Research Article

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T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) mediates natural killer cell suppression in chronic hepatitis B

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Background & Aims: T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) has been shown to influence autoimmune diseases; however, its function in viral infection has not been well-defined. We therefore investigated the expression and regulatory function of Tim-3 in natural killer (NK) cells in chronic Hepatitis B (CHB) infection.

Methods: Seventy-six CHB patients, 38 healthy controls, and 18 patients with fatty liver disease (FLD) were tested for Tim-3 expression on peripheral blood mononuclear cells (PBMCs) and in the liver tissue by flow cytometry and immunohistochemical stainning. The effects of HBV infection on Tim-3 expression in NK cells and the roles of Tim-3 in regulation of NK-cell function were also studied.

Results: There was a significant increase of Tim-3 expression in PBMCs, circulating NK cells and liver infiltrating lymphocytes (LILs) from CHB patients compared to that of healthy controls and FLD patients. Increased Tim-3 expression was also detected in NK92 cells that had been transfected with a HBV expression vector and NK cells isolated from the liver of HBV transgenic mice. Importantly, blockage of Tim-3 signaling with anti-Tim-3 antibodies or Tim-3-Fc fusion proteins resulted in an increased cytotoxicity for NK92 cells compared to HepG2 and HepG2.2.15 cells, as well as an elevated interferon-gamma (IFN-γ) production. Similarly, enhanced cytotoxicity was also observed in PBMCs or NK cells from CHB patients treated with the Tim-3 blockade *ex vivo*.

Conclusion: HBV infection can up-regulate Tim-3 expression in NK cells, which may in turn suppress NK-cell functions in CHB patients.

Keywords: Tim-3; Natural killer cells; Hepatitis B; Suppression.

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Abbreviations: HBV, hepatitis B virus; NK cells, natural killer cells; Tim-3, T cell immunoglobulin- and mucin-domain-containing molecule-3; IFN-γ, interferon-gamma; NKT, natural killer T cells; CHB, chronic hepatitis B; FLD, fatty liver disease; HCV, hepatitis C virus; HDV, hepatitis D virus; HGV, hepatitis G virus; HIV, human immunodeficiency virus; MNC, mononuclear cell; RT-PCR, reverse transcription polymerase chain reaction; PBMC, peripheral blood mononuclear cell; LILs, liver infiltrating lymphocytes; MFI, median fluorescence intensity.

Introduction

More than 350 million people worldwide suffer from chronic Hepatitis B virus (HBV) infection, and approximately 1 million of them die annually from HBV-induced liver diseases [1,2]. The persistence of virus that resulted from the low antiviral immune response had been thought to contribute to the pathogenesis of HBV-induced diseases [1,3]. However, the precise mechanisms underlying the HBV-mediated immune suppression in chronic infection are not completely understood.

Previous studies have shown that activated NK cells display their antiviral effects by mediating elevated cytotoxicity during HBV infection as well as augmenting adaptive immune responses as a result of cytokine production [4]. Accumulated data support a role for NK cells in the defense against HBV infection [5–7]. Furthermore, it has been shown that natural killer activity differs in different stages of HBV infection [8–10]. Patients with acute HBV infection showed enhanced NK cytotoxicity early in the course of infection. On the contrary, natural killing activity was significantly inhibited in chronic HBV infection [9,10].

T cell immunoglobulin- and mucin-domain-containing molecule-3 (Tim-3) plays a pivotal role in immune regulation and tolerance induction [11–23]. Engagement of Tim-3 by galectin-9 negatively regulates IFN-γ secretion and influences the ability to induce T cell tolerance in both mice and humans [14,16]. Recent studies have shown that Tim-3 is differentially expressed in innate versus adaptive immune cells, and that the ligation of Tim-3 can induce distinct signaling events, which may influence a range of inflammatory conditions [15–30].

Recently, Tim-3 mRNA has been shown to be mainly expressed in NK and NKT cells in humans [15]. However, it remains unclear what role Tim-3 plays in NK cells during the course of CHB infection. In this study, we thus examined the

expression of Tim-3 in NK cells and assessed the role of Tim-3 in chronic HBV infection.

Patients and methods

Patients and controls

A total of 132 subjects, including 76 patients with CHB, 18 patients with fatty liver disease (FLD) and 38 healthy individuals, were enrolled in this study. The liver tissues from 16 CHB patients and 6 FLD patients were collected for immunohistochemical staining. The diagnosis of these CHB patients were made according to the criteria established in the National Viral Hepatitis Conference of China (2000). FLD was diagnosed on the basis of abnormal liver enzymes, a detailed history, and exclusion of other causes of liver disease by appropriate serological and biochemical tests as reported [31]. All patients were hospitalized or followed in Jinan Infectious Disease Hospital, and Department of Infectious Diseases of Taian Chinese PLA 88 Hospital from June 2007 to June 2009. Clinical characteristics of enrolled subjects are summarized in Table 1. All of the included subjects were negative for antibodies to hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis G virus (HGV), and human immunodeficiency virus (HIV), and had no autoimmune liver diseases. None of the enrolled subjects were taking antiviral therapy or immunosuppressive drugs over three months. The study was approved by the Ethics Committee of Shandong University, and informed consent was acquired from each individual.

Cell lines and animals

NK92 cells (ATCC CRL-2407) were cultured in Minimum Essential Medium Alpha Medium (Invitrogen, Shanghai, China) containing 12.5% horse serum (Invitrogen), 12.5% fetal calf serum (FCS) (Invitrogen), and 100 IU/ml recombinant human interleukin 2 (IL-2) (Changchun Changsheng Gene Pharmacentical, Changchun, China). HepG2 cells (ATCC HB-8065) were cultured in Minimum Essential Medium (MEM) (Invitrogen) supplemented with 10% FCS and sodium pyruvate. HepG2.2.15 cells, which contain 4 copies of HBV DNA and express all viral proteins [32] were cultured in MEM with 380 μg/ml G418 (Invitrogen). K562 cells (ATCC CCL 243) were cultured in RPMI 1640 medium containing 10% FCS. All the cell lines were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences.

Male HBV transgenic Balb/c mice and the litter mates mice, 6–8 week-old, were purchased from the Transgenic Animal Central Laboratory (458 Hospital, Guang Zhou, China). The transgenic mice, containing 1.3 copies of the HBV complete genome (ayw subtype), express high levels of HBV antigens and have HBV DNA in the blood [33]. Mice were housed under specific pathogen-free conditions. All procedures were pre-approved by the Animal Care and Use Committee of Shandong University.

Flow cytometric analysis

Single-cell suspensions of the mononuclear cells (MNCs) from mouse liver were prepared as described previously [34] and stained with anti-CD3-Cy5, anti-NK1.1-FITC and anti-Tim-3-PE (eBioscience, San Diego, CA). Human PBMCs were washed with PBS containing 2% FBS and stained with anti-CD3-Cy5 (eBioscience), anti-CD16/56-FITC (Serotec, Oxford, UK) and anti-Tim-3-PE (R&D, Minneapolis, MN). At least 10,000 cells were analyzed using a Beckman Coulter flow cytometer (FC500, Fullerton, CA, USA).

Table 1. Clinical characteristics of enrolled subjects.

Group	CHB	Healthy controls	FLD
Case	76	38	18
Sex (male)	55 (72.4%)	25 (65.7%).	17 (94.4%)
Age (years)[a]	34.5 ± 13.6	35.1 ± 8.8	41.1 ± 10.8
ALT (U/L)[a]	278 ± 348	28 ± 7	103 ± 84
HBV DNA positive ($>10^3$ copies/ml)	62 (81.6%)	0	0
HBsAg positive	76 (100%)	0	0
HBeAg positive	52 (68.4%)	0	0
HBeAb positive	23 (30.3%)	0	0
HBcAb positive	75 (98.7%)	0	0

Patients with chronic Hepatitis B (CHB); patients with fatty liver disease (FLD).
[a]Median ± SD.

Transfection

Recombinant pcDNA3-1.1HBV plasmid containing 1.1 copies of the HBV genome was generated as described previously [35]. Transient transfections were performed by electroporation using a Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA) at 200 mV and a capacitance of 975 μF in a 0.4 cm Electroporation Cuvette (USA Scientific, Ocala, FL) [36]. 1×10^7 of NK92 cells were transfected with either 100 μg pcDNA3-1.1HBV or pcDNA3 vectors. After transfection, the cells were cultured in α-MEM supplemented with 200 μg/ml of G418 for 1–2 weeks.

Conventional RT-PCR and real-time RT-PCR

Total RNA was extracted from human peripheral blood mononuclear cells (PBMCs) using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed to assay the expression of Tim-3 on PBMCs as described previously [17]. The mRNA levels of Tim-3, HBx and HBc were measured by RT-PCR in NK92 cells that had been transfected with pcDNA3–1.1HBV or pcDNA3 plasmid DNA. The primers used for Tim-3, HBx and HBc were as follows: Tim-3 Forward, 5′-CCTATATGCCCTGCTTCTAC-3′; Tim-3 Reverse, 5′-CTGGTGGTAAGCATCCTTGG-3′; HBx Forward, 5′-ATGGCTGC TAGGGTGT-3′; HBx Reverse, 5′-TTAGGCAGAGGTGAAAAAG-3′; HBc Forward, 5′-ATGGACATTGA CCCGT AT-3′; HBc Reverse, 5′-CTAACATTGAGAT TCCCGAG-3′.

IFN-γ detection

IFN-γ in the cell culture supernatant was measured with an enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA) according to the manufacturer's instructions. Assays were performed in duplicate.

Immunohistochemical staining and double immunofluorescence staining

Formalin fixed paraffin-embedded liver tissue sections were stained with Goat-anti-human Tim-3 (R&D Systems, Minneapolis, MN) and biotinylated rabbit anti-goat Ig (Maixin Co., Fuzhou, China) followed by streptavidin-conjugated peroxidase. DAB was used as substrate. Positive cells were counted in 50 different fields by 2 independent observers. For double immunofluorescence staining, PBMCs from CHB patients were first cytospun onto poly-L-lysine slides, fixed with ice-cold acetone for 10 min. The slides were then washed and incubated with anti-Tim-3-PE and anti-CD16/56-FITC antibodies for 45 min at room temperature.

Preparation of purified NK cells from CHB patients

NK cells were obtained from PBMCs of CHB patients by positive selection using magnetic cell separation (Miltenyi, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. The purity of isolated $CD16/56^+CD3^-$ cells were >95% as determined by flow cytometry (data not shown).

Cytotoxicity assays

HepG2 and HepG2.2.15 were used as target cells. For the *in vitro* cytotoxicity assay, NK92 cells were used as effector cells. For the *ex vivo* cytotoxicity assay, freshly isolated PBMCs or purified NK cells from CHB patients were used as effector cells.

In order to determine the effects of Tim-3 on NK cytotoxicity, effector cells were incubated with anti-Tim-3 antibodies or Tim-3-Fc fusion proteins (R&D system) at the indicated concentrations for 30 min before cultured with 5000 target cells at the indicated ratios (effector to target ratios). Four hours later, the cells were stained with a Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) and the absorbance at 450 nm of each well was measured according to the manufacturer's instructions. Cellular cytotoxicity was calculated as: [1 − (OD value of (effector and target cells) − OD value of effector cells)/OD value of target cells] × 100%. All experiments were done in triplicates.

Statistical analysis

All data were analyzed using the GraphPad Prism 4. The Kruskal–Wallis nonparametric *H* test and Mann–Whitney nonparametric *U* test were used for comparison between groups. Spearman correlation analysis was performed between the Tim-