Development of PCR-ELISA for the Detection of Hepatitis B Virus x Gene Expression and Clinical Application

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The presence of hepatitis B virus x (HBx) antigen/antibody is known to correlate with the well-established serological markers of ongoing viral replication in the chronic phase of HBV infection, and strongly suggests that the level and duration of HBx expression may influence the outcome of the chronic infection. In this research, we developed a polymerase chain reaction enzyme-linked immunosorbent assay (PCR-ELISA) method for the detection of HBx gene expression. We also investigated its relationship to the progress of the disease in HBV-related patients. Peripheral blood mononuclear cells (PBMCs) were purely isolated, and reverse transcription-PCR (RT-PCR) was performed for improved sensitivity. The PCR products were determined by ELISA, and we investigated

the relationship of the proposed method to the clinical status of the patients. The PCR-ELISA used in this work was found to be at least 100 times more sensitive than the conventional PCR method, and even 8,000-fold diluted PCR products could be detected. The HBx concentrations significantly differed among control subjects (0.36 ± 0.09 , [$P < 0.01$]) and patients with chronic hepatitis (1.13 ± 0.34 [$P < 0.01$ compared to control]), liver cirrhosis (LC; 1.37 ± 0.28 [$P < 0.01$ compared to control]), and hepatocellular carcinoma (HCC; 1.48 ± 0.95 [$P < 0.01$ compared to control]). These findings suggest that monitoring of HBx could be useful for early diagnosis and prognosis in patients with chronic HBV infection, LC, and HCC. *J. Clin. Lab. Anal.* 19:139–145, 2005. © 2005 Wiley-Liss, Inc.

Key words: hepatitis B virus x (HBx); PCR-ELISA; peripheral blood mononuclear cell (PBMC)

INTRODUCTION

Hepatitis B virus (HBV) infection has various sequelae. Most infected patients remain asymptomatic for long periods, while others develop acute hepatitis, chronic liver disease (CLD), and/or primary hepatocellular carcinoma (HCC) (1). In many patients, the prevalence and persistence of liver disease during chronic infection correlate with serum markers of ongoing virus replication (2). One of the HBV genes, x, encodes a polypeptide of 154 amino acids with a molecular weight of 17-kDa, and has been implicated in the carcinogenicity of HBV because of its ability to induce the transformation of rodent cells (3) and to induce HCC in transgenic mice (4). The HBV x (HBx)

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBx, hepatitis B virus x; HBs Ag, hepatitis B surface antigen; PBMC, peripheral blood mononuclear cell; BSA, bovine serum albumin; DIG, digoxigenin; PBST, phosphate-buffered saline containing 0.05% (v/v) Tween-20.

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protein activates transcription of many viral and cellular genes, and plays a major role in viral replication in animals (5–7). These findings strongly suggest that the level and duration of HBx expression may influence the outcome of chronic infection. Therefore, in the progression of the chronic state of the disease to the malignant transformation of infected hepatocytes, it may be important to monitor the expression of the x gene as a prognostic marker (8). Although liver inflammation is the pathological consequence of HBV infection, and hepatocytes are the site of the most active viral replication, the virus also invades the host immune system. HBV DNA, including replicative intermediates and covalently closed circular DNA, as well as virus RNA transcripts and DNA sequences integrated with the cellular genome, have been detected in peripheral blood mononuclear cells (PBMCs) from patients with active chronic hepatitis B (11–14). For a practical application as a diagnostic tool, we used PBMCs, which are easily obtained, to detect hepatitis B virus x gene expression. To detect even low levels of potential replication, we developed a polymerase chain reaction enzyme-linked immunosorbent assay (PCR-ELISA) method for the detection of HBx gene expression, and elucidated the relationship of HBx to the disease progress in HBV-related patients.

MATERIALS AND METHODS

Subjects

In this study, 84 subjects were examined, including 77 hepatitis B surface antigen (HBs Ag)-positive patients (44 with chronic hepatitis, 27 with liver cirrhosis (LC), and six with primary HCC). Seven subjects who were negative for HBV markers were used as controls. The protocol was approved by the Ethics Committee of Dankook University Hospital, and informed consent was obtained from all of the subjects involved in the study. Blood samples were obtained from Dankook University Hospital, Cheon-An, ChungNam, South Korea. Classic serological markers of HBV infection were determined by commercial assays (Dade Behring, Marburg/Lahn, Germany).

RT-PCR

PBMCs were harvested from the whole blood of patients by centrifugation over a Ficoll-Hypaque (Seromed Biochrom KG, Berlin, Germany) gradient (9,10). Total RNA was isolated from the PBMCs with the use of RNazol B reagent (Tel-Test, Friendswood, TX) according to the manufacturer's instructions, and RT-PCR was carried out to identify expressions

of GAPDH and HBx from the total RNA. RT was performed in a 20 µl reaction mixture containing 2 µl of 10× buffer, 0.5 µl of Rnase block ribonuclease inhibitor (40 U/µl), 2 µl of 100 mM dNTPs, and 1 µl of MMLV-RT (50 U/µl). The mixtures were incubated at 37°C for 1 hr. The cDNA was amplified using GAPDH and HBx specific primers (as described below) under the following PCR conditions: For GAPDH, the PCR reaction mixture was made in a final volume of 50 µl containing 4 µl of cDNA, 5 µl of 10× buffer, 4 µl of 10 mM dNTP, 1 µl of 10 pmole primers (sense and antisense), and 0.5 U of Taq polymerase. The PCR reaction was performed with a 30-cycle amplification (1 min at 95°C, 1 min at 60°C, and 1 min 30 sec at 72°C). To establish the HBx optimal PCR condition, we increased the concentration of 10× buffer and 5× band doctor (SolGent Inc., Daejeon, Korea), and sequentially changed the number of cycles.

Primers

The primers were designed according to one of the X gene region, position 1382–1520, in the HBV sequence (EMBL accession number Y07578) (11). The biotin-labeled HBx sense primer was 5'-GCT AGG CTG TGC TGC CAA CTG G-3', and the digoxigenin (DIG)-labeled HBx antisense primer was 5'-GGT CGG TCG AAA CGG TAG ACG G-3'. As a control, a house-keeping gene, GAPDH, was used. The primers for GAPDH were based on the published sequences of human GAPDH (15). The biotin-labeled sense GAPDH primer was 5'-AGA ACA TCA CAT CAG GAT TCC TAG G-3', and the DIG-labeled antisense GAPDH primer was 5'-CTA GTA TGC CCT GAG CCT GAG-3'.

PCR-ELISA

The biotin/DIG-labeled PCR amplification products bind to the well of streptavidin-coated microtiter plates. Detection of anti-DIG-HRP-labeled amplified products was carried out by means of a PCR-ELISA detection system. Ninety six-well ELISA plates were coated with 50 µl of 50 mM carbonate buffer containing 20 µg/mL streptavidin, at 4°C overnight. After the wells were washed three times with phosphate-buffered saline containing 0.05% (v/v) Tween-20 (PBST), they were incubated at RT for 2 hr with PBS containing 1% bovine serum albumin (BSA) and 0.01% sperm-DNA. The RT-PCR amplified products were diluted fivefold with 1% BSA in PBS. Then 50 µl of the diluted PCR products were added to each well, incubated for 2 hr at RT, and washed four times with PBST. The reaction mixture was incubated with 50 µl of dilution solution

(1% BSA, 5% FBS, and 0.3 M NaCl in PBS) containing anti-DIG conjugated horseradish peroxidase for 1 hr at 4°C. Color was developed by the addition of o-phenylenediamine (0.4 mg/mL in 0.05 M citric acid phosphate buffer, pH 5, containing 0.006% H₂O₂). Absorbance at 490 nm was measured with a Microplate Reader (Titertek Multiskan Mcc/340; Labsystems, Huntsville, AL) after the reaction was stopped by the addition of 50 µl of 2.5 N H₂SO₄ to each well.

Southern Blotting

Ten microliters of PCR product were loaded into a 2% agarose gel containing 0.5 g/mL ethidium bromide. The gel was soaked in depurination solution (0.2 N HCl) for 20 min, and denaturation solution (0.4 M NaOH, 1.5 M NaCl) for 20 min, and then incubated in neutralizing solution (0.5 M Tris-HCl, 1.5 M NaCl). This was followed by blotting overnight with 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate H₂O, pH 7.0). The membrane was prehybridized at 42°C for 1 hr and hybridized with a ³²P-labeled oligonucleotide HBx probe (5'-ACG TCC TTT GTT TAC GTC CC-3') at 42°C for 18 hr. After the membrane was washed, it was exposed to X-ray film for 24 hr at -70°C and developed.

RESULTS

Optimization of the PCR Condition

An optimal PCR condition that does not make nonspecific bands was established with the use of a 10 × buffer and a 5 × band doctor. Specific bands appeared in lane 4 of the 1 × buffer and the 2 × band doctor (Fig. 1A). For the 2 × buffer, the greater the amount of 5 × band doctor used, the stronger the band appeared (Fig. 1A, lanes 5–8). The optimum condition is shown in lanes 9 and 10 (Fig. 1B). To rule out any possible impairment by DNA contamination, we subjected an RT-minus control to PCR. No signal was observed (data not shown). The primers used in this work were designed to recognize the HBx region, as described in Materials and Methods. Nested PCR using outer and inner primers was performed, and confirmatory Southern blot hybridization using an internal primer for PCR amplification products demonstrated the presence of low-molecular-weight bands, indicating specific HBV-x gene (Fig. 2).

Sensitivity of PCR-ELISA

The sensitivity of PCR-ELISA using anti-DIG-HRP labeled amplified products was compared with the

Fig. 1. Effect of buffer conditions (A) and cycling numbers (B) on the performance of PCR for HBx. A: Lane 1: 1 × buffer; lane 2: 1 × buffer, 0.5 × band doctor; lane 3: 1 × buffer, 1 × band doctor; lane 4: 1 × buffer, 2 × band doctor; lane 5: 2 × buffer; lane 6: 2 × buffer, 0.5 × band doctor; lane 7: 2 × buffer, 1 × band doctor; lane 8: 2 × buffer, 2 × band doctor. B: Lanes 1 and 2: 24 cycles, lanes 3 and 4: 26 cycles, lanes 5 and 6: 28 cycles, lanes 7 and 8: 30 cycles, lanes 9 and 10: 32 cycles, lanes 11 and 12: 34 cycles.

Fig. 2. Southern blot analysis of the HBx PCR product. Aliquots of each PCR reaction mixture were separated on a 2% agarose gel in TAE buffer.

sensitivity of the conventional ethidium bromide-staining method after the PCR product was loaded into an agarose gel. The PCR/ELISA used in this work was found to be at least 100 times more sensitive than the conventional ethidium bromide-staining method for PCR products in agarose gel, and even 8,000-fold diluted PCR products could be detected (Figs. 3 and 4).

Diagnostic Application of PCR-ELISA

Chronic infection with hepatitis B virus is associated with a high risk of developing HCC. Several studies have suggested that the x gene product(s) of HBV are important factors in the pathogenesis of HCC. We assayed clinical samples from patients with chronic hepatitis, LC, and HCC for HBx by PCR-ELISA. The HBx concentrations differed significantly among control subjects (0.365 ± 0.096 , [$P < 0.01$]) and patients with chronic hepatitis (1.13 ± 0.348 [$P < 0.01$ compared to control]), LC (1.37 ± 0.285 [$P < 0.01$ compared to control]), and HCC (1.475 ± 0.95 [$P < 0.01$ compared to control]). These findings suggest that monitoring of HBx can be useful for early diagnosis and prognosis in patients with chronic HBV infection, LC, and HCC (Fig. 5).

DISCUSSION

This report describes the detection of HBx gene expression in PBMCs from chronic hepatitis patients, as

well as the relationship between HBx and the progression of the disease. Generally, HBV is considered to be highly hepatotropic. However, small amounts of HBV-specific nucleic acids can be detected in many extrahepatic tissues during acute and chronic infection (16–18). Also, there is evidence that extrahepatic HBV persists in HBV carriers after they undergo liver transplantation (19,20). Infection of PBMCs by HBV may interfere with host's immune defense against the virus and support the establishment of HBV persistence (21,22). Several earlier studies reported the presence of viral DNA, RNA, and replicative intermediaries in a wide range of extrahepatic sites, including PBMCs (23,24). HBV has a unique fourth open reading frame, termed the HBx gene (25,26). The HBx gene can activate the transcription of various viral and cellular genes, and induce liver cancer in certain transgenic mouse models (3,27,28). The presence of HBx antigen/antibody is known to correlate with the well-established serological markers of ongoing viral replication in the chronic phase of HBV infection, and it has been strongly suggested that the level and duration of HBx expression may influence the outcome of the chronic infection (29–31).

Previous studies reported that anti-HBx positivity was detected most often in sera containing markers of viral replication, such as HBe antigen, viral DNA, or both, which supports a close relationship between serum x antigen and markers of viral replication (2). Serological demonstrations of HBx have been reported in

Fig. 3. Optimization for efficient detection of PCR products by PCR-ELISA. A: PCR products were electrophoresed on 2% TAE agarose gel. B: PCR-ELISA for quantitative detection with GAPDH (1 μ g/well Streptavidin coating, PCR product dilution (0.1% BSA in PBS), Dig-HRP (1% BSA/5% FBS/0.1% Chaps/0.3 M NaCl in PBS), and OPD chromophore). A: GAPDH. B: HBx.

Fig. 4. ELISA for the serially diluted PCR products of HBx.

Fig. 5. Detection of HBx in various liver diseases using PCR-ELISA (GAPDH: 1000 × diluted; HBx: 200 × diluted): 1) normal liver, 2) CLD, 3) LC, and 4) HCC.

human sera. Moriarty et al. (32) observed a close relationship between the presence of anti-HBx antibodies and HBV-related HCC. It has also been reported that the frequency of anti-HBx found in HBe antigen-positive patients with chronic hepatitis is less than in the later stage of HBV carriers, such as in the case of anti-HBe positive chronic hepatitis or LC (2). These findings suggest that the frequency of anti-HBx increases with the duration of HBV infection, and that HBx may play a role in disease progression. We developed a simple and rapid PCR-ELISA method for the detection of HBx gene expression, and evaluated the relationship of HBx to the disease progress in HBV-related patients.

Conventional EtBr staining methods followed by gel electrophoresis for detecting HBx limits not only the number of samples used, but also the sensitivity of the method (33,34). In our PCR-ELISA method, the amplified products are immobilized on the streptavidin-coated surface of a microtiter plate via the strong affinity of the avidin-biotin interaction, and then the amplicons are detected with an anti-DIG-peroxidase conjugate. The PCR-ELISA showed 100 times higher sensitivity than the conventional PCR method, and even 8000-fold diluted PCR products could be detected. In addition to the high sensitivity and specificity of this

PCR-ELISA, it offers a further advantage in that with this procedure, detection of the PCR product is rapid, easy, and objective, and requires no electrophoresis apparatus, UV light, or darkroom. Furthermore, the use of toxic chemical agents, such as ethidium bromide, can be avoided. The technique also allows the simultaneous handling of a large number of samples, and can be automated, making it very attractive for use in any clinical laboratory (35–38). However, while the PCR-ELISA for HBx showed higher sensitivity and corresponded with clinical status, only a limited number of HBV-infected patients were examined in this study. Therefore, further studies involving more cases are needed before any conclusions can be drawn. Furthermore, before this PCR-ELISA method can be clinically applied, the relationship between HBx and the progression of HCC must be further examined.

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