Interferon-γ Inhibits Hepatitis B Virus–Induced NF-κB Activation Through Nuclear Localization of NF-κB–Inducing Kinase

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Background & Aims: **Nuclear factor-κB (NF-κB) signaling pathway is an important regulating pathway in liver diseases, including hepatocellular carcinoma. In our study, immunohistochemical analysis showed that NF-κB–inducing kinase (NIK), an upstream kinase of IκB kinases, nuclear localization occurs only in liver tissues obtained from hepatitis B surface antigen (HBsAg)(+) patients but not in tissues from HBsAg(−) patients. The aim of the present study was to identify the inducer of NIK nuclear localization and determine whether the NIK nuclear localization affects the hepatitis B virus (HBV)-mediated NF-κB activation.** ***Methods:*** **The experiments were performed on HepG2.2.15 cells and on HepG2 cells transfected with pHBV1.2×, a plasmid encoding all HBV messages, using NF-κB–dependent luciferase reporter gene assay, electrophoretic mobility shift assay, immunoblot analysis, and fluorescent microscopy analysis.** ***Results:*** **HBV induced NIK-dependent NF-κB activation. However, interferon (IFN)-γ induced NIK nuclear localization and inhibited NF-κB activation in HepG2.2.15 cells and in HepG2 cells transfected with pHBV1.2×. When NIK nuclear localization was inhibited by deletion of nuclear localization signal on NIK, IFN-γ did not induce the NIK nuclear localization and did not inhibit NF-κB activation.** ***Conclusions:*** **IFN-γ selectively inhibits HBV-mediated NF-κB activation. This inhibition is accomplished by NIK nuclear localization, which is a novel mechanism of NF-κB inhibition.**

Hepatitis B virus (HBV), a member of the hepadnavirus family, has infected 350 million people worldwide.[1] In 5%–10% of patients, the infection advances to lifelong chronic hepatitis B infection, which is a frequent precursor to cirrhosis and hepatocellular carcinoma (HCC).[2–4] HBV is an enveloped, partially double-stranded DNA virus that encodes 4 unspliced overlapping messages that terminate at a common polyadenylation signal.[5] Each messenger RNA encodes HBc and HBs structural proteins, HBV pol for genome replication, and HBx, which induces variable signal transduction[5]; HBV can trigger changes in variable intracellular signal transduction.[6] Among these changes, nuclear factor-κB (NF-κB) activation is one of the most important events because it is closely related to tumor development.[7,8] The transcription factor NF-κB is a key regulator in oncogenesis. By promoting proliferation and inhibiting apoptosis, NF-κB tips the balance between proliferation and apoptosis toward malignant growth in tumor cells.[7] Generally, in host cells, NF-κB is activated by pathogenic viruses, including Herpesvirus saimiri,[9] human immunodeficiency virus type 1,[10] Epstein-Barr virus,[11] hepatitis C virus,[12] and human T-cell leukemia virus type 1.[13] HBV also activates NF-κB in host cells, and HBx alone can activate NF-κB as well.[12,14–16] Previous report suggested that HBx activates NF-κB through oxidative stress.[17] In addition, other components (eg, large HBs) can activate NF-κB.[18,19] The activation of NF-κB by pathogenic viruses is also severely inhibited by the expression of NF-κB–inducing kinase (NIK) dominant-negative form.[9–12] In previous studies, NIK was found to function as a tumor necrosis factor receptor–associated factor 2 binding protein[20] and was recognized as an upstream kinase of IκB kinases (IKKs).[21–23] Recent studies indicate that NIK is essential for NF-κB activation induced by lymphotoxin-β via IKK-α but is not essential for NF-κB activation by tumor necrosis factors (TNFs) via IKK-β.[24,25] In contrast, many reports indicate that NIK participates in the overall activation of NF-κB, including activation occurring via IKK-α and IKK-β, although NIK is not essential for NF-κB activation dependent on IKK-β.[9,26–29] Activated IKKs, especially IKK-β, then phosphorylate IκBα, which releases NF-κB bound to IκBα,[30] and

Abbreviations used in this paper: DAPI, 4′,6-diamidino-2-phenylindole; EMSA, electrophoretic mobility shift assay; HBV, human hepatitis B virus; HCC, hepatocellular carcinoma; NF-κB, nuclear factor κB; NIK, NF-κB–inducing kinase; IKK, IκB kinase; IFN, interferon; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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IκBα are targeted for degradation by ubiquitination. Released NF-κB is translocated to the nuclear region and functions as a transcription factor that regulates genes needed for cell survival.[31] However, other studies have shown that the NF-κB/IκBα complex, NIK, and IKKs exist in nuclear regions through nucleocytoplasmic shuttling, in which continuous shuttling is very significant for NF-κB activation processes.[32–35]

A recent study has shown that NF-κB activation by HBx expression can be inhibited by interferon (IFN)-α and that this activation is not efficiently inhibited by IFN-γ in HuH-7 cells.[15] Nevertheless, in acute infections, IFN-γ is expressed by activated $CD4^+$ T cells,[36,37] and the failure of IFN-γ is suggested to be closely related to development of the chronic phase.[38] In the chimpanzee model, IFN-γ noncytolytically eliminated the viral DNA in HBV-infected chimps.[39,40] In addition, adoptively transferred HBV-specific $CD8^+$ T cells inhibit viral replication in HBV transgenic mice by a noncytopathic IFN-γ–mediated mechanism.[41]

In this study, we report HBV-specific IFN-γ inhibition of NF-κB activation. This inhibition is associated with NIK nuclear localization, which is a novel mechanism of NF-κB inhibition. In addition to IFN-γ suppression of HBV, selective suppression of the NF-κB activated by HBV is expected to play a significant role in preventing HCC development.

Materials and Methods

Plasmids

Construction of pHBV1.2×, a plasmid from which all HBV messenger RNAs can be transcribed, was previously described.[42] The pHBV1.2×(-X) mutation vector, which contains an ochre termination signal (CAA to UAA) after codon 7 in the HBx open reading frame, was generated by site-directed mutagenesis; no mutation was introduced in the overlapping HBV pol open reading frame. pCMV-HA/HBx was constructed by inserting an HBx gene fragment, generated by polymerase chain reaction (PCR), into the *Eco*RI site of pCMV-HA (Clontech, Mountain View, CA). The pCMV-HA/Pol and pCMV-HA/HBs plasmids were constructed by the same techniques used for constructing pCMV-HA/HBx. For constructing pCMV-FLAG/HBc, a PCR-generated HBc gene fragment was fused to a FLAG epitope and inserted into pRc/CMV (Invitrogen, Carlsbad, CA). The pFLAG-NIK (wild type), pFLAG-NIK/DN (dominant-negative mutation), and pNF-κB-luci plasmids were described previously.[43] pSTAT-1 (wild type) and pSTAT-1/DN (dominant-negative mutation) have been described previously.[44] pFLAG-IKK-α and pFLAG-IKK-β have also been described previously.[21] pCMV-β-Gal was constructed by inserting a β-galactosidase gene fragment into pRc/CMV. For expressing NIK deletion-mutation proteins, the deletion fragments of NIK were generated through PCR-mediated mutagenesis, and the fragments were inserted into pCMV-HA. As nomenclature for each construct, the starting and terminating amino acids are specified and combined with the following construct names: pCMV-HA/NIK(121-947) and pCMV-HA/NIK(152-947).

Cells and Transfections

HepG2.2.15 cells[45] (HBV-producing cells) and HepG2 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). Cells were transfected by using the FuGENE 6 transfection reagent (Roche, Mannheim, Germany) as instructed by the manufacturer. For transfections, the total DNA amount was normalized with backbone DNA, such as pCMV-HA, pRc/CMV, and pUC119.

Immunohistochemistry

Liver tissue specimens were fixed with 10% neutral-buffered formalin and embedded in paraffin. Sections were cut, deparaffinized, and developed in the immunostaining system TechMate 500 Plus (Dako, Carpinteria, CA). Blocking antibody was applied for 20 minutes at room temperature. The primary anti-NIK rabbit polyclonal antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) was added and incubated for 90 minutes at room temperature. Control slides were incubated with either buffer or nonimmunized rabbit IgG (1:1000, negative control). The secondary peroxidase-labeled antibody was applied and incubated for 30 minutes at room temperature. Sections were washed and then stained with 3′,3′-diaminobenzidine. Micrographs at magnification ×400 were acquired with a BX51 system equipped with DP70 (Olympus, Tokyo, Japan). The nuclei were stained with hematoxylin. For controls, each section was stained with H&E. Informed consent was obtained from the patients, and the local ethics committee approved this study.

Fluorescent Microscopy

HepG2 or HepG2.2.15 cells were seeded on poly-L-lysine-coated coverslips and either transfected or treated, as appropriate. Slides were washed with 1× phosphate-buffered saline (PBS), fixed with ice-cold methanol for 10 minutes, and blocked with 1% body surface area in 1× PBS for 20 minutes. Slides were then incubated for 90 minutes at room temperature with anti-NIK rabbit polyclonal antibody (1:400, Santa Cruz Biotechnology), anti-FLAG (M2) mouse monoclonal antibody (1:100, Sigma), or anti-HA mouse monoclonal antibody (1:100, Sigma). HBV gene expression was confirmed by costaining cells with the anti-HBc rabbit polyclonal antibody (1:100, DAKO). Next, slides were incubated for 30 minutes at room temperature with either fluorescein isothiocyanate-conjugated secondary antirabbit antibody (Sigma) for the rabbit polyclonal antibody or with rhodamine-conjugated secondary antimouse antibody (Chemicon, Temecula, CA) for the mouse