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Ubiquitin-hepatitis B core antigen-cytoplasmic transduction peptide enhances HBV-specific humoral and CTL immune responses *in vivo*

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ABSTRACT

Therapeutic strategies based on an enhanced hepatitis B virus (HBV)-specific cytotoxic T lymphocyte (CTL) activity may eradicate HBV. We previously verified that a fusion protein ubiquitin (Ub)-hepatitis B core antigen (HBcAg)-cytoplasmic transduction peptide (CTP) can enter the cytoplasm of dendritic cells and enhance T cell response to generate HBV-specific CTLs efficiently *in vitro*. Ub, a marker of protein degradation, may promote the generation of peptides appropriate for major histocompatibility complex class I presentation. In the present study, the specific immune responses of the fusion protein Ub-HBcAg-CTP in BALB/c mice were evaluated and the underlying mechanisms were investigated. Results showed that Ub-HBcAg-CTP increased the anti-HBcAg titer and produced the cytokines IFN-γ and IL-2. This fusion protein also induced higher percentages of IFN-γ^+CD8^+ cells and specific CTL responses. Ub-HBcAg-CTP could also upregulate the expressions of Jak2, Tyk2, STAT1, and STAT4 in T lymphocytes. In conclusion, Ub-HBcAg-CTP enhanced cellular and humoral immune responses and induced robust HBV-specific CTL activities in BALB/c mice.

1. Introduction

Hepatitis B virus (HBV) infection remains a serious health problem with approximately 350 million individuals infected worldwide. HBV infection is also one of the major causes of end-stage liver diseases, such as cirrhosis and hepatocellular carcinoma [1,2]. Although considerable antiviral drugs, such as interferon, lamivudine, and adefovir, have been used to eradicate this virus, no significant progress on the treatment of patients with chronic hepatitis B (CHB) has been achieved [3]. Hence, new strategies should be developed to increase therapeutic efficacy for these patients. Appropriate humoral and cellular immune responses are also necessary to eradicate HBV infection. Studies have demonstrated that $CD8^+$ T cells function as cytotoxic T lymphocytes (CTLs) that eliminate HBV; in general, $CD8^+$ T cells recognize antigenic peptides presented by major histocompatibility complex (MHC) I molecules on the surface of antigen-presenting cells (APCs) or target cells [4–6]. Therefore, strategies to enhance or broaden specific CTLs of patients with CHB may be an efficient approach to eradicate this virus.

Ubiquitin (Ub), a 76-amino acid peptide, was discovered in early 1980s. It is an important component of the Ub–proteasome system (UPS) and covalently attached to numerous cellular and pathogen-derived proteins via a highly regulated process [7,8]. Proteasomal degradation generates peptides that are presented by MHC class I molecules at the cell surface to CTLs, and this process is rapid and efficient [9]. Hepatitis B core antigen (HBcAg) exhibits unique immunological characteristics. For example, HBcAg as a natural or recombinant antigen can induce strong adaptive immune responses. During chronic HBV infection, HBcAg is the only antigen that elicits prominent immune responses [10,11]. Tang et al. [6] found that patients who successfully survived this infection usually possess efficient HBcAg-specific CTL responses. On the basis of these theories, we aimed to investigate whether or not Ub-modified HBcAg can be presented by MHC-I molecules to elicit robust CTL responses.

However, HBV-specific CTL responses induced by exogenous antigens, such as HBV-encoded antigens, are usually weak because of the prohibition nature and selective permeability of biological membranes [12]. Cytoplasmic transduction peptide (CTP), which is derived from the protein transduction domain of the HIV-1 trans-activator of transcription protein, is a novel, deliberately designed transduction protein that is used to ensure an efficient cytoplasmic delivery of biomolecules [13]. Thus, these properties of CTP may stimulate Ub-modified HBcAg to elicit robust and specific HBV immune responses.

We previously showed that the purified recombinant fusion protein Ub-HBcAg-CTP can enter the cytoplasm of dendritic cells (DCs) and enhance T cell responses to generate specific CTLs efficiently *in vitro* (data not shown). On the basis of these findings, we aimed to identify whether or not the Ub-HBcAg-CTP fusion protein enhances HBV-specific CTL immune responses *in vivo*.

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2. Materials and methods

2.1. Reagents and cells

All of the fluorescent antibodies and isotype controls were purchased from eBioscience (San Diego, USA). All of the antibodies were obtained from Abcam (Cambridge, UK). ELISA kits for IFN-γ and IL-2 and anti-HBcAg ELISA kits were purchased from R&D Co., Ltd. (Minneapolis, USA) and BD Biosciences (San Jose, CA, USA), respectively. Phorbol 12-myristate 13-acetate (PMA), ionomycin, monensin, Concanavalin A (ConA), and HBcAg were obtained from Sigma (St Louis, MO, USA). The H-2^d mastocytoma cell line P815/c, which expresses the HBV core antigen, was generated according to a previously published method with some modification [14]. It was maintained in our lab and cultured in Dulbecco's modified Eagle's medium (Invitrogen, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under a humidified condition of 5% CO_2.

2.2. Protein expression, purification, and western blotting

The plasmid pcDNA3.1(−)-Ub-HBcAg was constructed and maintained in our lab. The Ub-HBcAg cDNA sequence was generated via PCR to obtain an 820 bp PCR product. The Ub-HBcAg-CTP gene was inserted into the pMAL-c2X prokaryotic expression vector, as well as the control genes (HBcAg-CTP and Ub-HBcAg). These plasmids were further identified via restriction enzyme digestion and bidirectional DNA sequencing. The recombinant plasmids were transformed into the *Escherichia coli* BL21 (DE3) bacterial strain to induce the expression of the recombinant fusion proteins. After being lysed by sonication and centrifugation, the supernatants containing Ub-HBcAg-CTP, HBcAg-CTP, and Ub-HBcAg fusion proteins were purified by amylase resin column according to the manufacturer's instructions and were analyzed via western blotting. The maltose binding protein (MBP)-tag was ultimately cleaved by the TEV protease (data not shown; Fig. 1A). All proteins were stored at 4 °C until used.

2.3. Animals and immunization

Forty BALB/c mice (H-2^d), half male and half female, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (China) and maintained under standard pathogen-free conditions in the Experimental Animal Center of Shanghai No. 6 Hospital. These mice were cared for and treated in accordance with the guidelines established by the Shanghai Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Mice aged 6 weeks to 8 weeks were randomly divided into five groups with eight mice in each group. Ub-HBcAg-CTP, HBcAg-CTP, Ub-HBcAg, and HBcAg (50 μg each) were then dissolved in 100 μl volume of PBS. The mice were immunized intramuscularly in the left tibialis with 100 μl of mixed PBS thrice at an interval of 1 week. Afterward, the mice were sacrificed. Serum samples and splenocytes were collected at 7 days after the third immunization was administered.

2.4. Detection of anti-HBcAg antibodies

ELISA was performed to detect the antibodies of HBcAg. Microtiter plates were pre-coated with HBcAg (10 μg/ml) and blocked with 5% fetal calf serum. Approximately 100 μl of mouse serum was added to

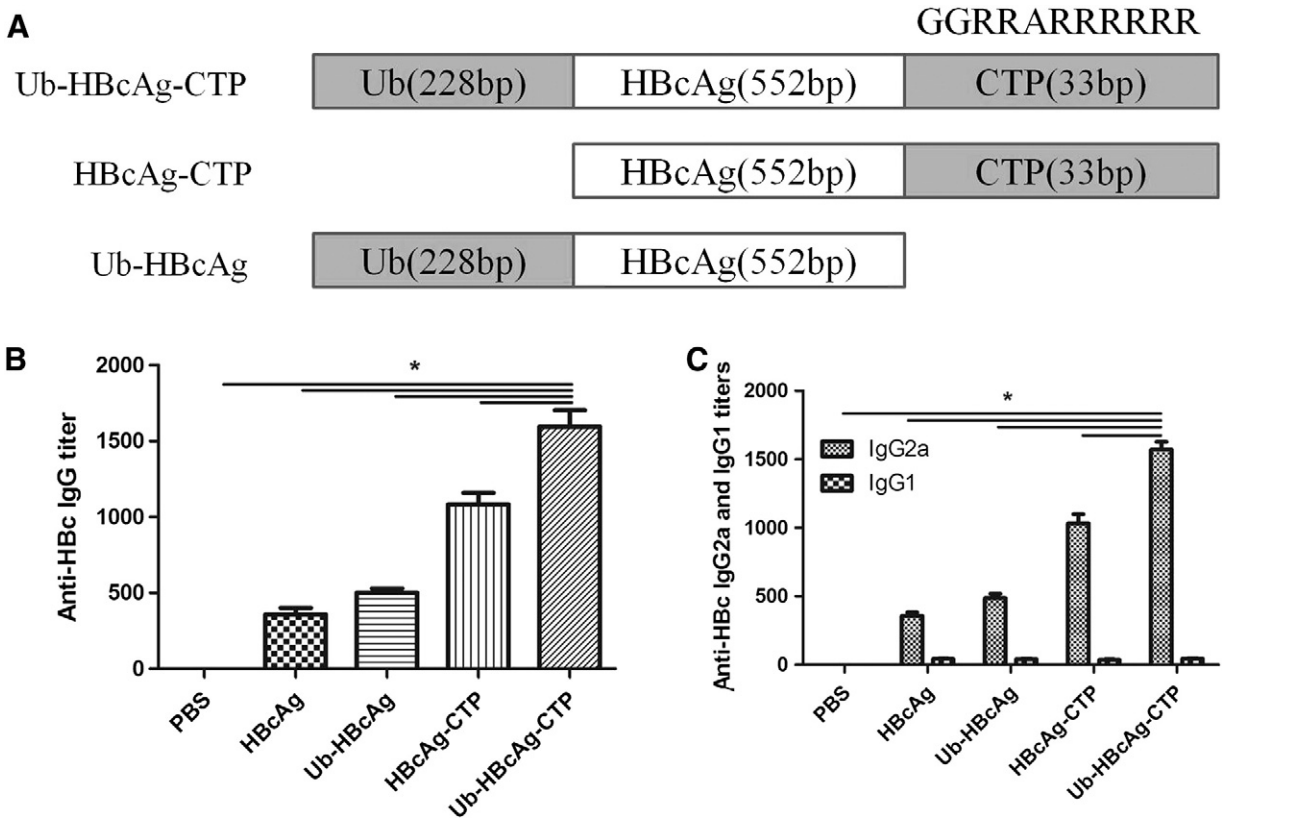

Fig. 1. Schematic of fusion protein and humoral immune responses in immunized mice. (A) Schematic of Ub-HBcAg-CTP, HBcAg-CTP, and Ub-HBcAg fusion proteins. Ub gene, HBcAg gene, and CTP sequence were spliced by PCR and cloned into pMAL-c2x expression vector. *Escherichia coli* BL21 (DE3) cells were transformed with constructed vectors, and soluble fusion proteins were expressed in the supernatants. (B) Blood samples were obtained after the mice were immunized thrice. Anti-HBc IgG antibody titers in sera were detected by ELISA. The titer of a given serum sample is defined as the reciprocal value of the highest dilution yielding a positive result. Mean values and standard deviation were calculated for each group of eight mice. *$P < 0.05$. (C) Blood samples were obtained after the mice were immunized thrice. The HBcAg-specific subtypes IgG2a and IgG1 titers in sera were detected by ELISA. Mean values and standard deviation were calculated for each group of eight mice. Anti-HBc IgG2a antibody titers in the Ub-HBcAg-CTP group were significantly higher than in the other groups (*$P < 0.05$), whereas the IgG1 isotype was very low in all of the groups.