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Effects of polysaccharides (FI_0-c) from mycelium of *Ganoderma tsugae* on proinflammatory cytokine production by THP-1 cells and human PBMC (II)[1]

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KEY WORDS *Ganoderma tsugae*; polysaccharides; interleukin-1; tumor necrosis factor

ABSTRACT

AIM: To study the effects of water-soluble polysaccharides, FI_0-c, and its sulfated derivative, FI_0-c-S, on production of human proinflammatory cytokines, interleukin-1α (IL-1α) and tumor necrosis factor α (TNFα). **METHODS**: The herbal polysaccharides were modified by chlorosulfonic acid in dimethyl sulfoxide (Me_2SO). Cytokine production was measured by radioimmunoassay. mRNA for the cytokines was measured by semi-quantitative RT-PCR. **RESULTS**: FI_0-c 4 mg/L itself induced IL-1α production by THP-1 cells without stimulants, such as lipopolysaccharides (LPS) and phorbol myristate acetate (PMA). On the other hand, FI_0-c and FI_0-c-S inhibited the IL-1α production by THP-1 cells with these stimulants. FI_0-c and FI_0-c-S significantly upregulated TNFα production by THP-1 cells without stimulants or at a low dose of LPS 10 mg/L and PMA 200 nmol/L, whereas these polysaccharides markedly downregulated the TNFα production by a high dose of LPS 100 mg/L and PMA. Human peripheral blood mononuclear cells (PBMC) responded to FI_0-c and FI_0-c-S in IL-1α and TNFα production in a fashion similar to THP-1 cell responses. FI_0-c 4 mg/L downregulated high-dose LPS- and PMA-induced IL-1α or TNFα mRNA and their protein production by THP-1 cells. **CONCLUSION**: The water-soluble polysaccharides of *Ganoderma tsugae* mycelium have bidirectional immunomodulatory effects on cytokine production in different cell stimulatory conditions. Chemical modification of this polysaccharide changed the intensity of regulatory effect on cytokine production.

INTRODUCTION

Ganoderma tsugae, Songshan Lingzhi, a Chinese herb, has been used as a liver-protector, minor tranquilizer and for enhancement of vitality, and other remedies in China for centuries. Currently, it is the most widely used medicinal mushroom for the enhancement of immune function. In our first study, in order to elucidate the mechanism of the immunomodulatory effects of the polysaccharide from cultured mycelium of *G tsugae*, a β-*D*-(1, 3) linked glucan (M_r 10 000), FI_0-b, and its formic acid-modified derivative, FI_0-b-H, were selected. The results showed that FI_0-b-H had more obvious regulatory effects on human proinflammatory cytokine production than FI_0-b. Chemical modification was observed to sometimes reverse the direction of cytokine production from up to down regulation and *vice versa*. Some reports showed that polysaccharides with sulfate groups were potent lymphocyte mitogens, desulfated polysaccharides lost their mitogenic activities and sulfation of neutral polysaccharides induced lymphoblastic transformation. Thus, sulfated polysaccharides were thought to be immunomodulators. In addition, the sulfated polysaccha-

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rides were seem to possess potent anti-HTLV-III activity. Some reports showed that the sulfated water-soluble polysaccharides, derived from water-insoluble pachymanan, had an antitumor activity[1,2]. Thus, we considered that sulfate group might play a key role in immunomodulation such as lymphocyte proliferation and antiviral or antitumor activities. We compared the water-soluble polysaccharides and the sulfated polysaccharides in this aspect.

We previously established a systematic procedure for the extraction, fractionation and the purification of antitumor polysaccharides from *G tsugae*[3,4]. Based on these results, Fl_0-c (tumor inhibition ratio 32.3 %), a homoglucan protein complex (M_r 10 000), was selected. In this study, Fl_0-c was chemically modified by chlorosulfonic acid and triethylamine in Me_2SO. We attempted to compare the effects of Fl_0-c with sulfated Fl_0-c (Fl_0-c-S) on the production of proinflammatory cytokines, interleukin-1α (IL-1α) and tumor necrosis factor-1α (TNFα) and their gene expression by human myelomonocytic leukemic cells, THP-1, and human peripheral blood mononuclear cells (PBMC). IL-1 and TNFα are produced and secreted from a variety of cells, especially of the macrophage/monocyte lineage, and are the principal mediators in many inflammatory diseases. Particularly, IL-1 induces production of not only nitric oxide synthetase, type-2-cyclooxygenase or prosglandin E_2 but also proinflammatory cytokines such as IL-1, IL-6, IL-8, TNFα, and interferon-γ (IFNγ)[5].

MATERIALS AND METHODS

Chemical modification of polysaccharides from cultured mycelium of *G tsugae* Sulfation of the polysaccharides (Fl_0-c) was carried out with chlorosulfonic acid in Me_2SO according to a modification of the method of sulfated pachymanan[1,2]. Degree of sulfation was determined by quantitative sulfation analysis. It was proved that one half of the sulfate groups were attached to primary alcohol groups and the sulfation degree of primary alcohol groups of Fl_0-S was 52 %. In this study, endotoxin contamination level in the two samples was below 2.5 μg/L measured by *Linmulus Amobocyte* Lysate (LAL) assay (Seikagaku Kogyo, Tokyo, Japan).

Cell culture Human myelomonocytic leukemic cells, THP-1, were cultured in RPMI-1640 medium (Gibco NY, USA) supplemented with 10 % heat-inactivated new-born bovine serum (Da Lian Biological Reagent Factory, Dalian, China), benzylpenicillin 1×10^5 IU/L, streptomycin 100 mg/L, HEPES 10 mmol/L and L-glutamine 5 mmol/L. The cells (3.2×10^8 cells/L) were cultured in the 96 well microtiter plates and stimulated with lipopolysaccharide (LPS) (*E coli* serotype 0111:B4, Sigma Chemical Co, MO, USA) 1 or 10 mg/L and phorbol myristate acetate (PMA) (Sigma Chemical Co, MO, USA) 200 nmol/L for induction of IL-1α or TNFα. Fl_0-c or Fl_0-c-S was administered 30 min before treatment with LPS and PMA. The final volume was 200 μL/well. As a control experiment, culture medium with the same volume of the *G tsugae* polysaccharide solution or LPS-PMA solution was added. After incubation at 37 ℃, 5 % CO_2 for 20 h, the cells were lysed by three times freeze and thaw cycles. It was reported that THP-1 cell did not secrete cytokines without stimulants such as LPS, PMA, silica gel or hydroxyurea[6]. LPS or PMA alone did not induce cytokine production.

Separation of human PBMC Blood was obtained from three healthy adult volunteers for PBMC. Human PBMC were separated by density gradient centrifugation in a Ficoll-Hypaque solution (Histopaque® 1.077 kg/L) at $400 \times g$ for 10 min, washed three times with phosphate buffer salined (PBS), then suspended in RPMI-1640 medium containing 2 % human AB serum. PBMC (5×10^9 cells/L) were added to the 24 well culture plates, and stimulated by LPS 1 mg/L. Fl_0-c or Fl_0-c-S was added 30 min before LPS treatment. The final volume was 1 mL/well. After incubation at 37 ℃ with 4 % CO_2 for 20 h, the cell suspension was lysed by three times freeze and thaw cycles.

Cytokine quantitation After cell lysis and centrifugation at $12\,000 \times g$ in 1.5 mL Eppendorf tube, IL-1α and TNFα in the supernatants were quantitated by human cytokine specific raidoimmunoassay (RIA). Recombinant human cytokines were kind gifts from Banyu Pharmaceutical Company Tsukuba Research Institute (Tsukuba, Japan). All the rabbit polyclonal antibodies for above cytokines were prepared in our laboratory. $Na^{125}I$ was purchased from China Institute of Atomic Energy, Beijing, China. Methods for RIA for human cytokines were followed as previously reported[7,8].

Analysis of mRNA production for IL-1α and TNFα by THP-1 cells using RT-PCR method THP-1 cells (3.2×10^8 cells/L) were added to the 24-well culture plates. The cells were cultured as previously described and were stimulated with LPS 10 mg/L or 100

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mg/L and PMA 200 nmol/L. FI_0-c or FI_0-c-S was administered 30 min before addition of LPS and PMA. The final volume was 1 mL/well. We obtained maximal production of TNFα mRNA 3 h after incubation and IL-1α mRNA after 5 h. THP-1 cells were collected by centrifugation at 450 × *g* for 10 min. Total RNA was extracted by using ISOGEN™ (Nippon Gene, Toyama, Japan) cell lysis buffer. The production of human IL-1α, TNFα and β-actin mRNA was detected by RT-PCR method[8]. The actual DNA sequences of primers (Life Tech Oriental Co, Tokyo, Japan) for IL-1α, TNFα, and β-actin cDNA amplification were as follows:

IL-1α (sense) 5′-GTAAGCTATGGCCCACTCCAT;
IL-1α (antisense) 3′-TGACTTATAAGCACCCATGTC;
TNFα (sense) 5′-ATGAGCACTGAAAGCATGATC;
TNFα (antisense) 3′-TCACAGGGCAATGATCCCAA-AGTAGACCTGCCC;
β-actin (sense) 5′-TCCTGTGGCATCCACGAAACT;
β-actin (antisense) 3′-GAAGCATTTGCGGTGGACGAT

Statistics Data were expressed as $\bar{x} \pm s$ and assessed by unpaired Student's *t*-test.

RESULTS

Effect of FI_0-c and its chemically-modified derivative, FI_0-c-S, on production of proinflammatory cytokines by THP-1 cells We summarized the results of IL-1α production by THP-1 cells with or without FI_0-c or FI_0-c-S stimulated by two LPS doses and PMA (Tab 1). Non-stimulated THP-1 cells produced low amounts of IL-1α (0.13 ± 0.04) μg/L. Addition of FI_0-c or FI_0-c-S without stimulants, generally did not induce marked changes in IL-1α production. Only at FI_0-c 4 mg/L, 100-fold increase (14 ± 6) μg/L was observed. FI_0-c or FI_0-c-S had almost no effects on LPS (1 mg/L)- and PMA (200 nmol/L)-induced IL-1α production. On stimulation with a higher dose of LPS 10 mg/L and PMA 200 nmol/L, THP-1 cells secreted a considerable amount of IL-1α (4.8 ± 2.5) μg/L, and addition of FI_0-c or FI_0-c-S blocked the production of IL-1α.

FI_0-c 4 mg/L itself significantly induced TNFα production without any stimulants, however the production of TNFα had no obvious change after addition of FI_0-c 4 mg/L at a lower-dose of LPS 10 mg/L and PMA (Tab 2). On the other hand, FI_0-c 4 mg/L blocked TNFα production at a higher dose of LPS 100 mg/L and PMA 200 nmol/L. Without stimulants, FI_0-c-S 4 mg/L and 40 mg/L significantly upregulated TNFα production from (0.22 ± 0.04) μg/L (medium control) to (1120 ± 133) μg/L or (400 ± 111) μg/L respectively. However, at the highest dose of stimulation with LPS 100 mg/L and PMA 200 nmol/L, they downregulated TNFα production from (640 ± 25) μg/L to (3.9 ± 0.4) and (8.0 ± 1.5) μg/L, respectively.

Effects of FI_0-c and FI_0-c-S on proinflammatory cytokine production by PBMC Non-stimulated PBMC produced low amounts of cytokines. When PBMC were stimulated by LPS 1 mg/L, elevated levels of IL-1α production were detected in some samples (92 μg/L ± 18 μg/L, Volunteer # 1), whereas FI_0-c and FI_0-c-S inhibited IL-1α production (1.1 μg/L ± 0.6 μg/L and 10 μg/L ± 4 μg/L, respectively). However, when IL-1α production by LPS-induced PBMC was relatively at a low level (0.520 μg/L ± 0.011 μg/L, Volunteer # 2; 2.4 μg/L ± 1.4 μg/L, Volunteer # 3), upregulation of IL-1α production by FI_0-c and FI_0-c-S proved significant (Tab 3).

Both FI_0-c and FI_0-c-S further upregulated produc-

Tab 1. Effect of FI_0-c and FI_0-c-S on IL-1α production by THP-1 cells (μg/L). $n = 3$ wells. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Polysaccharide/ mg·L^{-1}	Control	LPS 1 mg/L PMA 200 nmol/L	LPS 10 mg/L PMA 200 nmol/L
Control	0.13 ± 0.04	0.5 ± 0.3	4.8 ± 2.5
FI_0-c 4	14 ± 6^b	0.118 ± 0.020	0.320 ± 0.008^b
FI_0-c 40	0.8 ± 0.6	0.098 ± 0.007	0.240 ± 0.022^b
FI_0-c 400	0.102 ± 0.022	0.25 ± 0.08	0.22 ± 0.04^b
FI_0-c-S 4	0.16 ± 0.03	0.10 ± 0.012	0.160 ± 0.019^b
FI_0-c-S 40	0.204 ± 0.013	0.02 ± 0.04	0.20 ± 0.08^b
FI_0-c-S 400	0.4 ± 0.3	0.32 ± 0.16	0.36 ± 0.11^b

Tab 2. Effect of FI_0-c and FI_0-c-S on TNFα production by THP-1 cells (μg/L). $n=3$ wells. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

Polysaccharide/ mg·L^{-1}	Control	LPS 10 mg/L PMA 200 nmol/L	LPS 100 mg/L PMA 200 nmol/L
Control	0.22 ± 0.4	2.16 ± 0.26	640 ± 25
FI_0-c 4	160 ± 36^c	1.60 ± 0.21^b	3.6 ± 0.3^c
FI_0-c 40	0.44 ± 0.05^c	120 ± 18^c	112 ± 14^c
FI_0-c 400	3.84 ± 1.14^c	800 ± 175^c	360 ± 34^c
FI_0-c-S 4	1120 ± 133^c	4.80 ± 0.27^c	3.9 ± 0.4^c
FI_0-c-S 40	400 ± 111^c	3.0 ± 0.5	8.0 ± 1.5^c
FI_0-c-S 400	4.8 ± 1.1^c	2.4 ± 0.4	2.96 ± 0.29^c

Tab 3. Effect of FI_0-c and FI_0-c-S on proinflammatory cytokine production from human PBMC (μg/L) stimulated by LPS 1 mg/L. $n=3$ wells. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

Polysaccharide/ mg·L^{-1}	Volunteer # 1	Volunteer # 2	Volunteer # 3
		IL-1α	
Control	2.2 ± 0.3	0.010 ± 0.005	0.31 ± 0.03
FI_0-c 400	0.31 ± 0.03	5.20 ± 0.27^b	2.50 ± 1.25^b
FI_0-c-S 400	0.90 ± 0.23	2.4 ± 0.6^c	0.47 ± 0.03^c
LPS	92 ± 18	0.520 ± 0.011	2.4 ± 1.4
FI_0-c 400 + LPS	1.1 ± 0.6^c	3.1 ± 0.4^b	45 ± 19^b
FI_0-c-S 400 + LPS	10 ± 4^c	2.2 ± 1.6	2.20 ± 0.27
		TNFα	
Control	0.23 ± 0.05	0.48 ± 0.03	0.23 ± 0.05
FI_0-c 400	1.2 ± 0.3^c	1.20 ± 0.29^b	0.258 ± 0.005
FI_0-c-S 400	1.92 ± 0.14^c	1.14 ± 0.09^c	0.673 ± 0.016^c
LPS	5.1 ± 2.2	19.8 ± 1.8	0.57 ± 0.13
FI_0-c 400 + LPS	37 ± 8^c	14.1 ± 1.0^c	2.5 ± 0.3^c
FI_0-c-S 400 + LPS	20 ± 7^b	6.0 ± 0.5^c	1.082 ± 0.016^c

tion of TNFα by LPS 1 mg/L treated PBMC (Volunteer # 1, Volunteer # 3) whose TNFα production level was originally low. However, when LPS (1 mg/L)-induced TNFα levels were relatively high at (19.8 ± 1.8) μg/L (Volunteer # 2 PBMC), FI_0-c or FI_0-c-S reduced TNFα production.

Effects of FI_0-c and FI_0-c-S on cytokine mRNA expression by THP-1 cells In order to investigate the immunomodulation mechanism of FI_0-c and FI_0-c-S, first, we selected the samples that could significantly change the amount of cytokine production by THP-1 cells, then studied their effects on TNFα and IL-1α mRNA expression by THP-1 cells. FI_0-c 4 mg/L and FI_0-c-S 4 mg/L almost completely blocked IL-1α protein production by LPS 10 mg/L plus PMA. FI_0-c 4 mg/L remarkably upregulated TNFα protein production by 5000-fold at non-stimulatory conditions. On the other hand, it obviously downregulated TNFα protein production stimulated by LPS 10 mg/L plus PMA. Therefore, there was some tendency that the SongShan LingZhi water-soluble polysaccharides had bidirectional immunomodulatory effects in different stimulatory conditions. Twenty-four and 30 cycles of cDNA amplification were tried by RT-PCR. At 30 cycles, each DNA band on agarose-gel electrophoresis proved more obwious than that at 24 cycles, (Fig 1, 2), suggesting that at 24 cycles, cDNA amplification had not reached the plateau stage. Therefore, at 24 cycles we could compare the effect of FI_0-c and FI_0-c-S on the amount of cDNA which might reflect original mRNA amount. FI_0-c 4 mg/L decreased TNFα

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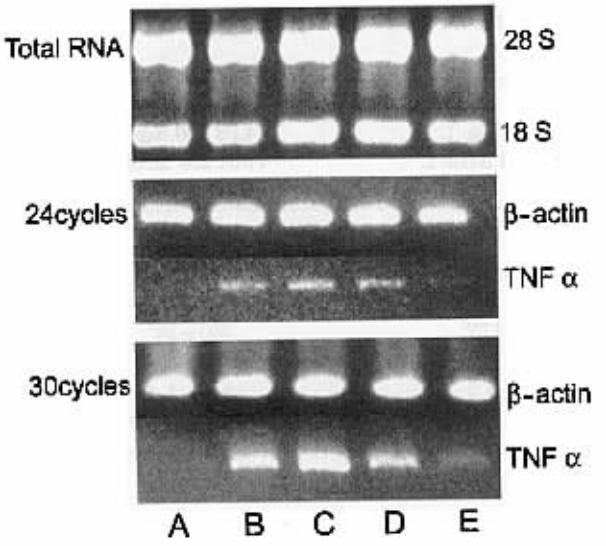

Fig 1. TNF_α mRNA expression. Lane A: FI_0-c 4 mg/L; Lane B: LPS 10 mg/L + PMA 200 nmol/L; Lane C: LPS 10 mg/L + PMA 200 nmol/L + FI_0-c 4 mg/L; Lane D: LPS 100 mg/L + PMA 200 nmol/L; Lane E: LPS 100 mg/L + PMA 200 nmol/L + FI_0-c 4 mg/L;

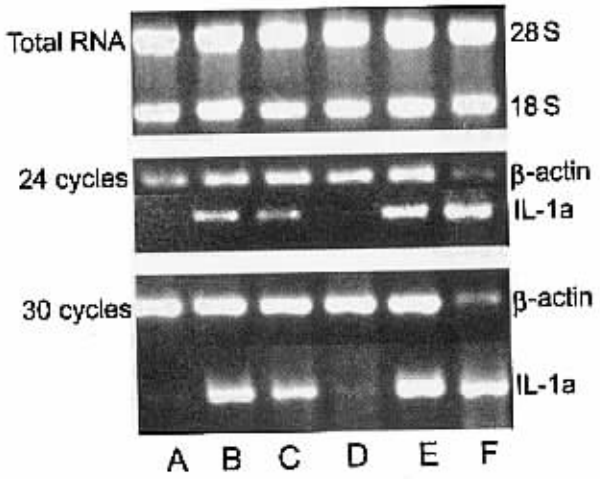

Fig 2. IL-1α mRNA expression by THP-1 cells with LPS 10 mg/L and PMA 200 nmol/L. Lane A: FI_0-c 4 mg/L ; Lane B: LPS + PMA; Lane C: FI_0-c 4 mg/L + LPS + PMA; Lane D: FI_0-c-S 4 mg/L ; Lane E: LPS + PMA; Lane F: FI_0-c-S 4 mg/L + LPS + PMA.

mRNA expression by THP-1 cells at higher doses of LPS 100 mg/L and PMA, and it also reduced TNFα protein production measured by RIA in the same cell stimulatory condition (Tab 2 and Fig 1, Lane E). FI_0-c 4 mg/L increased TNFα mRNA amount at a lower dose of LPS 10 mg/L plus PMA, whereas it did not cause significant change in TNFα protein production in the same condition (Fig 1, Lane C). FI_0-c or FI_0-c-S did not cause any significant changes in mRNA production by LPS and PMA-stimulated THP-1 cells (Fig 2). Results from RT-PCR did not necessarily coincide with that of RIA for proinflammatory cytokines.

DISCUSSION

Recent reports have begun to pay attention to the possible effects of Chinese medicinal herbs on the human immune system[9]. We compared FI_0-c with FI_0-c-S on proinflammatory cytokine production by human THP-1 cells. THP-1 cell line has human myelomoncytic leukemic cell origin and its reactions to a variety of stimulants have some similarity to those of human macrophages[10,11]. Both LPS and PMA induced significant amount of IL-1α and TNFα production by THP-1 cells. LPS and PMA by distinct pathways regulate the cytokine gene expression in monocytic cells at both the transcriptional and post transcriptional levels.

Our previous study[3,4] demonstrated that the water-soluble extract of *G tsugae* mycelium (FI_0-c) had a low level of anti-proliferative effect on murine S-180 sarcoma cells *in vivo*. In this study, FI_0-c was chemically modified by chlorosulfonic acid for increasing hydrophilicity. Our previous study (manuscript in preparation) demonstrated that SongShan LingZhi polysacchrides increased the production of proinflammatory cytokines after the chemical modification by formic acid. It also showed that the formylized then formolysed polysaccharide had more obvious bidirectional regulatory effects on proinflammatory cytokine production compared to original untreated compound. Therefore, in this study, we tried another chemical modification method, chlorosulfonic acid modification. This study showed that FI_0-c and FI_0-c-S downregulated TNFα production by THP-1 cells at higher stimulation with LPS and PMA, whereas they significantly upregulated TNFα production without stimulants. These herbal polysaccharides have bidirectional regulatory effects in the different stimulatory conditions. In addition, chemical modification caused the change in regulatory direction from up to down in the case of low level of stimulation of TNFα production. In order to show its value in clinical use, we further investigated the effects of FI_0-c and FI_0-c-S on proinflammatory cytokine production by human PBMC. Although each PBMC had different individual responses to FI_0-c and FI_0-c-S, bidirectional regulation was also demonstrated in IL-1α and TNFα syntheses. Different responses of THP-1 cells and PBMC to

these herbal polysaccharides might be attributed to the fact that THP-1 is an established monocytic cell line and PBMC consist of monocytes, T-cells, B-cells, and other cell populations with different individual genetic background.

FI_0-c 4 mg/L upregulated IL-1α mRNA, on the other hand, it downregulated IL-1α protein production, indicating post-transcriptional regulation at a higher dose of LPS and PMA. FI_0-c 4 mg/L reduced TNFα mRNA amount induced by a higher dose of LPS and PMA and also downregulated TNFα protein production in the same stimulatory condition, indicating that TNFα protein production was reflected by its mRNA quantity. Cytokine gene expression might be regulated by SongShan LingZhi polysaccharides at both transcriptional and post-transcriptional stages.

Since FI_0-c-S is generally more effective in regulating immunological function than FI_0-c, sulfation of FI_0-c might change this polysaccharide's immunological function. Effects of FI_0-c or FI_0-c-S on other proinflammatory cytokines, IL-6 and IL-8, were not marked (data not shown). Pharmacological effects of *G tsugae* might be mainly attributed to the induction of high amount of TNFα in the low or non-stimulatory conditions.

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松杉灵芝菌丝体多糖(FI_0 – c)对人组织瘤细胞和人外周血白细胞产生炎症性细胞因子的影响 (II)[1]

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关键词 松杉灵芝; 多糖类; 白介素-1; 肿瘤坏死因子

目的: 比较研究水溶性多糖(FI_0-c)及其氯磺酸修饰产物(FI_0-c-S)对人炎症性细胞因子产生的影响. **方法**: 应用氯磺酸修饰法对多糖进行化学修饰. 用放射免疫分析法(RIA)及逆转录聚合酶链反应(RT-PCR)测 FI_0-c 和 FI_0-c-S 对人组织瘤细胞(THP-1)和人外周血单核细胞(PBMC)分泌各种与炎症有关的细胞因子, 白介素-1(IL-1α)和肿瘤坏死因子 α (TNFα)的影响及对 mRNA 表达的影响. **结果**: FI_0-c 和 FI_0-c-S (浓度分别为 4, 40, 400 mg/L)显著提高

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低剂量组 LPS 10 mg/L 协同 PMA 200 nmol/L 诱导的 THP-1 细胞产生 TNFα 的量，然而，这些多糖明显地抑制高剂量组 LPS 100 mg/L 协同 PMA 诱导的 THP-1 细胞产生 TNFα. 在无刺激的条件下 FI_0-c 能够诱导比较多量的 IL-1α 产生，但是 FI_0-c 或 FI_0-c-S 却都明显抑制高剂量或低剂量 LPS 和 PMA 诱导的 THP-1 细胞产生 IL-1α. 低浓度 FI_0-c 4 mg/L 显著抑制高剂量组 LPS 100 mg/L 协同 PMA 诱导的 THP-1 细胞产生 IL-1 或 TNFα mRNA 及蛋白质的量. **结论**：松杉灵芝菌丝体水溶性多糖在不同的刺激条件下具有双向免疫调节作用. 化学修饰的多糖可改变原多糖对细胞因子产生的调节方向.

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