Targeting hepatitis B virus antigens to dendritic cells by heat shock protein to improve DNA vaccine potency

Qin-Long Gu, Xue Huang, Wen-Hong Ren, Lei Shen, Bing-Ya Liu, Si-Yi Chen

AIM: To investigate a novel DNA vaccination based upon expression of the HBV e antigen fused to a heat shock protein (HSP) as a strategy to enhance DNA vaccine potency.

METHODS: A pCMV-HBeAg-HSP DNA vaccine and a control DNA vaccine were generated. Mice were immunized with these different constructs. Immune responses were measured 2 wk after a second immunization by a T cell response assay, CTL cytotoxicity assay, and an antibody assay in C57BL/6 and BALB/c mice. CT26-HBeAg tumor cell challenge test \textit{in vivo} was performed in BALB/c mice to monitor anti-tumor immune responses.

RESULTS: In the mice immunized with pCMV-HBeAg-HSP DNA, superior CTL activity to target HBV-positive target cells was observed in comparison with mice immunized with pCMV-HBeAg (44% ± 5% vs 30% ± 6% in E: T > 50:1, \(P < 0.05\)). ELISPOT assays showed a stronger T-cell response from mice immunized with pCMV-HBeHSP than that from pCMV-HBeAg immunized animals when stimulated either with MHC class I or class II epitopes derived from HBeAg (74% ± 9% vs 31% ± 6%, \(P < 0.01\)). ELISA assays revealed an enhanced HBeAg antibody response from mice immunized with pCMV-HBe-HSP than from those immunized with pCMV-HBeAg. The lowest tumor incidence and the slowest tumor growth were observed in mice immunized with pCMV-HBe-HSP when challenged with CT26-HBeAg.

CONCLUSION: The results of this study demonstrate a broad enhancement of antigen-specific CD4\(^+\) helper, CD8\(^+\) cytotoxic T-cell, and B-cell responses by a novel DNA vaccination strategy. They also proved a stronger antigen-specific immune memory, which may be superior to currently described HBV DNA vaccination strategies for the treatment of chronic HBV infection.

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Key words: Hepatitis B virus antigen; Dendritic cell; Heat shock protein; DNA vaccine

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection continues to be a major human health problem, and there are about 350 million chronic HBV carriers worldwide\(^1\). Chronic HBV infection is associated with serious complications as a result of long-term sequelae such as liver cirrhosis or hepatocellular carcinoma\(^2\). The host immune response to HBBeAg and HBeAg appears critical in both viral clearance and clinical resolution. The ultimate objective for rational vaccine design is the induction of pathogen immunity. In laboratory animals, DNA vaccine has proven to be a simple and effective method to generate protective immunity against a variety of pathogens, including HBV\(^3\). DNA vaccination strategy. They also proved a stronger antigen-specific immune memory, which may be superior to currently described HBV DNA vaccination strategies for the treatment of chronic HBV infection.

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agents. The abilities of HSP include: (a) to chaperone peptides, including antigenic; (b) to interact with antigen presenting cells through a receptor; (c) to stimulate antigen presenting cells to secrete inflammatory cytokines; and (d) to mediate maturation of DCs, making them a one-stop shop for the immune system[8]. These properties also permit to use of HSP for developing a new vaccine. HSP has been reported to activate innate immune responses, to mediate the maturation of DCs, to upregulate proinflammatory cytokines[8,12], and to induce specific CTL responses[13,14].

In this study, we describe a novel DNA vaccination strategy to enhance uptake and presentation of antigens by DCs. Specifically, we developed a DNA vaccination based upon expression of the HBV e antigen fused to HSP, which are versatile immune regulators that chaperone antigenic peptides for MHC class I and II presentation by DCs. After vaccination, DNA is taken up by various cells that produce and secrete the antigen-HSP fusion proteins. The secreted fusion proteins, in addition to inducing B-cells, are efficiently captured and processed by DCs via receptor-mediated endocytosis, and then presented via MHC class I and class II molecules. This study demonstrates a broad enhancement of antigen-specific CD4+ helper, CD8+ cytotoxic T-cell, and B-cell responses by this DNA vaccination strategy, which may be superior to currently described HBV DNA vaccination approaches for the treatment of chronic HBV infection.

MATERIALS AND METHODS

Mice and cell lines
The mice used were female C57BL/6 and Balb/c mice, aged 4-5 wk. All mice were maintained in the animal facility at Baylor College of Medicine with approval of the Institutional Animal Care and Use Committee. The tumor cell lines EL-4, Tramp-c-2, and CT26 were purchased from the ATCC. EL-4 and Tamp-c-2 cells were cultured in DMEM medium and CT26 cells in RPMI 1640 medium both containing 10% heat-inactivated FBS (GIBCO) at 37°C in an humidified 5% CO2: atmosphere.

DNA constructs

The pCMV-HBeAg-HSP construct was generated by inserting HBeA (be derived from the precore open reading frame by cleavage of its C-terminus, nucleotide: 1901-2452 of HBV genome) & HSP-70 (StressGen Biotechnologies, Victoria, British Columbia, Canada) plasmid into a pCMV vector (Invitrogen, Carlsbad, CA, USA) with the cloning site HindIII & XbaI. Two control vectors, pCMV-HBeAg & pCMV-HSP, were also generated.

DNA preparation and immunization
Plasmid DNA was amplified in Escherichia coli DH5α and purified using an endotoxin-free purification kit (Qiagen) according to a standard protocol. Concentration was determined using the UV/Visible Spectrophotometer (Pharmacia Biotech) at 260 and 280 nm, and the material was adjusted to a final concentration of 1 mg/mL with endotoxin-free PBS (Sigma) and stored at -20°C. Mice were divided into 4 groups, which were immunized with different DNA vaccines including pCMV-HBeAg-HSP, pCMV-HBeAg, and pCMV-HSP. Controls were injected with PBS (C57BL/6 mice) or pCMV (Balb/c mice). Immunization method: mice were injected s.c. (C57BL/6) and i.m. (Balb/c) in quadriceps with 100 μg of DNA in 100 μL, two inoculations were carried out with an interval of 2 wk. Two weeks after the second immunization blood and spleens were collected, and BALB/c mice were challenged with CT26-HBeAg tumor cells.

Elispot for T-cell response assay

Elispot assays were used as a measure for T-cell response. Ninety-six well filtration plates (Milipore, Bedford, MA, USA) were coated with AN18 (anti-mouse IFN-γ, Mebtech) at the concentration of 10 μg/mL and kept at 4°C overnight. Splenocytes were cultured in 96-well plates (1 × 10^6 cells/mL and 2 × 10^6 cells/mL) with RPMI 1640/10% FBS containing HEPES, 2MC, and NEAAS. Splenocytes from mice of different groups were stimulated with HBeAg class I peptide (HBeAg93-100 peptide), HBeAg class II peptide (HBeAg120-131), or HBV protein (HBsAg, 227 amino acids, 24kD) (BD Pharmingen, SD, CA, USA) for comparing the effect of different DNA vaccinations on the T-cell response. The splenocytes derived from mice vaccinated with HBeAg-HSP were also stimulated with Tramp-c-2 class I peptide (P117-139, WT1), CT26 class I peptide (peptide AHI), Tyrosinase protein, Tyrosinase class I peptide (Ty-4), Tyrosinase class II peptide (Ty-5), and PMSA class II peptide as controls. Proteins were added at a final concentration of 60 μg/mL, peptides at a final concentration of 30 μg/mL. All assays were performed in triplicates. After stimulation for 20 h at 37°C, the plate was washed with PBS and the second antibody (anti-mouse IFN-γ, Mebtech Mab R4-6A2 biotin) was added for a further incubation at 37°C for 2 h. Avidin-HRP was added for 1 h at room temperature after washing, then 100 μL AEC was added to each well for coloring for 4 min after washing. The reaction was stopped by drying the membrane. The results were sent to Zellnet Consulting, Inc. (NY, USA) for test.

CTL cytotoxicity assays

CTL cytotoxic activity was determined using a 51Cr-release assay. In brief, splenocytes obtained from mice 2 wk after the second immunization were cultured in 24 well plates with RPMI 1640/10% FBS containing HEPES, 2MC, NEAAS, and IL-2 (50 U/mL). Splenocytes were stimulated with HBeAg class I peptide (HBeAg93-100 peptide) for 7 d, with changing half of the medium every 2 d. Target cell lines were cultured with IFN-γ (100 U/mL) for 24 h. EL-4 cells were pulsed with HBeAg class I peptide and HBV protein (HBsAg, 227 amino acids, 24 kDa) as target cells. EL-4 cells pulsed with HBV non-related class I peptide such as CT26 and Tramp-c-2 class I peptides, non-pulsed EL-4 cells, and CT26 cells pulsed with HBeAg class I peptide served as controls. All target and control cells were labeled with 51Cr for 90 min. Cells were added to the wells at effector-to-target ratios ranging from 100:1 to 6.25:1 in triplicates. Plates were incubated at 37°C for 5 h, before supernatants were collected and activity was assessed in a Gamma counter (Beckman, Fullerton, CA, USA).
Enhancement of T cell response and CTL activity by HBeAg-HSP DNA vaccine

To evaluate whether HBeAg-HSP DNA vaccine can enhance immune response in vivo, splenocytes were obtained for T cell response and CTL activity. These immune responses were first stimulated with HBV protein and were compared between C57BL/6 and Balb/c mice immunized with different DNA vaccines. In CTL assay, EL-4 cells were pulsed with HBV protein as target cells. ELISPOT showed a stronger T-cell response from the mice immunized with HBeAg-HSP than that from HBeAg immunized mice after stimulation with HBV protein (Figure 1A, spots 74 ± 5 vs 31 ± 6, P < 0.01). A specific T-cell response was obtained in HBV protein stimulation in comparison with Tyrosinase protein stimulation (Figure 1B). Superior CTL assay to HBV protein pulsed target cell was also observed in mice immunized with HBeAg-HSP in comparison to those immunized with HBeAg (Figure 1C, 46% ± 10% vs 35% ± 8% in E: T > 50:1, P < 0.05).

We also evaluated the specific stimulating effect of HBV MHC class I peptide on T cell response and CTL activity. After splenocytes were stimulated with HBV MHC class I peptide, ELISPOT showed a stronger T-cell response from the mice immunized with HBeAg-HSP than from those which had been immunized with HBeAg (Figure 2A, 76 ± 6 vs 29 ± 5, P < 0.01). CTL activity to HBV class I peptide pulsed target cells is also stronger in mice immunized with HBeAg-HSP than in mice immunized with HBeAg (Figure 2B, 44 ± 5 vs 30 ± 6 in E: T > 50:1, P < 0.05). A specific effect on T cell response is also obtained after stimulation with HBV class I peptide in comparison with CT26 class I peptide and TrampC-2 class I peptide stimulation (Figure 2C). A stronger CTL activity to HBV class I peptide pulsed target cells was shown in comparison with target cells pulsed with CT 26 class I peptide and TrampC-2 class I peptide (Figure 2D). T cell response to HBV class I peptide and CTL activity to HBV class I peptide pulsed target cells proved cytotoxic T cell activity.

To evaluate helper T cell activity, the effects of HBV MHC class II peptides on T cell response and CTL activity were studied. Splenocytes were stimulated with HBV class II peptides for T cell response. A stronger T cell response was obtained from HBeAg-
HSP DNA vaccine immunized mice in comparison with that in mice immunized with HBeAg (A, spots 74 ± 9 vs 31 ± 6, P < 0.01) and HSP DNA vaccine (Figure 3). A specific stronger T cell response by HBV class II peptide stimulation was shown in comparison with that by PSMA4 class II peptide, Tyrosin-4 and Tyrosin-5 class II peptide stimulation (Figure 3B).

We also studied whether HBV related proteins and class I peptides can increase target cell antigenicity. Results suggested that splenocytes from mice immunized with HBeAg-HSP DNA vaccine have a stronger CTL activity to target cells pulsed with HBV protein and HBV class I peptide in comparison to non-target cells and CT 26 cells (Figure 4).

**Serum antibody response to HBeAg antigen after DNA vaccination**

To determine whether HBeAg-HSP DNA vaccination can also induce an antibody response to HBeAg antigen, we measured serum anti-HBeAg antibody responses by ELISA assay. As shown in Figure 5, antibody levels detected in mice immunized with the HBeAg-HSP DNA vaccine were markedly higher than those immunized with...
Comparison of antibody titers to HBV between different groups after Tumor size DNA vaccine has been popularly studied for prophylaxis against HBV infection to induce a strong humoral and cell-mediated immunity specific immune responses and specific for HBV antigen vaccination is an efficient new technique to stimulate thus poor induction of a CTL response or absent presentation of antigen on class intracellular synthesis of antigen because there is poor individuals; whole-killed or subunit vaccines do not induce to a virulent form, especially in immunocompromised some disadvantages, such as the possibility of reversion with interferon-α (IFN-α). Antigen-based vaccines have some disadvantages, such as the possibility of reversion to a virulent form, especially in immunocompromised individuals; whole-killed or subunit vaccines do not induce intracellular synthesis of antigen because there is poor or absent presentation of antigen on class I MHC and thus poor induction of a CTL response. DNA-based vaccination is an efficient new technique to stimulate specific immune responses and specific for HBV antigen to induce a strong humoral and cell-mediated immunity against HBV infection. HBV(HBsAg, HBeAg) DNA vaccine has been popularly studied for prophylaxis or therapy against HBV infection.

DNA vaccine has made an attractive alternative to conventional methods of vaccination. HBV DNA vaccination can induce CD8+ T cells as well as a dominant Th1 phenotype among the splenic lymphocytes, so eliciting strong CTL and protective levels of antibody. Antigen-presenting cells (APCs) play a key role in induction of immune responses by DNA vaccines. DNA vaccines express native protein antigens in situ which can be recognized by B cells and presented by MHC class I and II molecules to prime helper T cells and CTLs. Dendritic cells (DCs) are usually thought of as a specific APCs for T cell and B cell activation and regulation of antibody synthesis, presentation of antigen by DCs is a potent stimulus to immune response, particularly to cell-mediated immunity and the development of CTLs. Thus, DCs are critical for initiating and modulating B and T cell responses elicited by DNA vaccination. However, only a very limited fraction of injected DNA molecules is taken up by DCs, the intracellular antigens expressed by DCs are difficult to be processed and presented to MHC class II.

In this study, we designed a novel DNA vaccination strategy to enhance uptake and presentation of antigen by DCs, specifically, we developed a DNA vaccine based on the expression of the HBV e antigen fused to HSP, which are versatile immune regulators that chaperone peptides for MHC class I, which are versatile immune regulators that chaperone peptides for MHC class I and II presentation by DCs. The HSP-based DNA vaccine has been shown to elicit CD4+ helper T cells and CD8+ cytotoxic T cells and has been used for inducing antitumor immunity and for therapy of infectious diseases. The novel vaccination strategy-HBeAg-HSP we developed in vivo by HBeAg-HSP DNA vaccine

To evaluate systemic immunity enhancement in vivo by the HBeAg-HSP DNA vaccine, Balb/c mice (10 mice/group) were challenged by CT26 cells transfected with HBeAg to observe the antitumor effect after different DNA vaccine immunizations. CT26-HBeAg cells were injected s.c. at 5 × 10⁶/mouse and tumor incidence and tumor growth were monitored. Results showed that there is a low tumor incidence in mice immunized with HBeAg DNA vaccine and a lower tumor incidence in HBeAg-HSP DNA vaccinated mice (Figure 6A), the incidence of tumor are 6/10 in HBeAg-HSP group, 8/10 in HBeAg group and 10/10 in the other two groups. Tumor growth was slowest in mice immunized with the HBeAg-HSP DNA vaccine (Figure 6B). The results suggested that HBeAg-HSP DNA vaccination can induce a stronger immune response to the related antigen.

**DISCUSSION**

HBV infection is a major human health problem and it is associated with a risk of developing liver cirrhosis or hepatocellular carcinoma. Thus, effective preventive and therapeutic strategy to chronic HBV infection has been a major exploration. Only a small proportion of patients with chronic HBV infection benefit from a treatment with interferon-α (IFN-α). Antigen-based vaccines have some disadvantages, such as the possibility of reversion to a virulent form, especially in immunocompromised individuals; whole-killed or subunit vaccines do not induce intracellular synthesis of antigen because there is poor or absent presentation of antigen on class I MHC and thus poor induction of a CTL response. DNA-based vaccination is an efficient new technique to stimulate specific immune responses and specific for HBV antigen to induce a strong humoral and cell-mediated immunity against HBV infection.

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