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Biological Effects of HBV X Protein on Hepatocellular Carcinogenesis in Association with Cellular Factors*

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Abstract: The X protein (HBx) of Human hepatitis B virus (HBV) acts as an indirect transcriptional transactivator to regulate the expression of many viral and cellular genes, as well as playing a critical role in pathogenesis and the development of Hepatocellular carcinoma (HCC). Here we described the biological effects of HBx in association with four cellular factors, including inflammatory factors (COX-2 and iNOS), oncoprotein (Ras), and a newly identified tumor suppressor (YueF). The characteristics of these effectors, which might be associated with hepatocellular carcinoma, are also discussed.

Key words: Human hepatitis B virus (HBV); Ras; YueF; Hepatocellular carcinoma

The genome of hepatitis B virus (HBV) is approximately 3 000 to 3 300 nucleotides in length, and molecular studies have suggested the presence of at least four genes in the viral genome: the S gene coding for the glycosylated envelope protein, C gene for the core protein, P gene for the HBV polymerase, and X gene for the X protein that is associated with transactivating properties (19). HBV infection causes liver diseases that vary greatly in severity from person to person. Some subjects control infection efficiently and eliminate the virus from circulation, while others are with an acute inflammation of the liver. A proportion of patients fail to clear the virus and develop chronic infection (2).

There is accumulating evidence suggesting a close association between chronic HBV infection and an increased risk for the development of primary hepatocellular carcinoma (HCC). Persistent infection by HBV results in sustained low level liver damage of infected hepatocytes. Almost all such damage can be attributed to attack by the host's immune system. The rate of hepatocyte proliferation must increase in order to compensate for cell loss. It is generally accepted that such an increased rate of proliferation over long periods is a major contributor to the development of liver cancer (7, 23, 25). In addition, the inflammation and phagocytosis that are integral to the immune response can lead to increased local concentrations of

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superoxides and free radicals that are highly carcinogenic (1).

In addition, the viral X region appears to have pleiotropic effects that could also be involved in the oncogenic processes, including transcriptional activation of cellular growth regulatory genes, modulation of apoptosis and inhibition of nucleotide excision repair of damaged cellular DNA (8, 14, 16). The effects of HBx are mediated by interaction with cell proteins and activation of cell signaling pathways. Here we focus on the roles of four representative cellular factors (COX-2, iNOS, Ras, and YueF) that are associated with HBx and discuss their contributions to hepatitis B virus-associated hepatocarcinogenesis according mainly to our recent work.

RESULTS AND DISCUSSIONS

Cyclooxygenase-2 (COX-2), a rate-limiting enzyme in the pathway of prostaglandin (PG) synthesis, is one of the important cellular factors that have been suggested previously to be associated with inflammation caused by microbes infections (5,15,18). Our previous studies proposed that the nucleocapsid (N) protein of SARS-associated coronavirus (SARS- CoV) activates COX-2 gene expression by recognizing directly to a nuclear factor-kappa B (NF-κB) binding site and a CCAAT/enhancer binding protein (C/EBP) binding site in the COX-2 gene promoter regions, which results in the induction of inflammatory responses through multiple COX-2 signaling cascades (24). In addition, we also demonstrate that the spike (S) protein of SARS-CoV can upregulate COX-2 expression through both calcium-dependent pathway, PKC alpha/ERK/NF-kappaB, and the unusual calcium-independent pathway, PI3K/PKC epsilon/JNK/CREB (12).

Recently, COX-2 has been categorized as an immediate-early gene associated with cellular growth, differentiation, and tumorigenesis. COX-2-expressing cells can induce neovascularization by upregulating proangiogenic enzymes and growth factors, such as inducible nitric oxide synthase (iNOS) and TGF-β. Moreover, COX-2 expression results in increased apoptosis resistance (14, 20, 22). All the above properties are shared by HBx protein.

We have investigated the regulation of COX-2 in chronic hepatitis B persisted patients and examined the relationship between HBx and COX-2 in hepatitis B virus-associated chronic liver diseases. Results showed that the CCAAT/enhancer binding protein (C/EBP) binding site in COX-2 promoter region is crucial for the induction of COX-2 by HBx (unpublished data). It has been demonstrated that NF- AT, but not NF-κB, is necessary for the induction of COX-2 by HBx. Their results suggest a role for NF-AT, oxidative stress, and calcium signaling in the induction of inflammatory mediators and tumor development induced by HBx (11).

Nitric oxide (NO) is a small potent lipophilic gas with multiple biological functions, which plays critical roles in modulating tissue injury, immune response, and carcinogenesis. Three distinct forms of nitric oxide synthase (NOS), endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS), catalyze the formation of NO. eNOS and nNOS are constitutively expressed in different tissues, whereas iNOS is associated with signal transduction pathway and is responsible for various pathological processes

(9). The expressions of iNOS and VEGF are closely related to tumor angiogenesis, and are also involved in the tumor relapse, expansion and the lymph node metastasis (3, 4).

The specific example of biological effects of viral protein in association with iNOS can be enumerated from our previous work that focused on the study of the P protein of Borna disease virus (BDV) (17). BDV is one of the potential infectious agents involved in the development of central nervous system (CNS) diseases. Neurons and astrocytes are the main targets of BDV infection. We showed that BDV P protein inhibits the activation of iNOS in murine astrocytes induced by bacterial LPS and PMA. The nuclear factor kappa B (NF-κB) binding element, AP-1 recognition site, and interferon-stimulated response element (ISRE) in the iNOS promoter regions are involved in the repression of iNOS gene expression regulated by the P protein (22). These data suggested that P protein may suppress signal transduction pathways, which resulted in the inhibition of iNOS in astrocytes.

We have recently shown that there is an enhanced intrahepatic expression of the iNOS gene during chronic hepatitis B virus infection, and that HBx transcriptionally activates this cellular gene (unpublished data). The molecular mechanism for such activation is currently under investigation. The biological significance of upregulated iNOS expression in cancers remains controversial and elusive.

Oncogenes are altered genes whose product can act in a dominant fashion to help make a cell cancerous. Typically, an oncogene is a mutant form of a normal gene (proto-oncogene) involved in the control of cell growth or division. However, the vast majority of the cells in the transgenic mouse that express the Myc or Ras oncogene, do not give rise to cancers, showing that the single oncogene is not enough to cause malignancy.

In liver tissues, HBx can activate Src-Ras signal transduction pathways, which are critical for the activation of many transcription factors, HBV reverse-transcription, and viral DNA replication (26). The relationship between HBx-Ras interaction and HBV tumorigenesis or cellular transformation is not quite sure, as opposite results are frequently reported by investigators in different laboratories. These discrepancies could not always be explained by different experimental designs or conditions. Maybe cooperation among research groups are needed to solve this enigma in the near future.

It has been showed that HBx collaborates with activated H-ras to transform immortalized rodent cells. REF52 cells transfected by both HBx and activated H-ras were morphologically transformed and were able to grow in soft agar. Nude mice injected with REF52 cells transfected by both HBx and activated H-ras developed tumors, whereas the mice injected with REF52 cells transfected by either gene alone did not. They concluded that HBx could contribute to neoplastic transformation of cells in collaboration with other oncogenes, such as H-ras, that renders cells to overcome the HBx-mediated apoptosis (10).

Our work demonstrated that co-transfection of mouse embryo fibroblasts (STO) with HBx and activated Ras triggered apoptotic cell death, while HBx or activated Ras individually failed to induce apoptosis (21). In addition, STO cells were able to form colonies on soft agar after transfected with HBx

or Ras, and cells co-transfected with both genes failed to transform. Moreover, nude mice injected with STO cells carrying either HBx or Ras could develop tumor, but tumor growth was inhibited by the injection of both STO cells harboring HBx and carrying Ras. These results suggested that HBx plays a role as a tumor inducer and stimulates neoplastic transformation of normal cells, but shifts its function to the induction of apoptosis in association with Ras (21). It seems that Ras-related cell signaling pathway is most important for regulating HBx-induced cellular events. We, therefore, examined the mechanism of HBx-triggered cell death in associated with Ras. Results showed that coexpression of HBx and activated Ras resulted in the decrease of mitochondrial membrane potential and release cytochrome C from mitochondria, while HBx or activated Ras individually failed to do so. Furthermore, caspase-8 was found to be activated when HBx and ras were both expressed in cells, and caspase-8 inhibitor (Z-IETD-FMK) repressed such cells apoptosis. Subsequently, it was found that HBx induced FasL expression in association with activated ras. These results provide a novel mechanism of HBx-induced cells apoptosis in association with the ras signaling pathway (unpublished data).

Tumor suppressors are a specific group of proteins that appear to prevent formation of cancer and loss-of-function mutations in related genes enhance susceptibility to cancer. In a previous study, we used HBV X protein as a bait protein for screening a human liver cDNA library using a yeast two-hybrid system (27). Several cell proteins were identified as new HBx interacting partners, including a salient tumor suppressor protein YueF. Direct interaction between HBx and YueF proteins was confirmed by *in vitro* and *in vivo* co-immunoprecipitation assays. HBx also co-localized with YueF in human cells as determined by confocal immunofluorescence microscopy. YueF is expressed at high levels in normal human hepatic cells and tissues, but scarcely found in hepatoma cells or other tumor tissues. Over-expression of YueF, or YueF and HBx could induce cell apoptosis and enhance a well known tumor suppressor, p53, expression in hepatoma cells, whereas overexpression of HBx alone behaved contrarily. These results confirm that YueF has tumor suppressor activity and affects the functions of HBx in cell apoptosis and p53 expression in hepatoma cells (9). Thus, further intensive investigation of this newly detected important cellular factor is currently carried out for the mechanisms involved in its tumor suppression faction and for its potential application in tumor diagnostic and cancer gene therapy.

CONCLUSIONS

The exact role of a cancer associated virus such as the hepatitis B virus is often hard to decipher because there is a delay of many years from the initial viral infection to the development of cancer. Moreover, the virus is responsible for only one of a series of steps in the progression to cancer, and other genetic accidents and environmental factors may be also involved. Viruses can either alter a cell's DNA directly or act as tumor promoter. In some other cancers, viruses seem to have additional indirect tumor promoting actions. Many human hepatocellular carcinomas contain integrated HBV, and the viral X protein appears to have pleiotropic effects that could be involved in the

oncogenic process. Through interaction with cell proteins, HBx also alter mechanisms of apoptosis and interfere with nucleotide excision repair of damaged DNA. Together with an influence on cellular signaling, these mechanisms may favor the cell's malignant clonal expansion. But we cannot forget that tumor formation in mammals is a much more complex process and we must make the best endeavors to make more apparent the role of viruses in the development of human cancer.

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References

1. **Asare G A, Mossanda K S, Kew M C, *et al.*** 2006. Hepatocellular carcinoma caused by iron overload: a possible mechanism of direct hepatocarcinogenicity. **Toxicology,** 219: 41-52.
2. **Bertoletti A, Gehring A J.** 2006. The immune response during hepatitis B virus infection. **J Gen Virol,** 87: 1439-1449.
3. **Chen C N, Hsieh F J, Cheng Y M, *et al.*** 2006. Expression of inducible nitric oxide synthase and cyclooxygenase-2 in angiogenesis and clinical outcome of human gastric cancer. **J Surg Oncol,** 94: 226-233.
4. **Cullis E R, Kalber T L, Ashton S E, *et al.*** 2006. Tumour overexpression of inducible nitric oxide synthase (iNOS) increases angiogenesis and may modulate the anti-tumour effects of the vascular disrupting agent ZD6126. **Microvasc Res,** 71:76-84.
5. **Dionne R.** 1999. COX-2 inhibitors--IBC conference. 12-13 April 1999, Coronado, CA, USA. **Idrugs,** 2: 664-666.
6. **Dooley A, Low S Y, Holmes A, *et al.*** 2008. Nitric oxide synthase expression and activity in the tight-skin mouse model of fibrosis. **Rheumatology (Oxford),** 47: 272-280.
7. **Fabregat I, Roncero C, Fernández M.** 2007. Survival and apoptosis: a dysregulated balance in liver cancer. **Liver Int,** 27: 155-162.
8. **He Y, Yang F, Wang F, *et al.*** 2007. The upregulation of expressed proteins in HepG2 cells transfected by the recombinant plasmid-containing HBx gene. **Scand J Immunol,** 65: 249-256.
9. **Huang J, Wu K, Zhang J, *et al.*** 2008. Putative tumor suppressor YueF affects the functions of hepatitis B virus X protein in hepatoma cell apoptosis and p53 expression. **Biotechnol Lett,** 30: 235-242.
10. **Kim Y C, Song K S, Yoon G, *et al.*** 2001. Activated ras oncogene collaborates with HBx gene of hepatitis B virus to transform cells by suppressing HBx-mediated apoptosis. **Oncogene,** 20: 16-23.
11. **Lara-Pezzi E, Gómez-Gaviro M V, Gálvez B G, *et al.*** 2002. The hepatitis B virus X protein promotes tumor cell invasion by inducing membrane-type matrix metalloproteinase-1 and cyclooxygenase-2 expression. **J Clin Invest,** 110: 1831-1838.
12. **Liu M, Yang Y, Gu C, *et al.*** 2007. Spike protein of SARS-CoV stimulates cyclooxygenase-2 expression via both calcium-dependent and calcium-independent protein kinase C pathways. **FASEB J,** 21: 1586-1596.
13. **McClain SL, Clippinger A J, Lizzano R, *et al.*** 2007. Hepatitis B virus replication is associated with an HBx-dependent mitochondrion-regulated increase in cytosolic calcium levels. **J Virol,** 81: 12061-12065.
14. **McGinty A, Chang Y W, Sorokin A, *et al.*** 2000. Cyclooxygenase-2 expression inhibits trophic withdrawal apoptosis in nerve growth factor-differentiated PC12 cells. **J Biol Chem,** 275: 12095-12101.
15. **Miyamoto T, Saika S, Ueyama T, *et al.*** 2006. Cyclooxygenase 2 expression in rat corneas after ethanol exposure. **J Cataract Refract Surg,** 32:1736-1740.

16. **Oishi N, Shilagardi K, Nakamoto Y, *et al.*** 2007. Hepatitis B virus X protein overcomes oncogenic RAS-induced senescence in human immortalized cells. **Cancer Sci,** 98: 1540-1548.
17. **Peng G, Zhang F, Zhang Q, *et al*.** 2007. Borna disease virus P protein inhibits nitric oxide synthase gene expression in astrocytes. **Virology,** 366: 446-452.
18. **Pesce C, Grattarola M, Menini S, *et al*.** 2006. Cyclooxygenase 2 expression in vessels and nerves in reversal reaction leprosy. **Am J Trop Med Hyg,** 74: 1076-1077.
19. **Seeger C, Mason W S.** 2000. Hepatitis B virus biology. **Microbiol Mol Biol Rev,** 64: 51-68.
20. **Tsujii M, Kawano S, Tsuji S, *et al.*** 1998. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. **Cell,** 93: 705-716.
21. **Wei W, Huang W, Pan Y, *et al.*** 2006. Functional switch of viral protein HBx on cell apoptosis, transformation, and tumorigenesis in association with oncoprotein Ras. **Cancer Lett,** 244: 119-128.
22. **Williams C S, Tsujii M, Reese J, *et al.*** 2000. Host cyclooxygenase-2 modulates carcinoma growth. **J Clin Investig**, 105: 1589-1594.
23. **Wullaert A, van Loo G, Heyninck K, *et al.*** 2007.Hepatic tumor necrosis factor signaling and nuclear factor-kappaB: effects on liver homeostasis and beyond. **Endocr Rev,** 28: 365-386.
24. **Yan X, Hao Q, Mu Y, *et al.*** 2006. Nucleocapsid protein of SARS-CoV activates the expression of cyclooxygenase-2 by binding directly to regulatory elements for nuclear factor-kappa B and CCAAT/enhancer binding protein. **Int J Biochem Cell Biol,** 38: 1417-1428.
25. **Yang Q, Ito S, Gonzalez F J.** 2007. Hepatocyte-restricted constitutive activation of PPAR alpha induces hepatoproliferation but not hepatocarcinogenesis. **Carcinogenesis,** 28: 1171-1177.
26. **Yun C, Cho H, Kim S J, *et al.*** 2004. Mitotic aberration coupled with centrosome amplification is induced by hepatitis B virus X oncoprotein via the Ras-mitogen-activated protein/extracellular signal-regulated kinase-mitogen-activated protein pathway. **Mol Cancer Res,** 2: 159-2169.
27. **Zhang J L, Zhao W G, Wu K L, *et al.*** 2005. Human hepatitis B virus X protein promotes cell proliferation and inhibits cell apoptosis through interacting with a serine protease Hepsin. **Arch Virol,** 150: 721-741.