Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of hepatitis B virus-specific CTL epitopes presented by HLA-A*33:03 in peripheral blood mononuclear cells from patients and transgenic mice

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ARTICLE INFO

Article history:
Received 21 April 2014
Available online xxxx

Keywords:
Hepatitis B virus
HLA-A33
Transgenic mice
Epitope

ABSTRACT

Cytotoxic T lymphocyte (CTL) epitopes in the HBV protein of hepatitis B virus (HBV) may play a key role in viral control and liver damage. The aim of this study was to identify and study the function of HLA-A*33:03-restricted CTL epitopes in HBV protein of the HBV genotypes B and C, which are epidemic in China. Sixteen HBV peptides were predicated by computational analysis, and synthesized peptides were examined for their affinity to HLA-A*33:03 using a stable cell line. After being analyzed by enzyme-linked immunospot and cytolytic activity assays, as well as the tetramers staining method using peripheral blood mononuclear cells isolated from HBV-infected patients, five peptides (Hbs245–253, HBs335–343, HBc119–127, HBc104–112, and HBp391–399) were chosen to further confirm their HLA_A*33:03 restriction in transgenic mice.

1. Introduction

Hepatitis B virus (HBV) infection is a global challenge, with an estimated worldwide distribution of millions of chronic carriers. All of these patients are at risk of developing adverse sequalae, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. Cellular immune responses play an important role in viral clearance and disease pathogenesis. The epitopes bound by the main type of HLA-A site, such as A2, A11, and A24, have been comprehensively investigated [2], but related data concerning HLA-A33 are almost completely lacking.

HLA-A33 is very rare, with a phenotypic frequency of 0–1% in Caucasian population. However, the phenotypic frequency of HLA-A33 is 6–23% in Asian populations, including Chinese (6–20.9%, $n = 133{,}046$), Japanese (7.7%, $n = 1024$), Korean (12.8%), Singaporean (9.3%, $n = 769$), and Malaysian peoples (12.9%, $n = 1445$). Further, hepatitis B patients in these areas account for larger proportion [3–5]. A*33:03 is dominant in Chinese, Korean, Singaporean, and Japanese populations. Identification of HLA-A*33:03-restricted HBV cytotoxic T lymphocyte (CTL) epitopes is therefore necessary to investigate the immmunopathogenesis of HBV in these areas and plan proper vaccine development.

2. Materials and methods

2.1. Patients

This study involved 322 HBV patient donors who all gave informed consent before blood donation. The diagnosis of chronic HBV infection was made according to the Chinese consensus criteria [6]. After blood was collected, genomic DNA was extracted using a Genomic DNA isolation kit (Promega), and HLA typing was performed using sequence-specific primers (S3; Protrans, Ketsch, Germany). Additionally, five chronic hepatitis B (CHB), four acute hepatitis B (AHB), and 15 liver cirrhosis patients were chosen for the experiment.

2.2. Cell lines

The HLA_A*33:03 gene was cloned into the pLentiLox3.7 (pLL3.7) viral vector using *Nhe*I and *Xho*I, and 15 μg of pLL3.7_HLA*33:03

Abbreviations: AHB, acute hepatitis B; CHB, chronic hepatitis B; CTL, cytotoxic T lymphocyte; ELISPOT, enzyme-linked immunospot; HBV, hepatitis B virus; HC, HLA heavy chain; HLA, human leukocyte antigen; IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cell; TH1, helper T cell type 1; WB, Western blotting; β2m, β 2microglobulin.

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http://dx.doi.org/10.1016/j.bbrc.2014.05.001
0006-291X/

(GenBank ID: DQ327719.1) from a positive clone was co-transfected by Lipofectamine™ 2000 (Invitrogen, CA, USA) into HEK293T cells in a 10-cm dish with packaged plasmids VSVG, RSV-REV, and pMDLg/pRRE (5 μg each). Four hours after transfection, the culture medium was replaced with puromycin-containing medium, and 16 h later, transfected HEK293T cells were incubated at 32 °C overnight to increase the viral titer. Forty-eight hours after transfection, the lentiviral supernatant of the culture was collected, filtered through a 0.45-μm Millipore filter, and stored at −80 °C.

RMA-S cells or RD cells were infected using the lentiviral supernatant and selected with 4 μg/mL puromycin over five rounds of selection. Positive clones were identified using flow cytometry with a FITC-Flag antibody and named RMAS/A*33:03 and RD/A*33:03.

2.3. Peptides

The amino acid sequences of the S, C, X, and P proteins from HBV strains B and C were screened for HLA-A*33:03 binding peptide motifs (the detail is in Supplement). Candidate peptides were synthesized and shown to be 95% homogenous by high-performance liquid chromatography.

2.4. Temperature induction and peptide stabilization assays

HLA_A*33:03 surface expression with lowered temperature was performed as described [7]. RMAS/A*33:03 (10^6/mL) were incubated at 26 °C for 24 h, further incubated at 37 °C with or without 0.5 μM peptide and 1 nM human β 2microglobulin (hβ2m), and then examined by flow cytometry using a FITC-labeled W6/32 antibody at the indicated time points. FI values were calculated by the following formula: FI = (mean FITC fluorescence for a given peptide − mean FITC fluorescence without peptide)/(mean FITC fluorescence without peptide). Peptides with an FI > 2 were regarded as candidate epitopes with a capacity to bind to HLA-A*33:03 molecules.

2.5. Tetramer staining of virus-specific $CD8^+$ T cells

Soluble tetrameric human HLA-A*33:03 peptide complexes were constructed as previously reported [8].

2.6. Th1 polarization assay via the IFN-γ enzyme-linked immunospot (ELISPOT) method

Commercially available ELISPOT assays in IFN-γ kits (Dakewe biotech company, China) were used to assess type 1 cytotoxic T cell responses [9]. Briefly, A series of cell dilutions starting at 2.5×10^5 cells/well of PBMCs in 100 μl AIM-V medium (Life Technologies Inc., NY, USA) were co-cultured with individual HBV peptides (0.5 μM) for 18 h. The assay procedures then followed the manufacturer's instructions, and the spots were automatically counted using a Biospot counter (Cellular Technology, USA) and Immunocapture® software.

2.7. Generation of transgenic mice

Chimeric human-mouse class I cDNA contained the leader, α1, and α2 domains from HLA-A*33:03 (GenBank ID: DQ327719.1) fused to the murine H-$2K^d$ α3, transmembrane, and cytoplasmic domains (GenBank ID: KF831067.1). To easily trace the protein, StrepII and FLAG tags were added to the C-terminus. The chimeric gene sequence was optimized by the RNAfolding prediction program, and 10 silent mutations were made in the gene sequence. The class I hybrid construct was subsequently cloned into the pCAGG expression vector (encoding a CAG promoter and a polyadenylation signal) using *Nco*I and *Cla*I. Purified DNA was linearized with *Eco*RV and *Pst*I, and the 4.5-kb fragment containing the target gene was injected in fertilized C57BL/6 eggs to produce transgenic mice, as previously described [10]. The resulting progeny were screened by PCR, RT-PCR, Western blotting, and FACS using the B9.12.1 mAb (Beckman Coulter, CA, USA), which binds to the all HLA_A proteins [11].

2.8. CTL assays

RMAS-A*33:03 cells were incubated at 26 °C for 16 h, 10 μg/mL peptides and hβ2m were added, and then incubated for an additional 5 h. Then, the cells were labeled with 2.5 μmol/L PHK-26 (Sigma–Aldrich) and 2.5 μmol/L CFSE (Sigma–Aldrich) as target cells, as previously described [12]. The effect cells were added at different ratios: 10:1, 20:1, 40:1, or 80:1. After incubation for 6 h at 26 °C, the cells were counted by flow cytometry. Each assay was performed in triplicate. When target cells shifted to RD/A*33:03, the incubation temperature was adjusted to 37 °C.

2.9. Statistical analyses

Statistical analyses (analysis of variance, *t*-test, χ^2 analysis) were performed with SPSS software version 19. Differences with a p value <0.05 were considered statistically significant.

3. Results

3.1. Temperature induction and peptide stabilization assays

The RMAS/A*33:03 stable cell line presented the positive peptide P+ (FFVDGAANR, FI = 6.5) well compared to the negative peptide P− (FDVDGAANR, FI = 0.7), which only differ by one (the second) amino acid (Fig. 1A) [13]. Sixteen HBV type B and C candidate peptides were selected as described in the methods and screened in the same manner as P+ and P−. We found that five of them had strong affinity for HLA_A*33:03 (FI > 5). Representative flow cytometry results are shown in Fig. 1B, and the FI values >5 are shown in Table 1.

3.2. IFN-γ ELISPOT assays

The PBMCs from seven HLA-A*33 HBV patients were successfully obtained and measured. Two of the patients suffered from acute hepatitis, and five patients were in the CHB stage. The result shows that: (i) comparing AHB with CHB, the AHB patients had more HBV-specific CTL activity than CHB patients on peptide HBp391–399 ($p < 0.05$), though other peptides show higher CTL responses in AHB with no statistical difference ($p > 0.05$). (ii) Comparing the five candidate peptides in CHB patient samples with or without stimulation for 6 h, all five peptides generated higher specific CTL activity compare to the unstimulated state ($p < 0.05$). (iii) As for the difference between the peptides, combining the seven patients' data, HBp391–399 showed greater CTL activity ($x = 119.8$) than the other peptides (Table 2).

3.3. Tetramer staining of virus-specific $CD8^+$ T cells

Based on the ELISPOT results, five positive peptides and one negative peptide HBp835–843 were used to generate tetramers, and five CHB patient samples were analyzed. Fig. 2A describes one typical PBMC sample that was stained by six HLA_A*33:03 tetramers (HBp835–843 is not a qualified tetramer; we apply it as a native control), and Fig. 2C shows the results from all five patients. To validate the tetramer staining results, we compare the peptides'

Please cite this article in press as: X. Pan et al., Identification of hepatitis B virus-specific CTL epitopes presented by HLA-A*33:03 in peripheral blood mononuclear cells from patients and transgenic mice, Biochem. Biophys. Res. Commun. (2014), http://dx.doi.org/10.1016/j.bbrc.2014.05.001