Hepatitis B virus (HBV) induces the expression of interleukin-8 that in turn reduces HBV sensitivity to interferon-alpha

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ABSTRACT

High levels of serum interleukin-8 (IL-8) have been detected in chronic hepatitis B (CHB) patients during episodes of hepatitis flares. We investigated whether hepatitis B virus (HBV) may directly induce IL-8 production and whether IL-8 may antagonize interferon-alpha (IFN-α) antiviral activity against HBV. We showed that CHB patients had significantly higher IL-8 levels both in serum and in liver tissue than controls. In HBV-replicating HepG2 cells, IL-8 transcription was significantly activated. AP-1, C/EBP and NF-κB transcription factors were concurrently necessary for maximum IL-8 induction. Moreover, HBx viral protein was recruited onto the IL-8 promoter and this was paralleled by IL-8-bound histone hyperacetylation and by active recruitment of transcriptional coactivators. Inhibition of IL-8 increases the antiviral activity of IFN-α against HBV. Our results indicate that HBV activates IL-8 gene expression by targeting the epigenetic regulation of the IL-8 promoter and that IL-8 may contribute to reduce HBV sensitivity to IFN-α.

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Introduction

Hepatitis B virus (HBV) infection is a major health problem worldwide with estimates of nearly 400 million people currently infected (Dienstag, 2008). HBV infection may associate with a large spectrum of clinical forms, ranging from very mild and asymptomatic clinical pictures to the most severe liver diseases, including fulminant hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Dienstag, 2008). Chronic HBV infection is a dynamic condition in which the interaction between virus and the host immune response influences the outcome of the disease. Its natural history is complex and variable. It includes different – not always sequential – phases that are distinguishable on the basis of the presence in the serum of HBV “e” antigen (HBeAg) or its antibody (anti-HBe), of the HBV DNA serum levels, of alanine aminotransferases (ALT) values and of the liver histology (EASL, 2012). The anti-HBe positive phase may be characterized by persistently low viremia levels < 2000 UI/ml, normal ALT and minimal histological lesions (inactive chronic HBV carrier state) (EASL, 2012) or by stable or fluctuating high levels of viral replication and ALT values, with active liver necroinflammation (HBeAg-negative chronic hepatitis). Patients with HBeAg-negative chronic hepatitis B (CHB) may undergo recurrent, spontaneous “hepatic flares,” characterized by wide fluctuations of viremia levels, liver inflammation and a propensity to a rapid progression towards cirrhosis (EASL, 2012).

The HBV replication cycle is not directly cytotoxic to cells and the pathogenesis of liver disease has conventionally been attributed to cytolytic killing of infected hepatocytes by virus-specific T cell response (Chisari and Ferrari, 1995; Perrillo, 2001). However, important data have shown that the high frequencies of HBV-specific CD8+ T cells in patients with chronic HBV infection are associated with HBV control rather than with hepatic injury.

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http://dx.doi.org/10.1016/j.virology.2013.06.028
Previous data had demonstrated that the HBV-encoded regulatory proteins can modulate the immune response to HBV infection and that HBV replication can be inhibited by IFN-α (Girard et al., 2002; Green et al., 2006; Hisatsune et al., 2007) and very recent data have shown that HBV-specific T cells maturing in the intrahepatic inflammatory environment can produce IL-8, which in turn can contribute to the development of liver damage through the recruitment of granulocytes (Kakimi et al., 2001; Sittia et al., 2004). Of interest, it has been recently shown that in chronic HBV infected patients flares of liver inflammation (either spontaneous or induced by anti-viral withdrawal) are preceded by a parallel increase of interleukin-8 (IL-8) production and serum HBV DNA levels (Dunn et al., 2007; Tan et al., 2010). IL-8 may synergize with interferon-α (IFN-α) to activate NK cells, (Dunn et al., 2007) and very recent data have shown that HBV-specific T cells involved in the activation of IL-8 gene expression and verified whether IL-8 might reduce HBV sensitivity to IFN-α.

Results

IL-8 amounts in liver tissues and sera of CHB patients, inactive HBV carriers and HBV-neg subjects

Real-time PCR analysis on liver tissue specimens showed that CHB patients had significantly higher median amounts of IL-8 mRNA (0.71 [range: 0.01–4.9] versus 0.06 [range: 0.01–0.07]; P=0.003) compared with the subjects of the control group, including both inactive HBV carriers (IBC) and HBV-neg subjects (Table 1 and Fig. 1A). Moreover, among CHB patients intrahepatic IL-8 mRNA levels were significantly higher in HBeAg-negative patients (1.7 [range: 0.5–4.7] versus 0.1 [range: 0.01–4.9]; P=0.028) than in HBeAg-positive ones. Both HBeAg-negative and HBeAg-positive CHB patients had much higher amounts of IL-8 mRNA in the liver than controls (P=0.004 and P=0.015, respectively). Concentrations of circulating IL-8 chemokine, assayed by a quantitative ELISA kit, were also significantly increased in CHB patients compared with controls (median, 29.5 pg/ml [range: 7.7–278.3 pg/ml] and 10.5 pg/ml [range: 2–20 pg/ml], respectively; P<0.0001) (Table 1 and Fig. 1B) and this statistical significance was maintained also when serum IL-8 amounts in CHB patients were separately compared with the amounts in IBC (median, 9.78 pg/ml; range, 4.2–19.9 pg/ml; P<0.0001) and in HBV-neg individuals either CHB or inactive carriers. Moreover, by using a cell-based HBV replication system that makes it possible to recapitulate the HBV replication cycle including the nuclear generation of covalently closed-circular DNA (cccDNA) molecules (Pollicino et al., 2006), we studied the molecular mechanisms implicated in the activation of IL-8 gene expression and verified whether IL-8 might reduce HBV sensitivity to IFN-α.
subjects (median, 11 pg/mL; range, 2–20 pg/mL; \( P < 0.0001 \)) of the control group. Instead, no statistically significant difference was found between serum concentrations of IL-8 chemokine in HBeAg-negative and HBeAg-positive CHB patients (median, 31.5 versus 28 pg/mL, respectively; \( P = 0.637 \)). As expected, serum HBV DNA levels were significantly higher in CHB patients (median, \( 3.2 \times 10^6 \) IU/mL; range, \( 2.1 \times 10^4 – 1.7 \times 10^8 \) IU/mL) compared with levels detected in IBC (median, \( 6.6 \times 10^2 \) IU/mL; range, 12–18 \( \times 10^3 \) IU/mL; \( P < 0.0001 \)). Of note, a significant correlation was found between serum IL-8 concentrations and HBV DNA amounts (\( r = 0.519; P = 0.002 \)) in HBV infected individuals.

IL-8 expression is upregulated in HBV-replicating hepatoma cell line

We have shown that transient transfection of plasmid-free linear HBV DNA into HepG2 or Huh7 cell lines makes it possible to recapitulate the HBV replication cycle, including the nuclear generation of viable cccDNA (Pollicino et al., 2006). Thus, to investigate the role of HBV in the induction of IL-8 expression, HepG2 cells were transiently transfected either with 0.5 \( \mu \)g of linear wild-type (WT) HBV DNAs of genotype D or genotype A (WTHBV-D and WTHBV-A, respectively) or with pcDNA3.1 empty vector as a control at 24 h postplating. Total RNA was extracted at 48 h after transfection, and expression of IL-8 was analyzed by quantitative real-time PCR. As shown in Fig. 2A, the replication of HBV (both of genotype D and A) in HepG2 cells was associated with a significant increase of IL-8 transcription (\( P = 0.038 \) and \( P = 0.046 \), respectively) when compared with the control cells. Moreover, when increasing amounts (0.1, 0.2, 0.5 and 1 \( \mu \)g) of WTHBV-D or WTHBV-A were transfected, higher amounts of viral replicative intermediates and transcripts were produced in a dose-dependent manner (Fig. 2B, D) and these events were paralleled by an increased production of IL-8 transcripts (Fig. 2B). In addition, to analyze the contribution of HBx viral regulatory protein in the induction of IL-8 gene expression, HepG2 cells were transfected with the same increasing amounts of either the pcHBx recombinant vector or of the mutHBxHBV-A genome that does not express HBx because of a stop codon for amino acid 8 of the HBx open reading frame (Belloni et al., 2009). Furthermore, to assess the pattern of IL-8 expression when the X protein is provided in trans to the HBx-mutant HBV, HepG2 cells were cotransfected with mutHBxHBV-A genome and pcHBx vector. Real-time PCR quantifications showed that IL-8 mRNA amounts were increased up to threefold in cells expressing HBx and up to fourfold in cells replicating the mutHBxHBV-A genome than in cells transfected with an empty vector (Fig. 2E, F). Thus, though IL-8 mRNA levels in
cells replicating the mutHBxHBV-A were lower compared to WTHBV-A transfected cells, they were higher compared to control cells, indicating that, aside from HBx, other viral factors are implicated in HBV-mediated IL-8 induction. Interestingly, in HepG2 cotransfected with mutHBxHBV-A genome and pcHBx vector IL-8 mRNA amounts were comparable to those detected in WTHBV-A replicating cells (Fig. 2G).

Transactivation of the IL-8 promoter by HBV

The impact of HBV transcription/replication on IL-8 promoter activity was investigated by using the HBV cccDNA-driven replication system (Polllicino et al., 2006). A 1.4-kb IL-8 promoter-luciferase construct (wtIL-8 LUC) was transfected into HepG2 cells along with increasing amounts of WTHBV-A, WTHBV-D, or mutHBxHBV-A genomes or of pcHBx vector. Measurement of IL-8 promoter-mediated luciferase activity in HBV-replicating cells showed that both genotype A and D WT HBV genomes were able to stimulate basal IL-8 promoter activity up to 100-fold in a dose-dependent manner (Fig. 3A), whereas transfection of equal amounts of mutHBxHBV-A virus led to significantly lower activation of the IL-8 promoter (up to 30-fold; \( P < 0.0001 \)) (Fig. 3B). HBx expression stimulated the basal IL-8 promoter activity up to threefold when 0.2 \( \mu \)g of the pcHBx were transfected (Fig. 3C). Moreover, when the HBx-mutant virus was trans-complemented with the wild type HBx gene, in co-transfection experiments, its ability to transactivate the IL-8 promoter was again largely restored (Fig. 3D).

Subsequently, to determine the minimal IL-8 promoter domains required for HBx and HBV-mediated transcriptional activation, a series of IL-8 reporter gene constructs containing mutated AP-1, C/EBP or NF-κB transcription factor binding sites were utilized (Caristi et al., 2005; Venza et al., 2007). In accordance with previously reported results, we found that HBx-induced transcriptional activation of the IL-8 promoter requires intact C/EBP and NF-κB binding sites (Fig. 4A). In HBV replicating cells, mutation in the AP-1, in the C/EBP or in the NF-κB binding sites reduced promoter activity by ∼50%, ∼40% and ∼46% respectively (Fig. 4B). These data indicate that all three binding sites, NF-κB, C/EBP, and AP-1, are involved in HBV-induced IL-8 promoter activity.

To determine signal pathways implicated in HBV-mediated transcriptional activation of the IL-8 gene, HBV-replicating cells and control cells were treated with inhibitors of PI3K/Akt (LY294002), p38 MAPK (SB203580), JNK (JNKi II), IkB kinase (Bay 11-7082), Erk (PD98059), SRC tyrosine kinase (PP2) and AP-1 (LY294002), p38 MAPK (SB203580), CREB, JUN, ATF4, MAPK11 and MAPK13) (Fig. 4E). HBV and indicate that PIK3/AKT, p38 MAPK and JNK signaling pathways also have a role in this induction.

The analysis of the expression of a selected number of genes by TaqMan Low-Density Arrays in HBV-replicating cells and control cells confirmed the ability of HBV to significantly induce the production of IL-8 transcripts and, consistently, showed that viral replication may induce the upregulation of numerous genes (as, NFKB1, IKKB, IL1B, TNFα, TNFRSF1A, TBK1, IRAK1, IRAK4, TRAF6, PIK3CB, PIK3R2, CREB, JUN, ATF4, MAPK11 and MAPK13) (Fig. 4E), involved – directly or indirectly – in the activation the IL-8 promoter (Hoffmann et al., 2002).

Fig. 3. The IL-8 promoter is activated by HBV. (A) HepG2 cells were cotransfected with 0.5 \( \mu \)g of a reporter plasmid containing 1.4 kb of the IL-8 promoter (wtIL-8 LUC) along with 0.1, 0.2, 0.5 or 1 \( \mu \)g of monomeric linear HBV genomes (both WTHBV-D and WTHBV-A were used), (B) 0.1, 0.2, 0.5 or 1 \( \mu \)g of monomeric linear mutHBxHBV-A or of WTHBV-A genomes, (C) 0.1, 0.2, 0.5 or 1 \( \mu \)g of pcHBx expression vector, (D) 0.1, 0.2, 0.5 or 1 \( \mu \)g of mutHBxHBV-A and 0.2 \( \mu \)g of pcHBx. The basal activity of cells cotransfected with the wtIL-8 LUC reporter and empty vectors was set at 1 (Control). Luciferase activities were determined 48 h after transfection, and the results are the average of three independent experiments carried out in duplicate. Means ± S.D. are presented.
The HBx viral protein is recruited onto the IL-8 promoter in liver tissues of chronic HBV carriers and in HBV-replicating HepG2 cells

It is known that HBV may induce epigenetic changes in host cells and HBx seems to play a central role in these epigenetic modifications. (Herceg and Paliwal, 2011; Paschos and Allday, 2010) Indeed, it has been shown that this protein may interact with different components of epigenetic complexes and may induce a modulation of transcriptional activity of several target genes (Kew, 2011; Park et al., 2007).

To analyze the recruitment of chromatin-modifying enzymes onto the IL-8 gene promoter, we applied the ChIP assay to HBV-replicating cells and liver tissues from two CHB patients, two IBC and two HBV-neg subjects. Chromatin was immunoprecipitated using antibodies that specifically recognize acetylated histone H4, the acetyltransferases PCAF and p300, the histone deacetylase HDAC1 and HBx viral protein. As shown in Fig. 5, histone H4 bound to the IL-8 promoter was highly acetylated and the transcriptional coactivators PCAF and p300 as well as HBx were recruited onto the IL-8 promoter both in HepG2 cells replicating HBV and in liver tissues from CHB patients with high viral loads. Interestingly, in IBC (subjects characterized by a state of persistently low HBV replication) the HDAC1 histone deacetylases was recruited onto the IL-8 promoter. HADC1 recruitment in these subjects was paralleled by the reduced binding to IL-8 of HBx viral protein, p300 and PCAF co-activators and by the presence of variable levels of IL8-bound histone acetylation. As expected, no HBx recruitment onto the IL-8 promoter could be detected in HBV-neg subjects. Moreover, in these subjects the recruitment on the IL-8...
promoter of PCAF, and p300 acetyltransferase was highly reduced and the binding of HDAC1 was associated with the hypoacetylation status of H4 histone. Thus, in HBV infected patients the differential recruitment of coactivators and corepressors onto the IL-8 promoter appeared to be correlated with HBV replication activity.

Altogether, these results indicate that HBx viral regulatory protein produced during HBV replication in the infected hepatocytes is recruited onto the IL-8 promoter and that its recruitment is associated with IL-8 gene expression.

**IL-8 impairs IFN alpha-inhibition of HBV replication**

It has been shown that IL-8 may inhibit in vitro the antiviral action of IFN-α (Khabar et al., 1997a) and an inverse correlation has been found between serum IL8 levels and the sensitivity to interferon treatment among patients infected with hepatitis C virus (Lee et al., 2011; Polyak et al., 2001b).

By the use of the cell-based HBV replication system, we verified whether IL-8 might weaken the action of IFN-α against HBV. Thus, HBV replication was examined in HepG2 cells co-transfected with HBV genomes and IL-8 siRNA (or siRNA-control) and then exposed or not to IFN-α treatment. Results from the ELISA assay ascertained that IL-8 protein released in the supernatants of transfected cells was significantly reduced by treatment with siRNA-IL-8 but not affected by siRNA-control (Fig. 6A).

Results from both Southern and Northern blotting showed that siRNA-directed inhibition of IL-8 expression improved the efficacy of IFN-α treatment against HBV (Fig. 6D and E, respectively). Indeed, HBV/IL8-siRNA co-transfected HepG2 cells showed a stronger reduction of both HBV replicative intermediates (Fig. 6D) and viral transcripts (Fig. 6E) compared to cells co-transfected with HBV genomes and siRNA-control, after IFN-α treatment. Interestingly, even in the absence of IFN-α treatment the suppression of IL-8 in HBV/IL8-siRNA co-transfected cells induced a reduction of the steady-state amounts of capsid associated HBV DNA and of all the viral transcripts (Fig. 6B and C), thus suggesting that IL-8 is able to induce HBV replication. To further confirm these results, viral transcription/replication was evaluated in HBV transfected-cells pretreated or not pretreated with recombinant human IL-8 (rIL-8) and then exposed or not exposed to IFN-α treatment. As shown in Fig. 6F and G, when HBV replicating-cells were pretreated with rIL-8 (10 ng/mL), a significant reduction of
Fig. 6. IL-8 weakens IFN-α antiviral action against HBV and induces viral transcription/replication in HBV-replicating HepG2 cells. (A) Determination of the effectiveness and specificity of siRNA-IL-8 on IL-8 gene expression. HepG2 cells were transfected with empty pcDNA3.1 vector monomeric or with linear HBV genomes (WTHBV-A) alone or along with siRNA-control or with siRNA-IL-8. IL-8 protein in culture supernatants was detected by ELISA. (B) Analysis of the effect of siRNA-IL-8 on HBV replication by Southern blotting of HBV replicative intermediates at 48 h post transfection. (C) Analysis of the effect of siRNA-IL-8 on HBV transcription by Northern blotting of viral mRNAs from HepG2 cells 48 h after transfection with WTHBV-A. (D) Southern blot analysis of HBV replicative intermediates from HepG2 cells transfected with WTHBV-A alone or co-transfected with WTHBV-A and IL-8 siRNA (or siRNA-control) and then exposed or not to IFN-α treatment (1000 U/ml) for 48 h. (E) Northern blot analysis of HBV transcripts extracted from HepG2 cells transfected with WTHBV-A alone or co-transfected with WTHBV-A and IL-8 siRNA (or siRNA-control) and then exposed or not to IFN-α treatment. (F) Northern blot analysis of HBV transcripts isolated from HBV transfected-cells pretreated or not pretreated with recombinant human IL-8 (rIL-8) and exposed or not exposed to IFN-α treatment. (G) Northern blot analysis of HBV transcripts from HBV transfected-cells treated with equal increasing amount of rIL-8. In all panels densitometric quantification of HBV replicative intermediates or of HBV transcripts is shown. Signal intensity of the single-strand (SS) band underneath the linear double-stranded (DS) HBV DNA band or of pregenomic RNA (pgRNA) band was quantified with Quantity One 1-D Analysis Software (BioRad Laboratories). Data are expressed as relative arbitrary units (mean ± SD) from three independent experiments. Abbreviations: OC, open circular HBV DNA; DS, double-stranded HBV DNA; SS, single-stranded HBV DNA.
IFN-α efficacy was consistently observed. Moreover, treatment of HBV transfected cells with rIL-8 in the absence of IFN-α was able to induce a dose dependent increase of HBV replication and transcription (Fig. 6H and I).

Discussion

The IL-8 chemokine is an important mediator of the innate immunity with well-defined immunomodulatory effects on T-cell function and inflammatory response (Mukaida, 2003; Taub et al., 1996). Recent evidence has suggested that this chemokine may play an important role in the immunopathogenesis of HBV infection (Bertoletti et al., 2010). High levels of serum IL-8 have been detected in CHB patients with active liver inflammation as well as in patients with acute hepatitis B (but not in patients with other acute viral infection) (Dunn et al., 2007; Gehring et al., 2011; Tan et al., 2010; Zimmermann et al., 2011). Moreover, it has been recently demonstrated that during the episodes of HBV reactivation, the increase of viremia levels is paralleled by an increment of serum IL-8 amounts and that these events precede the onset of hepatic flare (Dunn et al., 2007; Tan et al., 2010).

In the current study, IL-8 was quantified in paired serum and liver tissue samples from chronically HBV-infected and HBV-negative subjects. Through this analysis, we found that in CHB patients the presence of significantly higher serum concentrations of IL-8 chemokine was paralleled by an upregulation of IL-8 gene expression in the paired liver specimens compared to controls, suggesting that in CHB patients the high circulating amounts of IL-8 are likely derived from the liver. We also found that HBeAg-negative patients had significantly higher levels of IL-8 transcripts in the liver than HBeAg-positive patients, and this observation is consistent with the fact that HBeAg-negative patients are much more susceptible to flares of liver inflammation associated with rapid changes in HBV viremia levels than HBeAg-positive patients (EASL, 2012). Our data are also in accordance with the body of evidence indicating that HBeAg-positive infection is usually associated with lower levels of pro-inflammatory cytokines and in general with a more efficient inhibition of the host’s innate immune response (Chen et al., 2004; Visvanathan et al., 2007; Walsh and Locarnini, 2012).

By using a plasmid-free HBV transfection approach (Pollicino et al., 2006), we were able to demonstrate that IL-8 gene transcription is specifically induced in response to HBV replication and that the increased amounts of IL-8 directly correlate with the levels of HBV transcription/replication. In accordance with these in vitro results, we also found a significant correlation between IL-8 chemokine concentrations and HBV viremia levels in the HBV infected patients. Altogether, these data indicate that active HBV replication is able to directly induce IL-8 production and provide new evidence in support of an important role of the magnitude of viral replication in the virus–host interplay, particularly in the activation of the innate immune response (Ait-Goughoult et al., 2010; Bertoletti et al., 2010). It has been shown that the X protein of HBV is able to transactivate IL-8 gene expression through NF-kB and C/EBP-like cis-elements (Mahe et al., 1991). By the use of our HBV replication system, we demonstrated that binding sites for NF-kB, AP-1, and C/EBP are required for maximum HBV-induced IL-8 promoter activity as well as for maximal IL-8 protein production. In this context, our finding that in cells replicating HBx-defective HBV the activation of IL-8 gene promoter was significantly reduced, but not abolished, suggests that other HBV gene products might also be involved in the regulation of IL-8 gene expression. This hypothesis is supported by available data showing that in addition to HBx other viral proteins, such as the large and middle envelope proteins or the core and HBE proteins, have the ability to deregulate the cellular transcription program (Ait-Goughoult et al., 2010; Hildt et al., 2002; Locarnini et al., 2005). Our results showing that CHB patients infected with HBeAg-negative HBV had higher intrahepatic levels of IL-8 compared to patients infected with HBeAg-positive viral strains further confirm our hypothesis. Thus, the control of IL-8 gene expression by HBV appears to be complex and multifaceted with the involvement of different signaling pathways, as also indicated by the results obtained from the PCR arrays showing that HBV upregulates the expression of a number of transcription factors and protein kinases, which in principle have the ability to modulate NF-kB or AP-1 activity. In addition, in our HBV replication system the upregulation of IL-1 and TNF-alpha proinflammatory cytokines – which are known to strongly induce IL-8 production (Hoffmann et al., 2002) – indicates that different mechanisms synergize to induce IL-8 overexpression during active HBV replication.

By the use of ChIP assay, we found that active HBV replication was associated – both in vivo and in vitro – with a hyperacetylation status of IL8 promoter and with the recruitment on this promoter of PCAF and p300 acetyltransferases as well as of the HBx viral protein. Of interest, the presence of the class I deacetylase HDAC1 on the IL-8 promoter was associated with a low HBV replication state in vivo indicating that HBV may exert an epigenetic control of IL-8 gene by favouring the recruitment of chromatin modifying enzymes onto the IL-8 promoter. The HBx regulatory protein might have an important role in this control since it has been shown that it is able to interact directly with the CREB-binding protein (CBP)/p300 to synergistically enhance its activity and to modify chromatin dynamics of target genes (Cougnot et al., 2007). Altogether our results suggest that HBV might actively contribute to the constitution of the “enhanceosome”-like structure known to control IL-8 gene expression (Hoffmann et al., 2002).

Cytokines and chemokines released in response to virus infections have the main role of recruiting inflammatory cells, of constraining virus replication and spread, and of inducing adaptive immunity (Biron, 2001; Guidotti and Chisari, 2000; Redpath et al., 2001). However, when the production of chemokines (like IL-8) in the context of viral infections is continuous it may become harmful to the host (Luster, 1998; Mukaida, 2003). The existence of a kind of vicious circle between IL-8 production and several viral infections has been described (Alcorn et al., 2001; Girard et al., 2002; Khabar et al., 1997a; Khabar et al., 1997b; Murayama et al., 1994; Wagoner et al., 2007). It has been shown that human cytomegalovirus (CMV) can induce IL-8 gene transcription in various types of cells and that IL-8 may enhance human CMV replication (Murayama et al., 1994). In the present study, we found that IL-8 is able to induce HBV replication in a dose dependent manner and that the induction of IL-8 gene expression by HBV is also dose-dependent. These results suggest the presence of an amplifying loop between IL-8 production and HBV activity, which in part might explain both the amplification of liver damage that occurs during the phase of immune reactivation of HBV and the difficulties in finding a temporal correlation between immunological parameters and the kinetics of HBV viremia in the course of hepatic flares (Bertoletti et al., 2010).

Accumulating evidence indicates that IL-8 induced by viruses may also contribute to counteract IFN-α antiviral action (Girard et al., 2002; Lee et al., 2011; Polyak et al., 2001a, 2001b). Our results showing that the specific inhibition of IL-8 increases the potency of IFN-α against HBV in vitro and that the addition of recombinant human IL-8 almost totally rescues HBV replication during IFN challenge strongly suggest that IL-8 expression induced by HBV can impair the ability of endogenous IFN-α to inhibit early stages of viral replication, thus favoring viral persistence, and can also contribute to the poor response to IFN-α treatment.
This hypothesis is consistent with a great deal of evidence indicating that viruses have evolved strategies to turn chemokine activity to their own advantage (Alcamí, 2003; Finlay and McFadden, 2006; Kotwal et al., 2012). In that respect, the general inflammatory microenvironment induced by HBV can be considered an effective stratagem for manipulating the host’s immune response to achieve viral persistence and the establishment of CHB.

In conclusion, HBV is able to directly induce the production of the IL-8 chemokine which in turn may contribute to the persistence of the infection, to the pathogenesis of viral related liver damage as well as to the mechanisms of resistance to IFN. Consequently, inhibition of IL-8 production or activity could be instrumental to the suppression of viral activity, to the reduction/block of liver inflammation and to the improvement of IFN-α antiviral action and it might open novel avenues for the development of effective immunotherapeutic approaches against HBV.

Materials and methods

Study Patients

We studied serum samples from 48 subjects who were seen in the outpatient service of the Liver Unit at the Messina University Hospital (Table 1). Twenty-six of the 48 subjects (23 men and three women; mean age, 45 ± 17 years) had a histologically proven HBV-related chronic liver disease whereas 22 (16 men and six women; mean age, 37 ± 14.6 years) had no clinical, biochemical and ultrasound signs of liver disease (control group). Of these 22 subjects, seven were inactive HBV carriers (IBC) – with persistently serum HBV-DNA levels < 2000 IU/mL and normal ALT (< 40 U/L) over a 10-year median period of follow-up – and 15 were individuals negative for all HBV serum markers and without any sign of liver disease (HBV-neg subjects).

Eight of the 26 CHB patients were HBV “e” antigen (HBeAg) positive and 18 were HBeAg-negative and positive for the corresponding antibody (anti-HBe). All the seven inactive HBV carriers were HBeAg-negative/anti-HBe positive.

Frozen liver biopsies were available for molecular analyses from 14 CHB patients (seven HBeAg-positive and seven anti-HBeAg positive). In addition, frozen liver tissues were also available from five subjects of the control group, two inactive HBV carriers and three HBV-neg individuals who underwent surgery for benign liver tumor or metastasis from extra-hepatic tumors. Liver samples from the control subjects were obtained from unaffected areas of liver resections. All the subjects were negative for hepatitis delta virus, hepatitis C virus and human immunodeficiency virus serum markers and all CHB patients were treatment-naive.

The study protocol was performed according to the principles of the Declaration of Helsinki. The collection of blood and tissue samples for research was approved by the ethics committee of the University Hospital of Messina, Messina, Italy. Written informed consent was obtained from all patients.

Chemicals and Antibodies

Phosphatidylinositol 3′-kinase (PI3K)-Akt inhibitor LY294002, p38 kinase inhibitor SB203580, c-Jun N-terminal kinase (JNK) inhibitor JNKII, mitogen-activated protein/ERK kinase/extracellular signal-regulated kinase (MEK) inhibitor PD98059, SRC tyrosine kinase inhibitor PP2 and nuclear factor of kappa light polypeptide gene enhancer in B-cell (NFκB) inhibitor BAY 11–7082 were from Calbiochem-Novabiochem Corp (San Diego, CA, USA). The activator protein-1 (AP-1) inhibitor Tanshinone IIA was from Enzo Life Sciences (Farmingdale, NY USA). Recombinant human CXCL8/IL-8 (208-IL) was from R&D SYSTEMS (Minneapolis, USA) and recombinant human IFN-α2b was from Schering-Plough Corporation (Kenilworth, NJ, USA). Rabbit polyclonal antibody against ACh4 (IgG recognizing histone H4, which is tetra-acetylated at lysines 6, 9,13, and 17) (06-598) and rabbit polyclonal antibody against HDAC1 (06-720) were from Upstate (Waltham, MA USA), rabbit polyclonal antibody against p300 (sc-584) and rabbit polyclonal antibody against PCAF (sc-8999) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse monoclonal antibody against HBx (MAI-081) was from Affinity Bioreagent (Rockford, IL USA).

Plasmid Constructs and full-length HBV DNA Genomes

Plasmid constructs containing the full-length wild-type (wtIL-8 LUC) or truncated IL-8 promoters (IL-8-AP-1 mutant (IL-8-mAP1), IL-8 C/EBP mutant (IL-8-mC/EBP), IL-8 NF-kB mutant (IL-8-mNF-kB), and IL-8 double mutant for C/EBP and NF-kB (IL-8-C/EBP-NF-kB)) controlling the expression of the luciferase gene were generated as described previously (Caristi et al., 2005; Venza et al., 2007).

Monomeric linear full-length wild type (WT) and HBx mutant HBV genomes of genotype A (WTHBV-A and mutHBxBV-A, respectively) were released from the pCR.HBV.A.EcorI (Pollicino et al., 2006) and pCRmHBxBxA.EcorI (Belloni et al., 2009) plasmids using EcoRI-PvuI restriction enzymes (New England Biolabs GmbH, Frankfurt, Germany). Monomeric linear full-length WT HBV genome of genotype D (WTHBV-D) was released from pUC. HBV.D plasmid using SapI restriction enzyme (New England Biolabs) (Pollicino et al., 2006).

The X coding region of HBV genotype A was amplified by PCR and the resulting 465 bp fragment (positions 1374–1838 on the HBV map) was cloned into pcDNA3.1 HA cloning vector (Invitrogen, Milan, Italy) (pHBx-HA). Cloned HBx coding region was verified by sequencing.

IL-8 ELISA

Patients sera and supernatants of transfected cells were tested for IL-8 using the BD OptEIA human IL-8 ELISA kit II (BD Biosciences, San Jose, CA USA) where 100 μl of patient serum, cell culture supernatant or standard dilutions were analyzed according to the manufacturer’s protocol. This assay has a lower limit of detection of 0.8 pg/ml.

Cell lines, Transfections, Treatments and luciferase reporter Assays

The HepG2 cell line was maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Life Technologies, Grand Island, NY USA), 1% penicillin/streptomycin, and 1% glutamine (Sigma-Aldrich S.r.l., Milan, Italy). For reporter assays, semi-confluent cells in 60-mm-diameter Petri dishes were transiently transfected with 0.5 μg of monomeric linear wild-type HBV genomes (WTHBV-A, mutHBxBV-A or WTHBV-D) or 0.2 μg the pHBx-HA expression vector and 0.5 μg of different pIL-8-LUC constructs using the FuGENE transfection reagent (Roche, Indianapolis, IN). Luciferase activity was determined 48 h later. All experiments were performed in duplicate and repeated at least three times. For transfection control, cells were cotransfected with the plasmid pRL-TK (Clontech, Mountain View, CA), which expresses Renilla luciferase under the control of the thymidine kinase promoter. Luciferase levels in cell lysates were determined by using the Dual-Luciferase Reporter Assay System (Promega Corp, Madison, WI). The total amount of transfected DNA was kept constant by adding pcDNA3.1 vector.
For the kinase and AP-1 inhibition assay, drugs were added to cell culture medium 24 h post-transfection. The carrier dimethyl sulfoxide (DMSO) was added as control. The concentrations of drugs used are as follows: SB203580 (25 μM), LY294002 (30 μM), JNKI II (25 μM), Bay 11-7082 (10 μM) PD98059 (30 μM), PP2 (20 μM) and Tanshinone IIA (25 μM). At 16 h post-treatment, cells and supernatants were harvested to test luciferase activity by luciferase reporter assay and IL-8 production by ELISA test, respectively.

For treatments with IFN-α, HepG2 cells were seeded in a 6-well plate at a density of 2 × 10⁵/well and transfected 24 h later with 0.5 μg of monomeric linear WTHBV-A genomes or cotransfected with 0.5 μg of WTHBV-A genomes and 40 μg of IL-8 small interfering RNA (IL-8 siRNA: sc-39631, Santa Cruz Biotechnology) by using Lipofectamine 2000 (Invitrogen). IFN-α was used at a final concentration of 1000 IU/ml and was added directly to the culture medium starting at 3 h after transfection. HepG2 cells transfected with WTHBV-A genomes were also exposed to recombinant human IL-8 (rIL-8) which was used at 10, 20 and 30 ng/ml. Transfection experiments included 0.5 ng of pCMV-α-Gal reporter plasmid. IFN-α interferon protein, IFN-α was used at 1000 IU/ml and was added directly to the culture medium at 1 h after transfection. Cells were transfected with pCRII-IL-8 plasmid and the IL-8 sequence was gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI USA). The concentration of purified IL-8 DNA was determined with the ND-1000 spectrophotometer and the corresponding copy number was calculated. A series of 10-fold dilutions of the plasmid pCMV-α-IL-8 was used as standard for IL-8 DNA quantification.

For gene expression analysis by TaqMan Low-Density Arrays (TLDAs) (Applied Biosystems, Foster City, CA), total RNA from transfected HepG2 cells was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instruction. RNA (5 μg) was reverse transcribed using the Transcriptor First Strand cDNA Synthesis kit and 200 ng of each cDNA were loaded in double on a customized TDLA with 18S RNA used as control. TLDAs were run on an ABI 7900HT Sequence detection system and, real-time PCR data were collected and analyzed with the SDS2.2 software (Applied Biosystems).

ChIP assays

Forty-eight hours after transfection with linear HBV monomers, HepG2 cells were fixed in 1% formaldehyde, resuspended in 1 ml of ChIP lysis buffer (50 mM Tris–HCl, pH 7.4, 1 mmol EDTA, and 1% NP-40). Nuclei were pelleted by centrifugation for 1 min at 10,000 g. The supernatant was adjusted to 100 mM MgCl₂ and treated with 100 mg/ml of DNase I for 30 min at 37 °C. The reaction was stopped by adding EDTA to a final concentration of 25 mM. Protein was digested with 0.5 mg/ml proteinase K and 1% SDS for 2 h at 50 °C. Nucleic acids were purified by phenol/chloroform (1:1) extraction and ethanol precipitation after adding glycogen and examined by Southern blot following standard procedures as previously described (Pollicino et al., 2007).

HBV RNAs and cellular mRNA analysis

Total RNA was extracted from liver tissue specimens and HepG2 cells at 48 h post-transfection with the TRIzol reagent (Life Technologies) as recommended by the manufacturer. RNA concentration was measured using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 nm. RNA quantity and quality were monitored by ethidium bromide staining and by UV absorbance.

Northern blot analysis was performed following standard procedures as previously described (Pollicino et al., 2007). Radioactive probes were prepared by random priming protocol, using either full-length HBV DNA or 18S cDNA templates and [³²P]α-dCTP (Amersham).

For quantification of IL8 mRNA in liver specimens and transfected HepG2 cells, 5 μg of extracted RNA for each sample were treated with RQ1 RNase-free DNase (Promega, Madison, WI) for 1 h at 37 °C, and used as a template for first-strand cDNA synthesis with Transcriptor First Strand cDNA Synthesis kit (Roche, Indianapolis, USA) and oligo(dT) primers according to manufacturer’s protocol. Five μl of each cDNA were then quantified by real-time PCR analysis using the following IL-8 mRNA specific primers and probes: forward primer 5′-ATGACTTCAAGCTGCCGGTG3′, reverse primer 5′-TGTGATAATTTGGGCGTGAAGGAAAG.

TT-3′. FRET hybridization probes 5′-AGCTTCTGTATTGTCG–CACGCTCTGTTGAGAAAG-FL–3′; 5′-LCG40-AGTTTGGCAGGATCTCATAA-GAAGTTAGATG-PH-3′. The LightCycler h-GFPDH housekeeping Gene Set (Roche) was used to normalize the RNA samples. Real-time PCR by the use of the “utility channel” of COBAS TaqMan 48 was performed as follows: 95 °C for 10 min then 60 cycles of 95 °C for 30 s, 57 °C for 20 s, 72 °C for 20 s. The plasmid pCRII-IL-8, containing one copy of the IL-8 coding sequence (nt 1 to 1551) was used as standard for IL-8 cDNA quantification. The plasmid was digested with EcoRI (New England Biolabs) and the IL-8 sequence was gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI USA). The concentration of purified IL-8 DNA was determined with the ND-1000 spectrophotometer and the corresponding copy number was calculated. A series of 10-fold dilutions of the plasmid pCRII-IL-8 was used as standard for IL-8 cDNA quantification.

To perform ChIP assays on liver tissues from chronically HBV-infected patients, liquid nitrogen frozen biopsy specimens (from 0.5 to 1.0 cm) were lysed at 4 °C in 500 μl homogenization buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.2% NP-40, 150 mM NaCl) with a TissueRuptor instrument (Qiagen, Hilden, Germany). Nuclei were isolated by centrifugation and then fixed in 1% formaldehyde for 15 min at 4 °C. After sonication and dilution, the chromatin was
subjected to immunoprecipitation for 14–16 h at 4 °C using antibodies specific to act14, HDAC1, p300, PCAF and HBx. Immunoprecipitations with nonspecific immunoglobulins (Santa Cruz Biotechnology Inc.) were included in each experiment as a negative control. After the reverse cross-linking step, immunoprecipitated chromatin was analyzed by PCR amplification using cccDNA-specific primers.

**Statistical analysis**

Continuous variables were reported as median (range) and categorical variables as frequencies (percentages). A nonparametric approach was used to examine variables showing the absence of a normal distribution, as verified by the Kolmogorov–Smirnov test. The interdependence between numerical variables was performed by the use of the Spearman Rank correlation, whereas the Mann–Whitney test was applied to perform comparisons of continuously distributed variables between two independent groups. For in vitro experiments the non parametric ANOVA was applied and the Kruskall Wallis test was used. Bar graphs were plotted to show the mean ± standard deviation (SD) of at least three independent experiments. P values < 0.05 were considered as statistically significant. Statistical analysis was performed with SPSS version 12.0 software package (SPSS Inc, Chicago, IL).

**Acknowledgment**

This study was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) grant (IG 12022).

**References**


