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Interferon alpha inhibits the nuclear factor kappa B activation triggered by X gene product of hepatitis B virus in human hepatoma cells

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Abstract X gene product of hepatitis B virus (HBV) (HBx) regulates many transcription factors including nuclear factor kappa B (NF-κB) and plays a key role in hepatocarcinogenesis. In this study, we demonstrated that the expression of full HBV genome and HBx gene similarly stimulated the transcriptional activity of NF-κB in HuH-7 human hepatoma cells, and that interferon (IFN)-α as well as dominant negative mutant of IκB kinase-α effectively inhibited the HBx-mediated NF-κB activation, but IFN-γ did not. These results suggest that IFN-α may have a function to block the NF-κB activating pathway triggered by HBx in HBV hepatocytes.

Key words: Hepatitis B virus X protein; Nuclear factor kappa B; Interferon alpha

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignant diseases worldwide, especially in several areas of Asia and Africa where hepatitis B virus (HBV) is the major etiologic factor for HCC. HBV encodes four open reading frames. Of these, the X gene product of HBV (HBx) is believed to play a key role in the hepatocarcinogenesis [1,2]. HBx is able to transactivate numerous cellular genes through activating transcription factors such as AP-1 [3,4], NF-AT [5], and nuclear factor kappa B (NF-κB) [6–8], which appears to contribute its oncogenicity.

NF-κB is involved in a number of cellular processes, including immune cell activation and development, stress responses, expression of inflammatory cytokines, and the control of apoptosis [9–13]. Recently, it has been reported that constitutive activation of NF-κB is detected in HCC tissues but not in surrounding normal tissues [14], and that NF-κB localization in HBV-related HCC was closely related to the expression of HBx [15]. These findings suggest the possible involvement of NF-κB in HCC development.

Interferon (IFN)-α plays an essential role in both antiviral and antitumor host defenses and has been used clinically for the treatment of viral infections and malignancies [16]. IFN-α treatment improves the overall clinical outcome in patients with chronic HBV infection [17,18]. IFN-α also delays or prevents HCC in patients with HBV-related cirrhosis [19]. In addition, Lai et al. [20] demonstrated a beneficial effect of IFN-α with a 31% response rate in patients with inoperable HCC caused by chronic HBV or hepatitis C virus infection. These observations assume that IFN-α may exhibit an antitumor activity in HBV-related HCC through inhibiting the HBx-mediated cellular responses.

In the present study, we aimed to clarify whether IFN-α can repress the transacting activity of NF-κB which is stimulated by transfection of either full HBV genome or HBx expressing vector in human hepatoma cells.

2. Materials and methods

2.1. Cell culture and biological reagents

The HuH-7 human hepatoma cell line was maintained in a chemically defined medium, IS-RPMI with 5% fetal bovine serum. Recombinant human IFN-α2a was provided by Nippon Roche Co. (Tokyo, Japan) and recombinant human IFN-γ1a was provided by Shionogi Co. (Osaka, Japan). In some experiments, varying concentrations of recombinant human IFN-α2a or recombinant human IFN-γ1 were added to the cell cultures.

2.2. Plasmids

The full length HBV DNA was cloned into the pGEM11Zf[+] vector (Promega, Madison, WI, USA) as described previously [21], registered in GenBank (accession number AB050018), and digested with Sap1 (New England BioLabs, Beverly, MA, USA) before transfection. Transfection of the full length linear monometric HBV genome can initiate a full HBV replication cycle, including production of viral RNAs, translation of viral proteins and release of virions. [21–23]. The HBx expression vector was generated from adr 125 subtype HBV DNA (JCRB Gene Bank registry no. VG024). The *Nco*I–*Bgl*II fragment of HBV DNA containing the entire coding sequence of the X gene was cloned into the expression vector, pHβApr-1, which contains the human β-actin promoter, in the sense or reverse orientation [24]. IκB kinase (IKK)α dominant negative form expression vector was kindly provided by Tularik Inc., South San Francisco, CA, USA. IκBα dominant negative form expression vector was purchased from Clontech Laboratories, Inc., Palo Alto, CA, USA.

2.3. Luciferase assay

The pNFκB-Luc (Stratagene, La Jolla, CA, USA) containing four copies of the binding sequence of NF-κB and firefly luciferase gene

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Abbreviations: IFN-α, interferon alpha; IFN-γ, interferon gamma; NF-κB, nuclear factor kappa B; IKK, IκB kinase; NIK, NF-κB-inducing kinase; HBV, hepatitis B virus; HBx, X gene product of HBV; HCC, hepatocellular carcinoma

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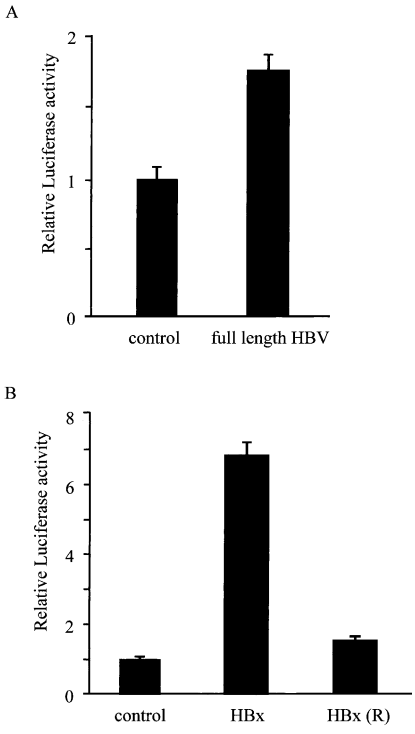

Fig. 1. Effect of HBV genome expression or HBx on the transcriptional activity of NF-κB in HuH-7 cells. 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with 0.3 μg of pGEM11Zf[+] control vehicle or 0.3 μg of full length HBV DNA into HuH-7 (A). 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with 0.3 μg of pHβApr-1 control vehicle, 0.3 μg of HBx expression vector or 0.3 μg of reverse-oriented HBx vector, HBx(R), into HuH-7 (B). Two days after transfection, luciferase activity in the cells was analyzed as described in Section 2. Data are expressed as mean ± S.D. of five separate experiments.

and pRL-TK-luc (Promega, Madison, WI, USA) containing the herpes simplex virus thymidine kinase promoter and expressing renilla luciferase gene were used in the assay. HuH-7 cells were grown in 24-well plates, and 0.3–0.5 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were transfected with 0.3 μg of full length HBV DNA or pGEM11Zf[+] into the cells using FuGENE™-6 (Roche Molecular Chemicals) according to the manufacturer's protocol. Similarly, 0.3–0.5 μg of pNFκB-Luc and 50 ng of pRL-TK-luc, or 0.3 μg of pLuc-MCS (Stratagene) which contains a basic promoter element (TATA box) but not NF-κB site and 50 ng of pRL-TK-luc were transfected with 0.3–0.5 μg of HBx expression vector, reverse-oriented HBx vector or pHβApr-1 into the cells. In some experiments, dominant negative IκBα expression vector or dominant negative IKKα expression vector was cotransfected into the cells. Two days after transfection, cell lysates were prepared and used for luciferase assay. The luciferase activities in the cells were determined by a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega). Data represent the ratios of firefly-luc activity derived from pNFκB-Luc or pLuc-MCS over renilla-luc activity derived from pRL-TK-luc relative to the control (pGEM11Zf[+] or pHβApr-1).

2.4. Western blot analysis

Immunoblotting with anti-HBx (Affinity Bioreagents) or anti-human β-actin (Sigma, Chemicals Co., St. Louis, MO, USA) was performed as described previously [25]. Cells were lysed by addition of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml PMSF (phenylmethanesulfonyl fluoride), 1 μg/ml of aprotinin, 1% NP40, 0.5% sodium deoxycholate) for 10 min at 4°C, and insoluble material was removed by centrifugation at 14 000 rpm for 30 min at 4°C. The same amount of protein from each lysate (20 μg/well) was subjected to 15% SDS–polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes which were then blocked for 1.5 h using 5% non-fat dried milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T), washed with PBS-T and incubated at room temperature for 1 h in the presence of each antibody. The membranes were washed with PBS-T and incubated with sheep anti-mouse IgG or donkey anti-rabbit IgG coupled with horseradish peroxidase (Amersham Life Science, Buckinghamshire, UK). The enhanced chemiluminescence system (ECL; Amersham) was used for detection.

3. Results

3.1. The expression of full HBV genome as well as HBx stimulates transcriptional activity of NF-κB

The effect of full HBV genome expression on the transcriptional activity of NF-κB was determined by transient transfection assay using luciferase reporter plasmid, pNFκB-Luc, which contains four repeats of the binding sequence of NF-κB. The full length HBV DNA transfection stimulated the luciferase activities from NF-κB-Luc at a maximal value 1.8 times higher than that of the control. (Fig. 1A). Similarly, the transfection of HBx expression vector but not reverse-oriented HBx vector stimulated the luciferase activities from NF-κB-Luc at a maximal value 6.9 times higher than that of the control (Fig. 1B).

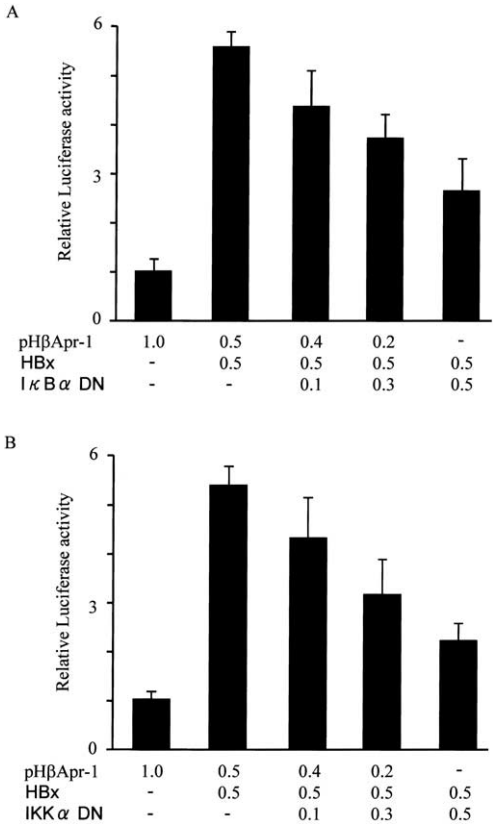

Fig. 2. Effect of dominant negative IκBα or IKKα on the HBx-mediated NF-κB activation. 0.5 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with or without 0.5 μg of HBx expression vector into HuH-7, together with indicated amounts (μg) of dominant negative IκBα expression vector (A) or dominant negative IKKα expression vector (B). To adjust the amounts of transfected DNA, indicated amounts of pHβApr-1 were also cotransfected. Two days after transfection, luciferase activity in the cells was analyzed as described in Section 2. Data are expressed as mean ± S.D. of five separate experiments.

3.2. HBx-mediated NF-κB activation is reduced by dominant negative IKKα and IκBα

Since the stimulus-induced degradation of IκBα is essential for NF-κB activation, we examined the effect of dominant negative form of IκBα on HBx-mediated NF-κB activation. As shown in Fig. 2A, The luciferase activity from NF-κB-Luc which was stimulated by HBx was dose dependently inhibited by the transfection of IκBα dominant negative expression vector. It is well known that IKK, a multicomponent protein complex composed of IKKα, IKKβ and IKKγ phosphorylates IκB and leads its degradation. Therefore, we examined whether dominant negative mutant of IKKα inhibit HBx-mediated NF-κB activation. As shown in Fig. 2B, the luciferase activity from NF-κB-Luc which was stimulated by HBx was significantly inhibited by the transfection of IKKα dominant negative expression vector in a dose-dependent manner. Taken together, it is possible that NF-κB activation by HBx is mediated by activation of IKKα and sequential IκBα degradation.

3.3. IFN-α but not IFN-γ effectively inhibits the HBx-mediated NF-κB activation

Next, we examined the effects of IFN-α on the HBx-mediated NF-κB activation in HuH-7 cells. As shown in Fig. 3C, the luciferase activity from NF-κB-Luc which was stimulated by HBx was significantly inhibited by the addition of IFN-α

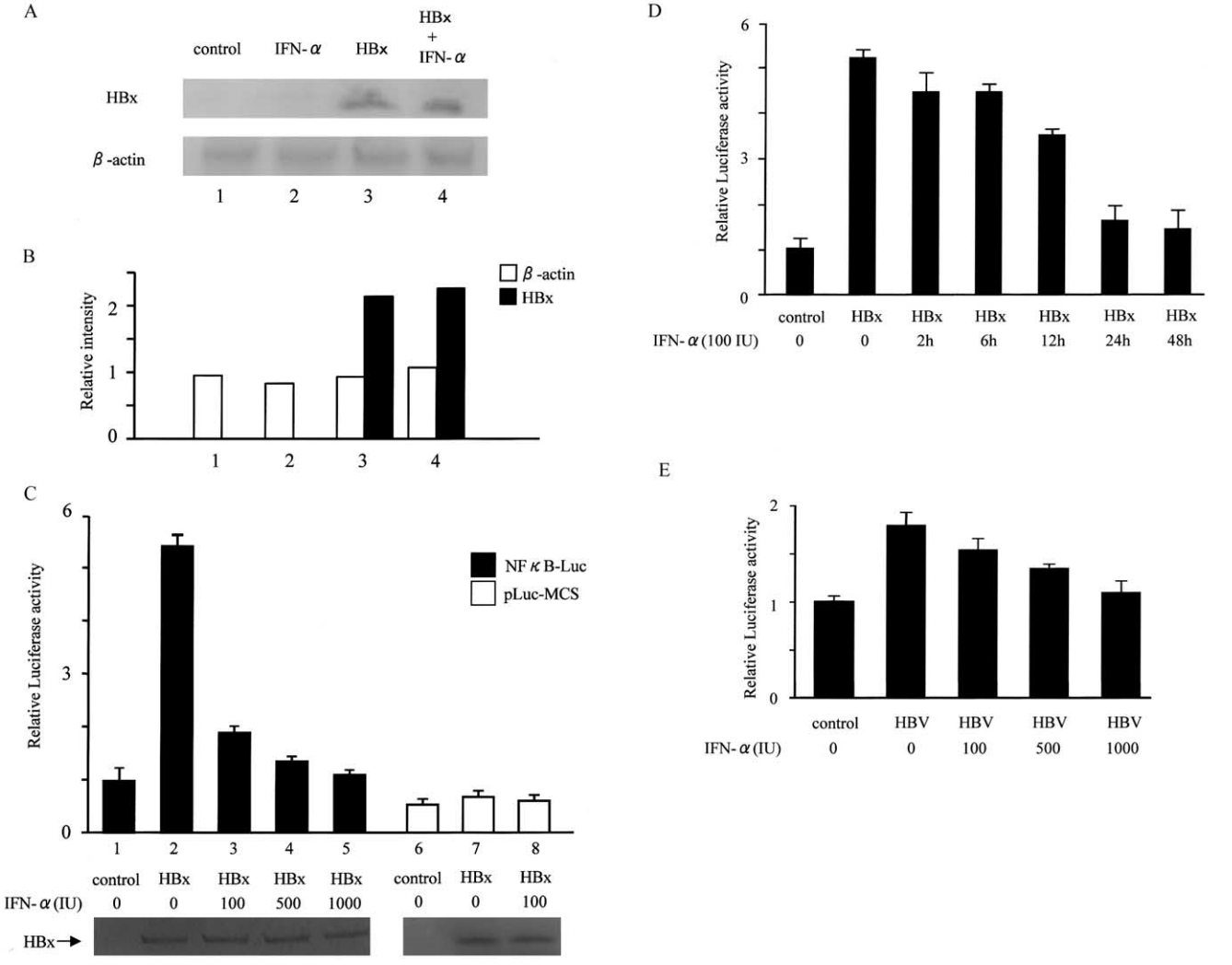

Fig. 3. Effect of IFN-α on the HBx-mediated NF-κB activation. A: HuH-7 cells were transfected with 0.3 μg of pHβApr-1 (lanes 1 and 2) or HBx expression vector (lanes 3 and 4). Twenty-four hours later, the cells were incubated with (lane 2 and 4) or without (lanes 1 and 3) 100 IU/ml of IFN-α for 24 h, then HBx expression was analyzed by Western blotting. β-actin was used as an internal control. B: The signal intensities shown in A were quantified with NIH image analysis software. Data are expressed relative to the intensity of β-actin (lane 1). The open and closed bars indicate relative intensities of β-actin and HBx, respectively. C: Combination of 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc (lanes 1–5) or combination of 0.3 μg of pLuc-MCS and 50 ng of pRL-TK-luc (lanes 6–8) were cotransfected with 0.3 μg of pHβApr-1 control vehicle or 0.3 μg of HBx expression vector into HuH-7. Twenty-four hours later, the cells were incubated with varying concentrations (0–1000 IU/ml) of IFN-α for 24 h, then luciferase activity in the cells was analyzed as described in Section 2. Data are expressed as mean ± S.D. of five separate experiments. A representative HBx expression in each cell lysate is also shown at the bottom. D: 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with 0.3 μg of pHβApr-1 control vehicle or 0.3 μg of HBx expression vector into HuH-7. Twenty-four hours later, the cells were incubated with 100 IU/ml of IFN-α for varying periods (2–48 h), then luciferase activity in the cells was analyzed. E: 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with 0.3 μg of pGEM11Zf[+] control vehicle or 0.3 μg of full length HBV DNA into HuH-7. Twenty-four hours later, the cells were incubated with varying concentrations (0–1000 IU/ml) of IFN-α for 24 h, then luciferase activity in the cells was analyzed.

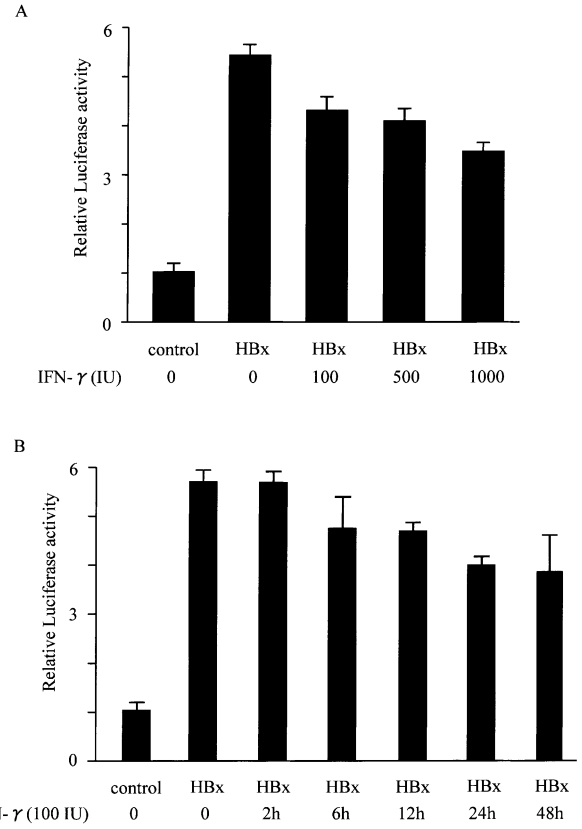

Fig. 4. Effect of IFN-γ on the HBx-mediated NF-κB activation. 0.3 μg of pNFκB-Luc and 50 ng of pRL-TK-luc were cotransfected with 0.3 μg of pHβApr-1 control vehicle or 0.3 μg of HBx expression vector into HuH-7. Twenty-four hours later, the cells were incubated with varying concentrations (0–1000 IU/ml) of IFN-γ for 24 h (A) or incubated with 100 IU/ml of IFN-γ for varying periods (2–48 h) (B), then luciferase activity in the cells was analyzed as described in Section 2. Data are expressed as mean ± S.D. of five separate experiments.

in a dose-dependent manner (lanes 1–5), however, these changes were not observed in the assay using pLuc-MCS reporter plasmid lacking the NF-κB binding site (lanes 6–8). In addition, inhibitory effect of IFN-α on the HBx-mediated NF-κB activation became obvious in parallel with the incubation periods (Fig. 3D). Western blot analysis showed that the IFN-α had no effect on the HBx expression in HuH-7 cells (Fig. 3A–C). Therefore, it is likely that IFN-α could block the NF-κB activating pathway triggered by HBx. Similarly, IFN-α also dose dependently repressed the NF-κB activation induced by full length HBV DNA transfection (Fig. 3E). However, in contrast to IFN-α, IFN-γ only slightly suppressed the HBx-mediated NF-κB activation even in the higher concentrations or longer incubation periods (Fig. 4A,B).

4. Discussion

In the current study, the vector-based HBx expression activated transcriptional activity of NF-κB in HuH-7 cells in accordance with other in vitro studies [6–8]. In addition, transfection of full length linear monometric HBV genome could weakly but significantly activate NF-κB, which was probably due to the very low level of HBx expression from full length HBV genome (data not shown). Recently, Guo et al. [15] reported that NF-κB was expressed diffusely in HCC tissues and its localization in the cells was closely related to the expression of HBx. They detected that NF-κB was expressed in both the cytoplasm and nuclei of HCC cells in which HBx was positive, whereas NF-κB was expressed only in the cytoplasm of HCC cells in which HBx was negative. They further confirmed that HBx could stimulate the translocation of NF-κB into nucleus in cultured HCC cells. These findings suggested that activation of NF-κB is also linked to HBx in vivo.

However, how HBx stimulates NF-κB activity has yet to be determined. HBx has been reported to activate NF-κB through turning on the ras-raf-MAP kinase cascade [6,7] and inducing the degradation of IκBα and NF-κB precursor/inhibitor p105 [7]. Alternatively, HBx could directly interact with IκBα and prevent its association with NF-κB and induce its disassociation from NF-κB [8]. In this study, we showed that HBx-mediated activation of NF-κB was repressed by expression of dominant negative IKKα and IκBα. These results are consistent with the recent report by Kim et al. [26] that dominant negative NF-κB-inducing kinase (NIK), IKK or IκB markedly attenuated the HBx-mediated NF-κB activation, and that the target disruption of tumor necrosis factor-α (TNF-α) receptor1 (TNFR1) gene completely abolished the HBx-mediated NF-κB activation. Taken together, it is likely that TNFR1 and following NIK-IKK-IκB signaling play essential roles in HBx-mediated NF-κB activation.

IFN-α are known to have antiproliferative effects on human HCC cells, both in vivo and in vitro [27,28]. The effectiveness of IFN-α in treating HCC patients has been reported positively in a few previous clinical trials [20,29]. It was also shown that IFN delays or prevents HCC in patients with HBV-related cirrhosis [19]. In addition, combination of IFN-α and 5-FU significantly prolonged the survival rate of patients with HCC [30]. These observations suggest that IFN-α has a capability of anticancer agents against HCC although its precise mechanisms are not fully understood. Recently, it was reported that IFN-α suppressed the activation of NF-κB composed of RelA-p50 and potentiated TNF-α-induced apoptosis in Jurkat cells and human cervical cancer cells [31,32]. Similarly, Shigeno et al. [33] reported that IFN-α pretreatment repressed the TNF-related apoptosis inducing ligand (TRAIL)-mediated activation of NF-κB composed of RelA-p50, and decreased its transcriptional activity in HuH-7 cells, resulting in sensitizing these cells to TRAIL-induced apoptosis. Moreover, Wen et al. [34] recently reported that overexpression of p202, an IFN-α-inducible protein, was capable of sensitizing breast cancer cells to TNF-α-mediated apoptosis through the inactivation of NF-κB by its interaction with p202. In the present study, IFN-α significantly inhibited the HBx-mediated NF-κB activation in dose- and time-dependent manner. Since HBx activates NF-κB signaling similar to TNF-α [27], it is conceivable that IFN-α can inhibit HBx-mediated NF-κB activation as well as TNF-α-mediated NF-κB activation. Whereas, IFN-γ only weakly inhibited the HBx-mediated NF-κB activation, suggesting that the IFN-α specific signaling molecules such as an IFN-stimulated gene factor-3 may mediate an inhibitory effect of IFN-α.

In conclusion, we have demonstrated that IFN-α could inhibit the HBx-mediated activation of NF-κB in human hepatoma cells. These results suggest that IFN-α may exhibit

antitumor activity in HBV-related HCC through inhibiting the NF-κB activation triggered by HBx.

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