Review Article

Pathogenesis and Molecular Mechanisms of Hepatitis B-Induced Hepatocellular Carcinoma

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ABSTRACT

Despite the existence of Hepatitis B vaccination, hepatitis B virus (HBV) infection is still prevalent worldwide and accounts for significant rates of morbidity and mortality. Hepatitis B virus infection can be influenced by many factors such as host immune status, age at infection, and level of viral replication. Majority of patients do recover from acute form of the infection; however, those that progress to chronic disease state is at great risk of developing complications such as hepatocellular carcinoma, cirrhosis and liver failure. Although the mechanisms by which chronic hepatitis B viral infection results in hepatocellular carcinoma are unclear, there is good evidence that the virus itself exerts a direct hepatocarcinogenic effect. With an estimated incidence of 500,000 – 1,000,000 new cases and 600 000 deaths annually, hepatocellular carcinoma due to HBV is said to be the third leading cause of cancer deaths globally. Despite the discoveries in cancer biology in respect with physiological and pathological factors in relation to prognosis, HCC remains still a fatal disease due to late diagnosis. The incidence is highest in Asia and Africa, where the endemic high prevalence of the virus strongly predisposes to the development of chronic liver disease and subsequent development of hepatocellular. Better approaches to universal HBV vaccination and identification and treatment of chronic hepatitis B are needed to allow for the application of the many advances that have been made in the understanding, prevention, and treatment of hepatocellular carcinoma.

Key words: Hepatocellular carcinoma, hepatitis B infection, hepatitis B proteins.

INTRODUCTION

Hepatocellular carcinoma (HCC), characterized by rapid tumor growth and a high propensity of vascular invasion, is one of the most common malignancies, ranking fifth in frequency among all malignancies in the world[1]. More than 80% of cases can be attributed to chronic infection with HBV in hyper-endemic regions, suggesting that chronic hepatitis B is a major risk factor for development of the disease[2]. Globally, HCC is increasingly becoming a major health concern with estimates of 500,000 – 1,000,000 new cases and 600 000 deaths reported annually[3]. Due to differences in the prevalence of viral hepatitis, the incidence of HCC in low and middle income countries is considered to be much higher than that of high income countries[4].

Increasing evidence points out to two major HBV-specific mechanisms that contribute to the development of HCC. The first is the integration of the viral genome into the host chromosome causing cis-effects, resulting in loss of tumor suppressor gene functions, and/or activation of tumor-promoting genes. The second mechanism involves the expression of trans-activating factors derived from the HBV genome, which have the potential to influence intracellular signal transduction pathways and alter host gene expression[5].

HBV can be transmitted through percutaneous or mucosal exposure to infected blood or other body fluids. The transmission has been observed with numerous forms of human contact including perinatal/mother-to-child; household (nonsexual); sexual; needle-sharing; and occupational/health-care-related. The highest concentrations of infectious HBV are found in blood and serum. However, other serum-derived body fluids, such as semen and saliva could also be infectious. Persons with chronic HBV infection are the major reservoir for transmission, although any person testing positive for hepatitis B surface antigen (HBsAg) is potentially infectious to both household and sexual contacts[6].

HBV is a partially double stranded DNA virus belonging to the Hepadnavirus family. The viral genome is a relaxed circular DNA molecule of 3.2 kb. It is organized in a compact manner with four partially-overlapping open reading frames (ORF): S, C, P, and X. Four separate viral promoters drive the expression of several mRNAs which give rise to various viral proteins, with the longest mRNA (pregenomic RNA, 3.5 kb) also serving

as the template for viral genome synthesis. HBV shows a high degree of species specificity and infects only humans and higher primates. Other members of the Hepadnavirus family, namely the duck hepatitis virus, woodchuck hepatitis virus and ground squirrel hepatitis virus, are also species-specific[7].

Eight HBV genotypes have been identified (A–H)[8] based on the variation of the entire genome (>8% of nucleotide divergence)[5]

Strategies to limit the impact of HCC include primary prevention against new cases of viral hepatitis, secondary prevention in susceptible individuals, and early detection. Universal hepatitis B vaccination has resulted in dramatic reduction in incidence cases of chronic hepatitis B and HCC in children and adolescents. In addition, randomized controlled trials and meta-analysis has shown that successful treatment of chronic hepatitis B and C can reduce the risk of HCC and cirrhotic complications. Furthermore HCC surveillance by regular ultrasound examination and alpha-fetoprotein testing could lead to early cancer detection and offers the opportunity for curative treatment[4].

Pathogenesis

Hepatitis B virus primarily interferes with the functions of the liver by replicating in liver cells or hepatocytes. The receptor is not yet known, though there is evidence that the receptor in the closely related duck hepatitis B virus is carboxypeptidase D[9]. HBV virions (DANE particle) bind to the host cell via the preS domain of the viral surface antigen and are subsequently internalized by endocytosis. PreS and IgA receptors are responsible for this interaction. HBV-preS specific receptors are primarily expressed on hepatocytes; however, viral DNA and proteins have also been detected in extrahepatic sites, suggesting that cellular receptors for HBV may also exist on extrahepatic cells[10].

During HBV infection, the host immune response causes both hepatocellular damage and viral clearance. Antiviral cytokines, such as interferon alpha, beta, and gamma (IFN-ά, IFN-β, IFN-γ) as well as tumor necrosis factor alpha (TNF-ά), have been implicated as the major contributors to viral clearance. Although the innate immune response does not play a significant role in these processes, the adaptive immune response, particularly virus-specific cytotoxic T lymphocytes (CTLs), contributes to most of the liver injury associated with HBV infection. CTLs eliminate HBV infection by killing infected cells and producing antiviral cytokines, which are then used to purge HBV from viable hepatocytes[11].

Although liver damage is initiated and mediated by the CTLs, antigen-nonspecific inflammatory cells can worsen CTL-induced immunopathology, and platelets activated at the site of infection may facilitate the accumulation of CTLs in the liver[12].

An individual can develop hepatitis B infection that is acute and achieve complete immune clearance of virus yielding lifelong immunity; however, an alternate fate of the host is the development of chronic hepatitis B[13].

There are three stages of HBV infection based on viral-host interaction, namely, the immune tolerance phase, the immune clearance phase, and the inactive carrier phase with or without reactivation[13].

After acute infection of HBV, some patients may remain HBeAg positive with high levels of serum HBV DNA, with little or no symptoms, normal alanine aminotranferase (ALT) levels and minimal histological activity in the liver. This phenomenon is known as the immune tolerance phase. This phase is typical of infection in children and young adults. It usually lasts for 2-4 weeks, but can last for years in those who acquired the infection during the perinatal period[13]. Individuals in this group are highly contagious and can transmit HBV easily. When the tolerogenic effect is lost during the immune tolerant phase, immune-mediated lysis of infected hepatocytes may become active and patients may enter the second stage defined as immune clearance phase in which the HBV DNA level decreases and ALT level increases. The duration of the clearance phase may last from months to years[14]. This is followed by the carrier stage, in which seroconversion of HBeAg to HBeAb occurs and HBV DNA becomes non-detectable or at low level while ALT usually remains normal, reflecting very low or no replication of HBV and mild or no hepatic injury. The inactive carrier stage may last for years or even lifetime. Patients in this stage can have spontaneous resolution of hepatitis B and develop HBsAb, but a portion of them may undergo spontaneous or immunosuppression-induced reactivation of chronic hepatitis, featuring elevated ALT, high level of DNA, moderate to severe liver histological activity, and with or without HBeAg seroconversion[14].

In patients with self-limited, acute HBV infection, viral clearance may occur through a polyclonal and multispecific peripheral blood mononuclear cell response mounted against the epitopes of several HBV proteins, including the viral envelope, polymerase, and core proteins[15]. This response involves human leukocyte antigen class II–restricted $CD4^+$ helper T lymphocytes and $CD8^+$ cytotoxic T

lymphocytes. A predominantly type-1 helper T-lymphocyte response may develop and lead to the secretion of cytokines such as interleukin (IL)-2 and IFN-γ. These cytokines may contribute to liver cell injury as well as to recovery from disease[16].

On the hand, in patients with chronic HBV infection, the peripheral cytotoxic T-lymphocyte response may be weak or undetectable and narrow in focus. An activated humoral response may develop, characterized by the production of IL-4, IL-5, and IL-10 secreted by type-2 helper T lymphocytes. This response may promote antibody production rather than viral clearance. Low levels of intrahepatic HBV-specific cytotoxic T lymphocytes have been detected in such patients and are probably responsible for the hepatic flares that occur in patients with chronic disease. However, these activated cytotoxic T lymphocytes are unable to clear HBV[17].

Both the adaptive and the innate immune responses may play critical roles in viral clearance[18]. Activation of innate immunity may occur early in HBV infection. Animal studies have highlighted the important role of IFN-γ and TNF-ά in controlling the bulk of viral replication without necessarily inducing the perforinor Fas-dependent apoptotic pathway of cell killing[18]. This marked reduction in viral replication occurs typically before the peak infiltration of T cells and the onset of liver injury. Thus, activation of innate immunity during the early stages of infection appears to be essential for the control of HBV infection and accounts for the bulk of the antiviral activity of the host's immune response[18].

Occult HBV infection

Many studies have clearly demonstrated that HBV infection may persist also in the absence of circulating HBsAg[19]. This occult infection mostly associates with individuals positive for antibodies to HBV antigens (anti-HBc ± anti-HBs), but it also occurs in a considerable number of individuals negative for all serum markers of HBV infection[20]. Moreover, it is now evident that these patients with occult infection may carry both integrated and free episomal forms of HBV-DNA, in analogy to subjects belonging to the HBsAg positive categories[21]. In some cases, lack of HBsAg detection is due to rearrangements in the HBV genome that interfere with gene expression or lead to the production of an antigenically modified S protein that is not recognized by commercially available kits[22]. However, in most cases HBV has no relevant genomic modification and the occult infection is a consequence of a profound suppression of viral replication and gene expression[23,24]. The molecular/immunological mechanisms involved in this inhibition are at present largely undefined[25].

The essential question in occult HBV infection issue is whether or not it has any clinical impact. In this connection, its potential role in HBV transmission and as a risk factor for HCC development has been sufficiently documented, although many aspects in terms of pathogenic mechanisms and frequency of occurrence still need to be clarified. Undoubtedly, carriers of occult infection may transmit HBV in the event of blood transfusion or organ transplantation and the recipients may develop a type B hepatitis that may have all the possible outcomes of the typical HBV infection, including the fulminant course and the development of a chronic hepatitis[26]. Moreover, many epidemiological and molecular studies performed since the '80s indicate that a persisting HBV infection might be involved in the development of HCC also in HBsAg-negative patients[27]. This evidence is confirmed by data in animal models showing that both woodchucks and ground squirrels, once infected by the corresponding hepadnaviruses (WHV, GSHV), are at high risk of developing HCC also after the apparent clearance of the virus[28,27.]

Occult HBV contribute to hepatocellular transformation through the same mechanisms traditionally considered the basis of the tumorigenic properties of the HBV[27]. Therefore, occult HBV infection appears to have relevance in terms of infectivity and contribution to HCC development, but whether it may induce liver injury is still unsolved[29].

Several reports have shown that HBV genomes may persist for decades after recovery from self-limited acute hepatitis[30,31], suggesting that the occult infection is inoffensive in itself and it might become injurious only in the event of viral reactivation occurring under immunosuppressive circumstances[32]. Nevertheless, there is growing evidence that HBsAg sero-clearance does not necessarily imply a good prognosis [33]. Furthermore it has been shown that patients with either idiopathic or HCV-related chronic liver disease have a high prevalence of occult HBV infection: among HCV patients, those carrying the occult HBV appear to have a more severe and precocious liver disease and a poorer response to interferon-ά therapy. All these data provide further support to the hypothesis that occult HBV infection might be responsible for liver injury, although much more research is needed to fully clarify the

biological basis and clinical meaning of this peculiar kind of infection[22].

Chronic inflammation and cirrhosis

A potential element of HBV-related hepatocarcinogenesis is the dynamic course of chronic hepatitis B. It is characterized by periodic down-regulations of viral titers accompanied by immune-mediated liver injuries known as ‘‘flares”. Such periodic injuries result in repeating cycles of death and proliferation of hepatocytes[34]. Liver injuries caused by chronic hepatitis B are considered to be immuno-mediated and are mainly due to the activity of HBV-specific T cells. However, some data suggests important roles of non-specific chemokine-mediated infiltration of neutrophils, nature killer cells and activated bystander lymphocytes in causing HBV-related liver damage. These inflammatory cells release cytokines and chemokines which may favor cancer growth. High hepatocyte proliferation rate is a major risk factor for hepatocellular carcinoma development in the cirrhotic liver[13].

Chronic inflammation also results in increased production of reactive oxygen species (ROS) which can cause DNA damage and lead to gene mutations[35]. Increased intracellular ROS levels can also activate several signal transduction pathways that regulate proliferation, differentiation and apoptosis, including the MAP-kinase/AP-1 and NF-kapaB pathways[35]. Inflammation-induced oxidative stress and influx of Kuppfer cells can promote the activation of stellate cells. The latter are the main producers of extracellular matrix in the liver[36]. Their persistent activation can finally lead to cirrhosis, which is characterized by the co-existence of regenerative nodules, irreversible fibrosis and severe liver injury[35].

Cirrhosis is an important predisposing state for HCC development. About 80–90% of HCCs of all etiological backgrounds arise in cirrhotic livers. In HBV endemic areas, infected patients with cirrhosis have an approximately 3-fold higher risk for HCC than those with only chronic hepatitis B, and a 16-fold higher risk than asymptomatic carriers[37]. The incidence of HCC development is approximately 3–5% per year in HBV associated cirrhosis. The long-term persistence of occult might also exert an indirect oncogenic role by chronically sustaining a mild necroinflammation contributing to cirrhosis and HCC development [38].

A higher rate of cirrhosis has been reported in HBeAg-negative as compared to HBeAg-positive patients. Also, older age and persistent viral replication are predictors for development of cirrhosis as well as mortality. Genotype C is associated with a higher risk of cirrhosis than genotype B based on studies in Asian patients[39]. The presence of any other independent hepatotoxic factors such as alcohol ingestion and HCV co-infection can contribute to progression to cirrhosis. Once cirrhosis is established, individuals can decompensate over time. In a EUROHEP cohort study, a 5-year cumulative incidence of hepatic decompensation was 16%, the incidence per 100 person years was 3.3 and the mean interval between the time of diagnosis of cirrhosis and the onset of first episode of decompensation was 31 months (range 6-109)[40]. After decompensation, the survival may drop to 55% to 70% at 1 year and to 14% to 28% at 5 years. Interestingly, an improvement in liver function activity has been observed in those individuals who subsequently lose their HBsAg positivity[37].

Molecular mechanisms

Integration of Hepaptitis B DNA into host genome

The extent by which HBV genome insertions contribute to hepatocarcinogenesis is still controversial[5]. Integration events are thought to precede the development of the tumor, since they are also found in individuals with chronic hepatitis B and are even observed during the acute stage of infection[41]. The integrations of HBV DNA into the genome of host hepatocytes are found in 85–90% of HBV-related HCCs[42].

Viral genome insertions have been shown to be facilitated by cellular conditions that disrupt host genomic stability or increase cellular DNA replication, such as exposure to oxidative stress or DNA-damaging agents, deficiency of the DNA repair machinery, co-infection with other viruses and chronic liver injuries that increase hepatocyte turnover[42]. Unlike the woodchuck hepatitis B virus (WHV), which preferentially inserts its DNA genome into c-Myc or N-Myc genes in the woodchuck genome, no specific genes in humans have been identified so far to be the preferential target for HBV insertion[42]. However, the insertion itself can induce general genomic instability in host cells, leading to chromosome deletions, duplications, amplifications, translocations and transpositions of viral and flanking host sequence between chromosomes[41]. In addition, the regulatory proteins HBx and the PreS2 activators can exert a tumor promoter-like function, resulting in positive selection of cells producing a functional regulatory protein[43].

Indeed, in HCC cells and liver tissues from HCC patients, a series of cancer-relevant cellular genes has been shown to be altered by HBV DNA insertion, in particular those regulating cellular immortalization (hTERT), proliferation (MAPK1, cycline A), and viability (TNF receptor-associated protein 1), suggesting that hepatocytes harboring these insertions are preferentially selected during hepatocarcinogenesis[42]. The rearrangement of viral and host DNA sequences during integration can lead to the production of altered protein products whose functions may render hepatocytes prone to transformation. For example, a C-terminal truncated form of the X protein resulting from a deletion of inserted viral DNA has been reported to enhance oncogene-induced transformation, whereas the wild-type full-length protein suppresses such transformation[43]. HBx mutations have been frequently described among occult HBV infections[44].

Taken together, though the insertion of HBV DNA into the host genome might be a random event that contributes to the formation of an unstable environment, and occasionally induces genomic alterations that facilitate hepatocyte transformation even in the case of occult HBV infections. Therefore, HBV integration may contribute, at least in part, to the development of most of HBV induced HCCs[5].

Role of hepatitis B virus proteins

In addition to the integration of viral genome into host DNA, another direct role of HBV in hepatocarcinogenesis involves the long-term expression of viral proteins such as surface proteins and the X protein (HBx). This is supported by the observation that in a large portion of HCCs, viral DNA sequences encoding these proteins are found to be integrated in the host genome[41].

HBV surface protein

the oncogenic potential of HBV surface proteins have been suggested by experiments showing that a truncated form of the large surface protein can increase hepatocyte proliferation by upregulating cyclin A expression, and that a truncated M surface protein can specifically activate c-Raf-1/Erk2 signaling an increased hepatocyte proliferation [45].

In addition, HBV surface proteins may contribute to HCC development by accumulating in the endoplasmic reticulum (ER) and inducing ER stress. ER stress is defined by the imbalance between the quantity of unfolded or misfolded proteins that enters the ER, and the capacity of the ER machineries to handle the folding of these proteins. Such imbalance activates intracellular signaling pathways that are often referred to as unfolded protein response (UPR)[47]. Activation of UPR can lead to various biological outcomes depending on the intensity and duration of ER stress. Under mild and temporary ER stress, UPR lowers the ER burden by attenuating protein synthesis and increasing protein degradation, as well as by increasing the expression of chaperones that refold misfolded proteins[47,46]. In the case of severe or prolonged ER stress, UPR triggers apoptosis through yet poorly understood mechanisms[46].

An important side effect of UPR relevant to carcinogenesis is the induction of oxidative stress. UPR has been shown to increase the production of intracellular ROS through upregulating the oxidative folding machinery [48]. The latter is responsible for oxidizing thiol groups to form disulfide bonds, and has been shown to be an important source of intracellular ROS[49]. UPR-mediated upregulation of ROS has been reported to contribute to ER stress induced apoptosis[50]. Similarly, the oxidative stress caused by increased ROS can lead to various oncogenic consequences, such as DNA damage, activation of mitogenic response and the deregulation of signaling pathways that control proliferation, cell survival and cell death[13].

During viral infection, the ER serves as an essential organelle for viral replication and maturation. Recent evidence suggests that infection by many types of virus, including HBV and HCV, can induce ER stress due to the large amount of unfolded and misfolded viral proteins synthesized in infected cells[49]. In human liver tissues with chronic HBV infection, cytoplasmic accumulation of HBsAg is often observed and has long been associated with the formation of ‘‘ground–glass” hepatocytes [50]. These hepatocytes often demonstrate ER proliferation and distortion[51]. Several studies have shown that ground–glass hepatocytes contain mutations in the pre-S region of the HBV genome, which may lead to the overproduction of the large surface protein that blocks the secretion of all forms of surface proteins from the ER[42]. Accumulation of these viral proteins in the ER triggers ER stress, leading to the induction of oxidative stress and DNA damage, and to the predisposition of hepatocytes to transformation[52].

The above scenario has been reproduced in transgenic mice containing the entire HBV envelope coding region. Four transgenic mice

lineages presenting progressively higher intrahepatic concentration of HBsAg were studied. Similar to human chronic HBV infection, these mice also demonstrated ground–glass alteration in their hepatocytes due to the ER accumulation of the large surface protein. Such accumulation results in a series of oncogenic events, including liver dysplasia. Severe prolonged liver damage, regenerative hyperplasia nodule formation and incidence of HCC was directly related to the amount of HBsAg accumulated in the hepatocytes[53]. Similarly, increased levels of oxidative DNA damage has been observed in the liver of these mice[54]. These observations, together with data obtained using human HCC cells described above, support the notion that HBV surface proteins harbor oncogenic effects, and that such effects are mainly mediated by the induction of ER stress and the consequent elevation of intracellular ROS levels[51].

HBV X protein

HBx has been shown to interfere with numerous cellular activities, in particular, proliferation, apoptosis, senescence and DNA repair. However, controversial or even opposite effects of HBx on the same cellular function are often reported, depending on the experimental models used[55].

HBV X protein (HBx) is a small protein of 154 amino acids, and is encoded by the X ORF which is highly conserved among mammalian Hepadnaviruses. HBx has been shown to be required for infectivity in vivo in the woodchuck model, and in some experimental conditions it may enhance viral DNA replication in tissue culture. Contradictory observations have been reported concerning the intrinsic tumorigenic effect of HBx in vivo, with HCC developing in some, but not all of transgenic mice carrying the X gene. However, in these mice, HBx was shown to increase HCC development in the presence of c-Myc expression or upon exposure to low levels of carcinogens, suggesting that the viral protein may assist hepatocarcinogenesis in the context of a cancer-prone environment as it has been shown to activate moderately in trans, a variety of cellular gene expression[55].

Furthermore, HBx was reported to inhibit apoptosis induced by p53, TNF, Fas, or TGF-beta, while it was shown to promote cell death by inducing mitochondrial aggregation and cytochrome C release, or by sensitizing cells to TNF-mediated killing. Several groups also reported its interaction with DNA repair proteins (UVDDB1). HBx has been observed to increase proliferation by inducing G0–G1 transition, and stimulating cell cycle progression in differentiated hepatocytes, whereas it causes S phase arrests in dedifferentiated cells. HBx may also play a major role in the epigenetic control of viral and host gene expression[56].

CONCLUSION

HBV is the single most common cause of HCC worldwide. HBV-related HCC is most common in developing countries, particularly in Eastern Asia and sub-Saharan Africa. Although the pathogenesis of HBV-related HCC remains uncertain, there is strong evidence of a direct effect of HBV itself in causing HCC. Population-based vaccination programs against HBV have been associated with reductions in incidence of HCC and it is expected that widespread programs of universal infant vaccination will have the potential to dramatically reduce the incidence of HCC in the future. Although the impact of antiviral therapy is also uncertain, there is good evidence that prolonged suppression of HBV replication with nucleoside or nucleotide analogues may reduce the risk of HBV in patients with chronic hepatitis B.

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