Hepatotropic viruses: New insights in pathogenesis and treatment

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ABSTRACT

Hepatitis B virus (HBV) can be detected in peripheral blood mononuclear cells (PBMCs), mainly B lymphocytes and monocytes. The frequency of PBMC infection is higher in patients with ongoing HBV replication, but can persist for years after the complete resolution of an acute episode of hepatitis B. Infected PBMCs can act as reservoirs for the cell-to-cell transmission of the virus, and vertical transmission studies indicate that the HBV-infected PBMCs of mothers may act as a vector for intrauterine HBV infection. Recent data evaluated whether HBV occult infection could co-operate with HCV infection in the pathogenesis of mixed cryoglobulinemia (MC) and lymphoma and/or whether it may be implicated in the pathogenesis of MC and malignant diseases -B-cell non-Hodgkin's lymphoma (NHL) also independently from HCV.

The treatment of chronic HBeAg-negative hepatitis B is intended to ensure the long-term suppression of HBV replication with the aim of halting the progression of liver damage and preventing the development of liver-related complications. This can be done by means of short-term "curative" treatment or long-term "suppressive" therapy. The first approach requires a 48-week course of peginterferon, which controls viral replication (HBV DNA <10.000 copies/ml) in 20-30% of patients; the second requires the long-term (possibly lifetime) administration of nucleoside and/or nucleotide analogues. As none of the currently available drugs alone suppresses viral replication (HBV DNA <200 copies/ml) for five years in all patients, some require a rescue therapy based on the addition of a non-cross-resistant drug, which should be given as early as possible ("on demand" combination therapy). However, the currently available anti-HBV analogues can easily suppress HBV replication for five years in most HBeAg-negative patients. As both strategies have their pros and cons, the best approach needs to be carefully evaluated on an individual basis.

Introduction

Viral hepatitis is a diffuse inflammatory reaction of the liver caused by hepatotropic viruses. Among the hepatitis viruses, only hepatitis B virus and hepatitis C virus are able to persist in the host and cause chronic hepatitis. In the course of persistent infection, besides nodular fibrosis, cirrhosis and, eventually, hepatocellular carcinoma (HCC), several extrahepatic manifestations can be observed. In chronic HBV infection, glomerulonephritis (of the membranous and of the membranoproliferative type) and polyarteritis nodosa have been observed. The pathogenesis of both conditions involves the deposition of circulating immune complexes. During the last few years, many autoimmune manifestations have been correlated with HCV infection; namely, mixed cryoglobulinemia sicca syndrome, chronic polyarthritis, polydermatomyositis, autoimmune thyroiditis, lung fibrosis, and diabetes mellitus. It is often difficult to verify whether the above associations are coincidental or a pathogenetic link actually exists. In this review, new insights in the pathogenesis of hepatotropic infections and HBV treatment will be discussed.

HBV and lymphocytes

Immunological abnormalities are often detected in patients infected with hepatitis B virus (HBV) and may be responsible for hepatocellular injury and chronic viral persistence. Furthermore, the functional and morphological lymphocyte alterations that occur during other viral infections are also

found in patients with acute viral hepatitis B. Various mechanisms may be responsible for these immunological abnormalities, and both extrinsic and intrinsic factors have been identified; the cytokine profile may be partially responsible for the extrinsic effect, but it has also been suggested that a direct effect of HBV on lymphocytes may play a major role.

HBV is not strictly hepatotropic, and early studies found that it can infect peripheral blood mononuclear cells (PBMCs) (1), as well as bone marrow cells (2) and lymphoblastoid cell lines (3). Animal studies have confirmed that replicative viral DNA intermediates and viral transcript proteins can be detected in PBMCs under certain conditions. Molecular hybridisation studies have found viral DNA in the majority of the patients with chronic hepatitis B, often as multimers of free HBV DNA, free monomers, or HBV DNA forms integrated into high molecular weight cellular DNA (4). PBMCs infection is more frequent in patients with ongoing HBV replication than in HBsAg carriers with inactive infection, and the state of the virus in mononuclear cells reflects the replicative profile of the virus in the liver.

The mononuclear cell compartment represents an extra-hepatic viral reservoir not only at the time of infection, but also for a long time after the resolution of acute hepatitis B. Molecular analyses of viral DNA from patients who have clinically recovered from acute infection have revealed that HBV DNA can persist in PBMCs for up to 70 months after the complete resolution of an acute episode of hepatitis B (5), and immunohistochemical analyses indicate that viral DNA is transcriptionally active in mononuclear cells long after serological, biochemical and clinical recovery from acute viral hepatitis. Similar data have also been detected in experimental animals, in which heapdna-virus DNA, RNA and proteins have been detected in woodchuck PMBCs. Furthermore, infectious woodchuck hepatitis virus (WHV) has been produced by mitogen-stimulated mononuclear cells, thus suggesting that they may be able to support virus replication and act as a reservoir for the cell-to-cell transmission of the virus (6).

The risk of HBV transmission from HBV seronegative blood has been clearly documented in humans and, in the setting of liver transplantation, *de novo* HBV infection has been found in up to about 80% of liver grafts from HbsAg-negative but anti-HBc-positive donors. Furthermore, despite liver negativity, the presence of viral DNA in PBMCs after liver transplantation has been associated with the reappearance of overt infection. In the hemodialysis setting, encapsulated and transcriptionally active HBV DNA has been detected in the PBMCs of up to 54% of HbsAg-negative patients (7).

Vertical transmission studies indicate that the HBV-infected PBMCs of mothers may act as a vector for intrauterine HBV infection, and it has been shown that HBV DNA can be found in the PBMCs of about 30% of the newborns of HbsAg-positive mothers, and is mainly related to the mothers' HBV viremia level and PBMC HBV DNA status. Mononuclear cell infection in newborns can last for a long time despite negative serum HBsAg and HBV DNA, and may give rise to a failed response to HBV vaccine (8).

Few studies have investigated the subpopulations of peripheral blood mononuclear cells infected by HBV. Highly sensitive limiting dilution PCR has been used to assess the presence of viral DNA in the PBMCs of patients with acute and chronic hepatitis, separated by means of magnetic beads into monocytes, B cells, $CD4^+$ and $CD8^+$ T cells, and NK cells, and HBV DNA sequences were detected in each PBMC subpopulation in all of the examined patients. The highest infection rates in terms of HBV-positive cells and viral load were found in monocytes and B cells, followed by $CD8^+$ T cells, NK cells and $CD4^+$ T cells, thus suggesting selective viral uptake within particular cell subsets. The frequencies of HBV positive cells in all of these subsets were 50-500 times higher in the patients with chronic hepatitis B than in those with the acute form; viral loads were mainly estimated as about one HBV genome per HBV-positive cell. Other studies have confirmed the presence of HBV DNA mainly in monocytes and B cells (9).

The site of the attachment of the virus to PBMCs has been investigated using recombinant particles containing different HBV surface proteins. It was found that the recombinant particles containing the S gene products or the preS2 and S derived protein did not bind peripheral blood mononuclear cells, whereas those carrying the preS1, preS2 and S encoded proteins did. These results recall findings observed in the liver, in which the preS1 encoded protein of the surface antigen has been identified as the major binding site responsible for attaching the virus to the surface of liver cells. In order to define the exact specificity of the binding site on recombinant particles, inhibition experiments were carried out using monoclonal antibodies: PBMC binding was completely inhibited by increasing amounts of anti-preS1 monoclonal antibody, but was not affected by monoclonal antibodies against preS2 and S genomic regions (10). These results revealed that the binding involves the 21-47 sequence of the preS1 protein domain, the same site as that involved in HBV virus attachment to hepatocytes.

PreS1-carrying recombinant particles preferentially bind to monocytes and B lymphocytes and, to a lesser extent, T lymphocytes and granulocytes. As preS1-encoded protein is typically expressed on the surface of complete HBV virions, whereas small, non-infectious 22 nm HBsAg particles contain very small amounts, the binding of virus particles to B cells and monocytes via the preS1 domain may initiate cell infection or be related to particle uptake by antigen-presenting cells.

The nature of the cell site of attachment involved in virus binding to peripheral blood cells is still being investigated, but it might be a cell receptor as the virus mimics the physiological ligand. Previous studies indicate that the serpin squamous cell carcinoma antigen (SCCA) is the cell protein involved in binding HBV to the surface of liver cells, an interaction with the preS1-encoded surface protein of the virus. As

recent findings indicate that the surfaces of B lymphocytes and, to a lesser extent, monocytes also express this serpin, it is likely that viral entry into peripheral blood cells occurs using the same mechanisms as those previously described for liver infection.

HBV and HCV infection: autoimmune manifestations and lymphoproliferation

Hepatitis C virus (HCV) infection, due to its diffusion and severity of sequelae, is a primary medical problem with about 170 million HCV carriers worldwide. It has been demonstrated that HCV can infect not only hepatocytes, but also cells of the immune system (11, 12) and, according to some reports, preferentially B lymphocytes (12). The possible clinical manifestations of chronic HCV infection reflect this dual tropism; in fact, they include not only liver diseases, but also autoimmune and/or lymphoproliferative disorders (LPDs) including both benign (mixed cryoglobulinemia: MC) and malignant diseases – B-cell non-Hodgkin's lymphoma (NHL). HCV-associated manifestations also include porphyria cutanea tarda, lichen planus, nephropathies, thyreopathies, sicca syndrome, and chronic polyarthritis (13). A pathogenetic link between HCV and some lymphoproliferative disorders was confirmed by their responsiveness to antiviral therapy, which is now considered the first choice treatment (14).

MC is considered the prototype of HCV-related LPDs due to the very strong association with HCV-infection as well as its possible evolution to NHL (15).

Cryoglobulins (CGs) are serum immunoglobulins (Igs) that reversibly precipitate below 37°C during blood tests. Mixed CGs are composed by a mixture of polyclonal IgG and monoclonal IgM (type II MC) or polyclonal IgM with rheumatoid factor (RF) activity (type III MC). By contrast, type I CGs are composed by a unique monoclonal component, usually sustained by an indolent B-cell lymphoma.

Different prevalence of serum CGs – ranging from 19 to >50% – have been reported in HCV patients. The difficulty in a correct determination of the presence of CGs, due to their thermolability, should account for an underestimation of such phenomenon.

Several studies have shown that a B-cell clonal expansion (in particular of RF B-cells) underlies MC, that this condition is associated with Bcl2/JH rearrangement, and that MC-II can evolve into a frank B-cell non-Hodgkin's lymphoma (NHL) in approximately 8-10% of cases (13, 16)

A monoclonal lymphoproliferation of uncertain significance (MLDUS) in liver and bone marrow infiltrates is a typical feature of subjects with clinico-laboratory features of MC-II (15).

MC clinical manifestations are secondary to a systemic vasculitis – involving medium and, more often, small-sized blood vessels – which is responsible for MC tissue injury. Such vasculitis is probably secondary to vascular deposition of circulating immune complexes (CIC), mainly CGs, and complement, with the possible contribution of both haemorheological and local factors (17-19).

MC-related symptoms are generally absent or very mild, whereas clinically evident MC – MC syndrome or MCS – would be evident in 10-15 to 30% of MC subjects and in 5-10% of all HCV infected patients (14). No standardized criteria are at present available for the diagnosis of MCS even if valuable classifications have been proposed. The most common symptoms of MCS are weakness, arthralgias, and palpable purpura that usually involves the lower extremities (Meltzer and Franklin triad). Different clinical manifestations include Raynaud's phenomenon, peripheral neuropathy, sicca syndrome, membranoproliferative glomerulonephritis (MPGN), as well as lung disorders, fever, hematocytopenia, and diffuse vasculitis (13, 20). Circulating mixed CGs, low C4, and orthostatic skin purpura are the hallmarks of the disease. Given its clinical polymorphism, the presentation of MCS may greatly vary in different subjects and in the same patient in different times. Consequently, the actual prevalence of MCS is probably underestimated.

MC is a "borderline" pathology, clinically benign, but evolving in about 10% of cases into a malignant lymphoma. Therefore, we hypothesized that HCV may be involved in the pathogenesis of B-cell lymphoma as well (12). This hypothesis was substantiated by several observations: the significantly high prevalence of HCV infection in B-cell non-Hodgkin's lymphoma (NHL) patients, the occurrence of the same pathological form in HCV patients and the association between HCV and splenic marginal zone lymphoma with circulating villous lymphocytes (13, 21).

The detection of HCV lymphotropism more than a decade ago led to the hypothesis of a causal link between infection of lymphatic cells and autoimmune-lymphoproliferative disorders (12) and different pathogenetic hypotheses have been proposed. Several studies highlighted the existence of an elevated frequency of proto-oncogene bcl-2 rearrangement-T (14, 18) translocation in subjects affected by chronic HCV infection (22, 24). T (14, 18) translocation was significantly more evident in cases of HCV-related cryoglobulinemic syndrome (24) with consequent over-expression of Bcl-2 anti-apoptotic protein in B-cells and altered bcl-2/bax ratio. Interestingly, high levels of Bcl-2 protein expression have been detected in bone-marrow and liver infiltrates of by histopatological analysis (25). Actually, malignancies occur after decades of persistent infection, suggesting that acquisition of a transformed phenotype requires accumulation of multiple genetic changes by the host cells. This accumulation may be facilitated by prolonged life due to the ineffective control of programmed cell death in lymphatic cells, suggesting that a key role in MC pathogenesis can be played by genetic changes that enhance B-cell survival (24).

Several studies focused on the importance of a sustained antigenic stimulation, partly analogous to mechanisms of *H. pylori*-related lymphomagenesis. In this light, the identification of the specific binding between the HCV E2 protein and the CD81 molecule, particularly abundant on the B-cell surface, led to the hypothesis of a possible role played by HCV in the promotion of a consistent polyclonal B-cell response

to viral antigens which favour the development of LPDs. According to this working hypothesis, the viral infection will support lymphomagenesis in a linear, progressive way until the possible malignant transformation.

A potential role of some HCV proteins in the pathogenesis of MC and NHL has been also suggested. HCV core showed several biological activities in *in vitro* studies performed in both hepatocyte and B-lymphocyte models. Recently, activation of PI3K/Akt pathway by HCV core in B-cell lines has been described (26). This phenomenon produced the p63 over-expression and an increased proliferation of HCV core expressing cells. Interestingly, high levels p63 were also detected in primary B-lymphocytes isolated from MC and NHL patients (24) suggesting that this mechanism could be involved in the pathogenesis of HCV-related LPDs also *in vivo*.

HCV and HBV are both parenterally transmitted agents. This implies the possibility of coinfection in the same patient. HBV was also shown to be lymphotropic and was implicated in a minority of MC cases (16). It has been shown that in patients with HCV infection a so called "occult" HBV infection (presence of markers of an apparently resolved infection, not determined by routine tests), may occur and that a variable combination of HCV and/or HBV replication can be observed, particularly after antiviral therapy (27, 28). The possible pathogenetic relevance of occult HBV infection in HCV-positive patients is generally admitted. In particular, evidence exists suggesting that occult HBV infection can play a role in hepatocellular carcinoma development (29). In consideration of these recent data it appears important to assess whether HBV occult infection could co-operate with HCV infection in the pathogenesis of MC and lymphoma and/or whether it may be implicated in the pathogenesis of MC/NHL also independently from HCV. Preliminary data support this hypothesis (30). However, further controlled studies are needed in order to confirm the role of HBV infection in lymphomagenesis.

Treatment of HBV

Lamivudine-naïve patients

Peginterferon. A large multinational trial has assessed the long-term virological and biochemical response to peginterferon alfa-2a with or without lamivudine, or lamivudine monotherapy, administered for 48 weeks (31, 32). After three years' follow-up, HBV DNA levels of <10,000 copies/ml were reached by 28% of the patients treated with peginterferon alfa-2a alone, and 25% of those treated with peginterferon alfa-2a + lamivudine; ALT levels normalised (≦30 U/L) in 31% of the patients treated with peginterferon alfa-2a with or without lamivudine, and 9% of patients in both groups lost HBsAg.

Lamivudine monotherapy. A virological response is obtained in 70% of patients after one year, but lamivudine-resistant strains emerge at the rate of 20% per year, which erodes virological, biochemical and histological responses in most patients (33, 34). By year 5, up to 80% of patients have become resistant to lamivudine.

Long-term lamivudine monotherapy is therefore not an option for the long-term treatment of HbeAg-negative patients.

Adefovir monotherapy. In a 5-year cohort study (35), 67% of the patients achieved HBV DNA levels of <1000 copies/mL, and 69% achieved normal ALT levels. Six patients (5%) lost HBsAg, five of whom had anti-HBs at the last available time point. The 5-year cumulative rates of genotypical resistance, virological resistance (defined as a >1 log rebound in comparison with the on-treatment nadir), and clinical resistance (defined as virological and biochemical rebounds) were respectively 29%, 20% and 11%. Creatinine levels were slightly high in only four patients (3%). These data indicate that adefovir 10 mg for five years lastingly suppresses HBV replication.

Adefovir was tentatively withdrawn in some patients in whom HBV DNA was persistently undetectable by means of PCR during the five-year treatment (36). They all experienced a virological relapse, but approximately 70% maintained HBV DNA levels of <10,000 copies/ml and ALT levels within the normal range over the following 12-18 months. It remains to be established whether these results will be confirmed by others, and whether this endpoint is acceptable for HbeAg-negative patients.

Entecavir monotherapy. A randomised controlled trial comparing 52 weeks' treatment with entecavir 0.5 mg/day or lamivudine 100 mg/day in nucleoside-naïve patients with HBeAg-negative chronic hepatitis B (37) found that significantly more patients in the entecavir group achieved HBV DNA levels that were undetectable by PCR (90% *vs.* 72%) and normalised ALT levels (78% *vs.* 71%), and showed improved histological findings (70% *vs.* 61%). They also less frequently experienced virological rebound (2% *vs.* 8%) or genotypically confirmed drug resistance (0% *vs.* 6%). The safety profiles of both drugs were similar.

The virological responders in the entecavir group (*i.e.*, the vast majority) stopped taking the drug after one year, and viral replication resumed in all cases; among the few who continued treatment for two (25 patients) and three years (16 patients), virological response was maintained (38).

There are no published long-term resistance data concerning HbeAg-negative patients, but the promising low resistance rates in HbeAg-positive partial responders strongly suggest that entecavir monotherapy may control viral replication in most HbeAg-negative patients for many years (39).

Telbivudine monotherapy. A randomised controlled trial comparing 104 weeks' treatment with telbivudine 600 mg/day or lamivudine 100 mg/day in nucleoside-naïve patients with HBeAg-negative chronic hepatitis B (40) found that 82% of the telbivudine-treated patients achieved HBV DNA levels that were undetectable by PCR as against 57% of those treated with lamivudine ($p < 0.01$); ALT levels normalised in respectively 78% and 70% of the patients (n.s.). Genotype resistance to telbivudine developed in 22% of the patients after two years. A 24-week virological response (HBV DNA < 2 or 3 log copies/ml) correlated with high rates of continued DNA undedectability and a negligible risk of telbivudine resistance after two years of therapy (41). These

data provide a rationale for adapting and tailoring antiviral therapy in patients who are suboptimal responders after 24 weeks.

De-novo combination. There are no studies assessing the long-term efficacy and safety of the *de novo* combination of two anti-HBV analogues in HbeAg-negative patients.

Lamivudine-resistant patients

Most studies investigating the management of lamivudine-resistant patients have used adefovir alone (in a "switch-to" strategy) or in combination with lamivudine ("add-on" strategy). Prospective cohort studies and one small, randomised, controlled study have clearly shown that the combination is superior to adefovir monotherapy as it minimises the emergence of adefovir resistance for at least the first three years (42-46).

Timing of adefovir administration is crucial because, as it is is not very potent, it should be started as soon as a virological rebound is identified: *i.e.*, when viremia starts to increase and ALT levels are still normal (HBV DNA < 5 log copies/ml) (45). This virological breakthrough phase precedes the clinical resistance phase, which is characterised by > 5 log copies/ml of HBV DNA and ALT levels above the normal range, by several weeks or months. The use of an early add-on strategy has been successfully tested in HBeAg-negative cirrhotic patients, in most of whom it led to the control of viral replication for more than six years (47).

Entecavir has not been tested as rescue monotherapy in HBeAg-negative and lamivudine-resistant patients, but the findings of a long-term rescue study in HbeAg-positive patients found low virological response rates, with high rates of genotype resistance, virologic breakthrough and clinical drug resistance, and thus do not support its first-line use in this setting (48, 39).

Adefovir-resistant patients

No studies have specifically assessed the management of adefovir resistance in large numbers of HbeAg-negative patients but, given the drug's resistance profile, *in vitro* cross resistance data and the few published case series, the use of a nucleoside analogue seems to be the most appropriate approach.

Conclusions

The treatment of chronic HBeAg-negative hepatitis B is intended to ensure the long-term suppression of HBV replication with the aim of halting the progression of liver damage and preventing the development of liver-related complications. This can be done by means of short-term "curative" treatment or long-term "suppressive" therapy. The first approach requires a 48-week course of peg-interferon, which controls viral replication (HBV DNA < 10.000 copies/ml) in 20-30% of patients; the second requires the long-term (possibly lifetime) administration of nucleoside and/or nucleotide analogues. As none of the currently available drugs alone suppresses viral replication (HBV DNA < 200 copies/ml) for five years in all patients, some require a rescue therapy based on the addition of a non-cross-resistant drug, which should be given as early as possible ("on demand" combination therapy). However, the currently available anti-HBV analogues can easily suppress HBV replication for five years in most HBeAg-negative patients. As both strategies have their pros and cons, the best approach needs to be carefully evaluated on an individual basis

Two remaining major therapeutic challenges are how to increase the rates of HBsAg seroconversion and how to prevent the development of liver cancer in cirrhotic patients on long-term HBV suppressant treatment.

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