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World J Gastroenterol 2007 January 7; 13(1): 74-81
World Journal of Gastroenterology ISSN 1007-9327

TOPIC HIGHLIGHT

Dieter Glebe, PhD, *Series Editor*

Hepatitis B virus-induced oncogenesis

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Received: 2006-07-27 Accepted: 2006-09-28

Abstract

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world with an annual incidence of more than 500 000 in the year 2000. Its incidence is rising in many countries. Recently, it has been estimated that about 53% of HCC cases in the world are related to hepatitis B virus (HBV). The epidemiological association of HBV with HCC is well established. In recent studies, it was revealed that HBsAg carriers have a 25-37 times increased risk of developing HCC as compared to non-infected people. At present, HBV-associated carcinogenesis can be seen as a multi-factorial process that includes both direct and indirect mechanisms that might act synergistically. The integration of HBV DNA into the host genome occurs at early steps of clonal tumor expansion. The integration has been shown in a number of cases to affect a variety of cancer-related genes and to exert insertional mutagenesis. The permanent liver inflammation, induced by the immune response, resulting in a degeneration and regeneration process confers to the accumulation of critical mutations in the host genome. In addition to this, the regulatory proteins HBx and the PreS2 activators that can be encoded by the integrate exert a tumor promoter-like function resulting in positive selection of cells producing a functional regulatory protein. Gene expression profiling and proteomic techniques may help to characterize the molecular mechanisms driving HBV-associated carcinogenesis, and thus potentially identify new strategies in diagnosis and therapy.

Key words: Hepatitis B virus; Hepatocellular carcinoma; Regulatory proteins; Signal transduction

Lupberger J, Hildt E. Hepatitis B virus-induced oncogenesis. *World J Gastroenterol* 2007; 13(1): 74-81

http://www.wjgnet.com/1007-9327/13/74.asp

INTRODUCTION

With an estimate of more than 500 000 incidences in the year 2000 hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors worldwide and its incidence is rising in many countries[1-4]. Despite being the 5th most frequent cancer in the world, HCC is the third leading cause of cancer death behind lung and stomach cancer. The high mortality associated with HCC is due to its unresponsiveness to treatment in many cases and symptoms of HCC often are recognized lately[5]. When viewed as estimated age-adjusted incidence rates of liver cancer per 100 000 men, the figures ranged as follows: in Asia, from 35.5 in Eastern Asia, 18.3 in South-eastern Asia to 5.6 in Western Asia; in Africa, from 24.2 in Middle Africa, 14.4 in Eastern Africa, 13.5 in Western Africa, 6.2 in Southern Africa to 4.9 in Northern Africa; in Europe, from 9.8 in Southern Europe, 5.8 in Eastern and Western Europe to 2.6 in Northern Europe; and to values of 4.8 in South America; 4.1 in North America; 3.6 in Australia/New Zealand and, finally, 2.1 in central America. In all regions, the rates recorded were two to three times higher in men than in women.

These significant differences in the geographic distribution of HCC incidence have led to identify chronic HBV infection as a leading risk factor for HCC[6-9]. Recently, it has been estimated that about 53% of HCC cases in the world are related to HBV[3]. The lifetime risk to develop a HCC was found to be increased even in patients that have cleared hepatitis B virus surface antigen (HBsAg) or with an occult HBV infection. Further risk factors include chronic HCV infection, exposure to aflatoxin B_1, alcohol abuse, obesity and diabetes. Aflatoxin B_1 (AFB_1) is a fungal metabolite that contaminates the food supply in certain areas of the world. It is produced by *Aspergillus flavus* and related fungi that grow on improperly stored foods, such as corn, rice and peanuts. AFB_1 requires metabolic conversion to its *exo*-8,9-epoxide in order to damage DNA. Coexistence of these risk factors, such as HBV and HCV infection or HBV infection and aflatoxin B_1, increases the relative risk of HCC development[11-13]. While a variety of risk factors have been identified in the last years, here a short review describing the current state of knowledge of the molecular pathogenesis of HBV-associated HCC

is given. A focus of this review will be on the role of the HBV-regulatory proteins in this process.

EPIDEMIOLOGY OF HBV-ASSOCIATED HCC

The epidemiological association of HBV with HCC is well established. In recent studies, it was revealed that HBsAg carriers have 25-37 times increased risk of developing HCC as compared to non-infected people[14,15]. Moreover, it was analyzed in more detail whether the viral status of the patients are correlated with the risk of developing HCC. HBV has been designated eight genotypes (A-H) based on genetic divergence. Each genotype has a distinct geographical and ethnic distribution. While genotypes B and C are prevalent in Asia, genotypes A and D occur frequently in Africa, Europe and India. There are conflicting data about the influence of HBV genotypes on HCC development[16-18]. Recent studies from Taiwan provide profound evidence for hepatitis B virus e antigen (HBeAg)-positive patients that HBV genotype C causes a more aggressive disease course as compared to genotype B[19-21]. On the other hand, there are reports from Taiwan describing that more than 50% of the HBV-related HCC patients are infected with genotype B. A study on Taiwanese pediatric patients with chronic HBV infection, who were followed for 15 years, showed that genotype B was identified in 74% of the children with HBV-associated HCC[22]. A further interesting observation is the prevalence of the T1762/A1764 mutation in the basal core promoter region which increases with the progression of liver disease. Since this mutation seems to be associated with HCC development, it might represent a helpful prognostic biomarker[23,24].

The risk of HCC seems to be elevated with increasing HBV viral load[25]. Therefore, it is important to consider that most epidemiological analyses were based only on HBsAg positivity. A recent study revealed that the relative risk of HCC was increased by 6-fold among patients who were positive for both HBsAg and HBeAg, compared to those who were positive for HBsAg alone[15]. Based on this, it can be concluded that HBeAg could be an additional useful marker for risk of developing HCC, since HBeAg reflects productive HBV replication.

DIRECT EFFECTS TRIGGERED BY THE INTEGRATION OF HBV-DNA INTO THE HOST GENOME

Integration is not essential for the viral replication but it allows persistence of the viral genome. Almost all of the HBV-associated HCCs harbor chromosomally integrated HBV DNA[26-28]. In many cases, these integrated viral genomes are characterized by rearrangements and/or partial deletions. HBV integration can induce deletions in the host chromosome at the integration site[29]. Based on these observations, it was tempting to speculate that the integration event *per se* causes a deregulation of key regulators of cell cycle control. This cis-hypothesis (place of integration = place of function) seems to be supported by the woodchuck hepatitis B virus (WHV)-related HCC. Here, insertions of WHV-DNA into the c-myc or, preferentially the N-myc2 gene, have been frequently detected[30-34]. However, in case of the HBV-associated HCC, site-specific integration of the HBV genome or integration of the HBV genome into known oncogenes seems to be a rare event. Interesting examples are the integration of HBV DNA in a cyclin A gene[35], in the retinoic acid receptor beta gene, in the mevalonate kinase gene or in the sarco/endoplasmic reticulum calcium ATPase1 gene[28,36].

It was recently confirmed, using a PCR-based approach, that HBV insertion into cellular genes is a frequent event that occurs early during HBV infection even after acute self-limiting hepatitis[37] and that integration can occur in genes regulating cellular signal transduction cascades, proliferation control and cell viability. Recently, hTERT (human telomerase reverse transcriptase) that is part of the telomerase ribonuclear protein complex was found to be targeted in different HBV-associated HCCs[28,38,39].

In light of these recent data, it will be an important issue to reconsider the role of the integration process for HBV-associated carcinogenesis. A helpful tool will be combining the analysis of putative HBV-specific integration sites with functional genomics of HBV-associated HCCs[40].

INDIRECT EFFECTS OF INTEGRATED HBV-DNA: HBX AND THE PRES2 ACTIVATOR FAMILY

HBx

In most integrated subviral HBV genomes, the open reading frame for HBx or PreS2 regulatory protein is conserved and can be transcribed[41]. The HBx gene is conserved among all mammalian hepadnaviruses. HBx is a small polypeptide (17 kDa) that is produced at very low levels during chronic and acute hepatitis. Recently, a HBx-like regulatory protein was identified for duck hepatitis B virus (DHBV)[42]. Since the time when HBx initially was described to act as a transcriptional activator[43,44], a variety of functions have been ascribed to the still enigmatic HBx[45,46]. While the X protein is essential for viral replication in case of WHV[47], there are conflicting results about the relevance of HBx for the viral life cycle in case of HBV. There are reports describing that expression of the viral genome occurs independently from HBx functionality[48-50]; other papers describe a relevance of HBx for HBV replication[51]. In transgenic mouse models harboring an overgenomic HBV integrate, it could be observed that HBV replication does not depend on the presence of a functional HBx[52]. Comparable results were obtained in cell culture models based on huh-7 cells[48,50] while in case of HepG2 cells a reduction in HBx-deficient HBV genomes could be observed[53,54]. Moreover, infection experiments of primary *tupaia* hepatocytes revealed that HBx-deficient HBV particles are infectious (J. Köck, personal communication).

HBx activates a broad variety of different promoter elements. Based on the pleiotropic nature of the HBx-dependent transcriptional regulation, it was concluded that HBx interferes with signaling cascades upstream from the transcription complex. These signaling cascades trigger activation of transcription factors like AP-1 (activator protein-1), NF-κB (nuclear factor kappa B), SP1, and oct-1[46,55]. HBx affects the expression of a variety of genes that are involved in the control of the cell cycle, proliferation or apoptosis. From the beginning, HBx was considered as a crucial viral protein for the process of HBV-associated carcinogenesis[45,56-58] and this might have affected the focus of HBx research. In light of the putative role of HBx for viral carcinogenesis, the major focus of many research projects has been and is the interference of HBx with signal transduction cascades that affect the control of the cell cycle, proliferation or apoptosis. However, one should consider that selective over-expression of HBx reflects a situation that is different from the situation in an infected cell expressing the complete HBV genome. For example, it is well established that HBx is able to promote cellular proliferation[59]. On the other hand, it was shown that expression of the complete HBV genome that harbors the HBx and the PreS2 regulatory protein inhibits cell cycle progression[60] (Figure 1).

The analysis of HBx/protein kinase C (PKC) interaction is such an example for many reports analyzing the interference of HBx with signaling cascades and correlating this with a putative role of HBx for HBV-associated carcinogenesis. There are conflicting reports about the interference of HBx with PKC signaling. On the one hand, there are reports describing an HBx-dependent activation of PKC, mediated by an elevated DAG level in HBx-producing cells. In these studies, PKC is considered as an essential factor for the HBx-dependent activation of NF-kB or AP-1[61,62]. Other reports provide evidence that HBx neither affects activity of PKC nor that PKC is essential for HBx-dependent transcriptional activation[63-65]. The interesting aspect of an HBx-dependent activation of PKC is that a conclusive model for the role of HBx in the process of HBV-dependent carcinogenesis can be deduced. According to the two-step model of carcinogenesis[66] (initiation and promotion), the HBx-dependent activation of PKC could exert a tumor promoter-like function[61]. Independent from the point whether or not the HBx-dependent activation of PKC exerts a tumor promoter-like function, there is profound experimental evidence from experiments with transgenic mice that HBx indeed could exert a tumor promoter-like function. Irradiation of HBx transgenics or exposure of these transgenics to mutagens (diethylnitrosamine) caused a significant increase in the amount of pre-neoplastic lesions as compared to the wild-type control animals[67,68]. Apart from this, a variety of HBx transgenic mouse models were established, but only in one model system so far direct formation of liver cancer could be observed[69].

A tumor promoter-like function of HBx does not necessarily require an activation of PKC. Other pathways as the activation of c-Raf-1-MEK/MAP2 (mitogen-activated protein kinase 2) kinase cascade could fulfill this function as well. Starting with the finding that HBx increases the Ras/GTP complex formation and thereby activates the c-Raf-1 signal transduction cascade[70], more and more data were collected elucidating the interference of HBx with signaling cascades upstream of Ras. One of the next steps was the observation that Src is activated in HBx-producing cells[71,72], followed by the observation that HBx is able to activate the cytosolic Ca^{2+}-dependent praline-rich tyrosine kinase 2 (Pyk)[54]. Pyk is able to activate Src. A recent report describes an HBx-dependent activation of FAK (focal adhesion kinase), a well known regulator of Src kinases[53]. The activation of these signaling cascades requires the presence of HBx in an extranuclear compartment. On the other hand, there is evidence that a fraction of HBx is localized within the nucleus. The subcellular distribution of HBx is still a matter of debate. There are reports providing evidence that HBx is localized in the cytoplasm as well as in the nucleus[49,73]. The different localizations are associated with different functions. HBx localized in the cytoplasm is able to modulate intracellular signal transduction cascades as described above. Moreover, an association of HBx with the outer membrane of mitochondria that induces oxidative stress was described[74-76]. HBx localized in the nucleus is suggested to interfere directly with transcription factors or to exert a transcription factor-like function. A direct interaction with CREB (cAMP responsive element–binding protein) and ATF-2 (activating transcription factor 2) resulting in their increased DNA binding affinity[77] was reported as well as an interaction with RNA polymerase Ⅱ in the transcription complex[78,79].

In addition to the interaction of HBx with the transcription machinery, there is evidence that HBx interferes at multiple steps with DNA repair and so confers to an increase of critical mutations. HBx was found to bind to DDB1[80,81], a subunit of the damaged DNA binding protein that is bound to damaged DNA, the first step in nucleotide excision repair. In cell culture experiments indeed the expression of HBx significantly inhibited the ability of cells to repair damaged DNA.

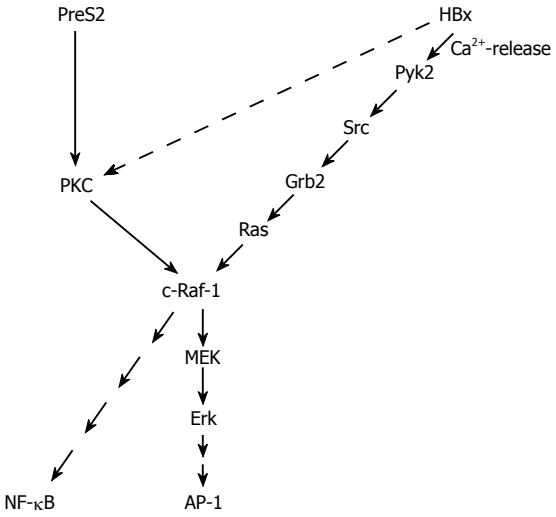

Figure 1 Major signaling pathways activated by the regulatory proteins HBx and PreS2 of HBV.

Therefore, it was tempting to speculate that HBx could confer by this to an increase in the amount of critical mutations in the host genome[80]. However, analysis of mutation frequency in HBx transgenic mice did not corroborate this hypothesis[82]. Other reports focus on the interaction of HBx with p53. On the one hand, it has been shown an indirect inhibition of p53 by HBx: HBx causes a transcriptional repression of the human p53 gene[83]. On the other hand, there is evidence for the capacity of HBx to bind to p53[84,85]. However, if the intracellular amounts of HBx and p53 are considered, there exists a tremendous excess of p53 as compared to HBx in the hepatocytes. The physiological significance of the direct p53/HBx interaction remains questionable.

The family of the PreS2 activators

Apart from the HBx-regulatory protein, the HBV genome encodes a second family of regulatory proteins: the PreS2 activators. Based on a subcloned HBV integrate of the human hepatoma cell line huH4[86] and of an integrate isolated from an HBV-associated HCC[87], preS/S genes that were truncated at the 3´ end were identified[41]. These preS/S^t genes encoded for C-terminally truncated surface proteins (MHBs^t) that display a regulatory protein function. Initial analysis revealed that generation of the regulatory protein function requires at least deletion of the last transmembrane region in the S-domain (transmembrane region 3)[88-91]. This results in C-terminally truncated MHBs molecules that are endoplasmic reticulum (ER)-membrane associated by the remaining transmembrane regions I and Ⅱ of the S domain[92,93]. A prototype of the ER-membrane-associated MHBs^t activator is encoded by the integrate isolated from the human hepatoma cell line huH4[86]. This integrate is truncated at ntHBV 221 of the HBV genome resulting in a C-terminally truncated MHBs protein at amino acid (aa) 76 (MHBs^{t76}). A detailed analysis revealed that a variety of differences exist between the structural protein MHBs and its C-terminally truncated variant MHBs^t. In contrast to the structural protein MHBs and the regulatory variants, MHBs^t are not secreted and lack the glycosylation at asparagine (asn) 4 of the PreS2 domain[92]. The intracellular retention of ER-membrane-associated MHBs^t proteins gave raise to the hypothesis that the observed activator function is due to ER stress, induced by intracellular retention and subsequent accumulation in the ER[93-96]. More detailed analysis revealed, however, that the structural protein MHBs and the regulatory protein MHBs^t differ in the topology of the PreS2 domain[97]. In case of the structural protein, the PreS2 domain faces the lumen of the endoplasmic reticulum and in accordance with this glycosylation at asn 4 can occur. In case of the activator protein MHBs^t, the PreS2 domain directs into the cytoplasm. This explains the lack of N-glycosylation at asn 4. The PreS2 domain facing the cytoplasm interacts with cytosolic binding partners, thereby triggering intracellular signal transduction cascades. In accordance with this, a minimal PreS2 activator was identified lacking any membrane insertion domain (MHBs^{t55})[88,67,98]. This minimal activator encompasses the complete PreS2 domain and is localized within the cytoplasm. Since the PreS2 domain is sufficient to exert the regulatory protein function, this class of regulatory proteins was designated PreS2 activator. The family of PreS2- regulatory proteins encompasses the membrane-associated regulatory proteins, such as MHBs^{t76} or MHBs^{t167}, and the non-membrane-associated short proteins, such as PreS2 domain (MHBs^{t55}). There is no functional difference between the ER and the cytoplasmatically localized Pres2 activators clearly arguing against the ER-overload hypothesis[97].

The PreS1-PreS2 domain of the large hepatitis B virus surface protein (LHBs) displays a dual membrane topology[99-101]. In one fraction of LHBs, the first transmembrane region that is located at the beginning of the S-domain (aa 8-21) is used: in this case, the PreS1-PreS2 domain of LHBs faces the lumen of the endoplasmic reticulum. In case of the other fraction, this transmembrane region is not used, resulting in a PreS1-PreS2 domain that directs into the cytoplasm. As described above, the cytoplasmic orientation of the PreS2 domain in case of the MHBs^t proteins is causative for their regulatory protein function. In accordance with this, LHBs displays a regulatory protein function[102] and belongs to the family of PreS2 activator proteins.

The PreS2 activators bind PKC-α in the cytoplasm. This interaction with PKC results in a DAG (1, 2, sn diacylglycerol)-independent activation of PKC and phosphorylation of the PreS2 domain. The activation of PKC is transduced by the c-Raf-1/MEK/ERK (extracellular signal-regulated kinase) signal transduction cascade[63]. This signal transduction cascade can exert a tumor promoter-like function according to the classical two-step model of carcinogenesis[66]. Indeed, transgenic mice expressing the PreS2 activator MHBs^{t76} develop liver tumors at an age above 10 mo. Although the MHBs^{t76} protein is produced in very small but clearly detectable amounts in the MHBs^{t76} transgenic mice, a permanent activation of the Raf-1/MEK/ERK signal transduction cascade can be observed, resulting in an increased proliferation rate of the hepatocytes. The fact that MHBs^{t76} is produced in very small amounts ensures that the observed effects are not due to any overload-associated effects. The tumor formation in these mice can be explained by the permanent activation signal transduction cascades that exert a tumor promoter-like function[63]. Since tumor formation is observed in older animals, it can be assumed that during the aging process critical mutations are accumulated (initiation) and then the tumor promoter function positively selects these cells.

In case of the LHBs-transgenics, tumor formation can be observed as well[103]. In these mice, a very strong overproduction of the LHBs protein occurs, resulting in an intracellular accumulation of the protein and subsequent formation of ground glass hepatocytes. This permanent accumulation results in a situation comparable to a storage disease. Tumor formation in these transgenics was explained by the resulting permanent inflammation[103-105]. In light of the observation that LHBs can act as a regulatory protein, however, the regulatory protein function that is immanent to LHBs should be considered as an additional factor conferring to tumor formation in these mice. The overload-associated stress

and inflammation results in the formation of critical mutations (initiation) and the permanent activation of the PKC/Raf/MEK/ERK signal transduction cascade which exerts a tumor promoter-like function.

Immune pathogenesis of HCC

A major factor in the process of HBV-associated HCC development is the immune system[104,106,107]. The relevance of a chronic, virus-specific immune response for development of HBV-associated carcinoma was shown in an elegant experiment from F. Chisari`s laboratory[108]. Transgenic mice that produce non-cytopathic amounts of HBsAg were used. In these mice, immunologic tolerance against the transgene product can be observed. In accordance with this, no evidence of the liver disease was observed. These mice were subjected to thymectomy and lethally irradiated. One group was reconstituted with the bone marrow and spleen cells derived from non-transgenic littermates that were vaccinated with a recombinant HBsAg encoding vaccinia virus resulting in HBsAg-specific cytotoxic T lymphocytes (CTLs) and antibodies. The other group was reconstituted with the bone marrow and spleen cells derived from transgenic donors that were immunologically tolerant.

In this animal model, the development of hepatitis and later of chronic hepatitis and finally HCC development could be exclusively observed in the mice that were reconstituted with the bone marrow and spleen cells derived from the vaccinated non-transgenic animals, but not in the control groups. Based on this, it was concluded that the immune system-mediated chronic inflammation of the liver, continuous cell death and subsequent cell proliferation might increase the frequency of genetic alterations and the risk of cancer[104,109-111]. This scenario is not exclusively restricted to HBV. Chronic inflammation, degeneration and regeneration are common to a variety of human liver diseases, such as glycogen storage disease or alcoholism or HCV infection, that can finally result in liver carcinoma development[5]. This means that an ineffective immune response can be the principal oncogenic factor during a chronic HBV infection in man. In other words, the same T-cell response can have complete different effects: if the T cell response is strong enough, HBV can be eliminated from the liver, if not, a pro-carcinogenic effect can be induced by permanently triggering necro-inflammatory disease without resulting in a final eradication of HBV from the liver. An interesting aspect is that the nucleoside analogue on lamivudine in patients with chronic hepatitis B can induce the recovery of antiviral T cell responses. However, restoration of HBV-specific T cell reactivity is only transient. The transient nature of the immune reconstitution may represent a favorable condition for virus reactivation once lamivudine therapy is withdrawn.

CONCLUSION

At present, HBV-associated carcinogenesis can be seen as a multi-factorial process that includes both direct and indirect mechanisms that might act synergistically. The integration of HBV DNA into the host genome occurs at early steps of clonal tumor expansion. The integration has been shown in a number of cases to affect a variety of cancer-related genes and to exert insertional mutagenesis. The permanent liver inflammation resulting in a degeneration and regeneration process confers to the accumulation of critical mutations in the host genome. In addition, the regulatory proteins HBx and the PreS2 activators that can be encoded by the integrate can exert a tumor promoter-like function, resulting in positive selection of cells producing a functional regulatory protein.

Based on new technologies, including gene expression profiling and proteomics, it should be possible to further reveal the molecular mechanisms underlying HBV-associated HCC development and to identify novel diagnostic markers as well as therapeutic and preventive targets.

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