Abstract
As a “stealth” virus, hepatitis B virus (HBV) is not directly cytopathic for infected hepatocytes. Hepatic injury is due primarily to the response of the body’s immune system to either acute or chronic infection with HBV. In general, two types of host’s responses occur to viral infection, i.e., innate immune response and adaptive immune response. Current evidence suggests that the innate immune response does not play an important role either in HBV clearance or in liver injury. In contrast, the adaptive immune response mediated by cytotoxic T-lymphocyte (CTL) cells is kinetically associated with viral clearance and liver injury. This observation suggests that the pathogenesis of HBV is closely related to the CTL-mediated immune response. One important way in which CTL cells mediate viral clearance is to secrete serine protease granzymes such as granzyme A and granzyme B which lead to the apoptosis of infected cells. However, HBV replication can upregulate the expression of apoptosis inhibitors such as serine protease inhibitor Kazal, or SPIK, resulting in the resistance of the cells to CTL-mediated immune killing. The inability of the immune system to clear HBV-infected cells can lead to chronic hepatitis B and development of HBV-cirrhosis and hepatocellular carcinoma.

Key Words:  hepatitis B virus; innate immune response; adaptive immune response; serine protease inhibitor Kazal; SPIK; granzyme A; granzyme B

Hepatitis B virus (HBV) is a major human pathogen responsible for acute and chronic liver disease. Over 95% of acutely infected adults recover completely and spontaneously from the infection, whereas most individuals who acquire neonatally transmitted infections develop persistent viral replication or chronic infection. Chronic HBV infection can progresses to life-threatening complications such as cirrhosis and hepatocellular carcinoma (HCC). Worldwide, more than 350 million people are chronically infected with HBV. More than one-third of these individuals will die from serious liver diseases such as cirrhosis and HCC if their conditions are left untreated. Therefore, it is important to improve our understanding of HBV pathogenesis, which will lead to the development of better treatments for HBV infection.

Because of lacking efficient animal models in which to study the pathogenesis of HBV infection, the most convincing data have come from studies done in chimpanzees. Chimpanzees injected with HBV developed an acute infection. Hepatic injury and the body’s immune response were then evaluated. Results showed that HBV is not directly cytopathic for the infected hepatocytes. During the early phase of infection of the chimpanzees, or before HBV-specific T lymphocytes enter the animal’s liver, 100% of the hepatocytes may be infected without histological or biochemical evidence of liver disease. Damage to the liver occurs only after the initiation of the immune response to clear the virus, which suggests that HBV does not directly cause hepatic injury. Further study suggested that damage to the liver is associated with the infiltration of activated T lymphocytes in HBV-infected liver. Moreover, if the cellular immune response mediated by T lymphocytes is deficient or pharmacologically suppressed, HBV can replicate at high levels in the liver of patients in the absence of cytological abnormalities or inflammation. These results further support the hypothesis that hepatic injury is triggered by the body’s immune response during clearance of virus-infected cells. In chronic HBV infection, usually only slight or no liver damage occurs, although active viral replication is found that may be due to the absence of an active immune response or to the fact that the immune system is overwhelmed.

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Innate Immune Response Does Not Play an Important Role in HBV-Mediated Liver Injury

The body’s immune response usually includes innate and adaptive immune responses. Unlike the adaptive immune response, the innate immune response is a cellular response to the virus or other pathogens. It is an immediate, nonspecific response that often results in a rapid induction of interferon alpha/beta by the infected cell, which triggers the transcriptional expression of a large number of interferon inducible genes (ISGs). The ISGs in turn initiate a variety of intracellular antiviral mechanisms that have the potential to minimize pathogenetic processes by limiting viral production and spread.10 Surprisingly, in acute hepatitis, no evidence indicates that HBV replication leads to apoptotic death of hepatocytes is mediated by an innate immune response. The fact that the existence of HBV does not trigger an innate immune response is further supported by the observation that high levels of HBV in stable cell lines such as HepG2.2.15 and AD38 cells (derived from Huh7 cells) do not induce cell death.15 It was reported that HBV replication triggered autophagy in Huh7 cells; however, it could not be excluded that this autophagy may be the result of artificially overexpressing HBV proteins in some in vitro systems. Other evidence supporting this hypothesis comes from analysis of Toll-like receptors in patients with chronic HBV infection. The Toll-like receptor pathway is an important route of the innate immune response. Activation of Toll-like receptors can suppress HBV replication.18 However, an obvious decrease in the levels of Toll-like receptors, such as TLR1, TLR2, TLR4, and TLR6, was found in patients with chronic HBV infection, suggesting that the innate immune response through the Toll-like receptor pathway does not play a role in virus clearance in chronic hepatitis B.19

The Role of the Adaptive Immune Response in HBV Infection

The clearance of infected virus during acute and probably also in chronic HBV infection is due to the body’s adaptive immune response, which usually prompts the death of infected hepatocytes leading to hepatic injury and damage.20-21 Serum glutamic-pyruvic transaminase (SGPT), known also as alanine transaminase (ALT), is one of the markers for diagnosing liver damage. Dead hepatocytes release ALT, increasing its level in the blood. In acute HBV infection, the death of hepatocytes is caused by an attack from the T lymphocytes, the intention of which is to remove virus-infected cells. In this immune response, both CD4 T cells (T helper cells) and CD8 T (cytotoxic T-lymphocyte (CTL)) cells are activated.6,9 CD4 T cells are robust producers of cytokines and are required for the efficient development of CTLs and B cells, which produce anti-HBV antibody to reduce the levels of circulating virus.9,22 Studies of HBV-infected chimpanzees suggest that CD4 T cells have no direct effect on viral clearance and liver disease. Depletion of CD4 T cells at the peak of HBV infection in chimpanzees does not affect the rate of viral clearance or the extent of liver damage, thereby supporting this hypothesis.20 However, CD4 T cells may be necessary to instruct and maintain anti-HBV CTLs. The specific CTL response plays a significant role in viral clearance and the pathogenesis of liver damage. In acute HBV infection, initial damage to the liver corresponds kinetically with the entry of HBV-specific CTLs into the liver. Furthermore, depletion of these cells at the peak of viremia delays the onset of liver damage and viral clearance in chimpanzees.8,20 The association of CTLs with liver injury is also observed in patients with acute viral hepatitis who successfully clear HBV.21 In patients with chronic HBV infection, CTLs seem to be suppressed, although low levels of CTLs exist in the infected liver.21,24 Reactivation of the killing mediated by CTLs usually leads to the clearance of HBV in patients with chronic infection.21 Adoptive transfer of HBV-specific CTL lines and clones into immunologically tolerant HBV transgenic mice triggers a noco-inflammatory liver disease that shares the same histologic features seen in acute viral hepatitis in man and results in the inhibition of HBV replication.25

The Granzymes and Viral Clearance and Liver Injury

The killing of virus-infected cells mediated by the adaptive immune response starts by activation of CTL and natural killer (NK) cells. They possess cytolytic granules that are secreted during interaction with infected cells and induce apoptotic death in the target cells (Figure 1).26-27 The granule-induced cell death pathway relies primarily on a family of structurally related serine proteases known as granzymes (Gzms) and the membrane-disrupting protein perforin. The Gzm family includes GzmA and GzmB as well as other lesser known Gzms (e.g., C and M).28-30 The roles of GzmA and GzmB as inducers of apoptotic cell death are well established; however, the role of other Gzms remains uncertain.31-33 Activated CTL and NK cells dominantly express GzmA and GzmB but not other Gzms, suggesting that GzmA and GzmB are important parts of the adaptive immune clearance process. GzmB induces apoptosis by activating caspase-dependent pathways that can be suppressed by the pan-caspase inhibitor Z-VAD. Therefore, this apoptotic pathway is known as caspase-dependent cell apoptosis.34-35 GzmA, however, acts in a caspase-independent manner and can be inhibited by serine protease inhibitors. GzmA-induced apoptosis is therefore known as serine protease-dependent cell apoptosis (SPDCA) (Figure 1).36-37 Neither GzmA nor GzmB alone can trigger cell apoptosis because neither one can pass the cell membrane. To induce cell apoptosis, they need assistance from perforin. The specific mechanism of action for perforin and its role in granzyme-mediated apoptosis are widely debated.27,38-39
Perforin itself cannot induce cell apoptosis; however, one hypothesis is that perforin pokes holes in the target cell, allowing the Gzms to enter and initiate apoptosis (Figure 1).\textsuperscript{26, 40-42} Suppressing or inhibiting either GzmA or GzmB can block CTL-induced apoptosis, resulting in the ability of infected cells to evade death by immune surveillance. GzmA is especially important in the challenge of viral infection. GzmA-deficient mice showed a compromised ability to maintain the ectromelia (mousepox) virus and herpes simplex neuronal infections even though GzmB and perforin were competently expressed.\textsuperscript{43-44} This finding suggests that GzmA plays an important role in viral clearance (see below).

**Figure 1.** Immune-killing mediated by CTL and NK cells. Granules: GzmA and GzmB are secreted by activated CTL and NK cells. GzmA induces cell apoptosis in a caspase-independent-serine protease-dependent manner (SPDCA). GzmB induces cell apoptosis in a caspase-dependent manner. CDCA, caspase-dependent cell apoptosis; CTL, cytotoxic T-lymphocyte; Gzm, granzyme; NK, natural killer; SPDCA, serine protease-dependent cell apoptosis.

HBV Replication Upregulates SPIK Expression, Resulting in the Ability of Infected Cells to Evade Gzma-Mediated Apoptosis

Our recent studies showed that HBV replication can upregulate the expression of a newly discovered apoptosis inhibitor, serine protease inhibitor Kazal (SPIK).\textsuperscript{45-46} SPIK is a small protein,\textsuperscript{47} also known as pancreas secretory trypsin inhibitor (PSTI) and tumor-associated trypsin inhibitor (TATI).\textsuperscript{48-50} It was first discovered in the pancreas as an inhibitor of autoactivation of trypsinogen.\textsuperscript{51} The expression of SPIK in normal tissues is limited or inactivated except in the pancreas. Studies have shown, however, that it can be activated as a reactant during hepatitis or liver inflammation.\textsuperscript{52-53} For example, SPIK was activated in rat liver cells to counter turpentine-induced liver inflammation.\textsuperscript{53} SPIK was also activated during human viral hepatitis in response to inflammatory cytokines.\textsuperscript{52} HBV replication can upregulate the expression of SPIK in cell culture systems.\textsuperscript{46} A high level of SPIK is found in HBV-infected human liver cells.\textsuperscript{49} As an apoptosis inhibitor, SPIK can suppress SPDCA induced by treatment with brefeldin A combined with cycloheximide.\textsuperscript{45} Overexpression of SPIK, either by transfection of the HBV genome or by direct transfection of the SPIK gene, prompts cellular resistance to SPDCA.\textsuperscript{46} Although what kind of serine protease is involved in brefeldin A/cycloheximide-induced SPDCA is still unknown, it is clear that SPIK can inhibit this kind of serine protease, preventing the apoptosis mediated by it.

Considering that GzmA and GzmB are serine proteases, it was hypothesized that SPIK inhibited GzmA- and GzmB-induced apoptosis. The first evidence to support this hypothesis came from the study of mouse SPIK. In 2003, Tsuzuki et al. reported that rat SPIK could directly bind GzmA and inhibit its ability to hydrolyze substrates such as N-\(\alpha\)-benzoyloxycarbonyl-L-lysine thiobenzyl ester.\textsuperscript{54} Although the structure of mouse SPIK has few similarities with that of human SPIK, we found that human SPIK could also bind GzmA (unpublished data). Moreover, we found that overexpression of SPIK in cells resulted in cellular resistance to apoptotic death mediated by GzmA (unpublished data).
These findings suggested that SPIK may function as a GzmA inhibitor, preventing the apoptosis mediated by GzmA. Considering that HBV replication can upregulate SPIK expression, the overexpression of SPIK probably protects HBV-infected cells from apoptotic killing mediated by CTLs via GzmA. As we mentioned previously, in patients with chronic viral hepatitis, CTL-mediated immune clearance is usually inefficient or suppressed. This observation may explain why HBV-infected cells cannot be cleared during chronic hepatitis despite the existence of CTLs in the liver. Even though CTL-mediated immune clearance not only relies on the induction of apoptosis by GzmA, inhibition of GzmA by SPIK definitely helps HBV-infected cells avoid CTL-mediated immune clearance.\(^{43-44}\)

**HBV Infection and Development of HCC**

Persistent HBV infection often leads to the development of HCC. The reason is still unknown. However, one important reason may be the inability of the immune system to remove virus-infected cells from the body. Failure to remove virus-infected cells often results in gradually accumulating cellular genetic changes that finally lead to the development of HCC.\(^{55}\) As we mentioned before, the failure of the immune system to remove malignant cells through apoptosis may be due to the upregulation of apoptosis inhibitors such as SPIK in these cells. Both persistent viral replication and necroinflammation of liver cells in patients with chronic HBV infection could increase the SPIK levels in infected cells. This process may then prevent the removal of these cells by GzmA-mediated immune clearance. This observation is supported by the fact that the development of HCC is closely associated with the increase of SPIK levels in cells. Lee and colleagues found that the levels of SPIK in HBV-infected patients were correlated with the progress of HCC, for example, with the malignant phase of the cancer.\(^{56}\) Moreover, the high levels of SPIK were closely related with early recurrence of HCC in these patients following surgical resection.\(^{56}\) Because recurrence of cancer often implies the inability of the immune system to clear lingering oncogenetic cells, early recurrence of HCC in patients with high levels of SPIK raises the possibility that overexpression of SPIK might interfere with the immune elimination of lingering oncogenetic cells. **Figure 2** summarizes the possible pathogenesis of HBV infection and liver disease.

![Figure 2](image)

**Figure 2.** Possible pathogenesis of HBV infection and liver disease. CTL, cytotoxic T-lymphocyte; HBV, hepatitis B virus; NK, natural killer; SPIK, serine protease inhibitor Kazal.
Suppression of SPIK Expression to Prevent the Development of Chronic Viral Infection and HCC

No effective methods are currently available to prevent or completely cure chronic HBV infection and HCC. Nine licensed treatments for chronic HBV are presently available: Two are based on interferon-α; the remaining seven are based on nucleoside/nucleotide analogues (NAs). Both types of interferon-based therapies are currently limited to certain well-defined populations, are expensive, arelogistically challenging for populations most in need, and are associated with many side effects that can lead to cessation of treatment. In general, NAs are long-term treatments with varying side effects that are costly to populations with the greatest need; they also are associated with slow cure rates, and their physiological impact is inhibited by both unique and common mutations. Over time, resistance develops that impacts the utility of changing from one NA to another. Recent efforts to thwart antiviral resistance led to pairing a nucleoside with a nucleotide; however, new mutants against such combinations continue to arise. Preliminary evidence indicates that current treatments may lead to a new viral mutant that is no longer susceptible to any of the approved NAs. Such a strain could become a serious health issue if it were sturdy enough to circulate in at-risk populations, or even worse, novel enough to infect those already vaccinated. Efforts to develop new classes of HBV therapeutics may help to mitigate these risks and thwart resistance to NAs. For treatment of HCC, only sorafenib has been approved. However, the nonspecific killing of normal cells limits its therapeutic use. Therefore, it is imperative to further develop anti-HBV drugs, particularly those drugs that work differently from existing drugs. If resistance to GzmA-induced apoptosis can prevent the elimination of HBV-infected cells, then reinstating sensitivity to GzmA-induced-apoptosis should allow clearance of infected cells, further preventing HCC formation. Our study suggests that suppressing overexpressed SPIK in HBV-expressing cells restored the sensitivity of these cells to apoptosis. Thus, it is feasible to develop a drug that can suppress overexpressed SPIK either in HBV-infected cells or HCC cells to treat chronic HBV infection and HCC by restoring the ability of the immune system via GzmA to kill HBV-infected cells.

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