Stimulation of cellular proliferation by hepatitis B virus X protein

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Chronic infection with the hepatitis B virus (HBV) is a known risk factor in the development of human hepatocellular carcinoma (HCC). The HBV-encoded X protein, HBx, has been investigated for properties that may explain its cancer cofactor role in transgenic mouse lines. We discuss here recent data showing that HBx is able to induce hepatocellular proliferation *in vitro* and *in vivo*. This property of HBx is predicted to sensitize hepatocytes to other HCC cofactors, including exposure to carcinogens and to other hepatitis viruses. Cellular proliferation is intimately linked to the mechanism(s) by which most tumor-associated viruses transform virus-infected cells. The HBx alteration of the cell cycle provides an additional mechanism by which chronic HBV infection may contribute to HCC.

1. Introduction

At present, chronic viral infections are linked etiologically with 15% of all human cancers (reviewed in [31,49]). One such association involves the link between chronic hepatitis B virus (HBV) infection and the development of human hepatocellular carcinoma (HCC) [1]. There are an estimated 300 million chronically infected people worldwide, and HBV-associated HCC is one of the most common cancers in humans [34]. Because liver cancer arises in only a subset of infected patients, it is important to identify early markers of viral infection that can accurately predict those patients at risk for cancer.

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2. HBV and liver cancer

The mechanism(s) by which HBV contributes to HCC formation is complex. Cycles of immune-mediated cell death and regeneration are central to the pathogenesis of chronic viral infection (reviewed in [11]). The release of oxidants from resident phagocytes can damage liver DNA [19], and the compensatory cell proliferation that follows cell death increases the opportunity for cells to incur mutations necessary for tumor development (reviewed in [11]). During the decades-long chronic infection, portions of the viral genome integrate randomly into host cell DNA. While on rare occasions, this may lead to the direct disruption of a growth regulatory gene (reviewed in [38]), more commonly it leads to chromosomal rearrangements that include the loss of tumor suppressor genes (reviewed in [36]).

Less is known about the contribution(s) of virus-cell interactions, which are thought to evolve for the benefit of virus replication. Such interactions are particularly critical for small viruses like HBV, which has a limited genome size and replicates in resting hepatocytes. Many indirect-acting human tumor viruses encode viral oncoproteins that are essential for viral replication and cell transformation (reviewed in [7]). Although HBV does not appear to encode a direct acting oncogene, the 17-kDa HBx regulatory protein possesses several properties consistent with a role in transformation. Indeed, HBx has a cancer cofactor role in several transgenic mouse lines (Table 1).

3. The HBV regulatory protein, HBx

HBx is a broadly acting transcriptional transactivator (reviewed in [6]) that is required for virus replication *in vivo* [9,48]. HBx can activate core gene expression *in vivo* [33], but additional functions of HBx important to the virus life cycle are not yet defined. In cell culture, HBx is reported to possess AMP kinase activity [14], to activate signaling pathways in the cell [4,12,30], and

Table 1
HBx is a cofactor in transgenic mice

Regulatory region[a]	Mouse strain	Pathology	Other observations	Role of HBx (Ref.)
α-1-anti-trypsin	Outbred (CD-1)	None		None [27]
HBV promoter	Outbred (CD-1)	HCC[b] (100%)	10% spontaneous HCC	Cofactor [22]
MUP	C57BL6 / x SJL	None		None [18]
α-1-anti-trypsin	Outbred (ICR)	None	HCC with DEN	Cofactor [37]
WHV promoter	Outbred (CD-1)	None	HCC with DEN	Cofactor [13]
HBV promoter	C57B1/6 /DBA2	None	HCC with activated *myc*	Cofactor [43]
Anti-thrombin III	C57B1/6 /DBA2	None	HCC with activated *myc*	Cofactor [43]

[a]Regulatory region controlling expression of the HBV X gene. WHV, Woodchuck hepatitis Virus; MUP, major urinary protein.
[b]HCC, hepatocellular carcinoma.

to inhibit the ability of cells to repair damaged DNA [2, 17,20,32]. The mechanism for the latter observation is unclear, and could be either direct (through protein-protein interactions) or indirect.

How HBx acts as a cofactor in transgenic mice is not known. HBx inhibition of damaged DNA repair suggests one possible pathway, and would be particularly important in geographical locations in which HBx expression coincides with exposure to environmental carcinogens [8,42]. In cell culture, HBx sensitizes cells to carcinogen-induced mutations [39], and it is predicted that HBx will similarly cause an accumulation of DNA mutations *in vivo*. A double transgenic mouse model has been developed to answer this question [29]. Although HBx expression in the absence of exogenous DNA damage does not alter the *in vivo* DNA mutation frequency [29], carcinogen experiments now in progress should reveal whether HBx inhibits DNA repair *in vivo* (C. Madden and B. Slagle, unpublished). A second mechanism to explain the HBx cofactor role in transgenic mice centers on the demonstration that HBx can activate the Ras-Raf-Map Kinase signaling pathway in cell culture [4,12,30]. This property of HBx *in vitro* has led to the suggestion that HBx may act as a tumor promoter *in vivo* by stimulating hepatocellular growth [4].

4. Induction of cellular proliferation by HBx

The induction of cell proliferation is intimately linked to the mechanism by which most tumor-associated viruses transform virus-infected cells. Several different tumor viruses (SV40, HPV-16, EBV) encode non-structural proteins that are able to stimulate resting cells to enter S phase, in a strategy that provides a cellular environment favorable for viral DNA replication. The ability of these viruses to usurp cell cycle control appears related to their ability to cause cancer.

Recent studies clearly demonstrate that HBx can stimulate the proliferation of cells grown in culture. Upon release from quiescence induced by low serum concentrations, Chang liver epithelial cells expressing HBx move through the cell cycle (from Go to S phase, and into DNA synthesis) at an accelerated pace compared to control cells similarly released from that block [3,4,39]. Similarly, dexamethasone-induced HBx expression is sufficient to overcome a serum-induced Go block in NIH 3T3 cells [24]. These studies establish that HBx may act as a mitogen to stimulate cell cycle progression in quiescent cells *in vitro*. It is likely that HBx-induced proliferation is under-reported, as the very nature of actively dividing cells in culture would diminish the ability to measure the proliferative effect of HBx.

The effect of HBx on hepatocyte proliferation *in vivo* is less clear. Most HBx transgenic mice do not develop tumors, indicating the need for additional cooperating events (Table 1). Analysis of HBx transgenic mice that are susceptible to spontaneous HCC [22] revealed an HBx-associated increase in hepatocellular proliferation [25], while other HBx mice that do not develop HCCs lack an HBx-associated proliferation [43, 44]. Our studies of transgenic mice in which a cofactor role for HBx has been established [27,37] revealed that livers from HBx (vs. nontransgenic) mice contained significantly more proliferating hepatocytes (Table 2; C. Madden, unpublished). These results indicate that HBx is able to induce hepatocellular proliferation under certain conditions *in vivo*. Interestingly, this property is conserved among at least three subtypes of HBx, which differ by over 10% in the sequence identity (Table 2) [21].

A delicate balance between cellular proliferation and apoptosis is required for normal liver homeostasis. Since HBx is able to induce hepatocellular proliferation (Table 1), there must be a compensatory effect on apoptosis. Indeed, several studies have demon-

Table 2
HBx effects on cellular proliferation

X Subtype[a]	Regulatory region[b]	Cell type	Effect of HBx expression (ref.)[c]
ayw	CMV	Chang liver epithelial	Increased entry into cell cycle [3,4]
adr	MMTV	Chang liver epithelial	Increased cell proliferation [24]
ayw	AP2	Chang liver epithelial	Increased entry into cell cycle [39]
adr	HBV	Murine hepatocyte	Increased proliferation and apoptosis [25]
adw2	AAT	Murine hepatocyte	Increased proliferation[d]

[a] X gene derived from different subtypes of HBV, as listed.
[b] Regulatory region driving expression of HBx: CMV, Cytomegalovirus promoter; MMTV, Mouse Mammary Tumor Virus LTR; AP2, promoter responsive to AP2 transcription factor; HBV, native X promoter; AAT, human α-1-anti-trypsin promoter.
[c] Ref., reference.
[d] C. Madden, manuscript submitted.

strated that HBx sensitizes cells to apoptosis in cell culture, although the mechanism by which HBx acts is unknown [5,10,23,40]. While HBx is reported to bind p53 [15,46], to inhibit p53 sequence-specific DNA binding [47], and to inhibit p53 transactivation activity [26,45,47], other studies have failed to support the idea that a p53 pathway is utilized in HBx-mediated effects on apoptosis or proliferation [16,41]. Using the inducible Cre/loxP expression system, HBx detection preceded apoptosis by at least one day [35], a result in agreement with the need for additional cooperating events. Consistent with that observation, the HBx-associated increase in apoptosis *in vivo* was not limited to those cells expressing detectable HBx [25].

Together, these results reveal that HBx does sensitize cells to apoptosis in some experimental systems. Although the biologic significance of this remains unclear, an imbalance between cell death and regeneration *in vivo* may contribute significantly to viral pathogenesis over time.

5. Implications of HBx-induced proliferation

It can be presumed, by analogy with known tumor viruses, that the ability of HBx to induce cell proliferation somehow benefits viral replication. In other tumor virus systems, the binding to and inactivation of cellular genes involved in negative growth regulation, e.g., tumor suppressor genes, is critical for virus-induced cellular proliferation (reviewed in [28]). Data showing that HBx either stabilizes p53 or promotes its degradation, as has been shown for other tumor virus-encoded proteins, would provide much support for the hypothesis that HBx acts through a p53 pathway. The lack of a cell culture system for HBV severely limits studies designed to understand the contribution of HBx-induced proliferation to virus replication.

The observation that HBx can induce hepatocellular proliferation in the absence of an immune response (Table 2) is significant. The immune-mediated induction of hepatocellular proliferation during chronic viral infection is well established (reviewed in [11]). Continuous cycles of cell death and compensatory regeneration are considered central to the mechanism(s) by which chronic hepatitis viral infections lead eventually to the development of liver cancer. The virus-induced proliferation, regardless of the molecular mechanism, may set the stage for the accumulation of genetic mutations that result in cancer.

In the woodchuck hepatitis B virus model, expression of the homologous WHx protein is found only in cells that are permissive for viral replication [13]. A similar expression pattern for HBx would predict its expression during the decades of chronic infection, concomitant with exposure to environmental carcinogens in certain geographical regions [8,42]. Therefore, HBx-induced cellular proliferation of cells containing unrepaired DNA lesions would lead to fixation of those lesions into DNA mutations. This scenario could explain, in part, the synergy between chronic HBV infection and other HCC cofactors such as environmental carcinogens.

The above hypothesis can be tested experimentally using a new HBx transgenic mouse model that additionally contains a transgene that permits measurement of *in vivo* DNA mutation frequency. In those mice, HBx expression per se does not lead to liver pathology nor does it influence the accumulation of spontaneous DNA mutations [29]. However, a 3-fold increase in hepatocellular proliferation in HBx (vs. nontransgenic) livers could explain the increased susceptibility of those mice to hepatocarcinogens [13,37]. In this scenario, HBx might act by inducing the proliferation of cells containing unrepaired DNA lesions, thereby providing a growth advantage for the selection of a mutated cell.

6. Conclusion

The cooperation between chronic HBV infection and other HCC cofactors in the etiology of HCC has been established. We review here data showing that HBx is able to induce hepatocellular proliferation *in vivo*, a property that presumably benefits some aspect of the virus life cycle. We propose that the ability of HBx to induce cellular proliferation may contribute to viral pathogenesis during the decades of chronic infection. The untimely proliferation of cells containing damaged DNA may lead to an increase in DNA mutations. In addition, HBx-induced proliferation may sensitize cells to additional cofactors, such as chronic hepatitis C virus and/or dietary factors. Understanding the underlying mechanism of HBx-induced hepatocellular proliferation may reveal a molecular marker by which early HCCs can be identified, and lead to the identification of novel targets for intervention and treatment.

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