HBx-induced reactive oxygen species activates hepatocellular carcinogenesis via dysregulation of PTEN/Akt pathway

Hye-Lin Ha, Dae-Yeul Yu

Abstract

AIM: To investigate the role of hepatitis B virus X-protein (HBx)-induced reactive oxygen species (ROS) on liver carcinogenesis in HBx transgenic mouse and HepG2-HBx cells.

METHODS: Cell growth rate was analyzed, and through western blotting, mitogenic signaling was observed. Endogenous ROS from wild and HBx transgenic mice and HepG2-Mock and HBx cells were assayed by FACS-calibur. Identification of oxidized and reduced phosphatase and tensin homolog (PTEN) was analyzed through N-ethylmaleimide alkylation, nonreducing electrophoresis.

RESULTS: We observed that the cell-proliferation-related phosphoinositide 3-kinase/Akt pathway is activated by HBx in vivo and in vitro. Increased ROS were detected by HBx. Tumor suppressor PTEN, via dephosphorylation of Akt, was oxidized and inactivated by increased ROS. Increased oxidized PTEN activated the mitogenic pathway through over-activated Akt. However, treatment with ROS scavenger N-acetyl cysteine can reverse PTEN to a reduced form. Endogenously produced ROS also stimulated HBx expression.

CONCLUSION: HBx induced ROS promoted Akt pathways via oxidized inactive PTEN. HBx and ROS maintained a positive regulatory loop, which aggravated carcinogenesis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality. Among other risk factors (including alcohol abuse, cirrhosis, and aflatoxin B1), chronic hepatitis B virus (HBV) infection plays a central role in the etiology of...
HCC[3]. About 53% of HCC cases are related to HBV, and the risk of HCC in chronic HBV carriers is approximately 100 times greater than in uninfected individuals[3]. Among the four proteins encoded by the HBV genome, X protein (HBx) is a multifunctional regulatory protein that is closely linked to HCC, but its role in tumor growth has not been fully clarified. Prior work from this laboratory has shown that HBx induces liver cancer in transgenic mice[3]. HBx does not bind directly to DNA, but affects transcriptional activation through interaction with nuclear transcription factors and by cytoplasmic modulation of signal transduction pathways[3,4]. HBx also mediates the activation of the Ras/Raf/extracellular signal-regulated kinase and mitogen-activated protein kinase kinase-1/c-Jun NH2-terminal kinase cascades, which leads to the induction of activator protein-1 and nuclear factor κB[5,6]. One of the most well-known pathways activated by HBx is phosphoinositide 3-kinase (PI3K)/Akt, which is associated with anti-apoptotic activity and cell proliferation[7,8]. Therefore, HBx is thought to be associated with the development of human HCC, but the precise function of HBx in the tumorigenic transformation of liver cells remains unclear.

Previous studies have indicated that HBx protein directly interacts with the membrane proteins of mitochondria, the major site of reactive oxygen species (ROS) production, and alters the mitochondrial membrane potential in a hepatoma cell line. HBx also increases the level of mitochondrial ROS and lipid peroxide production[9,10]. The results of many previous studies have shown that normal cells exposed to low levels of H2O2 can increase their proliferation[11,12]. In this context, many types of cancer cells manifest increased production of H2O2[13].

Protein tyrosine phosphatases (PTPs) are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. Together with tyrosine kinases, PTPs regulate the phosphorylation state of many important signaling molecules. They have been suggested to be direct targets of H2O2[13,14]. In general, PTPs exert an inhibitory effect on cancer signaling by opposing the tyrosine phosphorylation initiated by activated receptor kinases. Cell stimulation induces the transient activation of class-I PI3K, and the subsequent production of PI 3,4,5-triphosphate (PIP3) which is important for the activation of a variety of downstream signaling molecules, including the protein kinase Akt, that mediate promotion of cell proliferation and survival[15]. The reaction catalyzed by PI3K is reversed by phosphatase and tensin homolog (PTEN), which functions as a PIP3 3-phosphatase. Indeed, by negatively modulating the PI3K signaling pathway, PTEN acts as a tumor suppressor. PTEN is also a member of the PTP family. It has been previously demonstrated that Cys-124 in the catalytic site of human PTEN is readily oxidized by exogenous H2O2 to form a disulfide with Cys-71[16].

In the present study, we attempted to determine the effect of HBx on the activated Akt pathways. We showed that HBx-produced H2O2 induces reversible inactivation of PTEN and activation of Akt. We suggest that scavenging H2O2 could be a therapeutic target for abnormal cell signaling to reactivate PTEN.

MATERIALS AND METHODS

Transgenic mice

The production of HBx transgenic mice used in this study has been reported previously[3]. HBx homozgyous (+/+ ) transgenic mice were produced by mating HBx heterozygous transgenic mice with each other. To generate HBx homozygous transgenic mice on a mixed background of C57BL/6 and CBA strains, HBx homozygous mice with C57BL/6 backgrounds were crossed with CBA wild-type mice. The heterozygous transgenic offspring with a mixed background of C57BL/6 and CBA strains were crossed among. Among their offspring, HBx homozygous transgenic mice were selected by genotyping the next generation. Selected mice were then crossed up to F12, which is applicable for the study as an inbred strain with a mixed genetic background (C57BL/6 and CBA). In the current study, these F12 mice were used for in vitro analyses. HBx (+/+ ) transgenic mice were verified by polymerase chain reaction (PCR) analysis. The PCR primers used were as follows: one set was sense primer 5′-TTCTCTATCGCCG-GTCCGCTG-3′ and antisense primer 5′-GGGTCAAT-GTCCATGCCCCA-3′, and another set was sense primer 5′-GAAAAACACTCTAGTTTCAGAG-3′ and antisense primer 5′-GTAAGCGGTTTCTCTTTATGCAG-3′. The wild-type mice were derived from littersmates between HBx heterozygous transgenic male and female mice, with a mixed genetic background (C57BL/6 and CBA). Mice were housed in a specific pathogen-free environment. Mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea).

Cell lines and cell culture conditions

HepG2-HBx cells derived from HepG2 cells were stably transfected and expressed HBx. HepG2 cells were grown in an atmosphere that contained 5% CO2 at 37°C in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin.

Proliferation assay

Cell proliferation was determined by the crystal violet staining method, as described previously[17].

Western blotting analysis

Proteins (20 μg/sample) were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blotted at 4°C overnight with primary antibodies. The membranes were washed five times with 10 mmol/L Tris-HCl (pH 7.5) plus 150 mmol/L NaCl (Tris-buffered saline; TBS) that contained 0.2% Tween-20, and incubated with horseradish peroxidase (HRP)-conjugated IgG. After the removal of excess antibodies by washing with TBS, specific binding was detected using a chemiluminescence detection system (Amersham, Berks, UK) according to the manufacturer’s in-
The HBx protein is considered to be closely associated with the development of HCC. HBx transgenic mice, previously developed in this laboratory, developed dysplasia around 4 wk of age, and hepatocellular tumors developed from 6 mo of age. Several studies have shown that HBx stimulates cell proliferation and growth through the activation of signal transduction pathways such as Akt. To study the role of the HBx protein in cancer generation at the cellular level, HepG2-HBx cells were obtained by stably transfecting HepG2 cells with an HBx expression plasmid. The growth rate of the HepG2-HBx cells was approximately double that of the HepG2 control cells (Figure 1A and B). There were differences not only in cell growth, but also in morphology. HepG2-HBx cells showed aberrant actin bundling. Taken together, these results show that HBx has a role in the development of the liver tumor by activating proliferation and changing cell characteristics.

**Figure 1** Effect of hepatitis B virus X-protein on induction of aberrant cell growth. A: Cell growth analysis by crystal violet staining and *A*~550~ nm detection. 10^4~ cells were seeded, and stained at 1, 5, 6, 7 and 8 d after seeding. Values represent mean ± SD (n = 3). *P* ≤ 0.001 compared with mock transfectants; B: Morphology of the cells was observed by optical microscopy. HBx: Hepatitis B virus X-protein.

### Statistical analysis
Comparisons were analyzed for statistical significance by unpaired or paired Student’s *t* test using Microsoft Excel software. *P* < 0.001 was considered as significant. All data are reported as mean ± SD.

### RESULTS

**HBx promotes tumor formation**
The HBx protein is considered to be closely associated with the development of HCC. HBx transgenic mice, previously developed in this laboratory, developed dysplasia around 4 wk of age, and hepatocellular tumors developed from 6 mo of age. Several studies have shown that HBx stimulates cell proliferation and growth through the activation of signal transduction pathways such as Akt. To study the role of the HBx protein in cancer generation at the cellular level, HepG2-HBx cells were obtained by stably transfecting HepG2 cells with an HBx expression plasmid. The growth rate of the HepG2-HBx cells was approximately double that of the HepG2 control cells (Figure 1A and B). There were differences not only in cell growth, but also in morphology. HepG2-HBx cells showed aberrant actin bundling. Taken together, these results show that HBx has a role in the development of the liver tumor by activating proliferation and changing cell characteristics.

**Tumorigenesis in HBx transgenic mice and HepG2-HBx cells through activation of the Akt pathway**

The PI3K/Akt signaling pathway is crucial to many aspects of cell growth and survival. To determine whether HBx-associated HCC is also accompanied by activation of the Akt pathway, lysates from the mouse liver tissue and cells transfected with HBx or an empty vector were used. As expected, the lysates of HBx transgenic mice and HepG2-HBx cells displayed an activated Akt pathway. Accumulated β-catenin, phosphorylated Akt, and increased cyclin D1 were detected (Figure 2A). Even though cancer...
cell lines might have activated Akt, total Akt per p-Akt of HepG2 HBx cells was increased 1.4-fold compared with the HepG2 control cells (Figure 2B).

**HBx-induced endogenous ROS cause PTEN inactivation via cysteine oxidation**

Peroxides are known to modify PTPs by oxidation. PTEN is also known to be inactivated through H$_2$O$_2$-mediated oxidation$^{20}$. FACS analysis was used to verify HBx-induced ROS in mice and HepG2 cells. Primary hepatocytes were isolated from HBx transgenic and wild-type mice at the same age. ROS levels were significantly increased in HBx transgenic hepatocytes and HepG2-HBx cells compared to controls (Figure 3A and B). HBx expression was also associated with decreased mitochondrial membrane potential (data not shown). To examine the effect of HBx-induced ROS on PTEN inactivation, a PTEN oxidation assay was performed. HBx-expressing cells had higher ROS levels, and showed higher levels of oxidized PTEN when evaluated in primary hepatocytes and in HepG2 cells. HBx-induced ROS inactivated PTEN by promoting oxidation of cysteine residues within PTEN, thereby inactivating PTEN and promoting the function of Akt.

**Inactivated PTEN correlates with upregulation of the PI3 kinase/Akt pathway**

To investigate the activation of Akt in the presence of ROS-inactivated PTEN, we examined the Akt pathway.
activity, which was detected in 0 and 500 μmol/L H₂O₂. Increases in oxidized PTEN were associated with a higher p-Akt/total Akt ratio and increased cyclin D1 expression. To investigate further whether induced ROS is required for activation of the Akt pathway, HepG2 cells were treated with H₂O₂ in the presence or absence of NAC, a ROS quencher. Scavenging ROS through NAC were able to block the Akt pathway (Figure 4A and B). These observations are consistent with the hypothesis that HBx-mediated generation of ROS inactivates PTEN, thereby activating the Akt pathway in carcinogenesis. In addition, elevated ROS was also associated with elevated levels of HBx (Figure 4C).

**DISCUSSION**

One of the HBV-encoded proteins, HBx, is considered to be a major risk factor for HCC. It is well known that HBx activates cell signal transduction pathways, such as PI3K. Mutations or inactivation of the tumor suppressor, PTEN, regulates Akt activation. This is considered one of the reasons for activation of Akt signaling in cancer. For example, endogenously produced H₂O₂ has been shown to inactivate PTEN in a macrophage cell line and cancer cell lines. In this study, HBx-triggered ROS were associated with the oxidation and functional inactivation of PTEN. Although quantification of the extent of PTEN oxidation in the cells was not possible, the level of oxidized, inactivated PTEN was associated with several factors, such as Akt activation and accelerated HepG2 cell growth, and thus might be associated with hepatocarcinogenesis in HBx transgenic mice. Both cell growth and abnormal actin filaments were observed in HepG2-HBx cells. It has been reported that reorganization of actin filaments can cause loss of focal adhesions and cell-cell contact, which leads to an epithelial-mesenchymal transition that consequently disrupts monolayer integrity.

The HBx-induced ROS appear to stimulate HBx expression further, which suggests the existence of a positive feedback loop. Such feedback would be expected to cause a rapid increase in the abundance of H₂O₂. This localized H₂O₂ accumulation would be expected to result in the oxidation of only those PTEN molecules located nearby, possibly explaining the small proportion of PTEN molecules that undergo oxidative inactivation in HepG2-HBx cells and mouse livers.

The scheme presented in Figure 4D represents the HBx-induced generation of H₂O₂. H₂O₂ participates in

![Figure 4](image_url)
in intracellular signaling by targeting PTEN, and regulation of HBx gene expression, depending on the concentration. The results of the present study suggest that the HBx-mediated activation of Akt is regulated, at least in part, by the effects of HBx-induced ROS upon PTEN.

In summary, these studies further strengthen the case for a close relationship between oxidative stress and tumorigenesis. The studies reported herein have shown that HBx-induced generation of ROS can promote cellular transformation signaling by altering the function of PTEN. H2O2-oxidized PTEN leads to the activation of Akt. This is significant from a mechanistic as well as therapeutic point of view. Hence, drugs that scavenge endogenous ROS might slow down progression to HBx-induced liver cancer.

ACKNOWLEDGMENTS

The authors thank Dr. Mark Feitelson in Temple University, Philadelphia, PA, USA for proof reading the article.

REFERENCES

1. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer 2006; 6: 674-687
2. Tiollais P, Charnay P, Vyas GN. Biology of hepatitis B virus. Science 1981; 213: 406-411
3. Yu DY, Moon HB, Son JK, Jeong S, Yu SL, Hoon Y, Han YM, Lee CS, Park JS, Lee CH, Hyun BH, Murakami S, Lee K. Incidence of hepatocellular carcinoma in transgenic mice expressing the hepatitis B virus X-protein. J Hepatol 1999; 31: 123-132
4. Haviv I, Shamay M, Doish G, Shaul Y. Hepatitis B virus x-targets TSH-β in transcription activation. Mol Cell Biol 1998; 18: 1562-1569
5. Benn J, Su F, Doria M, Schneider RJ. Hepatitis B virus HBx protein induces transcription factor AP-1 by activation of extracellular signal-regulated and c-Jun N-terminal mitogen-activated protein kinases. J Virol 1996; 70: 4978-4985
6. Chirillo P, Falco M, Puri PL, Artini M, Balsano C, Levero M, Nottoli C. Hepatitis B virus x-targets NF-kappa B-dependent transcription through a Raf-independent pathway. J Virol 1996; 70: 641-646
7. Lee YH, Yun Y. HBs protein of hepatitis B virus activates Jak1-STAT signaling. J Biol Chem 1998; 273: 22551-22555
8. Lee YI, Kang-Park S, Do SI. The hepatitis B virus-x protein activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade. J Biol Chem 2001; 276: 16969-16977
9. Suzuki A, Hayashida M, Kawano H, Sugimoto K, Nakano T, Shiraiki K. Hepatocyte growth factor promotes cell survival from fas-mediated cell death in hepatocellular carcinoma cells via Akt activation and Fas-death-inducing signaling complex suppression. Hepatology 2000; 32: 796-802
10. Lee YI, Hwang JM, Im JH, Kim NS, Kim DG, Yu DY, Moon HB, Park SK. Human hepatitis B virus-x protein alters mitochondrial function and physiology in human liver cells. J Biol Chem 2004; 279: 15460-15471
11. Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic Biol Med 1995; 18: 775-794
12. Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 1991; 51: 794-798
13. Lee SR, Kwon KS, Kim SB, Rhee SG. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J Biol Chem 1998; 273: 15366-15372
14. Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K, Griendling KK. Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. J Biol Chem 1999; 274: 22699-22704
15. Maehama T, Taylor GS, Dixon JE. PTEN and myotubularin: novel phosphoinositide phosphatases. Annu Rev Biochem 2001; 70: 247-279
16. Lee SR, Yang KS, Kwon J, Lee C, Jeong W, Rhee SG. Reversible inactivation of the tumor suppressor PTEN by H2O2. J Biol Chem 2002; 277: 30334-30342
17. Kim YM, Chung HT, Simmons RL, Billiar TR. Cellular non-heme iron content is a determinant of nitric oxide-mediated apoptosis, necrosis, and caspase inhibition. J Biol Chem 2000; 275: 10954-10961
18. Wang AG, Moon HB, Lee MR, Hwang CY, Kwon KS, Yu SL, Kim YS, Kim M, Kim JM, Kim SK, Lee TH, Moon EY, Lee DS, Yu DY. Gender-dependent hepatic alterations in H-ras12V transgenic mice. J Hepatol 2005; 43: 836-844
19. Kim SY, Lee PY, Shin HJ, Kim Do H, Kang S, Moon HB, Kang SW, Kim JM, Park SC, Park BC, Yu DY, Bae KH, Lee SC. Proteomic analysis of liver tissue from HBx-transgenic mice at early stages of hepatocarcinogenesis. Proteomics 2009; 9: 5056-5066
20. Leslie NR, Bennett D, Lindsay YE, Stewart H, Gray A, Downes CP. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. EMBO J 2003; 22: 5501-5510
21. Keniry M, Parsons R. The role of PTEN signaling perturbations in cancer and in targeted therapy. Oncogene 2008; 27: 5477-5485
22. Kwon J, Lee SR, Yang KS, Ahn Y, Kim YJ, Stadtmann ER, Rhee SC. Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. Proc Natl Acad Sci USA 2004; 101: 16419-16424
23. Hirohashi S, Kanai Y. Cell adhesion system and human cancer morphogenesis. Cancer Sci 2003; 94: 575-581

S- Editor Tian L, L- Editor Kerr C, E- Editor Lin YP