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Original Research Article

Lentinan relieves hepatitis B surface antigen induced functional impairment of monocytes/macrophages

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Abstract

Purpose: *To investigate the efficacy of lentinan in relieving hepatitis B surface antigen (HBsAg)-induced functional impairment of monocytes/macrophages.*
Methods: *Monocytic cell line THP-1 was incubated with lentinan and HBsAg for 24 h and then stimulated with LPS (Lipopolysaccharide). The expression levels of interleukin-1β (IL-1β), IL-12 and tumour necrosis factor-α (TNF-α) were measured by enzyme-linked immunosorbent (ELISA) assays and quantitative reverse transcriptase-PCR (q-PCR). Protein levels of IkB-α, phospho-ERK, and phospho-p38 were measured by western blotting.*
Results: *THP-1 cells treated with lentinan and HBsAg showed higher IL-1β, IL-12, and TNF-α levels than cells treated with HBsAg alone. The underlying mechanisms were associated with NF-kB and MAPK signal pathways. Decreased expression of IkB-α and phospho-ERK and increased expression of phospho-JNK and phospho-p38 were observed in cells treated with lentinan and HBsAg when compared with cells treated with HBsAg alone ($p < 0.001$). THP-1 cells incubated with 500 μg/mL lentinan secreted lower levels of cytokines than did control cells after LPS stimulation, suggesting an anti-inflammatory effect for lentinan.*
Conclusion: *Lentinan shows both pro- and anti-inflammatory functions and may be a promising candidate for hepatitis B virus (HBV) treatment.*

Keywords: *Hepatitis B surface antigen, Lentinan, Immuno-suppression, Pro-inflammatory, Anti-inflammatory*

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INTRODUCTION

Hepatitis B virus (HBV), a non-cytopathic DNA virus with a partially duplex circular genome of 3.2 kb, infects more than 350 million people worldwide [1,2]. Chronic HBV infection is the main cause of hepatocellular carcinoma, which is prevalent in the Asian population [3]. Multiple immune-subversion strategies employed by HBV disrupt the host innate and adaptive immune responses and result in systemic immuno-suppression [1,4]. Hepatitis B surface antigen

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(HBsAg) is a leading cause of weak antiviral immune responses to HBV infection [5]. High levels of HBsAg are detected in peripheral blood and liver of patients chronically infected with HBV [6,7]. Therefore, an important concern with HBsAg is that HBsAg can directly repress the active progression of circulating and liver monocytes/macrophages [8,9]. HBsAg or HBV can inhibit the production of type I IFN and IL-12 by antigen presenting cells in a Toll-like receptor (TLR)-dependent manner. Furthermore, the functional activity of monocyte/macrophage were strongly impaired by HBsAg through TLRs, suggesting that HBsAg serves as a suppresser to hamper host innate immune responses [10,11].

Lentinan from shiitake mushrooms (*Lentinula edodes*) is a β-1,3 β-glucan with β-1,6 branching and a molecular weight of 500 kDa [12]. Since 1985, lentinan has been approved as an adjuvant for stomach cancer in Japan due to its effects on host immune systems. Lentinan can enhance the functional activity of monocytes/macrophages and the anti-tumour activity of cytotoxic T lymphocytes (CTLs) [13,14]. However, the potential of lentinan to treat HBV infection remains unstudied.

In this study, lentinan was used to relieve HBsAg-induced impairment of cytokine secretion by THP-1 cells. The NF-kB and MAPK signalling that was hampered by HBsAg was activated in the presence of lentinan. Lentinan also repressed cytokine production by THP-1 cells upon LPS stimulation. These data indicate that lentinan may be a promising candidate for HBV therapy.

EXPERIMENTAL

Reagents

HBsAg was purified from the serum of HBV patients using sucrose gradient centrifugation and affinity chromatography. Lentinan was purchased from Kanghaipharm (Shanghai, China). Polyclonal anti–phospho (p)-ERK, anti-ERK, and anti-IkB-α and monoclonal anti–phospho-JNK, anti–JUK, anti–p-p38, and anti–p38 were obtained from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidise (HRP)-conjugated goat anti-mouse/rabbit antibodies were obtained from Jackson Laboratory.

Cells and culture conditions

THP-1 (the human monocytic leukaemia cell line) cells were obtained from ATCC (Manassas, Virginia, USA) and cultured in complete RPMI 1640 medium which contained additional 10 % FBS, 1 % penicillin (100 IU/ml), and 1 % streptomycin (100 mg/mL) (Life Technologies, USA) at 37 °C with 5 % CO_2. To activate THP-1 cells for further assays, 50 ng/ml PMA were first added into the culture media for 24 h. Then with various concentrations of lentinan (100, 250 and 500 μg/mL), with or without 25 μg/mL HBsAg in fresh complete RPMI 1640 were used in the further culture for another 24 h. The cells were washed and stimulated with LPS (100 ng/mL) for 15 min or 6 h in complete RPMI 1640 medium.

Enzyme-linked immunosorbent assay (ELISA)

After LPS stimulation for 6 h, secreted IL-1β, IL-12, and TNF-α levels were measured by ELISA kits (R & D Systems, USA) under the guidance of the manufacturer's introduction. Supernatants were added to pre-coated plates in triplicate wells and incubated at 37 °C. After washed, HRP-labelled antibodies were added for 2 h. Substrate was added to washed plates. Chromogenic reactions were stopped with 10 % H_2SO_4. Absorbances450 nm were measured and the absolute amount of IL-1β, IL-12, and TNF-α were calculated based on standard curves.

Total RNA extraction and quantitative reverse-transcriptase-PCR (q-PCR)

A TRizol reagent (Invitrogen, USA) was used to extract total RNA in THP-1 cells. Then cDNA was synthesized using M-MLV (Promega, USA) under the guidance of the manufacturer's introduction. To assess the gene transcription levels, q-PCR was performed using an ABI Step One Plus Real-Time PCR System (Applied Biosystems, CA, USA) and an EzOmics SYBR qPCR kit (Vazyme Biotech Co., Ltd.) under the guidance of the manufacturer's introduction.

Western blot analysis

THP-1 cells were treated with 100 ng/mL LPS for 15 min. After two times of wash with ice-cold Phosphate Buffer solution (PBS), the cells were lysed in RIPA buffer containing 1 Mm phenylmethanesulfonyl fluoride and centrifuged. The supernatants were collected for protein quantification. Expression levels of IkB-α, p-p38, p-JNK, and p-ERK were analysed. The protein in samples were separated via 12 % SDS-PAGE and transferred to PVDF membranes. Membranes were gently rinsed in deionized water, blocked with skim milk, and incubated 12 h with the antibodies indicated above (diluted 1 : 1000 - 1 : 2000 in 1× PBS/0.05 % Tween 20) at 4 ℃. After washed, Membranes were incubated

with HRP-conjugated goat anti-rabbit/mouse antibodies for 2 h at room temperature. Membranes were washed and imaged using ECL detection reagents.

Statistical analysis

All data were analysed using SPSS 17.0 statistical package and presented as mean ± standard deviation (SD). ANOVA or Student's t-tests were used to determine significance levels; $p < 0.05$ was considered significant. The data were processed using GraphPad Prism version 5.

RESULTS

Lentinan relieves HBsAg-induced functional impairment of THP-1 cells

To investigate if lentinan affects monocytes/macrophages treated with HBsAg, the protein concentrations of IL-1β, IL-12, and TNF-α in THP-1 culture supernatants were measured by ELISA (Figure 1 A-C). The results show that HBsAg repressed the expression of these cytokines at the mRNA and protein levels, indicating potent immuno-suppressive activity by lentinan (Figure 1 D-F). In the present of HBsAg, addition of lentinan significantly enhanced the production of IL-1β, IL-12, and TNF-α in a dose-dependent manner. Interestingly, THP-1 cells incubated with 500 μg/mL lentinan alone showed a mild cytokine response to further stimulation with LPS, suggesting that lentinan induced a certain tolerance of THP-1 cells to LPS that coincided with the multiple functions of the polysaccharide. Thus, in response to further stimulation with LPS, lentinan relieved the functional impairment caused by HBsAg in monocytes/macrophages.

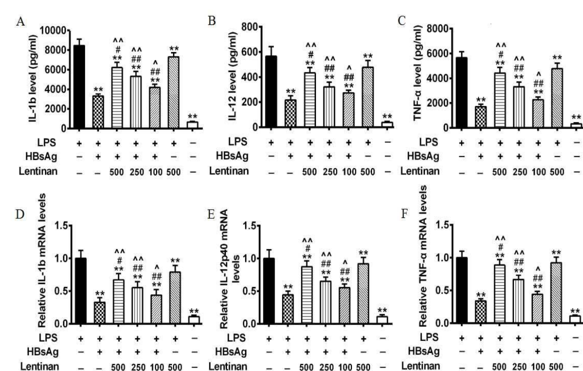

Figure 1: Lentinan relieves HBsAg-induced functional impairment of THP-1 cells. 50 ng/mL PMA were used to stimulate THP-1 cell differentiation. Stimulated cells were incubated with HBsAg (25 μg/mL) and/or lentinan (100, 250, or 500 μg/mL) for 24 h. Cells were harvested and stimulated with LPS for another 6 h. Protein levels of IL-1β (A), IL-12 (B), and TNF-α (C) in culture supernatants were measured by ELISA. RNA was extracted from the cells. Relative IL-1β (D), IL-12p40 (E), and TNF-α (F) mRNA levels were measured by q-PCR. Graph are presented as mean and SD. A significance level of $p < 0.05$, $p < 0.01$, and $p < 0.001$ were labelled with one to three asterisk respectively. (* vs. control; # vs. HBsAg; ^ vs. lentinan)

Lentinan restores the function of HBsAg-treated THP-1 cells by activating NF-kB signalling

Lentinan and HBsAg alter the function of monocytes/macrophages though the NF-kB signalling pathway. Therefore, NF-kB signalling were examined in this cell culture model using western blotting analysis. The engagement of HBsAg alone inhibited the phosphorylation of IKB-α (Figure 2 A-B). In contrast, addition of lentinan to HBsAg-treated cells promoted the phosphorylation of IKB-α, indicating that lentinan induced functional recovery of THP-1 cells at least in part through activation of NF-kB signalling. This result was further confirmed by THP-1 cells treated with lentinan alone. Expression of IKB-α was negatively associated with the production of IL-1β, IL-12, and TNF-α. These results suggest that, at least in part, lentinan protected the function of monocytes/macrophages from the effect of HBsAg through competitive activation of NF-kB signalling.

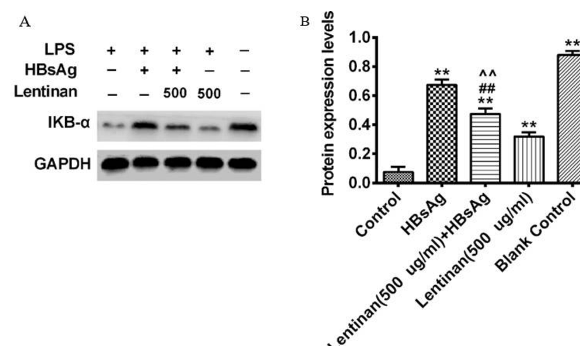

Figure 2: Lentinan reduces IkB-α expression to activate NF-kB signalling in THP-1 cells. (A) IkB-α protein expression was measured by western blotting. (B) Densitometric analysis of (A). The relative expression of IkB-α was normalized to GADPH. Data are presented as mean and SD; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with control; # vs. HBsAg; ^ vs. lentinan)

Lentinan restores the function of HBsAg-treated THP-1 cells by activating the MAPK signalling pathway

To investigate the role of the MAPK pathway in the process of immune-regulation induced by lentinan, activated THP-1 cells were treated with

HBsAg, lentinan and HBsAg+lentinan respectively, and then incubated with LPS. Reduced p-ERK expression and increased p-JNK and p-p38 expression were observed in the THP-1 cells that simultaneously incubated with 500 μg/ml lentinan and 25 μg/ml HBsAg when compared with cells treated with HBsAg alone (Figure 3A-B). The presence of HBsAg facilitated phospho-ERK expression, but dramatically repressed the phosphorylation of JNK and p38. Consistently, the addition of lentinan resulted in reduced expression of p-ERK, p-JNK, and p-p38 in the cells treated with HBsAg.

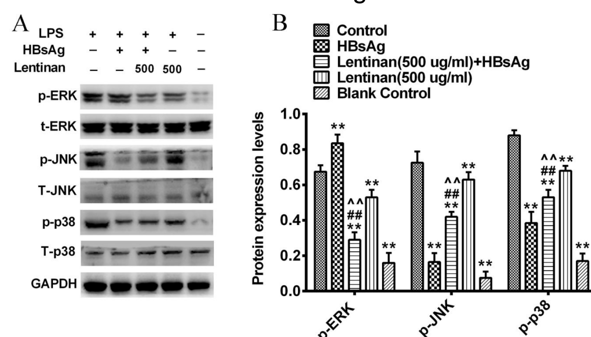

Figure 3: Lentinan regulates the MAPK signalling pathway to relieve HBsAg-induced functional impairment in THP-1 cells. (A) Phospho (p)-ERK, total (T)-ERK, p-JNK, T-JNK, p-p38, and T-p38 protein levels were assessed by western blot. (B) Densitometric analysis of the western blots. Expression levels were normalized to GADPH. Data are presented as mean and SD; **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 compared with control; # vs. HBsAg; ^ vs. lentinan)

DISCUSSION

Lentinan is an immune response modifier and an anti-cancer drug for stomach cancer [12,14]. Lentinan enhances host immunity by activating monocytes/macrophages and neutrophil antimicrobial functions [13,15]. The β-glucan structure of lentinan can directly bind to dectin-14 that is expressed on the surface of neutrophils, macrophages, and natural killer cells [16]. As a polysaccharide, lentinan also has various effects on inflammatory signalling pathways, such as NF-kB, MAPK, and the inflammasome [17]. Although lentinan promotes the activity of macrophages and CTLs, it may also inhibit the progress of inflammation *in vivo* by blocking pro-inflammatory cytokines, such as IL-1β and IL-18 [17].

Several studies have examined the immune-modulating effects of lentinan in cancer treatments. Tumour patients who received lentinan enhanced survival rates [14,18]. Administration of lentinan promoted the generation of CTLs, as well as the complement response that helped the CTLs recognize tumour cells. Moreover, macrophage cytotoxic activities and TNF production were enhanced after administration of lentinan [19].

Cancer patients show potent immuno-suppression in tumour microenvironments, as well as in the circulatory system [20]. Myeloid-derived suppressor cells (MDSCs) induce failure of host anti-tumour immune responses and repress the functions of CTLs, resulting in tumour survival and expansion [21]. Indeed, some MDSCs share the phenotype of a monocyte subset; thus, lentinan may function on these cells to promote host anti-tumour responses. Patients chronically infected with HBV have insufficient antiviral immune responses. These deficits presented as the impairment of myeloid subsets, indicating that the repression of the host innate immune arm may be a leading cause of systematic immuno-suppression [22]. These data suggested that lentinan might have a role in chronic HBV treatment.

In this study, addition of lentinan recovered the production of IL-1β, IL-12, and TNF-α in a dose-dependent manner by THP-1 cells that exposed to HBsAg. The underlying mechanisms were associated with activation of NF-kB and MAPK signal pathways. Upon LPS stimulation, THP-1 cells treated with HBsAg alone showed decreased IkB-α expression; co-incubation with lentinan significantly promoted IkB-α degradation. Thus, lentinan affected NF-kB signalling to recover cytokine production. Since the MAPK signal pathway was indicated in both HBsAg- and lentinan-induced inflammatory responses, the protein levels of p-ERK, p-JNK, and p-p38 protein levels were further measured. THP-1 cells incubated with lentinan and HBsAg showed decreased expression of IkB-α and p-ERK and increased expression of p-JNK and p-p38. Notably, the addition of 500 μg/ml lentinan alone inhibited the production of these pro-inflammatory cytokines, but significantly reduced the expression of IkB-α and repressed process of ERK, JNK, and p38 phosphorylation following LPS stimulation.

In addition to activating the NF-kB and MAPK signalling pathways, lentinan alters inflammasome activation and cytokine maturation [13]. It triggers expression of pro-inflammatory cytokines via the NLRP3 inflammasome and selectively inhibits cytokine secretion in response to AIM2 inflammasome activation in a colitis model [17]. These findings may explain why lentinan alone decreased cytokine production on further LPS stimulation.

The conventional treatments for HBV infection, including pegylated interferon-α and nucleotide

analogues, halt the progression of active liver disease, but do not clear HBV infections [23]. These treatments are costly and generally need long-term administration, though they are effective at reducing disease exacerbation. Compared to recombinant interferon-α or nucleotide analogues, lentinan is accessible and shows potential for HBV treatment.

CONCLUSION

The findings of this study demonstrate that lentinan activates NF-kB and MAPK signalling pathways *in vitro* to enhance the function of monocytes/macrophages in the presence of HBsAg. These results support the potentials of lentinan in HBV treatment and thus, warrant the need for further studies of its effects in an *in vivo* model of HPV infection.

DECLARATIONS

Conflict of Interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Le Qin and Pei-pei Fang designed all the experiments and revised the manuscript. Jie Li, Shan-shan Huang and Chen-wei Pan performed the experiments, and Guang-yao Zhou, Lingxiang Jin and Lu Zhuge wrote the manuscript.

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