Human Interleukin 6 Gene Is Activated by Hepatitis B Virus-X Protein in Human Hepatoma Cells

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ABSTRACT

Interleukin 6 (IL-6) is a pleiotropic cytokine that induces many biological activities, including some aspects of the immune reaction and inflammatory responses. In the liver, IL-6 regulates the synthesis of a broad spectrum of acute-phase proteins. IL-6 is also known to be a factor involved in the immunoregulatory perturbations in patients with chronic liver diseases (CLDs). Here, we involved in the immunoregulatory perturbations in patients with CLD proteins. IL-6 is also known to be a factor in acute-phase reaction and inflammatory responses. In the University Medical College, Seoul [S. K. Y., Y. M. P.], Korea of Bioscience and Biotechnology, KIST, Taejon 305-606 [Y. L., U.-Young 1k Lee2 Yoonik Lee, Ui-Sun Park, Inpyo Choi, Seung Kew Yoon, Young Min Park, and Young Ik Lee2

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INTRODUCTION

IL-6 is a multifunctional cytokine with both differentiation and growth-promoting effects for a variety of target cell types. In the liver, IL-6 regulates the synthesis of a broad spectrum of acute-phase proteins (1). Also, IL-6 is involved in the pathogenesis of many fibrogenic diseases. IL-6 is induced by a number of different signals, including cytokines, viral infections, bacterial endotoxins, serum, and double-stranded polynucleotides (2, 3). Thus, the expression of IL-6 gene is regulated by many substances that trigger inflammations. Up-regulated expression of the IL-6 gene appears to be involved in pathological conditions (4, 5), and it has also been hypothesized that activation of IL-6 gene might trigger initial events leading to oncogenic transformation (5). High levels of IL-6 have been detected in the sera of patients with alcoholic LC (6), HBV infection (7, 8), and acute hepatitis (9). HBV, one of the hepadnavirus group, has a partial double-stranded DNA genome with four overlapping open reading frames. The smallest open reading frame, called X, encodes a protein of 154 amino acids that acts as a viral transactivator on a wide variety of promoters, including cellular and viral promoters (10). The X-responsive elements in these promoters include many different transcription factors, such as AP1, AP2, and NF-kB. HBV-X protein does not bind to its response element on DNA; rather, it acts indirectly through protein-protein interactions with other transcription factors or the signal transduction pathway. Although the role of HBV-X in HBV pathogenesis is not clearly defined, almost all infected individuals have antibodies to HBV-X, suggesting that it is expressed during the replication cycle (11). In this experiment, we found high IL-6 levels in patients with CH-B, LC, and LC+HCC caused by HBV. Because all patients were infected with HBV, we checked whether HBV-X was involved in up-regulation of IL-6. We demonstrated that IL-6 can be induced by HBV-X. HBV-X transactivates the human IL-6 promoter through the NF-kB-binding site. The up-regulation of IL-6 by HBV-X protein can induce hepatic inflammation and can play an important role in the pathogenesis of fibrosis and diseases of the liver, which can eventually lead to LC and HCC.

The abbreviations used are: IL, interleukin; LC, liver cirrhosis; HBV, hepatitis B virus; NF-kB, nuclear factor-kB; HCC, hepatocellular carcinoma; LC+HCC, LC with HCC; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; HCV, hepatitis C virus; ALT, alanine aminotransferase; PT, prothrombin time; HBsAg, hepatitis B surface antigen; CAT, chloramphenicol acetyltransferase; CLD, chronic liver disease; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; AH, acute hepatitis.

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Activation of Human IL-6 Gene in Hepatoma Cells

The mean serum IL-6 levels measured from nine healthy subjects in all age ranges, implying that normal IL-6 levels in serum do not change with age. HBsAg, anti-HBx, anti-HBe, and ALT normal range, 0-35 units/liter. ALB, albumin (normal range, 3.0-5.6 g/dl); PT, normal range, 70-100%.

TABLE 1 Clinical features and serum IL-6 levels of patients studied

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No.</th>
<th>Age (yr)</th>
<th>Sex (M/F)</th>
<th>ALTa (units/liter)</th>
<th>PT (%)</th>
<th>ALB (g/dl)</th>
<th>IL-6 (pg/ml)</th>
<th>Etiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH-B</td>
<td>20</td>
<td>33 ± 9</td>
<td>13/7</td>
<td>156.7 ± 56.2</td>
<td>98.6 ± 2.7</td>
<td>4.0 ± 0.2</td>
<td>11.5 ± 19.1</td>
<td>HBV</td>
</tr>
<tr>
<td>CH-C</td>
<td>12</td>
<td>45 ± 10</td>
<td>8/4</td>
<td>134.7 ± 66.7</td>
<td>98.3 ± 3.3</td>
<td>4.2 ± 0.3</td>
<td>34.3 ± 28.9</td>
<td>HCV</td>
</tr>
<tr>
<td>LC</td>
<td>17</td>
<td>55 ± 7</td>
<td>12/5</td>
<td>44.8 ± 21.4</td>
<td>61.5 ± 18.3</td>
<td>2.7 ± 0.4</td>
<td>33.4 ± 29.4d</td>
<td>HBV</td>
</tr>
<tr>
<td>LC+HCC</td>
<td>17</td>
<td>56 ± 6</td>
<td>13/4</td>
<td>48.0 ± 27.6</td>
<td>76.0 ± 17.6</td>
<td>3.2 ± 0.7</td>
<td>24.0 ± 15.1d</td>
<td>HBV</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>28 ± 2</td>
<td>9/3</td>
<td>21.8 ± 4.0</td>
<td>98.5 ± 2.4</td>
<td>4.0 ± 0.2</td>
<td>8.3 ± 3.9</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05, compared to control.

Materials and Methods

Patients Studied. Studies were undertaken on 20 patients with CH-B, 12 patients with CH-C, 17 patients with LC, and 17 patients with LC+HCC, as shown in Table 1. Patients with liver diseases were grouped by the following criteria: patients who were positive for HBsAg and HBeAg and had elevated ALT levels for >6 months were biopsy-tested and were grouped as CH-B patients. Patients who had abnormally high ALT levels and were anti-HCV antibody-positive and negative for HBsAg for >6 months with liver biopsy tests were grouped as CH-C patients. Patients whose biopsy tests showed hepatic fibrotic character with nodular regeneration and disturbance of the normal hepatic architecture and portal hypertension with liver failure, with low GOT or GPT levels (<100), were grouped as LC patients. LC was also confirmed by ultrasonogram, liver scintigraphy, and/or abdominal computed tomography. Patients with HBsAg-positive LC with high serum levels of α-fetoprotein (400 ng/ml or higher) and α-GTP, confirmed by abdominal computed tomography, were grouped as LC+HCC patients. None of the LC or LC+HCC patients in this study were coinfected with HCV. Nine control individuals who lacked a history of previous exposure to HBV showed negative serological tests for HBsAg, anti-HBs, and anti-HBe; and had normal liver function tests were randomly selected for the study. The mean serum IL-6 levels measured from nine healthy subjects (8.3 ± 3.9 pg/ml) coincided well with the experiments of Sun et al. (12), in which they got <15 pg/ml from 21 healthy subjects in all age ranges, implying that normal IL-6 levels in serum do not change with age. HBsAg, anti-HBx, anti-HBc, HBeAg, and anti-HBe were assayed with commercially available EIA kits (Abbot Laboratories, Chicago, IL). Details of the patients studied are summarized in Table 1.

Measurement of Serum IL-6 Levels. All serum samples were obtained from these patients when they were admitted to hospital. These sera and control sera were kept at -80°C. Human IL-6 immunoassay kit (Quantikine human IL-6 immunoassay kit; R&D Systems, Minneapolis, MN) was used for checking IL-6 activity, and the whole procedure followed the manufacturer’s instructions.

Statistical Analysis. Statistical analysis was done by Student’s t-test. A P of <0.05 was reported as significant. An inverse correlation coefficient between serum IL-6 levels and PT level (%) was performed.

Cell Culture, DNA Transfection, and CAT Assays. MEM-α, PBS, fetal bovine serum, and trypsin-EDTA were purchased from Life Technologies, Inc. (Grand Island, NY). [14C]Chloramphenicol was purchased from Amersham Co. (Buckinghamshire, United Kingdom). Acetyl-CoA was purchased from Sigma Chemical Co. (St. Louis, MO). Human hepatoma cells Huh-7 (13), HepG2 (14), and X-expressing HepG2-4X were grown in DME-supplemented with 10% FBS. Levels of IL-6 in supernatant were measured by ELISA.

Fig. 1 IL-6 levels assayed by ELISA. After HBV-X transfection (0-20 μg), cells (5 × 10⁵ ml) were cultured for 48 h in RPMI 1640 supplemented with 10% FCS. Levels of IL-6 in supernatant were measured by ELISA.
HBV-X activates the human IL-6 promoter. A, diagram shows the IL-6 promoter (−1180 to +4) fused to the CAT gene. B, transactivation of IL-6 promoter by HBV-X in two different cell lines. The IL-6-promoter-CAT plasmids were cotransfected into HepG2 and Huh-7 cells with either a control vector (pMAM) or a HBV-X expression vector (pMAM-HX). C, dose dependence of HBV-X on transcriptional transactivation of the IL-6 promoter. The IL-6 promoter-CAT was activated by increasing the concentrations of pMAM-HX expression vector in HepG2 cells.

Gene was prepared from the pHBV 315-dimer plasmid, which has two copies of HBV DNA cloned into the BamHI site of pBR 322. The HBV-X expression plasmid, pMAM-HX, was constructed by cloning the X-open reading frame insert into the Sa/I site of pMAM-neo (Clontech, Palo Alto, CA; Ref. 18). Promoter regions (−1180 to +4) of IL-6 were isolated from pPBeta2 CAT plasmid (19) using BamHI and HindIII. After the ends were filled with Klenow fragment, the resulting fragment was inserted into the Sa/I site of plasmid pGEM41-CAT. The deletion mutant series of the IL-6 promoter (D1, D2, D3, and D4) was generated by PCR amplification using oligonucleotides designed to generate ends with HindIII and XbaI restriction sites (20). The PCR product was then cloned into pGEM41-CAT, which was cut with the same enzymes and purified in two sequential CsCl centrifugation steps. The vector pXT, which was purified from the CAT expression vector pCT (21) and contained the adenovirus major late promoter sequences from −50 to +33, linked directly to 60 nucleotides of the SV40 early region noncoding leader sequence (SV41 sequence 5235–5175). pXT-NFkB contains two NF-κB binding sequences, on pXT expression vector.

**RESULTS**

**Serum IL-6 Levels in Patients with LDs.** Serum IL-6 levels in the control and the patient studies are illustrated in Table 1. The mean value in the control was 8.3 ± 3.9 pg/ml. Serum IL-6 values in patients with CH-B (11.5 ± 19.1 pg/ml), CH-C (34.3 ± 58.9 pg/ml), LC (33.4 ± 29.4 pg/ml), and LC+HCC (24.0 ± 15.1 pg/ml) were observed. Serum IL-6 values in patients with CH-B were slightly higher than that of the control. On the other hand, patients with CH-C had increased activity of IL-6 compared to the control (P < 0.05). The most remarkable difference was observed in LC patients. The IL-6 value in LC patients was significantly higher than that of the controls (P < 0.001) and that of two patient groups (CH-C and CH-B; P < 0.001 in both studies). There was a significant correlation between serum IL-6 activity and serum ALT value in CH-B (P < 0.0001) and CH-C (P < 0.049), whereas there were no correlations in LC and LC+HCC (P > 0.05; data not shown). PT was negatively correlated with IL-6 in LC (P < 0.0001), whereas there were no correlations in CH-B.
Activation of Human IL-6 Gene in Hepatoma Cells

**Effect of HBV-X Gene Transfection on the Production and Secretion of IL-6.** We studied the effect of HBV-X on the production and secretion of IL-6 (Fig. 1). After transfection of pMAMneo (–HBV-X) or pMAM-HX (+HBV-X), the production and secretion of IL-6 by HepG2 cells in culture supernatants were detected by ELISA. As shown in Fig. 1, at 48 h after transfection, pMAM-HX induced a gradual increase of IL-6 production and secretion as the transfected pMAM-HX increased gradually. These results suggest that pMAM-HX may activate IL-6 transcription and production and may be secreted in the culture supernatant.

**HBV-X Protein Transactivates the Human IL-6 Promoter.** To determine whether HBV-X regulates the IL-6 promoter, a region of −1180 to +4 was fused to the bacterial CAT gene (Fig. 2A). Regulation of IL-6 promoter was monitored following transfection into HepG2 and Huh-7 cells with vector plasmid DNA (pMAM) or plasmid containing the insert of human HBV-X gene (pMAM-HX; Fig. 2B and C). Cotransfection of IL-6 promoter-CAT reporter construct with the expression vector (pMAM-HX) results in a 5–9-fold increase in the expression of CAT activity (Fig. 2B). When increasing amounts of HBV-X expression vector were cotransfected with the IL-6 promoter-CAT reporter construct, CAT activity increased progressively, suggesting that HBV-X activates IL-6 gene transcription in a dose-dependent manner (Fig. 2C).

**Mapping of the HBV-X Responsive Element in the IL-6 Promoter.** To determine whether HBV-X regulates IL-6 promoter through a specific response element, 5′ deletions of the promoter were constructed (Fig. 3A). Five plasmids were cotransfected with either a control expression plasmid (pMAM) or human HBV-X expression vector (pMAM-HX). The level of CAT activity was determined 48 h after transfections. The activities of the D0 and of deletion constructs D1 (−236), D2 (−137), D3 (−86), and D4 (−51) showed 8.6-, 8.8-, 8.9-, and 1.3-fold induction by HBV-X, respectively (Fig. 3B). Induction by HBV-X dropped rapidly when deletion reached −51. These results suggested that the sequence between −86 and −51 is important for the induction of IL-6 expression in the presence of HBV-X protein. This critical DNA region contains a sequence element (GGGATTTTCCC) with a high homology with the NF-κB motif which was originally identified on the immunoglobulin light chain gene (22).

**NF-κB Site Is Required for HBV-X Transactivation of IL-6 Promoter.** To ascertain the relevance of this NF-κB-binding site for the activation of IL-6 promoter by HBV-X, we
Fig. 4  HBV-X regulates IL-6 expression through the NF-κB binding sites. A, IL-6 promoter NF-κB-binding sites were analyzed after site-directed mutagenesis. The sequence of the NF-κB-binding sites was obtained from pIL-6D1(wt) or the pIL-6D1-mt plasmid. B, wild-type (pIL-6D1) plasmid was transfected into HepG2, and Huh-7 cells, mutant (pIL-6D1-mt) plasmid was transfected into HepG2 cells, and cell extracts were assayed for CAT activity. C, schematic diagram of (NF-κB) X 2 – CAT reporter (pXT-NF-κB) and control vector (pXT). In pXT-NF-κB, two NF-κB-binding sites (GGGATTTCCC) were inserted in CAT expression vector (pXT), and pXT was used as a control. D, effect of HBV-X on transcription of the NF-κB-binding sites in HepG2 cells. The parental plasmid pXF-NF-κB and the control plasmid pXT were cotransfected into HepG2 cells with either a control vector (pMAM) or an X-expression vector (pMAM-HX).

used a mutant IL-6 promoter-CAT construct, in which the NF-κB binding site was mutated. Three bp within the putative NF-κB binding sites were mutated and introduced into the IL-6 D1 plasmid (IL-6 D1mt; Fig. 4A). Transfection of the mutated IL-6/CAT plasmid into HepG2 cells with HBV-X expression vector showed that the activity of the mutant IL-6 promoter was completely lost (Fig. 4B, Lane 6), whereas the wild type was not (Fig. 4B, Lanes 2 and 4). The transcriptional activity of the NF-κB element was examined using a heterologous promoter-reporter construct containing the NF-κB sequence inserted upstream of a minimal promoter (Fig. 4C). When transfected into the HepG2 cells, the minimal promoter vector (pXT) was not inducible by HBV-X (Fig. 4D, Lane 2), whereas the construct containing two copies of NF-κB element (pXT-NF-κB) was strongly activated by HBV-X (Fig. 4D, Lane 4). pXT-NF-κB construct was also PMA-inducible when transfected into HepG2 cells (Fig. 4D, Lane 5). pXT-NF-κB construct showed a synergistic effect on DNA and HBV-X treatment experiments (Fig. 4D, Lane 6). These results indicate that the kB site is essential and critical for the activation of the IL-6 promoter by HBV-X.

Induction of NF-κB Binding by HBV-X in HepG2 Cells.

We tested the ability of HBV-X to modulate the NF-κB DNA-binding activity. Because of the small percentage of the cells that are transfected by HBV-X expression vector (pMAM-HX), we not only used the HBV-X-transfected HepG2 cells [HepG2(X)], but we also used the HBV-X-expressing cell line (HepG2-4X) that constitutively expressed large quantities of HBV-X. The nuclear extracts from HepG2, HepG2(X), and HepG2-4X were checked for NF-κB consensus sequence-binding activity (Fig. 5A, Lanes 2 and 3), whereas the control band was not (Fig. 5A, Lane 5). Two predominant protein-DNA complexes were bound in vitro with nuclear extracts (Fig. 5A, Lanes 1 and 2). In transiently HBV-X-transfected HepG2 cells [HepG2(X)] or constitutively HBV-X-producing cells (HepG2-4X; Fig. 5A, Lanes 3 and 4), the NF-κB binding to its consensus DNA probe increased compared to the untransfected HepG2 cells (Fig. 5A, Lane 2). Competition experiments with unlabeled NF-κB consensus sequences showed a decrease of both the bands (Fig. 5A, Lanes 1 and 2).

Characterization of HBV-X Inducible NF-κB Element.

To characterize further the NF-κB, activated by HBV-X, we used specific antibodies raised against some members of the NF-κB family. As verified in Fig. 5A, increased binding of two predominant protein-DNA complexes was found (Fig. 5A, Lanes 3 and 4), whereas competition experiments with unlabeled NF-κB consensus sequences showed a decrease of both bands (Fig. 5B, Lanes 3 and 4). Supershift of DNA-protein complexes was...
observed by antibodies against p52 and p65 NF-κB subunits (a and b; Fig. 5B, Lanes 7 and 8), in which no shifted bands were observed when treated with c-rel or p50 antibodies (Fig. 5B, Lanes 6 and 9).

**DISCUSSION**

We report here that the HBV-X trans-activates the IL-6 gene. This was evidenced by high level serum IL-6 (Table 1) in patients with CLD, as well as by enhanced production of IL-6 (Fig. 1) in HBV-X-transfected cells. Functional studies were also conducted to identify the X-responsive element on IL-6 promoter. We demonstrated that the HBV-X responsive cis element on IL-6 promoter is NF-κB. The transcriptional activity of the NF-κB element was demonstrated using a heterologous reporter construct containing the NF-κB sequence inserted upstream of the minimal reporter. The result showed that the κB site is essential and critical for the activation of the IL-6 promoter by HBV-X (Fig. 4). In our experiment, we demonstrated that HBV-X enhances the NF-κB-DNA-binding abilities. Both subunits, p65 and p52, showed the enhanced DNA-binding abilities.

Constitutive and high production of inflammatory cytokines, such as IL-1 and IL-6, that also participate in normal immune functions, may have deleterious effects on the host; therefore, their synthesis must be tightly controlled (19). IL-6 induces hepatic inflammation by inducing acute-phase protein synthesis in the liver. IL-6 gene transcription is induced in a variety of different normal tissues in response to RNA and DNA virus infection, endotoxin, serum, and inflammation-associated cytokines such as IL-1, TNF, platelet-derived growth factors, and IFNs (23–25). Based on extensive studies carried out in many different laboratories, the transcription regulatory elements present in the 5' flanking region of the human IL-6 gene have been identified (26–29). The NF-κB site in the IL-6 promoter appears to contribute to the activation of the gene in some cell types (30). Because of rapid induction of genes encoding defense and signaling proteins by NF-κB, it is believed that NF-κB is an immediate early mediator of immune and inflammatory responses. Also, IL-6 induces hepatic fibrosis characterized in the deposition of extracellular matrix, hepatocellular necrosis and induces collagen synthesis by the liver tissues and lipocytes. High levels of IL-6 were detected in the sera of the patients with alcoholic LC, HBV infection, and AH (12, 31). Elevated IL-6 levels in AH patients (16.5 ± 14.5 pg/ml in AH, 26.3 ± 19.0 pg/ml in severe AH, and 470.2 ± 261.4 pg/ml in FHF; control level, 5.2 ± 0.6 pg/ml) already suggested a possible marker for identifying the clinical status in AH, in particular, FHF, and that IL-6 may have some role in hepatic injury (12). During acute and chronic HBV infection, it is likely that the tissue damage is the result of a complex interplay of specific T-cell antigen reactions and inflammatory mediators, including the cytokines. Among these inflammatory mediators are TNF, IL-1β, and IL-6. So, in liver disease, cytokines are involved in the onset of intrahepatic immune responses, in liver regeneration, and in the fibrotic and cirrhotic transformation of the liver after chronic chemical injury or viral infections (32). Also increased production of IL-1β and IL-6 in chronic HBV infection is strongly associated with the amounts of fibrosis in

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**Fig. 5** Induction of DNA-binding activity of NF-κB by HBV-X in HepG2 cells. A, induction of DNA-binding activity of NF-κB by HBV-X. Nuclear extracts obtained form HepG2, HBV-X-transfected HepG2 [HepG2(X)], and HBV-X constitutively expressing HepG2 cells (HepG2-4X) were used. Purified nuclear extracts (mg) were incubated in a reaction mixture containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 2 μg of poly(dI)-poly(dC), and 1 μl of γ-32P-labeled double-stranded probe (5–10 × 106 cpm/ng) in a final volume of 20 ml. Reactions were incubated at room temperature for 20 min and then loaded onto 5% polyacrylamide gels and electrophoresed in 0.5 × 45 mM Tris-borate and 1 mM EDTA. Gels were then dried and autoradiographed. Competition experiments with a cold NF-κB probe are shown. Lane 1, control (probe); Lane 2, HepG2 extract; Lane 3, HepG2-4X extract; Lane 4, HepG2-4X + competitor; Lane 5, HepG2(X) extract; Lane 6, HepG2(X) + competitor. B, characterization of the NF-κB element of the HBV-X activated IL-6 gene. 32P-labeled NF-κB sequences on IL-6 gene were incubated with 10 μg of nuclear extracts obtained from HBV-X-treated or untreated cells. NF-κB subunit antibodies (anti-P50, P52, P65, and c-rel) were used for a DNA-protein complex supershift experiment. Lane 1, control (HepG2 extract); Lane 2, PMA-treated HepG2 extract; Lane 3, HepG2(PMA) + competitor; Lane 4, HBV-X-transfected HepG2 extract; Lane 5, HepG2(X) + competitor; Lane 6, HepG2(X) + p50Ab; Lane 7, HepG2(X) + p52Ab; Lane 8, HepG2(X) + p65Ab; Lane 9, HepG2(X) + c-relAb.
liver (32, 33). Muto et al. (34) reported that, in many HHF patients who have high serum IL-1 and TNF, levels that stimulated T cells resulted in hyperproduction of IL-6. This hyperproduction of IL-6 caused the rapid impairment of the hepatocytes by enhancing CTL cytotoxic activity. It is conceivable that the induction of the IL-6 gene by HBV-X in hepatoma cells also plays an important role in the pathogenesis and/or modifications of manifestation in HBV-associated CLD. Studies with transgenic mice carrying the HBV-X genome have shown that HBV-X could trigger chronic hepatitis (35). Therefore, it is important to clarify the regulatory mechanisms of the constitutive expression of IL-6 gene and how this expressed gene can play a role in HBV-associated CLD. Also, clinical studies of the in vivo significance of IL-6 activation by HBV-X will be necessary to define the disease process after HBV infection.

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