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CpG ODN pretreatment attenuates concanavalin A-induced hepatitis in mice

Hui Zhang [a,1], Quan Gong [a,b,1], Jun-hua Li [c], Xiao-ling Kong [a], Li Tian [a], Li-hua Duan [a], Jing Tong [a], Fei-fei Song [a], Min Fang [a], Fang Zheng [a], Ping Xiong [a], Zheng Tan [a,*], Fei-li Gong [a,*]

[a] Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, People's Republic of China
[b] Department of Immunology, School of Medicine, Yangtze University, Jingzhou 434023, People's Republic of China
[c] Institute of Organ Transplantation,Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, People's Republic of China

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ABSTRACT

T cell-mediated hepatic damage plays a key role in the pathogenesis of liver diseases such as autoimmune hepatitis, viral hepatitis and acute liver failure. CpG-containing oligodeoxynucleotides (CpG ODN), a ligand for toll-like receptor (TLR) 9, is widely used as an immunological adjuvant. In the present study, we investigated the effect of CpG ODN on T cell-mediated liver injury in a murine model of concanavalin A (Con A)-induced hepatitis. We found that the aminotransferase level was significantly decreased in CpG ODN pretreated mice and the survival of the mice was markedly prolonged. CpG ODN pretreatment inhibited NF-κB DNA binding activity. As a result, the systemic/liver levels of TNF-α and IFN-γ were significantly suppressed. Furthermore, the activation of inflammatory cells was diminished by CpG ODN pretreatment. These results suggest that CpG ODN pretreatment protects the mice from Con A-induced liver injury via inhibiting hepatocyte apoptosis, inflammation and activation of lymphocytes.

1. Introduction

Autoimmune hepatitis, viral hepatitis and other liver diseases are major threats to human health worldwide. Concanavalin A-induced hepatitis is primarily T cell-mediated hepatic damage [1] and has been widely used as a model in studies of etiopathogenisis, pathogenesis and clinical treatment of immunological hepatitis in humans. In Con A-induced hepatitis, inflammatory cells such as T cells, NK cells, NKT cells and eosinophils cells infiltrate into the liver and produce a variety of hepatotoxic cytokines, such as interferon gamma (IFN-γ) [2], tumor necrosis factor alpha (TNF-α) [3], interleukin 1 (IL-1) [4], IL-4 [5], and IL-5 [6]. The Con A-induced liver injury was characteristic of high serum level of transaminase and massive hepatocyte apoptosis/necrosis.

CpG-containing oligodeoxynucleotides (CpG ODNs) activate the innate/adaptive immune system through binding to Toll-like receptor 9 (TLR9) which is expressed in many immunological cells such as B cells, macrophages and plasmacytoid dendritic cells (pDCs) [7]. The TLR9 is then activated to induce Th1-based immune responses. To our current understanding, the potential therapeutic uses of these CpG ODNs have been focused on infectious disease, cancer and allergy therapy [8,9].

A recent study suggested that CpG ODN have a protective effect on the CpG ODN/D-GalN-induced hepatic injury [10]. However, the effects of CpG ODN on Con A-induced hepatitis are less known and need to be further investigated. In the present study, we demonstrated that CpG ODN pretreatment can protect the mice from Con A-induced liver injury. Furthermore, we explored the possible mechanisms underlying this protective effect.

2. Materials and methods

2.1. Animals

Six- to eight-week-old male C57BL/6 mice (20–25 g) were purchased from the Institute of Organ Transplantation, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The mice were housed in a specific pathogen-free room and were exposed to a 12-h light/12-h dark cycles. All animal studies were approved by the institutional animal care and use committee, Tongji Medical College, Huazhong University of Science and Technology.

2.2. Reagents

Phosphorothioate ODNs, biotin-labeled NF-kappa B probe and the primers used for RT-PCR were synthesized from Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai,

Abbreviations: ODN, synthetic oligodeoxynucleotide; CpG ODN, ODN containing CpG motifs; MNC, mononuclear cell; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha.

* Corresponding authors. Fax: +86 27 83693500/62851.
E-mail addresses: tanzhengwh@sina.com.cn (Z. Tan), flgong@163.com (F. Gong).
[1] These authors contributed equally to this work.

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China). The sequences of ODN were previously described [11] and are as follows: CpG ODN: GCT AGA CGT TAG CGT, and non-CpG ODN: GCT AGA TGT TAG CGT . Type IV Con A was purchased from Sigma-Aldrich (Saint Louis, MO, USA). The cytokine specific ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Murine phycoerythrin conjugated anti-CD3 Ab, fluorescein isothiocyanate conjugated CD69 Ab, allophycocyanin conjugated NK1.1 Ab and isotype Ab were purchased from BD Pharmingen (San Diego, CA, USA). TUNEL assays kits were purchased from Boster Biological Technology Co., Ltd (Wuhan, China).

2.3. Cell line

RAW 264.7, a murine macrophage-like cell line was cultured in RPMI 1640 with 10% fetal bovine serum (FBS).

2.4. Con A-induced hepatitis

CpG ODN and Con A were dissolved in pyrogen-free phosphate-buffered saline (PBS). Mice were administered intravenously with CpG ODN (100 μg/mouse) or PBS as controls. Three hours later the mice were challenged intravenously with Con A (15 μg/g body weight). Serum and livers were collected at the indicated time points after Con A injection. For survival experiments, a lethal dose of Con A (25 μg/g body weight) was intravenously injected.

2.5. Determination of liver injury

Alanine aminotransaminase (ALT) activities were determined using a multiple biochemical analyzer. Liver tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin–eosin. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assays were performed according to the manufacturer's instructions.

2.6. Isolation of hepatic mononuclear cells (MNCs)

Livers were harvested and pressed through a 200-gauge stainless steel mesh. After washed twice with PBS, the cells were resuspended in 40% Percoll. Single-cell suspension was overlaid gently onto 70% Percoll and centrifuged at 2300 rpm for 30 min at room temperature. Hepatic MNCs were obtained from the interphase, washed twice with PBS, and resuspended in RPMI 1640.

2.7. Flow cytometric analysis

Liver MNCs were preincubated with the Fc receptor blocker (anti-CD16/32 mAb, BD Pharmingen) at 4 °C for 10 min to avoid non-specific antibody binding. Then the cells were stained with fluorescence-labeled antibody (phycoerythrin conjugated anti-CD3 Ab, fluorescein isothiocyanate conjugated CD69 Ab, allophycocyanin conjugated NK1.1 Ab or isotype Ab) diluted in PBS containing 2% fetal bovine serum and 0.1% sodium azide at 4 °C for 30 min. After washed twice with PBS, the cells were analyzed by a LSR II flow cytometer (BD Bioscience, San Jose, CA, USA). The expression of CD69 on T cells, NK cells and NKT cells were analyzed on gated $CD3^+$, $CD3^-$ $NK1.1^+$, $CD3^+$ $NK1.1^+$ respectively.

2.8. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated from the livers at the indicated time points using TRIzol® Reagent (Invitrogen) according to the manufacturer's instruction. cDNA was synthesized from 2 μg of total RNA using PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Biotechnology, Co., Ltd., Da Lian, LiaoNing, China). PCR amplifications were performed with standard methods using following specific primers: for Bcl2: Sense: 5′-GGC ATC TTC TCC TTC CAG-3′, Antisense: 5′-CTA CCC AGC CTC CGT TAT-3′; for Bax: Sense: 5′-TTT CAT CCA GGA TCG AGC AGG-3′, Antisense: 5′-GCA AAG TAG AAG AGG GCA ACC AC-3′; for IFN-γ: Sense: 5′-GTG GCA TAG ATG TGG AAG AA-3′, Antisense: 5′-CCT CAA ACT TGG CAA TAC TC-3′; for TNF-α: Sense: 5′-CAT CTT CTC AAA ATT CGA GTG ACA A-3′, Antisense : 5′-TGG GAG TAG ACA AGG TAC AAC CC-3′; for β-actin: Sense: 5′-CTG TCC CTG TAT GCC TCT G-3′, Antisense: 5′-CAT CGT ACT CCT GCT TGC T-3′.

2.9. Electrophoretic mobility shift assay (EMSA) and western blot

Nuclear and cytoplasmic proteins were extracted from the liver tissues at the indicated time points using Nuclear and Cytoplasmic Protein Extraction Kit (Nanjing KeyGen Biotech. Co. Ltd., China) according to the manufacturer's instruction. Electrophoretic mobility shift assay was performed to determine the DNA binding activity of the transcription factor NF-κB. Biotin-labeled double stranded oligodeoxynucleotides (sequence: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′) that contains the NF-κB binding motif was used as a probe [12].

For western blot analysis, equal amounts of cytoplasmic extracts of liver tissues were subjected to electrophoresis a 10% SDS-PAGE directly. Proteins were then transferred to PVDF membranes (Hybond, Escondido, CA, USA) and then incubated overnight with various antibodies against phosphorylated IκBα or β-actin (Santa Cruz, CA, USA) at 4 °C. Blots were developed by SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, USA).

2.10. Enzyme-linked immunosorbent assay (ELISA)

Serum or culture supernatants were assayed for protein levels of IFN-γ and TNF-α by ELISA according to the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA).

2.11. Statistical analysis

Data are expressed as means ± SD. Student's *t* test (two groups) or one way ANOVA (multiple groups) were used. *P* values less than 0.05 were considered significant.

3. Results

3.1. CpG ODN pretreatment attenuates Con A-induced liver damage

To determine the effect of CpG ODN on Con A-induced hepatitis, CpG ODN (100 μg per mouse) was administered to mice through the caudal vein 3 h before Con A (15 μg/g body weight) injection. Serum aminotransferase levels were determined 12 h after Con A injection. As shown in Fig. 1A, serum ALT levels were slightly elevated in mice treated with CpG ODN alone. As expected, Con A administration significantly increased the serum level of aminotransferase. However, the Con A-induced ALT was markedly decreased in mice pretreated with CpG ODN, but not in mice pretreated with non-CpG ODN, which suggests that CpG ODN has protective effects on Con A-induced liver injury. Since serum ALT is released from necrotic hepatocytes, we examined if there is a difference in cell death between the livers of Con A and CpG ODN treated mice. As shown in Fig. 1B, Con A injection caused massive necrosis in the liver, which was nearly abolished by CpG ODN pretreatment. Next, we determined whether CpG ODN pretreatment protected mice from lethal dose of Con A (25 μg/g body weight). As shown in Fig. 1C, CpG ODN pretreatment dramatically increased mice survival. The protective effect of CpG ODN on Con A-induced liver injury was dose-dependent (Fig. 1D). Furthermore, CpG ODN pretreatment before Con A challenged (−12hCpG/Con A or −3hCpG/Con A) protected the mice from