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Kynurenine production mediated by indoleamine 2,3-dioxygenase aggravates liver injury in HBV-specific CTL-induced fulminant hepatitis

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ABSTRACT

Indoleamine 2,3-dioxygenase (IDO), an enzyme that is ubiquitously distributed in mammalian tissues and cells, converts tryptophan to kynurenine, and is also known as a key molecule that promotes apoptosis in lymphocytes and neurons. In this study, we established hepatitis B virus (HBV)-transgenic (Tg)/IDO-knockout (KO) mice and examined the influence of IDO in a murine fulminant hepatitis model induced by HBV-specific cytotoxic T lymphocytes (CTL). An increase of IDO expression in the livers of HBV-Tg/IDO-wild-type (WT) mice administered HBV-specific CTL was confirmed by real-time polymerase chain reaction, western blotting, and evaluating IDO activity. Plasma alanine aminotransferase (ALT) levels in HBV-Tg/IDO-KO mice after HBV-specific CTL injection significantly decreased compared with those in HBV-Tg/IDO-WT mice. An inhibitor of IDO, 1-methyl-D-tryptophan (1-MT), could also attenuated the observed liver injury induced by this HBV-specific CTL. The expression levels of cytokine and chemokine mRNAs in the livers of HBV-Tg/IDO-WT mice were higher than those in the livers of HBV-Tg/IDO-KO mice. The administration of kynurenine aggravated the liver injury in HBV-Tg/IDO-KO mice injected with HBV-specific CTL. Simultaneous injection of recombinant murine IFN-γ and kynurenine also increased the ALT levels in HBV-Tg/IDO-KO mice. The liver injury induced by IFN-γ and kynurenine was improved in HBV-Tg/TNFα-KO mice. *Conclusion*: Kynurenine and IFN-γ induced by the administration with HBV-specific CTL are cooperatively involved in the progression of liver injury in acute hepatitis model. Our results may lead to a new therapy for the acute liver injury caused by HBV infection.

1. Introduction

Fulminant hepatitis is a severe, rapidly progressive loss of hepatic function due to viral infection or other cause of inflammatory destruction of liver tissue [1]. Hepatitis B virus (HBV) infection can cause fulminant hepatitis; this disease has a high mortality rate despite intensive medical care and the implementation of the latest therapies, including liver transplantation. HBV is a non-lytic virus that does not cause direct cell damage [2]. Liver damage and viral clearance after HBV infection are thought to be mediated by the host cellular immune response to viral antigens. A fulminant hepatitis model has been examined in mice by using adoptive transfer of HBV-specific cytotoxic T lymphocytes (CTL) into HBV-transgenic (Tg) mice [3–5]. The mice develop a necroinflammatory liver disease that is histologically similar to acute viral hepatitis in humans. Various studies have been performed on viral clearance and liver diseases in HBV infection by using this murine fulminant hepatitis model [4,6,7].

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that is ubiquitously distributed in mammalian tissues and cells; this enzyme converts tryptophan to *N*-formylkynurenine, which is further catabolized to kynurenine. IDO is induced via an IFN-γ dependent and/or an independent mechanism as well as other pro-inflammatory cytokines in the course of an inflammatory response in various cell types, including macrophages, fibroblasts, and epithelial cells [8,9]. Previously, we reported that IDO expression in hepatocytes is increased after HBV-specific CTL injection into HBV-Tg mice [7]. In this model, IFN-γ induced by the CTL injection was involved in the observed increase in the IDO expression on the liver, which is also reportedly up-regulated in hepatitis virus-infected woodchuck [10]. Thus, IDO expression in the liver is strongly increased in acute and fulminant viral hepatitis. Kynurenine and other tryptophan metabolites produced by IDO promote cell death and tissue injury [11]. On the other hand, immune-mediated diseases such as GVHD are improved by the activation of IDO [12]. These findings indicate that IDO has conflicting function in tissue

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injury. The role of increased IDO expression is not defined clearly in the fulminant hepatitis model induced by HBV-specific CTL. In the present study, we established HBV-Tg/IDO-KO mice and examined the influence of induced IDO during acute liver injury caused by HBV-specific CTL.

2. Material and methods

2.1. Reagents

1-Methyl-D-tryptophan (1-MT) and L-kynurenine were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant murine IFN-γ was purchased from Peprotech (Rocky Hill, NJ).

2.2. Mice

The HBV-Tg mouse lineage 107-5D (official designation Tg [Alb-1, HBV] Bri66; inbred B10.D2, H-2d), in which the HBV envelope coding region is under the control of the mouse albumin promoter, was provided by Dr. F. V. Chisari (Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, CA). IDO1 gene knockout (IDO-KO) mice on a C57BL/6J background were obtained from Jackson Laboratory (Bar Harbor, ME) and backcrossed to B10.D2 (H-2d). HBV-Tg/IDO-KO mice were produced by backcrossing IDO-KO mice with 107-5D. TNF-α-KO mice were produced by gene targeting as described previously [13] and backcrossed onto B10.D2 (H-2d). HBV-Tg/TNF-α-KO mice were produced by backcrossing TNF-α-KO mice with 107-5D. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and guidelines for the care and use of animals established by the Animal Care and Use Committee of Gifu University.

2.3. Cell lines

P815 cells expressing HBV-preS1, 2, and S (P815preS1) were provided by Dr. F. V. Chisari. HBV-specific $CD8^+$ CTL clones were produced as described in a previous report [14]. The clones are H-2d restricted, and can recognize an epitope (IPQSLDSWWTSL) located between residues 28 and 39 of the hepatitis B surface antigen (HBsAg). The CTL were washed 5 d after a final stimulation with irradiated P815preS1 and then intravenously injected into HBV-Tg mice.

2.4. Fulminant hepatitis model and assessment of hepatocellular injury

HBV-specific $CD8^+$ CTL clones (1×10^6/mouse) were intravenously injected into HBV-Tg/IDO-WT, IDO-WT, HBV-Tg/IDO-KO, and IDO-KO mice. To monitor hepatocellular injury, plasma alanine aminotransferase (ALT) activity was measured using an automated clinical analyzer BM2250 (JEOL, Tokyo, Japan).

2.5. Histological examination

Histopathological examination of the liver was performed at 0 d and 2 d after CTL injection. The liver was fixed with 10% formalin in PBS for 48 h and then embedded in paraffin. Tissue sections were deparaffinized, stained with hematoxylin and eosin, and examined under light microscopy. TUNEL staining was performed using in situ apoptosis detection kit (Takara Bio, Shiga, Japan), according to the manufacturer's instructions. Necroinflammatory foci are defined as the existence of inflammatory cell infiltration and hepatocyte necrosis more than three, and inflammatory cell foci are defined as the existence of inflammatory cell more than ten in paraffin sections of the liver. The data are represented as the numerical value per area (1 mm^2).

2.6. Measurement of kynurenine

Kynurenine was measured using high-performance liquid chromatography (HPLC) equipped with a spectrophotometric detector (Tosoh ultraviolet-8000, Tosoh, Tokyo, Japan) as described in a previous study [8].

2.7. Assay of IDO activity

IDO activity was assessed using the methylene blue/ascorbate assay, as described previously [15]. Briefly, the liver lysate was centrifuged at 7000 ×*g* at 4 °C for 10 min. The supernatant (50 μL) was then reacted with the substrate solution (50 μL) at 37 °C for 60 min. The substrate solution comprised 100 mM potassium phosphate buffer (pH 6.5), 50 μM methylene blue, 20 μg of catalase, 50 mM ascorbate and 0.4 mM D-tryptophan. After incubation, the samples were acidified with 3% perchloric acid and centrifuged at 7000 ×*g* at 4 °C for 10 min. The concentration of kynurenine in the supernatant of the reaction solution was measured using HPLC, and IDO activity was expressed as the kynurenine content per hour per milligram protein.

2.8. Real-time RT-PCR analysis

Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to quantify the levels of TNF-α, IFN-γ, IL-6, IL-10, MCP-1, and MIP-2 mRNA in the liver. Total RNA in the liver was isolated using Isogen (Nippon Gene, Tokyo, Japan) and reverse-transcribed to cDNA by using a High capacity cDNA transcription kit (Applied Biosystems, Foster City, CA). Purified cDNA was used as the template for real-time RT-PCR conducted with pre-designed primer/probe sets for TNF-α, IFN-γ, IL-6, IL-10, MCP-1, MIP-2 mRNA and 18S rRNA (Applied Biosystems), according to the manufacturer's recommendations. 18S rRNA was used as an internal control. Real-time RT-PCR was carried out using a Light-Cycler 480 system (Roche Diagnostic Systems).

2.9. Western blot analysis

Protein (40 μg) from the liver lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk and incubated with anti-IDO and anti-β-actin antibodies for 24 h at 4 °C, followed by incubation with peroxidase-labeled anti-rabbit IgG for 60 min at room temperature. Immunoreactive protein bands were visualized using ECL plus (GE Healthcare UK Ltd, England).

2.10. Isolation of liver mononuclear cells and flow cytometry analysis

Hepatic mononuclear cells (MNCs) were isolated as outlined in our previous report [16]. Briefly, the mouse liver tissue was minced using a pair of scissors and the resultant liver homogenate was filtered through a stainless steel mesh. MNCs were obtained by the centrifugation of liver homogenate with Ficoll-Conray (IBL, Gunma, Japan). Flow cytometry was used to evaluate the cell phenotypes in the liver MNCs at 0 h and 24 h after CTL injection. The MNCs were then stained with below-mentioned antibodies: FITC-conjugated hamster anti-mouse CD3ε antibody (clone 145-2C11; BD Biosciences, Franklin Lakes, NJ), FITC-conjugated rat anti-mouse CD4 antibody (clone RM4-5; BD Biosciences), PE-conjugated rat anti-mouse CD8 antibody (clone 53-6.7; BD Biosciences), CD49b PE-conjugated rat anti-mouse CD49b antibody (clone DX5; BD Biosciences) and FITC-conjugated rat anti-mouse F4/80 antibody (clone BM8; eBioscience, San Diego, CA). The phenotypic characterization of the MNCs was carried out using a FACScan flow cytometer (BD Biosciences).

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