

Original Research

Nitric oxide donor andrographolide enhances humoral and cell-mediated immune responses

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Abstract: The present study was aimed to investigate the regulatory effect of Nitric oxide donor andrographolide (Q-1) on cellular immunity in patients with chronic hepatitis B. Peripheral blood mononuclear cells (PBMCs) were isolated from patients with chronic hepatitis B. Cell viability was assessed using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The levels of expression of interferon gamma (IFN- γ), interleukin 4 (IL-4), interleukin 10 (IL-10) and tumor necrosis factor α (TNF- α) in PBMCs of patients with chronic hepatitis B were determined using real-time quantitative polymerase chain reaction (qRT-PCR). Anti-HBV effect of isolated HBV DNA was also assessed *in vitro*. Q-1 had no significant effect on the viability of Vero and isolated PBMCs ($p > 0.05$). The expression of IFN- γ in PBMCs of control patients significantly and time-dependently increased after treatment with Q-1, but the expressions of IL-4 and IL-10 in PBMCs of patients with chronic hepatitis B were decreased significantly and time-dependently ($p < 0.05$). The function of Th1 cells was significantly enhanced by Q-1 treatment ($p < 0.05$). The mean replication of HBV DNA in HepG2 cells at the three concentrations of Q-1 and adefovir were 3.96×10^6 , 4.13×10^6 and 4.53×10^6 copies/mL, respectively. There was no significant difference in the expression of HBV DNA among the concentration levels. These results indicate that andrographolide enhances the function of HBV-specific T cells in patients with chronic hepatitis B.

Key words: Chronic hepatitis B; Andrographolide; Cytotoxic T lymphocytes; Cellular immunity; Expression.

Introduction

Chronic hepatitis B is caused by persistent HBV infection. Its pathogenesis is complex. It is caused by decline in antigen-specific cell function, leading to persistent infection (1, 2). Nucleoside analogues and interferons are employed in antiviral therapy for the treatment of chronic hepatitis B (3, 4). Interferons play an antiviral role via the regulation of cellular immunity, while nucleotide analogues directly inhibit viral replication. Studies have shown that activation of HBV antigen-specific T-cell in patients with chronic hepatitis B after antiviral therapy is due mainly to enhancement in T helper 1 cells (Th1 cells) and balance in Th1/Th2. However, antiviral therapy is limited by several factors (5, 6).

Some herbs used in Traditional Chinese Medicine (TCM) have shown potential in regulating internal cellular and humoral immunity (7). Andrographolide is a bioactive compound isolated from *Andrographis paniculata* Nees (8). It stimulates normal PBMCs to produce IFN- γ and TNF- α (9). Andrographolide promotes the secretion of IL-2 and proliferation of peripheral blood lymphocytes (10). Reports on the antibacterial and anti-inflammatory effects of andrographolide abound. However, it was only recently that the role of andrographolide on cellular immunity received attention (11). In phase I clinical trials of andrographolide in the treatment of

HIV, it was found that the number of cells stimulated by andrographolide after treatment was significantly higher than those before treatment, but it had no direct inhibitory effect on HIV. *In vitro* and *in vivo* studies involving BALB/c mice have shown that the expressions of IL-2 and IFN- γ were significantly upregulated after treatment with andrographolide, an indication that it may exert an antitumor effect (10-13). Nitric oxide (NO) is an important messenger molecule which has a wide range of physiological functions. *In vivo*, nitroglycerin, sodium nitroprusside (SNP) and other NO donors release high amount of NO capable of inducing apoptosis in tumor cells (14, 15). In a previous study, the secretion of IFN- γ by spleen cells in mice treated with andrographolide was significantly increased relative to the control, suggesting that andrographolide increases the specificity of cellular immunity (9, 16). Reports on the immunomodulatory effect of andrographolide in chronic hepatitis B patients are scanty or non-existent. The present study investigated the regulatory effect of NO donor andrographolide on cellular immunity in patients with chronic hepatitis B.

Materials and Methods

Materials and reagents

Vero and HepG2.2.15 cell lines, and SYBR Green I fluorescence quantitative PCR kit were obtained from

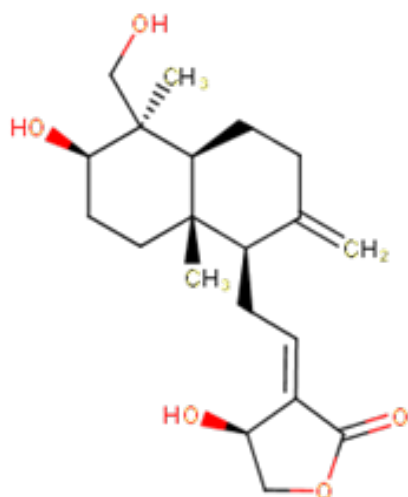


Figure 1. Chemical structure of andrographolide.

Shanghai Enzymatic Research Biotechnology Co. Ltd. Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin double antibody and FBS were products of Gibco Co. (USA). Chloroform, phenol and protease K were purchased from Shanghai Shenneng Gambling Biotechnology Co. Ltd. Boxun Super Clean Station, fluorescence quantitative PCR machine, Eppendorf 5810R centrifuge, CO₂ cell culture box, sterile Pasteur pipettes, pipetting guns, and Eppendorf tubes were purchased from Shanghai Boxun Industrial Co., Ltd. DNase I was obtained from Boehringer Mannheim, while RNase A and adefovir were products of Sigma-Aldrich (USA).

Cell culture

Vero and HepG2.2.15 cells were cultured in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin solution at 37 °C for 24 h in a humidified atmosphere of 5 % CO₂ and 95 % air. After attaining 60 - 70 % confluency, the cells were treated with serum-free medium and graded concentrations of Q-1 (1 - 100 mg/L) for 24 h. Cells in logarithmic growth phase were selected and used in this study.

MTT assay

The MTT assay was used to assess the cytotoxic effect of andrographolide on Vero cell line and isolated PBMCs. Peripheral blood of patients was collected in sodium citrate anticoagulant containers, and centrifuged at 3500 rpm for 15 min to obtain erythrocytes. The cells were seeded at a density of 1 × 10⁶ cells/well in 96-well plates and cultured in DMEM for 24 h. Then, Q-1 (1 - 100 mg/L) was added to the cells and incubated for 72 h. At the end of the third day, 20 μL of MTT solution (5 mg/mL) was added to the wells, followed by incubation at 37 °C for 2 h. The medium was finally replaced with 150 μL of 0.1 % dimethyl sulfoxide (DMSO) to completely dissolve the formazan crystals formed. The absorbance of the samples was read in a microplate reader at 485 nm. The assay was performed in triplicate. Cell viability at different time points: 0, 4, 12 and 24 h was calculated as shown in Equation 1:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of the experimental group} \times 100}{\text{Absorbance of the control group}} \quad (1)$$

Extraction of HBV DNA from intracellular viral particles

The extraction of HBV DNA from intracellular viral particles was performed as described below.

HepG2.2.15 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and 600 μL pyrolysis solution which comprised 10 mM Tris, pH 7.9, 1 mM EDTA, 50 mM NaCl, 1 % NP40 and 8 % sucrose. The cells were subsequently incubated at 37 °C for 10 min. The cell suspension was centrifuged at 12 000 rpm at 4 °C for 5 min to obtain a supernatant which was then incubated with 6 μL of 1 M MgOAc, 12 μL of 5 mg/mL DNase I, and 3 μL of 20 mg/mL RNase A at 37 °C for 30 min. The resultant mixture was centrifuged at 12,000 rpm at 4 °C for 5 min to obtain a supernatant. Then, ice-cold solution of EDTA, and 35 % PEG8000/1.75 M NaCl was added and incubated for 60 min, after which it was centrifuged at 9000 rpm for 5 min to obtain a pellet. Then, SDS/proteinase K buffer solution was added to the pellet containing HBV core particles and digested at 37 °C for 12 h. Finally, DNA was extracted from the digest using Trizol reagent, and resuspended in 20 μL triple-distilled water.

qRT-PCR

The levels of expression of IFN-γ, IL-4 and IL-10 in patients with chronic hepatitis B, and control were determined using qRT-PCR. Total RNA was extracted from Vero and isolated PBMCs using Trizol reagent, while cDNA synthesis kit was used to perform cDNA synthesis reaction according to the instructions of the manufacturer. Light Cycler 1536 RT-PCR detection system was used for the estimation of the mRNA expressions of IFN-γ, IL-4 and IL-10 genes. Variation in the cDNA content was normalized using β-actin. The PCR reaction mixture (20 μL) consisted of 6.4 μL of dH₂O, 1.6 μL of gene-specific primer (10 μM), 2 μL of synthesized cDNA and 10 μL of SYBR Premix Ex Taq™ II. The Ct value of U6 was taken as the internal parameter, and 2^{-ΔΔCt} was used to calculate the relative expression levels of the proteins. The primer sequences of PCR reaction were: Core 1: CTTCTGGAGTTACTC; Core 2: GGAAGTGTTGATAAGATAGG.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using SPSS (13.0). Groups were compared using Student *t*-test. Values of *p* < 0.05 were considered statistically significant.

Results

Levels of expression of IFN-γ, IL-4 and IL-10 in PBMCs of chronic hepatitis B patients and control before treatment with Q-1

Before treatment, the expression levels of IFN-γ and IL-10 in PBMCs of patients with chronic hepatitis B were not significantly different from those of control group (*p* > 0.05), but the corresponding IL-4 expression level was significantly higher in PBMCs of patients with chronic hepatitis B than in control group (*p* < 0.05; Figure 2).

Results of MTT assay

As shown in Table 1, Q-1 had no significant effect on the viability of Vero and isolated PBMCs ($p > 0.05$).

Effects of different concentrations of Q-1 on the expressions of related cytokines in control patients

Treatment of PBMCs of control patients with graded concentrations of Q-1 led to significant and time-dependent reductions in the expressions of IL-4, IL-10 and TNF- α , but significant and time-dependent increases in the expression of IFN- γ ($p < 0.05$). These results are shown in Figure 3.

Expressions of cytokines in PBMCs of control patients after treatment with Q-1

The expression of IFN- γ in PBMCs of control patients significantly and time-dependently increased after Q-1 treatment, but the corresponding expressions of IL-4 and IL-10 were decreased significantly and time-dependently ($p < 0.05$; Figure 4).

Effect of Q-1 on expression levels of IFN- γ , IL-4 and IL-10 in PBMCs of patients with chronic hepatitis B

Treatment of PBMCs with Q-1 led to significant increase in the expression of IFN- γ in patients with chronic hepatitis B, but the expressions of IL-4 and IL-10 were significantly reduced ($p < 0.05$) (Figure 5).

Effect of Q-1 on IFN- γ /IL-4 in PBMCs of patients with chronic hepatitis B

The IFN- γ mRNA/IL-4 mRNA ratio in PBMCs after Q-1 treatment was significantly higher in patients with chronic hepatitis B than in control group. The function

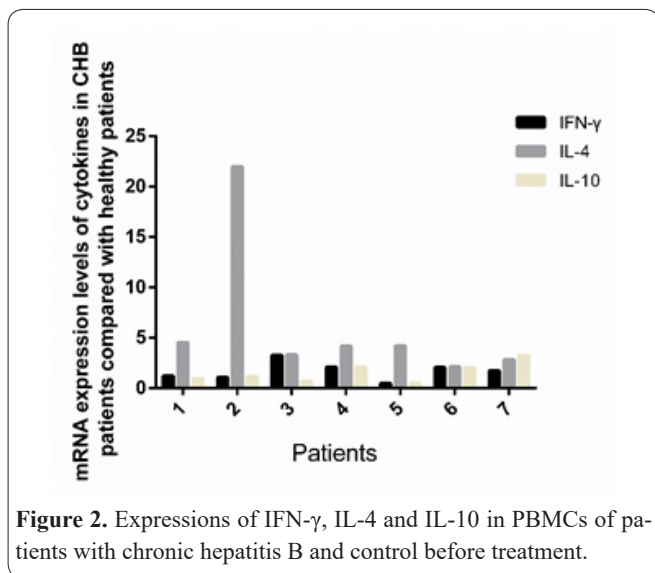


Figure 2. Expressions of IFN- γ , IL-4 and IL-10 in PBMCs of patients with chronic hepatitis B and control before treatment.

Table 1. Effect of Q-1 on viability of Vero cells.

Concentration of Q-1 (mg/L)	Viability (%)
0.00	100.00 \pm 3.84
3.13	99.00 \pm 2.51
6.25	98.00 \pm 3.22
12.5	96.00 \pm 3.11
25.00	95.00 \pm 2.98
50.00	94.00 \pm 2.98
100.00	96.00 \pm 3.55

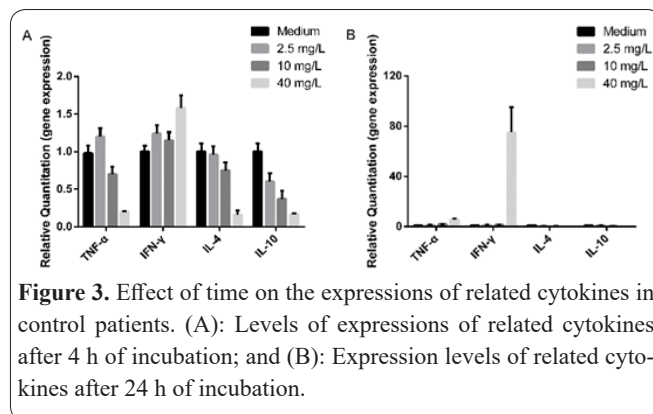


Figure 3. Effect of time on the expressions of related cytokines in control patients. (A): Levels of expressions of related cytokines after 4 h of incubation; and (B): Expression levels of related cytokines after 24 h of incubation.

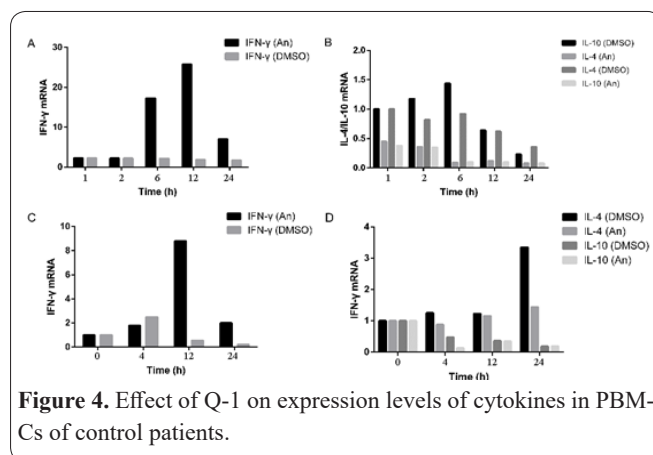


Figure 4. Effect of Q-1 on expression levels of cytokines in PBMCs of control patients.

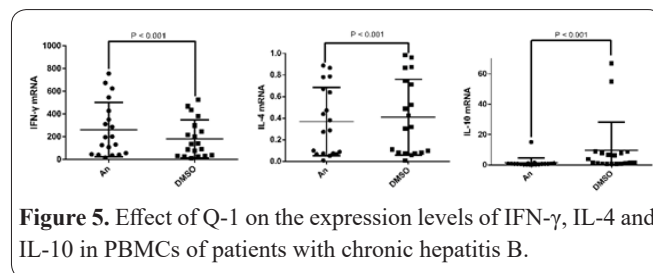


Figure 5. Effect of Q-1 on the expression levels of IFN- γ , IL-4 and IL-10 in PBMCs of patients with chronic hepatitis B.

of Th1 cells was significantly promoted by Q-1 treatment (Figure 6).

Effect of Q-1 treatment on HBV DNA replication

The mean replication of HBV DNA in HepG2.2.15 cells at the three concentration levels of Q-1 and adenofovir were 3.96×10^6 , 4.13×10^6 and 4.53×10^6 copies/mL, respectively. There was no significant difference in the expression of HBV DNA among the concentration levels (Figure 7).

Discussion

The pathogenesis of chronic hepatitis B is complex,

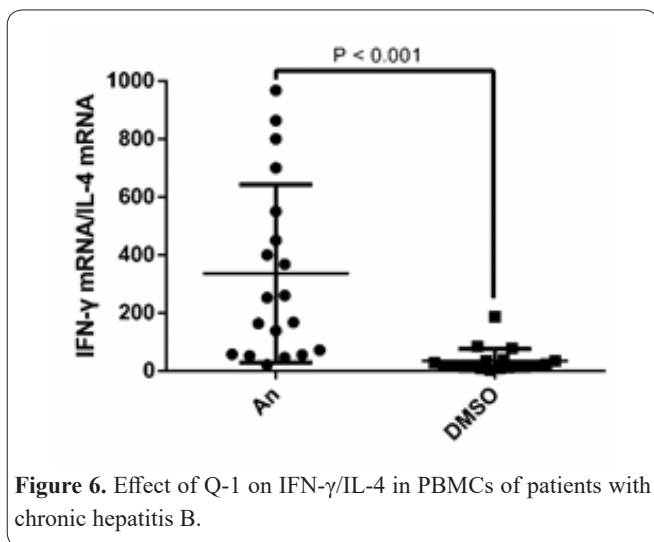


Figure 6. Effect of Q-1 on IFN- γ /IL-4 in PBMCs of patients with chronic hepatitis B.

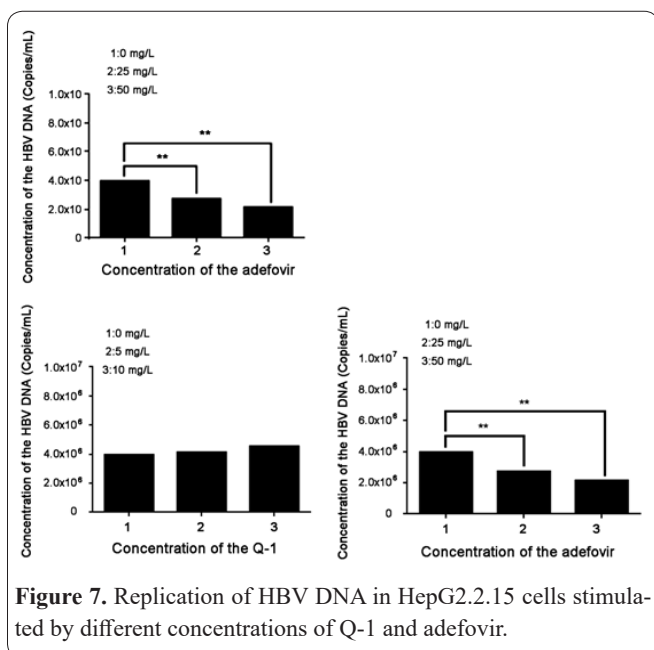


Figure 7. Replication of HBV DNA in HepG2.2.15 cells stimulated by different concentrations of Q-1 and adefovir.

and the resultant hepatic lesions are not directly caused by HBV, but by specific immune cells and cytokines produced during viral clearance (17). Specific cellular immune response to HBV antigen is key in viral clearance. The CD4⁺T cells (Th cells) are important regulators of specific cellular immune response. They differentiate into helper T cells 1 (Th1) and helper T cells 2 (Th2) on stimulation by antigens and cytokines. Interferon gamma (IFN- γ) is secreted by Th1 cells, and it inhibits the differentiation of Th2 cells. Similarly, IL-4 secreted by Th2 cells promotes the differentiation of Th1 cells. However, Th1 and Th2 cells under normal circumstance maintain immune balance via mutual regulation. An imbalance in the levels of both cells results in allergic and autoimmune diseases, tumors, organ rejection, and chronic infectious diseases. Studies have shown that acute self-limiting HBV infection promotes Th1 cell differentiation, HBV specific T cell function, and elimination of viral particles. In patients with chronic hepatitis B, the function of Th1 cells is weakened, and IFN- γ secretion is markedly reduced, leading to differentiation of the cells into Th2 cells with marked increase in IL-4 secretion. This reduces specific T cell function and slows the clearance of HBV. In addition, Th1 cells eliminate HBV infection via the regulation of

cellular immunity. In the liver, enhanced Th1 cell function leads to CTL activation, increased IFN- γ expression, liver damage and extensive inflammation of hepatocytes. Cytokine IL-10 is secreted by Th2 cells, and it inhibits Th1 cells, leading to persistent HBV infection. It also possesses potent anti-inflammatory effect. Chronic hepatitis B is treated with nucleotide analogues such as adefovir dipivoxil or interferons, thymosin and other regulatory immunotherapies. Results of *in vivo* and *in vitro* studies have shown that the balance between Th1 and Th2 cells is maintained by enhancement in the function of Th1 cells and inhibition of Th2 cells, and this leads to activation of HBV antigen-specific T cells (18, 19). The goal of anti-HBV therapy is the elimination of HBV infection via restoration of HBV-specific T cells function.

Andrographolide is a diterpenoid lactone isolated from *Andrographis paniculata*. It has been reported that treatment of hepatic mice with andrographolide promotes CTL function via stimulation of Th1 cells to secrete IL-2 and IFN- γ . Nitric oxide (NO) is a biological free radical with rapid diffusion through biofilm. It plays an important role in immune regulation, tunneling and cerebral vascular regulation, especially in the metastasis of tumors. The cytotoxic effect of NO at high concentration is due to DNA damage, peroxidation of membrane lipids, and inhibition of some proteins via S-nitrosation. The effect of endogenous NO on most cancer cells cannot be predicted due to its short half-life. Therefore, the development of exogenous NO donor drugs provides a highly regulated and predictable routes for the application of NO. Nitrate vinegar is an important and excellent NO donor in medicine. The NO produced by nitric acid vinegar causes DNA amination and breakage, tumor cell apoptosis, and down-regulation of the expression of signal proteins (STST3 and AKT). However, the regulatory effect of the NO donor andrographolide on cellular immunity in patients with chronic hepatitis B has not been reported.

In this study, the expression of Th1 cytokine IFN- γ in PBMCs of patients with chronic hepatitis B was significantly increased, while the expressions of Th2 cytokines IL-4 and IL-10 were significantly reduced, an indication of a well-regulated Th1/Th2 balance. The results also suggest that Q-1 has no direct inhibitory effect on HBV DNA *in vitro*.

The results obtained in this study indicate that andrographolide enhances the function of HBV-specific T cells in patients with chronic hepatitis B.

Acknowledgements

None.

Conflict of Interest

There are no conflicts of interest in this study.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Bo Yang; Bing Zhang, Bo Yang, Lijun Du, Yunfen Guo collected and analysed the data; Bing Zhang wrote the text and all authors have read and approved the text prior to publication.

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