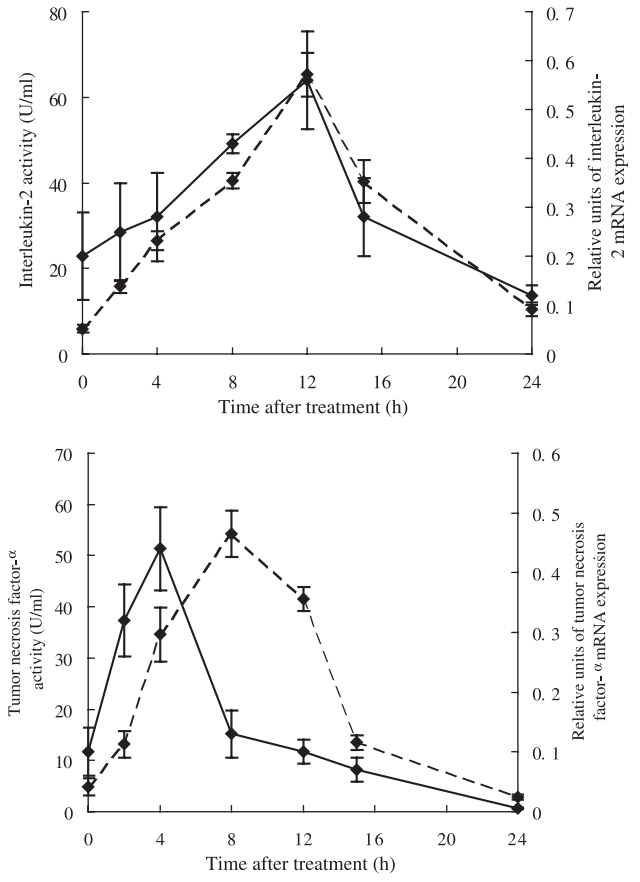

Fig. 1. Time kinetic profiles of interleukin-2 and tumor necrosis factor-α mRNA expression (—) and their secretion (- - -) in human peripheral blood mononuclear cells treated with LBP_{3p}. The cells were stimulated with LBP_{3p} 10 mg/l for different time. The levels of mRNA expression were determined by a semiquantitative RT-PCR. mRNA expression is presented as the ratio of the cytokine RT-PCR band intensity to the respective β-actin band intensity. Interleukin-2 activity was measured in a bioassay using a murine interleukin-2-dependent CTLL-2 cell line. Tumor necrosis factor-α activity was measured in a bioassay using a L929 cell line. Data represent means ± S.D. of four separate experiments.

3.2. Effects of LBP_{3p} 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 LBP_{3p} administration, we determined the effects of LBP_{3p} 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 LBP_{3p} 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 LBP_{3p} 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 LBP_{3p} for 4 h, tumor necrosis factor-α mRNA level was significantly increased in human peripheral blood mononuclear cells. Treatment with LBP_{3p} 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 LBP_{3p} 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).

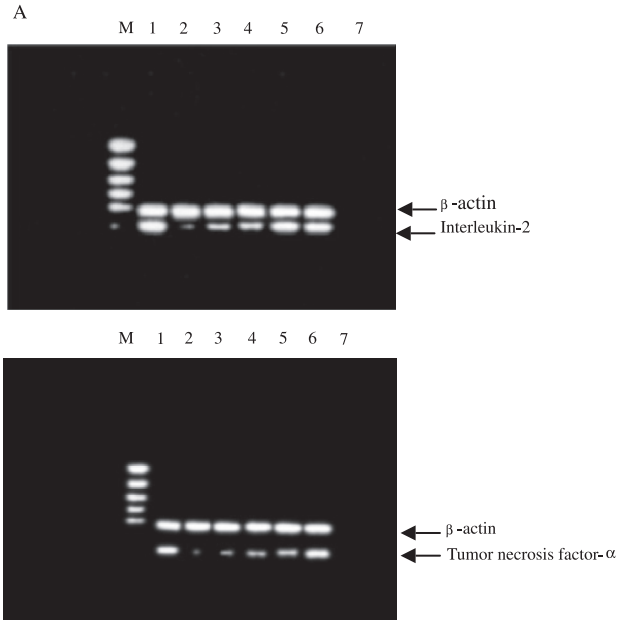

Fig. 2. Dose-dependent upregulation of interleukin-2 and tumor necrosis factor-α mRNA levels and their activity by LBP_{3p} in human peripheral blood mononuclear cells. (A) PCR products of interleukin-2 and tumor necrosis factor-α in human peripheral blood mononuclear cells after a 12- (interleukin-2) and 4-h (tumor necrosis factor-α) stimulation, respectively. Total cellular RNA was isolated and reverse transcribed using random primers to generate cDNA. cDNA (1 μg) was amplified by PCR using gene-specific primers. PCR products were separated by 1% agarose gel electrophoresis containing ethidium bromide. Lane M: PCR marker (1543, 994, 697, 515, 377 and 237 bp); Lane 1: cells stimulated with 10 mg/l phytohemagglutinin; Lane 2: negative control containing only the cells; Lanes 3–6: cells stimulated with 5, 10, 20 and 40 mg/l LBP_{3p}, respectively; Lane 7: total RNA control. (B) The expression of mRNA for interleukin-2 and tumor necrosis factor-α after 12 (interleukin-2) and 4 h (tumor necrosis factor-α) of stimulation in human peripheral blood mononuclear cells by a semiquantitative RT-PCR. An aliquot of PCR products was separated on a 1% agarose gel containing ethidium bromide and measured semiquantitatively using a Bio-Rad Multi-Analyst System. The mRNA levels are expressed as the ratio of interleukin-2 or tumor necrosis factor-α band intensity to the respective β-actin band intensity. (C) The activity of interleukin-2 and tumor necrosis factor-α in human peripheral blood mononuclear cells treated with LBP_{3p}. Supernatants were collected after 12 (interleukin-2) and 8 h (tumor necrosis factor-α) of stimulation, respectively. Interleukin-2 activity was measured in a bioassay using a murine interleukin-2-dependent CTLL-2 cell line. Tumor necrosis factor-α activity was measured in a bioassay using a L929 cell line. Data represent means ± S.D. of four separate experiments. $*P<0.05$ and $**P<0.01$ represent significant difference from negative control.

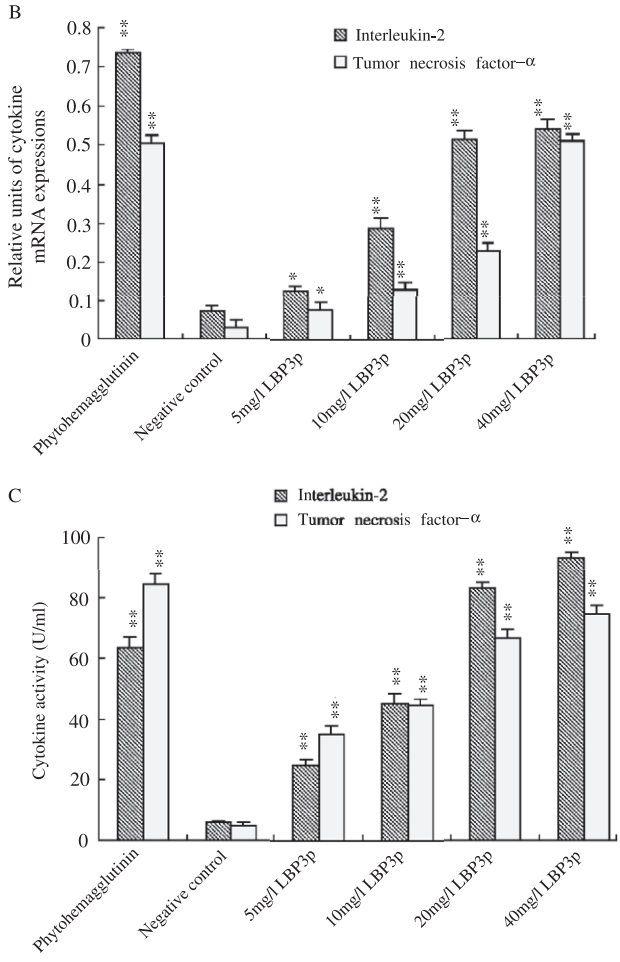

Fig. 2 (*continued*).

4. Discussion

The use of cytokines has a long history in immunotherapy, with interferen-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 LBP_{3p} can upregulate the expression of interleukin-2 and tumor necrosis factor-α at both transcription and protein levels. Our recent study showed that administration of LBP_{3p} could inhibit the growth of S180 solid tumor in experimental animals and exerted its antitumor activity through potentiation of the host animal's defense system (Gan and Zhang, in press). The immunomodulating and antitumor activity of LBP_{3p} 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 LBP_{3p} 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 LBP_{3p}, 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 LBP_{3p} on cytokine expression is similar to that of mushroom polysaccharide-protein complexes to some extent.

In conclusion, our results demonstrated that LBP_{3p}, 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 LBP_{3p}, 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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