Effects of hepatitis B virus X protein on human telomerase reverse transcriptase expression and activity in hepatoma cells

XIAODONG ZHANG, NAN DONG, HANG ZHANG, JIACONG YOU, HONGHUI WANG and LIHONG YE

TIANJIN, PEOPLE'S REPUBLIC OF CHINA

In subjects with hepatitis B, carcinogenesis has been associated with the hepatitis B virus (HBV) X protein (HBX) and human telomerase reverse transcriptase (hTERT). In the experiments reported here, we used immunohistochemical methods to study the expression of hTERT and HBV antigens (HBsAg, HBcAg and HBxAg) in 34 cases of HCC and corresponding paratumor tissues, 30 cases of liver cirrhosis, and 6 normal livers. To examine the effect of HBX on hTERT expression and activity in hepatoma cells, we transiently and stably transfected the pCMV-X plasmid cloned HBx gene into H7402 hepatoma cells, then measured the expression of c-Myc and hTERT in these cells with the use of Western-blot analysis. Telomerase activity was detected with the use of the telomerase repeat amplification protocol (TRAP) in transiently and stably transfected cells. We found that hTERT expression was 67.6%, 73.5%, and 100% in tumor, paratumor, and cirrhosis samples, respectively, but found no hTERT positivity in samples of normal liver. HBsAg, HBcAg, and HBxAg were expressed in 58.8%, 26.5%, and 76.5% of tumor tissues, respectively; in 64.7%, 41.2%, and 85.3% of the corresponding paratumor tissues; and in 76.7%, 66.7%, and 100% of cirrhotic tissues. The χ^2 test revealed no significant difference between the expression of hTERT and HBxAg in these tissues. Western-blot analysis revealed that expression of c-Myc and hTERT in the transiently transfected cells was much greater than that in the control cells. We elicited a similar result when we used the TRAP method to measure telomerase activity. Our data collectively demonstrate that HBX up-regulates the expression and activity of hTERT in hepatoma cells, suggesting that hTERT is associated with tumor development. (J Lab Clin Med 2005;145:98–104)

Abbreviations: anti–β-tubulin = antibody to β-tubulin; anti-HBc = antibody to hepatitis B core antigen; anti-HBe = antibody to hepatitis B e antigen; anti-HBs = antibody to hepatitis B surface antigen; anti-HBx = antibody to hepatitis B X antigen; anti-hTERT = antibody to human telomerase reverse transcriptase; cDNA = complementary DNA; DAB = 3, 3′-diaminobenzidine; HBcAg = hepatitis B core antigen; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus; HBX = hepatitis B virus X protein; HBxAg = hepatitis B X antigen; HCC = hepatocellular carcinoma; hTERT = human telomerase reverse transcriptase; mRNA = messenger RNA; PBS = phosphate-buffered saline solution; PCR = polymerase chain reaction; RT-PCR = reverse transcription–polymerase chain reaction; SABC = streptavidin-biotin complex; SDS = sodium dodecyl sulfate; SDS-PAGE = sodium dodecyl sulfate–polycrylamide gel electrophoresis; TPBS = 0.1% Triton X 100–phosphate-buffered saline solution

From the Department of Cancer Research, Institute for Molecular Biology, and the Department of Biochemistry, College of Life Sciences, Nankai University, Tianjin, People's Republic of China.

Supported by grant 043113411 from the Tianjin Natural Scientific Foundation.

Submitted for publication May 10, 2004; revision submitted October 20, 2004; accepted for publication November 29, 2004.

Reprint requests: Xiaodong Zhang, MD, PhD, Department of Cancer Research, Institute for Molecular Biology, College of Life Sciences, and Lihong Ye, MD, PhD, Department of Biochemistry, Nankai University, 94 Weijin Road, Tianjin, 300071, People's Republic of China; e-mail: zhangxd@nankai.edu.cn.

0022-2143/$ – see front matter

doi:10.1016/j.lab.2004.11.018

Hepatocellular carcinoma is one of the most common cancers in China, where approximately 90% of HCC cases are associated with HBV infection. However, the complex mechanism by which HBV infection leads to the development of HCC remains unclear. The HBV genome is a partially double-stranded 3.2-kb incomplete circular DNA molecule containing 4 overlapping open-reading frames that encode the structural and nonstructural viral proteins. Among them, the HBX encoded by open-reading frame X plays a central role in liver oncogenesis.[1–3] Previous transient-transfection experiments have demonstrated that HBX transactivates a wide variety of viral and cellular promoters. Several HBx-responsive *cis*-elements are present in RNA polymerase II promoters, including activator protein-1 (AP-1), activator protein-2 (AP-2), antithiamin factor (ATF), CCAAT/enhancer binding protein (c/EBP), and nuclear factor-κB sites and serum-responsive elements.[4–12] In addition, one of the RNA polymerase III promoters is known to be transactivated by HBX.[4,8,13,14] These observations indicate that HBX regulates a wide variety of genes, suggesting that HBX not only up-regulates HBV gene expression by transactivating the HBV enhancer but also modifies the environment by transactivating cellular genes in infected cells to facilitate viral replication. These observations also support the notion that HBX plays a role in hepatocellular carcinogenesis.[15,16]

Because HBX does not directly bind DNA, protein-protein interactions are crucial in HBX transactivation.[17] The reported HBX-binding proteins include transcription factors such as TATA-binding protein (TBP),[18] RNA polymerase II (RPB5),[19] cAMP response element binding (CREB/ATF2),[20] Oct-1,[21] putative DNA repair protein, ultraviolet damaged DNA binding protein (UV-DDB),[22] and the tumor suppressor p53.[23,24] It has been reported that HBX mediates transcriptional activation by modifying cytoplasmic signal-transduction pathways such as the AP-1/protein kinase C and Ras-Raf-mitogen–activated protein kinase signaling pathways.[5,6,25–28] HBX is localized in both the cell cytoplasm and nucleus,[17,29] suggesting that the protein plays a dual role in transcriptional regulation; nuclear HBX likely functions at the promoter level, whereas cytoplasmic HBX may influence the regulation of second-messenger systems.[29] However, the effect of HBX on the immortalization of hepatocytes is not yet known.

Telomerase is a ribonuclear protein enzyme composed of a template RNA and several proteins. In recent years, the genes encoding the RNA and protein subunits of telomerase have been cloned from a wide variety of species. In human beings, 3 such subunits have been identified and extensively analyzed: human telomerase RNA component, which encodes the RNA component of the telomerase[30,31]; telomerase-associated protein-1, which encodes a telomerase-associated protein of unknown function[32,33]; and hTERT, which encodes the catalytic subunit of telomerase.[34,35] It has been reported that hTERT mRNA expression is significantly correlated with telomerase activity.[34] Telomerase activity and apoptosis in hepatoma cells are both influenced by the HBx gene.[36]

In this study, we examined the expression of hTERT and HBV antigens by immunohistochemical means in hepatoma and cirrhotic liver tissues and in samples of normal liver. We found that hTERT expression correlated with that of HBX in the HCC and cirrhotic tissues. Furthermore, we transfected an HBX-expression construct into cultured hepatoma cells and used it to examine the effects of HBx on hTERT expression and activity.

METHODS

Specimens. Tissue samples from 34 cases of human HCC and corresponding paratumor tissues, specimens from 30 cases of liver cirrhosis, and samples from 6 normal livers were obtained from the Tianjin First Central Hospital. All sampling methods and protocols used in the study conformed to the relevant ethical guidelines, and informed consent for tissue collection was obtained from patients before surgery. All patients underwent total or subtotal hepatectomy followed by pathologic diagnosis. Patients, who ranged in age from 39 to 55 years, comprised 1 woman and 69 men. All specimens were fixed in 10% formalin and embedded in paraffin. Five-micrometer sections were cut and attached to glass slides with poly-lysine, after which the slides were transferred to a slide warmer at 70°C for 30 minutes and dried at room temperature.

Clinical detection of HBV infection. HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc were detected in the peripheral blood of 34 patients with HCC. In brief, serum samples were obtained before surgery and tested for HBV markers with the use of an enzyme immunoassay. All patients were positive for HBsAg and anti-HBc (IgG) and 4 were positive for anti-HBe, but none was positive for HBeAg.

Immunohistochemical staining. Mouse anti-HBsAg and anti-HBcAg and rabbit hTERT antibody were all purchased from Beijing Zhongshan Biotechnology (Beijing, China), and rabbit anti-HBxAg was obtained from the Fox Chase Institute for Cancer Research (Philadelphia, Pa). The SABC kit for immunohistochemical staining was purchased from Wuhan Boster Biological Technology (Wuhan, China). For immunohistochemical study, slides were enhanced in 10 mmol/L citrate buffer with the use of the microwave antigen-unmasking technique. The tissue sections were then blocked and incubated with antibodies against human HBcAg, HBsAg, HBxAg or hTERT (all diluted 1:100) overnight at 4°C. The tissue sections were then washed 3 times in PBS (5 minutes each) and incubated with a biotin-labeled secondary antibody at 37°C for 1 hour. Samples were then washed with 3 times in PBS (5 minutes each time) and incubated with a streptavidin-labeled tertiary antibody for 30 minutes at 37°C. The sections were washed 3 times in PBS, after which positive staining signals were detected with the use of a