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Pathogenesis of Hepatitis B Virus in Transgenic Mice

Giovanni Sitia[a,b]*, Matteo Iannacone*[a,c]*, Francis V. Chisari*[a]*, Luca G. Guidotti*[a]

[a]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, Calif., USA and Departments of [b]Infectious Diseases and [c]Internal Medicine, San Raffaele Scientific Institute, Milan, Italy

Hepatitis B virus (HBV) is a small, enveloped DNA virus that causes acute and chronic necroinflammatory liver disease and hepatocellular carcinoma [1]. HBV infection in immunocompetent adults usually results in transient liver disease and viral clearance. A small percentage of these patients (5–10%) develop chronic hepatitis associated with viral persistence. When neonates are infected, however, over 90% of them will become persistently infected, suffering different degrees of chronic liver disease. Unfortunately, cirrhosis and hepatocellular carcinoma are frequent complications of chronic HBV infection. Since HBV is not directly cytopathic for the hepatocyte, the immune response to viral antigens is thought to be responsible for both liver disease and viral clearance following HBV infection. Indeed, patients with acute viral hepatitis, who successfully clear the virus, mount a multispecific polyclonal CTL response to several HBV-encoded antigens [1]. In contrast, this response is absent or extremely weak in chronically infected patients who do not clear the virus [1, 2] and thus, it is believed that the outcome of HBV infection (viral clearance versus viral persistence) is determined primarily by the vigor and quality of the cellular immune response.

The experimental approaches to HBV pathogenesis have been difficult because the host range of HBV is limited to man and chimpanzees. Studies of HBV pathogenesis using models of HBV-related hepadnavirus infections in the woodchuck, ground squirrel and Pekin duck have also been difficult because the immune system of these outbred species has not been characterized.

Definitive analysis of the immunological mechanisms involved in HBV pathogenesis required the development of an inbred animal model with a

well-defined immune system, i.e. the HBV transgenic mouse. In the course of those studies many other previously unknown aspects of HBV pathogenesis have been elucidated because of the unique power of the transgenic mouse system to replicate HBV in the primary hepatocyte in vivo.

Two lineages of transgenic mice containing complete copies of the HBV genome have been produced whose hepatocytes replicate the virus at high levels without any evidence of cytopathology [3]. These mice were generated by microinjection of a terminally redundant viral DNA construct 1.3 HBV genomes in length, containing only viral regulatory elements and no cellular promoters. Out of all four HBV RNAs produced in the liver of these animals, the two most abundant transcriptional products of the transgene (as occurs during natural infection) are the 3.5- and 2.1-kb RNA. The 3.5-kb RNA (or pregenomic) RNA is reverse transcribed by the viral polymerase into the HBV DNA replicative intermediates inside of viral nucleocapsid particles more abundant in centrilobular hepatocytes. As a consequence of efficient viral replication, ultrastructurally complete and infectious [4] viral particles that are morphologically indistinguishable from human-derived virions are detected at high levels in the transgenic mouse serum (between 107 and 108 viral particles per ml), further indicating that the HBV life cycle can be efficiently completed in the transgenic mouse hepatocyte [3].

Antiviral Mechanisms

The antiviral and immunopathological consequences of antigen recognition in this model were examined by administration of virus-specific CTLs. Surprisingly, the antiviral potential of the CTLs was shown to be primarily mediated by non-cytolytic mechanisms that involve the intrahepatic production of type 1 inflammatory cytokines by the CTLs [5–7]. These cytokines activate two functionally independent virocidal pathways: an early pathway that eliminates HBV nucleocapsid particles and their cargo of replicating viral genomes from the hepatocyte [8, 9]; and a later pathway that post-transcriptionally downregulates the viral RNA [10, 11]. In recent studies, it was shown that IFN-γ mediates most of the antiviral effect of the CTLs [12] and nitric oxide (NO) mediates most of the antiviral activity of IFN-γ [13].

One might predict from the foregoing that HBV-non-specific inflammatory responses of the liver could facilitate the clearance of HBV if they induce the local production of antiviral cytokines (such as IFN-γ and IFN-α/β) to which HBV is susceptible. Precisely these events have been shown to occur in

the HBV transgenic mice during unrelated hepatotropic infections of the liver which include lymphocytic choriomeningitis virus (LCMV) [12, 14], adenovirus [12, 15], mouse cytomegalovirus (MCMV) [15], malaria [16] or *Schistosoma* [17] or after administration of recombinant murine IL-12 [18], a cytokine produced by antigen-presenting cells (APCs) that has the ability to induce IFN-γ secretion by T cells, natural killer (NK) and NKT cells. Along these lines, it was also shown that a single injection of α-galactosylceramide (α-GalCer), a glycolipid antigen presented to Vα14$^+$, NK1.1$^+$ T cells by the non-classical MHC class I-like molecule CD1d, inhibits HBV replication by directly activating NKT cells to produce IFN-γ in the liver [19, 20]. Furthermore, HBV replication was inhibited in the transgenic animals by systemic administration of IFN-α [17], the IFN-α/β inducer poly-inosinic-polycytidylic acid complex (Poly-I/C) [9, 17] or IL18 [21]. Finally, recent studies have shown that an anti-CD40 agonistic mAb (αCD40) was sufficient to activate APCs within the liver and inhibit HBV replication non-cytopathically by a cytokine-dependent process [22].

Importantly, previous work has also produced evidence suggesting that non-cytopathic antiviral mechanisms may contribute to viral clearance during acute viral hepatitis in chimpanzees, thus validating the transgenic mouse studies in a natural infection model [4]. Moreover, cytokines known to abolish HBV replication from the hepatocyte also clear a persistent LCMV infection from the hepatocyte non-cytopathically, indicating that, like HBV, LCMV is also susceptible to intracellular inactivation by cytokine-induced antiviral mechanisms that are operative in the hepatocyte [23].

Absolute clearance requires elimination of the episomal covalently closed circular (CCC) HBV DNA species that serves during natural infection as the viral transcriptional template in the nucleus of the hepatocyte [24]. For unknown reasons, wild-type HBV transgenic mice do not produce CCC DNA, but they very efficiently express and replicate HBV using the integrated transgene as template [3]. It must be noted that low levels CCC DNA have been found in the liver of these same HBV transgenic mice once they were crossed with hepatocyte nuclear factor 1α-null mice [25]. This suggests that the impairment on CCC DNA synthesis in the mouse hepatocyte is not absolute. The absent or very low levels of CCC DNA detected in the transgenic mice, however, do not allow to know whether this viral species is also susceptible to cytokine-mediated control in this model. Nonetheless, since CCC DNA is abolished in the chimp infection model in the absence of massive destruction or regeneration of hepatocytes and in the presence of inflammatory cytokines [4], it is possible that cytokine-dependent pathways may contribute to the elimination of CCC DNA from the resting hepatocyte as well.

Immunopathological Mechanisms

Liver disease in the CTL transfer model begins with antigen recognition by the CTLs and delivery of signals that trigger the death of the hepatocyte by apoptosis [26]. Following antigen recognition, the CTL become activated and recruit many host-derived inflammatory cells into the liver, thereby contributing to the formation of necroinflammatory foci in which apoptotic hepatocytes and CTL are outnumbered by host-derived lymphomononuclear (such as lymphocytes, NK cells and macrophages) and polymorphonuclear (such as neutrophils and eosinophils) inflammatory cells [27, 28]. These necroinflammatory foci are scattered throughout the liver parenchyma and cause a focal lesion histologically identical to classical viral hepatitis in man.

Recruitment of host-derived antigen non-specific inflammatory cells into the liver is a process that is associated with the intrahepatic production of chemokines and it is likely to contribute to the pathogenesis of liver disease. Indeed, a recent study showed that blocking the chemokines CXCL9 and CXCL10 reduces the intrahepatic recruitment of host-derived lymphomononuclear cells and the severity of liver disease [28]. In that study it was also shown that CXCL9 and CXCL10 are rapidly and strongly induced in the liver after CTL transfer and the transferred CTL produce neither chemokine; rather, they activate (via the secretion of IFN-γ) hepatocytes and non-parenchymal cells of the liver to produce them [28].

The association of reduced liver disease with reduced recruitment of antigen non-specific lymphomononuclear cells implies that these cells can amplify the liver damage initiated by the antigen-specific CTLs. Similar mechanisms may contribute to the pathogenesis of viral hepatitis in man, where, like in our system, the number of HBV-specific T cells detected in the liver is outnumbered by recruited non-virus-specific T cells [2, 29] and other inflammatory cells [30]. The pathogenetic mechanisms whereby antigen-non-specific lymphomononuclear cells may induce liver damage are not understood. Future studies will attempt to address this important issue.

We recently showed that depletion of Gr-1$^+$ cells also reduces the severity of liver disease in this model. Gr-1$^+$ cells include polymorphonuclear neutrophils (PMNs) [31], plasmacytoid dendritic cells (pDCs) [32–34] and a subset of monocytes/macrophages [35–37]. Interestingly, depletion of Gr-1$^+$ cells completely blocks the recruitment of all Gr-1$^-$ intrahepatic lymphomononuclear into the liver despite the fact that many chemokines (including CXCL9 and CXCL10) are induced at high levels in the organ [38]. These results indicate that Gr-1$^+$ cells are necessary for the intrahepatic recruitment of antigen non-specific Gr-1$^-$ lymphomononuclear cells and they suggest that

functions by Gr-1^{+} cells in addition to chemokine induction are necessary for the recruitment process to occur.

These functions may include the release of the matrix-degrading metallo-proteinases (MMPs) by PMNs or other Gr-1^{+} cells. While it is not known whether pDCs or Gr-1^{+} monocytes/macrophages produce MMPs, PMNs are known to produce high levels of collagenases (such as MMP-8, neutrophil collagenase) and gelatinases (such as MMP-9, gelatinase B) [39]. The major action of these enzymes involves the remodeling of the extracellular matrix, a process that is thought to facilitate leukocyte trafficking through endothelial barriers and solid organs [39].

In keeping with this, in preliminary studies we showed that following CTL transfer various MMPs are rapidly induced in the liver. Interestingly, MMP-8 and MMP-9 (known to be produced by PMNs [39]) are not induced in anti-Gr-1-treated mice, while MMP-2, MMP-3, MMP-7, MMP-12, MMP-13 and MMP-14 (known to be produced by many other myeloid and non-myeloid cell types [39]) are induced, suggesting that Gr-1^{+} cells, especially PMNs, are the likely source of MMP-8 and MMP-9 in our system. Since depletion of Gr-1^{+} cells also inhibits CTL-induced recruitment of antigen-non-specific inflammatory cells into the liver [38], the tight association between MMP-8 and MMP-9 activities and IHL recruitment is compatible with the hypothesis that these enzymes facilitate leukocyte trafficking through the endothelial barrier and entry into the liver parenchyma.

To test this hypothesis, we inhibited MMP activity in vivo (via the hydrodynamic injection of a plasmid encoding the tissue inhibitor of matrix metalloproteinases TIMP-1) and we monitored whether this altered the intra-hepatic recruitment and pathogenetic effector functions of HBV-specific CTLs and other inflammatory cells in our system (fig. 1). The enhanced expression of TIMP-1 inhibited the induction of MMP activity and reduced the CTL-induced recruitment of host-derived lymphomononuclear cells into the liver and the attending liver disease, indicating that the recruitment of these cells requires MMP activity. The data is compatible with the hypothesis that PMNs represent the first cell type to be recruited into the liver following antigen recognition by the CTLs. According to this hypothesis, the production of MMPs by PMNs could remodel the extracellular matrix and facilitate the trafficking of lymphomononuclear cells through the endothelial barrier and into the liver parenchyma in response to their own chemoattractants.

In conclusion, the production of transgenic mice has created the opportunity to examine several aspects of HBV pathogenesis that could not be approached in any other experimental system. Undoubtedly, the transgenic mouse model will help us to answer many more questions that still remain.
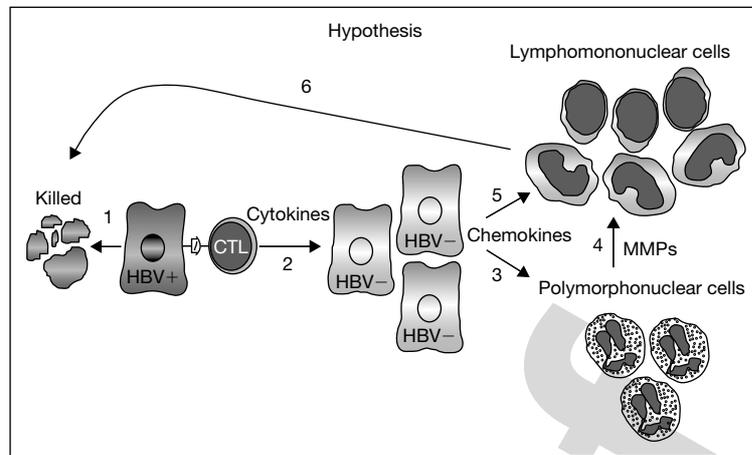
Fig. 1. Role of antigen-specific and non-specific inflammatory cells in the pathogenesis of HBV: *Hypothesis*. After antigen recognition, HBV-specific CTL kill a small number of hepatocytes (1), and produce antiviral cytokines (2) that inhibit HBV replication noncytopathically in a greater number of cells. The same cytokines can activate parenchymal and non-parenchymal cells of the liver to produce chemokines (3) that recruit antigen non-specific polymorphonuclear cells (i.e. neutrophils) into the organ. Production of MMPs by these cells (4) in addition to chemokine induction (5) may contribute to the migration of antigen non-specific lymphomononuclear cells (i.e. NK cells, T cells and macrophages) into the liver and the amplification of the liver disease initiated by the CTL (6).

Acknowledgements

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Luca G. Guidotti
The Scripps Research Institute, Department of Molecular and Experimental Medicine
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)
Tel. +1 858 7842758, Fax +1 858 7842960, E-Mail guidotti@scripps.edu