Upregulation of endoplasmic reticulum stress and reactive oxygen species by naturally occurring mutations in hepatitis B virus core antigen

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Hepatitis B virus (HBV) infection is associated with a broad spectrum of clinical manifestations, including cirrhosis and hepatocellular carcinoma (HCC). Endoplasmic reticulum (ER) stress and subsequent oxidative stress have been implicated in liver carcinogenesis and disease progression with chronic inflammation. In our previous study, several mutations in the precore/core region of HBV genotype C were identified from 70 Korean chronic patients, and the mutations were associated with HCC and/or HBV e antigen serostatus. Here, we found that the naturally occurring mutations P5T/H/L of the HBV core antigen induced ER stress. The upregulation of ER stress resulted in higher reactive oxygen species production, intracellular calcium concentration, inflammatory cytokines as well as surface antigen production and apoptosis of cells. This study suggested that these mutations may contribute to the progression of liver disease in chronic patients.

More than 350 million people suffer from chronic hepatitis B virus (HBV) infection worldwide (Kao et al., 2002; Löhrr et al., 1994; Safioleas et al., 2007). It has been estimated that 15–40 % of chronic hepatitis patients develop serious liver diseases, including liver cirrhosis and hepatocellular carcinoma (HCC) without proper treatment (Lavanchy, 2004, 2005). Despite the clear relationship between HBV infection and the development of liver cirrhosis and HCC, the underlying mechanism by which HBV causes liver cirrhosis and HCC remains unknown. Endoplasmic reticulum (ER) stress and subsequent oxidative stress have been implicated in liver carcinogenesis and disease progression in direct or indirect ways, together with chronic inflammation (Ji & Kaplowitz, 2006; Malli & Kaufman, 2011; Marra et al., 2011). Viral infections usually cause disturbances in ER homeostasis through the production of large amounts of viral proteins and increased protein trafficking (Wang & Kaufman, 2012; Zhang & Wang, 2012). The contribution of ER stress in HBV- or hepatitis C virus-related HCC developments has also been suggested (Benali-Furet et al., 2005; Chua et al., 2005; Kim, 2014; Kim et al., 2014; Pollicino et al., 2014). HBx, which is known to play a major role in hepatocellular carcinogenesis, along with the LHB large surface proteins of HBV have been shown to induce ER stress and activate the surface antigen promoter (Cho et al., 2011; Kim et al., 2008; Lee et al., 2011; Xu et al., 1997). In our previous study, several mutations (P5H/L/T, E83D, L100I and Q182K) in the precore/core region of HBV genotype C were identified from 70 Korean chronic patients, and the mutations were associated with HCC and/or HBV e antigen serostatus (Kim et al., 2007, 2012). To understand the physiological role of the mutations within the core region, HBV viral genomes harbouring each mutation were generated. Among the mutated HBV genomes, P5H/L/T HBV genomes induced higher HBV surface antigen (HBsAg) secretion from transiently transfected Huh7.5 cells and all the tested mutant HBV genomes induced higher reactive oxygen species (ROS) in transfected Huh7.5 cells (Fig. S1, available in the online Supplementary Material). In addition, during the attempt to determine the role of the mutations, significant increases in the amounts of ER stress-related proteins were observed in Huh7.5 human hepatoma cells transfected with P5H, P5L and P5T viral genomes compared with WT. Thus, we tested the hypothesis that these mutations are related to disease progression via ER stress induction. Transfection of all three HBV core mutants into Huh7.5 cells dramatically increased the level of GRP78 (Fig. 1a). In addition, phosphorylation of PERK (dsRNA-activated protein kinase-like ER kinase) and eIF2α (eukaryotic initiation factor 2α) was significantly increased by P5T and P5H mutations, suggesting that these mutations are related to the induction of the ER

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One supplementary figure is available with the online Supplementary Material.
stress response (Fig. 1a). An examination of WT and mutant HBV core antigens (HBcAg) by confocal microscopy showed the co-localization of HBcAg and ER. In particular, P5L and P5H showed significantly higher co-localization compared with WT (Fig. 1b), although it is not clear whether this co-localization is due to the movement of the mutant HBcAg protein by itself or whether this is caused by the budding into the ER of viral cores. To examine whether HBcAg itself can induce ER stress, WT and P5H expression plasmids (pcDNA3.3-WT and pcDNA3.3-P5H) were transfected into Huh7.5 cells. The transfection of both WT and P5H resulted in a clear increase of ATF6, and GRP78 and P5H exerted a stronger effect compared with WT, suggesting that the expression of mutated HBcAg sufficiently induces ER stress (Fig. 1c). DAPI staining of Huh7.5 cells transfected with the indicated HBV genome showed the typical pattern of apoptotic cells, i.e. nuclear membrane invagination and chromatin condensation (Fig. 1d). The increased level of cytochrome c released from the cells transfected with the HBcAg mutants supported the hypothesis that the mutations

**Fig. 1.** Induction of ER stress by HBV core mutations. (a) Huh7.5 cells were transfected with WT and the indicated mutant HBV genomes. At 48 h after transfection, ER-stress-related proteins were analysed by immunoblotting. Similar expression levels of WT and mutant core proteins were confirmed by quantitative real-time (qRT)-PCR. IRE1, inositol-requiring transmembrane kinase/endonuclease 1. The levels of GRP78, phosphorylated PERK, phosphorylated eIF2α and CHOP (C/EBP homologous protein) were analysed by densitometry and normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data represent mean ± SD; *P<0.05, ***P<0.001 versus mock-treated. (b) Co-localization of HBcAg (anti-HBc, green; 1:1000) and secondary antibody anti-mouse Alexa Fluor 488-conjugated HRP (1:2000) with ER (anti-calnexin, red) in Huh7.5 cells transfected with the indicated HBV genome was analysed by confocal microscopy. Bar, 10 μm. (c) Huh7.5 cells were transfected with WT (pcDNA3.3-WT) or P5H HBcAg (pcDNA3.3-P5H) expression plasmid. GRP78 and ATF6 levels were analysed by immunoblotting with a corresponding antibody. The levels of the proteins were analysed by densitometry (right). Data represent mean ± SD; *P<0.05, **P<0.01, ***P<0.001 versus mock-treated. (d) Huh7.5 cells transfected with the indicated HBV genome were stained with DAPI and observed under a confocal microscope. Cytosolic concentrations of cytochrome c were determined by cytochrome c ELISA assessments. Data represent mean ± SD; ***P<0.001 versus WT.
induce the ER stress response and subsequent apoptosis (Fig. 1d). Next, we examined the effect of the mutations on the production of ROS and on the intracellular calcium concentration which follow ER stress. These investigations were done with dichlorodihydrofluorescein diacetate (DCF-DA; Molecular Probes) staining and Rhod-2AM (Invitrogen) staining, respectively (Dolai et al., 2011). As depicted in Fig. 2(a), all three HBV mutants increased the level of intracellular ROS. In line with previous results, the ectopic expression of P5H HBcAg also resulted in significantly higher intracellular ROS levels compared with WT HBcAg (Fig. 2b). Subsequently, P5T/H/L HBV mutants induced a

![Fig. 2. Increased ROS production, intracellular calcium concentrations and NFκB-mediated proinflammatory cytokine production by the core mutations.](image-url)

(a) Huh7.5 cells transfected with WT or mutant HBV genomes were loaded with DCF-DA (25 μM) and the production of ROS was estimated using a fluorimeter. Mean values were calculated from three independent experiments. Data represent mean ± SD; *P<0.05, **P<0.01, ***P<0.001 versus WT. The levels of pre-genomic RNA (pgRNA) were determined by qRT-PCT to confirm the similar transfection efficiency (right). (b) Huh7.5 cells were transfected with WT (pcDNA3.3-WT) or P5H HBcAg (pcDNA3.3-P5H) expression plasmid. The production of ROS was measured as described in (a). Data represent mean ± SD; **P<0.01 versus WT. (c) Intracellular Ca^{2+} concentrations of Huh7.5 cells transfected with WT or mutant HBV genomes were determined. Cells were treated with Rhod-2AM (4 μM) and subjected to flow cytometric analyses. (d) Huh7.5 cells were transfected with WT or mutant HBV genomes together with NFκB firefly luciferase reporter and cytomegalovirus promoter/Renilla luciferase reporter plasmids. NFκB transcriptional activities were determined by dual-luciferase assays. Data represent mean ± SD; *P<0.05 versus WT. (e, f) Synthesis of IL-6 (e) and TGF-β (f) mRNA was analysed by qRT-PCR after transfection of WT or mutant HBV genomes into Huh7.5 cells. Data represent the mean ± SD; *P<0.05, **P<0.01, ***P<0.001 versus WT.
higher intracellular calcium concentration compared with WT (Fig. 2c). Given that ER-stress-mediated ROS production induces the activation of NFκB and the subsequent synthesis of inflammatory cytokines, we assessed the NFκB activation and synthesis of inflammatory cytokines. P5L and P5H mutants transfected into Huh7.5 cells induced significant NFκB activation, as determined by a NFκB luciferase assay (Fig. 2d). Consistently, the mRNA synthesis of IL-6 and transforming growth factor (TGF)-β was significantly increased (Fig. 2e, f). As TGF-β has been implicated in the development of both fibrosis and HCC (Benali-Furet et al., 2005; Ganem, 1999), this result suggests that the increased production of TGF-β by these HBCAg mutations contributes to the progression of disease. Previously, it was reported that the synthesis and secretion of HBsAg could be induced by the ER stress response (Wang et al., 2006). Thus, the secretion of HBsAg from Huh7.5 cells transfected with the P5T/H/L HBV genome was compared with cells transfected with WT (Fig. 3a). The effect of the mutation of P5H on HBsAg secretion was further confirmed using an in vivo hydrodynamic injection model (Huang et al., 2006; Wang et al., 2014; Yang et al., 2002). As shown in Fig. 3(b), the serum level of HBsAg from mice which received a hydrodynamic injection of the P5T HBV genome (10 μg) was significantly higher than mice injected with the WT HBV genome (10 μg). Granted that the secretion of HBsAg serves as an immune evasion mechanism of HBV (Ait-goughoulte et al., 2010; Arzumanyan et al., 2013; Wieland & Chisari, 2005), it is conceivable that the increment of HBsAg caused by these mutations contributes to the persistent infection of HBV and to disease progression. Although we showed that the ectopic expression of the mutant HBCAg alone is capable of inducing ER stress responses, the increased synthesis of HBsAg may also augment the ER stress responses. Here, we demonstrate that P5T/H/L mutations in the core region of HBV genotype C elicit the ER stress response. In turn, ER stress evokes several biological responses, such as ROS production, inflammatory cytokine production, TGF-β secretion, apoptosis and HBsAg secretion, all of which are related to liver disease progression. Thereby, prolonged inflammation, liver damage and increased HBsAg secretion by these naturally occurring mutations may contribute to the progression of liver disease.

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**References**


**Fig. 3.** Increased secretion of HBsAg by the core mutations. (a) After transient transfection with HBV WT or core mutants, cell culture supernatants were analysed by HBsAg ELISA. Data were analysed from three independent experiments using Tukey’s multiple-comparison test. Data represent the mean ± SD; *P*<0.05, **P**<0.01, ***P***<0.001 versus WT. (b) WT or P5H (10 μg) HBV genome was injected by means of a hydrodynamic tail vein injection into C57BL/6 (n=5 per group). The amount of HBsAg secreted into serum was determined by HBsAg ELISA assessments. The graph shows the maximum, minimum and mean values. Data were compared from three experiments using Tukey’s multiple-comparison test; *P*<0.05 versus WT. Whisker bars indicate SD.


