ELSEVIER

Life Sciences, Vol. 64, No. 12, pp. 1005-1011, 1999

0024-3205/99/$–see front matter

PII S0024-3205(99)00027-2

ANALYSIS OF IMMUNOMODULATING CYTOKINE mRNAs IN THE MOUSE INDUCED BY MUSHROOM POLYSACCHARIDES

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(Received in final form December 7, 1998)

Summary

The immunomodulating action of two mushroom antitumor polysaccharides, polysaccharide-protein complex (PSPC) and lentinan, was elucidated through analysing the expression profile of cytokines during a time course (0 h to 48 h) after their administration. Among the 5 cytokine genes, the induction of a marked increase in the mRNA levels of IL-1α, IL-1β, TNF-α, IFN-γ and M-CSF by PSPC and lentinan was observed in the peritoneal exudate cells and splenocytes. However, the time point of their increased production was different after PSPC and lentinan administration.

Key Words: polysaccharide, cytokine, splenocyte, immunomodulation, mushroom

Many mushroom polysaccharides and polysaccharide-protein complexes have been considered as antitumor and immunomodulating agents and their modes of immunomodulation, especially at the cellular level, have been proposed (1,2,3,4,5). It is generally accepted that polysaccharides enhance various immune responses *in vivo* and *in vitro* (6,7,8). However, molecular biological characterisation of antitumor activities of polysaccharides has not been fully elucidated. Some researches showed that the antitumor action of mushroom polysaccharide complexes, such as PSK from *Coriolus versicolor* (9,10,11), PSPC from *Tricholoma lobayense* (12), and OL-2 from *Omphalia lapidescens* (13,14), was due to the potentiation of the host immune system through the regulation of cytokines in the cytokine network.

A polysaccharide–protein complex (PSPC) has been isolated from the culture filtrates of a local edible mushroom, *Tricholoma lobayense*, by ethanol precipitation (15). PSPC contained

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40% polysaccharide which consisted of galactose, glucose, mannose, fucose, arabinose and rhamnose, and 30% protein which was composed of 15 amino acids. It significantly suppressed the growth of S-180 solid tumor *in vivo* and exhibited immunomodulating effect (16). Lentinan is a pure β-glucan and has been used clinically in Japan as a host immunopotentiator for cancer therapy (1,17,18,19). It was isolated from the water-soluble extracts of fruiting bodies of *Lentinus edodes* (20). However, the mechanism of antitumor action of PSPC and lentinan is still not fully understood at the molecular level. In the present study, the immunopotentiating activity of cytokines was elucidated by reverse transcription-polymerase chain reaction (RT-PCR) in response to PSPC and lentinan in the ICR mice.

Methods

Mushroom polysaccharides

Polysaccharide-protein complex (PSPC) was isolated and characterized from culture filtrates of *Tricholoma lobayense* as described elsewhere (15). The backbone of PSPC is not a glucan, but rather it is a heteroglycan-protein. Its protein portion predominantly consists of aspartic acid, glutamic acid and serine. The molecular mass of PSPC is about 150 kDa by gel-chromatography on Sepharose CL-4B (15).

Lentinan is commercially available. Lentinan was extracted from the fruiting body of *Lentinus edodes* with hot water at 80-100°C for 8-10 hours. The water-soluble extracts was then precipitated with ethanol (20). It is a pure (1→3)-β-D-glucan. Its molecular mass is about 400 kDa (1).

Both PSPC and lentinan powders were dissolved in double distilled water by boiling for 10 min. Before use for injection into experimental animals, PSPC and lentinan solutions were filtered using 0.2μm membrane and thus were free of bacteria.

Experimental animals

Male ICR mice (6 weeks old) were used in this study. 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. Mice were injected intraperitoneally with PSPC or lentinan respectively at the dose of 20 mg/kg, under aseptic conditions.

Preparation of peritoneal exudate cells and splenocytes

At 0, 3 h, 6 h, 12 h, 24 h and 48 h after PSPC or lentinan administration, three mice were sacrificed and peritoneal exudate cells (PEC) were recovered by lavage of the peritoneal cavity with ice cold Hank's balanced salt solution (HBSS) and the splenocytes were obtained by homogenizing spleen with ice cold HBSS.

RNA extraction

PEC or splenocytes of three mice were centrifuged at 5,000 rpm for 5 min, and the supernatant was discarded. The cells were lysed in 4 M guanidinium thiocyanate (GT) solution containing 0.7% mercaptoethanol. The cell lysate was incubated at 65°C for 5 min, then chilled for 5 min. 2.5 ml of cell lysate was overlaid to 1 ml of 5.7 M CsCl and subjected to ultracentrifugation at 18°C, 32,000 rpm with SW 60 Ti rotor of XL-80 ultracentrifuge for 18 h.

The supernatant was discarded, and the RNA pellet was dissolved in 400μl dd H_2O, and then 45 μl of 3 M NaAc and 1 ml ethanol were added. The RNA pellet was obtained after centrifugation at 15,000 rpm in a microcentrifuge for 30 min and washed with ethanol. RNA concentration was determined by measuring the optical density at 260 nm.

Reverse transcription (RT)

RNA sample was heated at 65°C for 5 min and reverse-transcribed at 37°C for 1 h in a 20 μl reaction mix (for 1 μg of total RNA) containing 40 units RNase inhibitor, 0.1 μg oligo $dT_{12\text{-}18}$, 0.5 mM of each dNTPs, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 200 units Moloney murine leukemia virus reverse transcriptase. Samples were stored at −70°C until subjected to PCR amplification.

Polymerase chain reaction (PCR)

The RT sample equivalent to 0.1 μg total RNA was boiled for 5 min and quickly chilled on ice. PCR cocktail was added to the RT sample to make up 50 μl reaction mix containing 1.5 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01% Tween 20, 0.2 mM of each dNTPs, 1 pmole/ml of each primers and 0.25 units Thermoprime plus DNA polymerase (Thermoprime plus 207 G). PCR was performed for 25 cycles, each cycle consisting of 1 min of denaturation at 94°C, 1 min of annealing at 56°C and 1 min of extension at 72°C. The reaction products were visualized by electrophoresis of 10μl reaction mixture at 100 V for 1 h in 2.0% agarose gel containing 0.5 μg/ml ethidium bromide. 1 kb DNA ladder (BRL) was run in parallel as a molecular weight marker (providing main bands at 1018, 506, 396, 344, 298, 220 and 201 bp). The glyceraldehyde-3-phosphate-dehydrogenase (GAPDH, 452 bp) was used as standard in this study. Sequences of the PCR primers were shown in Table 1.

Table 1. Sequences of the primers used in RT-PCR

Cytokines		Sequence (5' to 3')	Size (bp)
IL-1 α	upper primer	ACAGTATCAGCAACGTCAAGCAA	
	lower primer	CCGACTTTGTTCTTTGGTGGCA	546
IL-1 β	upper primer	GAGCTTCAGGCAGGCAGTATC	
	lower primer	GTATAGATTCTTTCCTTTGAGGC	382
TNF-α	upper primer	TCCCCAAAGGGATGAGAAGTTC	
	lower primer	TCATACCAGGGTTTGAGCTCAG	411
IFN-γ	upper primer	AGGAACTGGCAAAAGGATGGTG	
	lower primer	GTGCTGGCAGAATTATTCTTATTG	353
M-CSF	upper primer	GTAGCCACATGATTGGGAATGG	
	lower primer	TCATGGAAAGTTCGGACACAGG	304

Results

Peritoneal exudate cells (PEC) and splenocytes were collected at appropriate intervals after PSPC and lentinan administration. The cytokine gene expression of PSPC and lentinan was compared by RT-PCR using primers specific for IL-1α, IL-1β, TNF-α, IFN-γ and M-CSF. After

the RT reaction of each RNA, the concentration of the resulting cDNA was normalized by the intensity of the PCR products of GAPDH, which was used as a control in order to eliminate experimental variations.

Cytokine gene expression in PEC of ICR mice

In comparison with the mRNA concentration of control mice, PSPC and lentinan affected the expression levels of cytokine mRNAs in the PEC (Fig. 1). Both PSPC and lentinan could up-regulate the expression of M-CSF mRNA from 3 h onwards. IL-1α, IL-β and TNF-α were dramatically up-regulated in the lentinan and PSPC treated mice at 3 h, and then dropped at 24 h. Basically, PSPC had only mild effect on the up-regulation of IFN-γ expression in the PEC while lentinan could strongly up-regulate IFN-γ expression.

Cytokine gene expression in splenocytes of ICR mice

In comparison with the mRNA concentration of control mice, PSPC and lentinan changed the expression levels of cytokine mRNAs in the splenocytes (Fig. 2). IL-1α was not expressed in the splenocytes of the PSPC or lentinan treated mice. Nevertheless, IL-1β was strongly up-regulated at 6 h and 48 h of the PSPC treated mice while it was up-regulated at 24 h of the lentinan treated mice. The expression level of TNF-α mRNA was increased and remained high in the PSPC treated mice. However, in the lentinan treated mice, the expression level of TNF-α started to increase at 3 h, and then dropped at 24 h. IFN-γ and M-CSF were more dramatically up-regulated in the PSPC treated mice than in the lentinan treated mice.

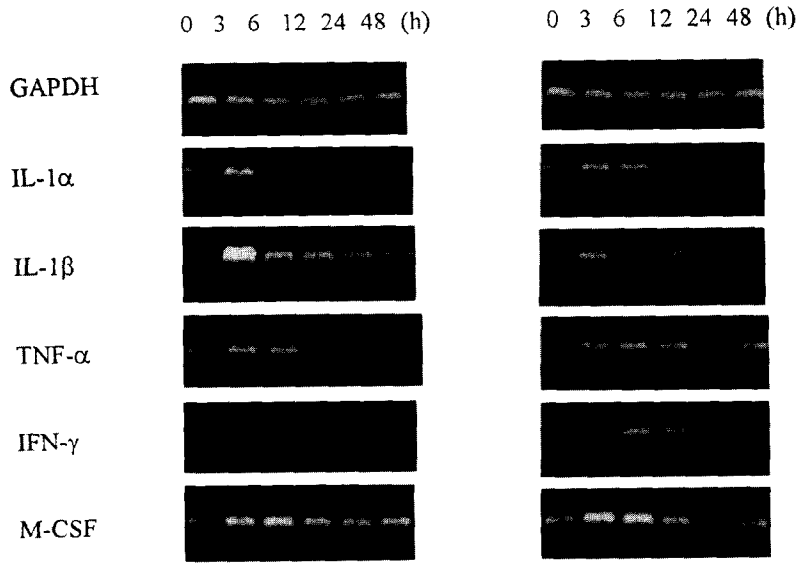

Fig. 1

RT-PCR amplification of GAPDH and cytokine mRNAs from the PEC of PSPC (left) and lentinan (right) administered ICR mice

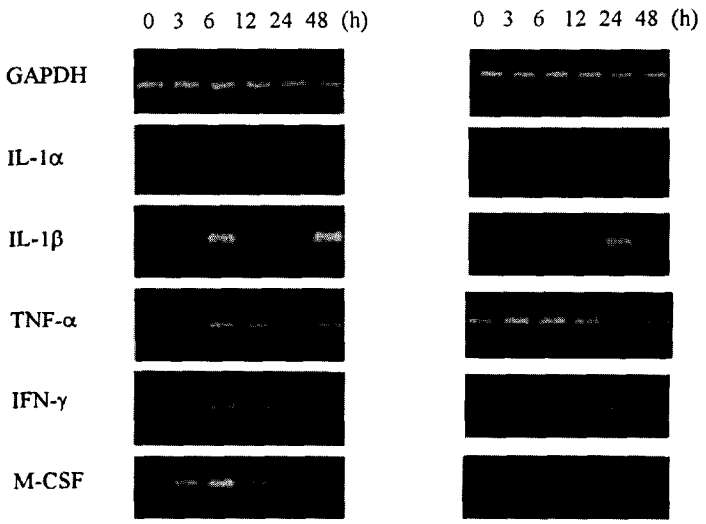

Fig. 2

RT-PCR amplification of GAPDH and cytokine mRNAs from the splenocytes of PSPC (left) and lentinan (right) administered ICR mice

Discussion

The immunomodulating mechanism of PSPC (16) and lentinan (1) has been studied at the cellular level. In the present study, when analyzing and comparing the cytokine gene expression in the ICR mice, after PSPC and lentinan administration, a significant difference in the expression profiles was observed in some of the cytokines. PSPC and lentinan increase the gene expression level of IL-1β in both peritoneal exudate cells (PEC) and splenocytes. However, the increased production of IL-1β induced by PSPC is higher than that by lentinan. In the PEC, both PSPC and lentinan induce the gene expression of IL-1α, whereas in the splenocytes, IL-1α is not detectable. This is in line with the well-known fact that IL-1 is produced primarily by macrophages and monocytes (12,21). Generally, IL-1 is not produced without stimulation, and it is clear that a variety of substances including lipopolysaccharide (LPS), immune complexes and bacteria can act as stimulants (21). In the present study, IL-1α and IL-1β were up-regulated in the mice treated with either PSPC or lentinan. The result shows that PSPC and lentinan are immune stimulants. In most instances, cells produce a mixture of IL-1α and IL-1β following stimulation, although usually not in equal proportion. For example, LPS-stimulated monocytes produce approximately ten times more IL-1β than IL-1α (21). In the present study, following PSPC and lentinan-stimulation, IL-1β mRNA expression increases many folds higher than the basal level whereas IL-1α mRNA expression only slightly increases in the PEC, but not in the splenocytes. This observation strongly suggests that regulation of production of the two IL-1 species is mainly controlled at the level of transcription. IL-1 is known to have a wide range of biological activities on many different target tissues, including B cells, T cells and monocytes. Hence, increased production of IL-1 results in augmented maturation capable of inducing various

other cytokines such as TNF-α (1,22). IL-1 was also found to have an effect on the differentiation of non-Hodgkin's lymphoma (23), B cell chronic lymphocytic leukemia (24) and myeloid leukemia cell line (25). PSPC and lentinan strongly induce the gene expression of TNF-α in both PEC and splenocytes. The increase of the gene expression of TNF-α stimulated by PSPC and lentinan is consistent with their antitumor activity (1,16). TNF-α may interact with activated T-cells and regulate both growth and functional activities of these cells. TNF-α also induces lysis of malignant cells and regression of some animal tumors (22). IFN-γ is a T cell derived cytokine with important anti-proliferation and anti-viral activity (22). The increased production of IFN-γ mRNA induced by PSPC and lentinan appears to correlate with their antitumor activity (1,16). Moreover, the anti-viral activity of lentinan is probably related with the increase of gene expression of IFN-γ stimulated by it (20). PSPC also increases the gene expression of IFN-γ, but it is not known whether it has any anti-viral activity or not. Similarly, the antitumor activity of PSPC and lentinan *in vitro* is consistent with the increased production of M-CSF in the PEC stimulated by them (1,16). Therefore, the immunomodulating and antitumor activity of PSPC and lentinan may be closely related to the expression level of cytokine genes.

In addition, the kinetics of each cytokine gene expression is different and 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 (12,13). In the present study, the induction of cytokine gene expression appeared at 3 h in the PEC and at 6 h in the splenocytes after PSPC and lentinan administration. It seems that the changes of cytokine expression level in the PEC is earlier than that in the splenocytes. On the other hand, the levels of mRNAs of M-CSF, IL-1β and TNF-α remain high for a long time, whereas the levels of mRNAs of IL-1α and IFN-γ remain high for only a short time, i.e. respectively 3 h or 6 h. Therefore, this sort of kinetic study may provide a possible means for improving immmnomodulating and antitumor action of PSPC and lentinan although the kinetics of cytokine gene expression is quite complicate.

Acknowledgements

The authors would like to thank Miss P. K. Lee for her technical help. This study was partially supported by an Earmarked Grant from the Research Grants Council of Hong Kong.

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