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Reactive oxygen species modulates the intracellular level of HBx viral oncoprotein

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Abstract

HBx (hepatitis B virus X) viral oncoprotein is a multifunctional protein of which the cellular level may be one of the important factors in determining HBV-mediated pathological progression of liver diseases, chronic hepatitis, and hepatocellular carcinoma. Our previous work revealed that adriamycin, a chemotherapeutic agent, caused a marked increase in the intracellular level of HBx by retarding its rapid degradation. In the present study, modulation of HBx expression was found to be confined to adriamycin but not to other chemotherapeutic agents, cisplatin and 5-fluorouracil. Interestingly, adriamycin caused a rapid increase of reactive oxygen species (ROS) and its accumulation continued until 24 h. In contrast, two other agents had little effect on ROS generation, suggesting the possible involvement of ROS in the HBx regulation. In fact, direct addition of H_2O_2 to the cells significantly increased the level of HBx protein in HBx-expressing ChangX-34 cells as well as in hepatitis B virus-related hepatoma cells, PLC/PRF/5 and HepG2.2.15 cells. Furthermore, antioxidants, *N*-acetyl-cysteine and pyrrolidinedithiocarbamate (PDTC), completely abolished the increase of HBx protein induced by adriamycin, indicating that adriamycin modulates the intracellular HBx level via ROS generation. Together, these findings provide a novel aspect of HBx regulation by cellular ROS level. Therefore, intracellular microenvironments generating ROS such as severe inflammation may aggravate the pathogenesis of liver disease by accumulating the HBx level.

Keywords: HBx; HBV; ROS; Adriamycin; Gene expression; Antioxidants

Human hepatitis B virus (HBV) belongs to a member of the family *Hepadnaviridae*, which includes woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV), and duck hepatitis B virus (DHBV) [1,2]. Mammalian hepadnaviruses showed a strong pathogenicity in liver, causing acute and chronic hepatitis, some of which are further progressed into cirrhosis and hepatocellular carcinoma [1]. Interestingly, these mammalian hepadnaviruses share a unique regulatory gene, X. The HBx (human hepatitis B virus X) protein comprises of 154 amino acids, which has drawn much attention due to its pleiotropic activities [3]. HBx in cytoplasm stimulates many different signal transduction pathways such as Ras, Src, c-Jun NH_2-terminal kinase (JNK), and phosphatidylinositol-3-kinase (PI-3-K) [4–7]. Some HBx in nucleus also directly interacts with the basal transcription machinery as well as several transcription factors, directly altering transcriptional activities [8,9]. The pleiotropic activities of HBx can result in several different biological activities such as proliferation, transformation, anti-apoptosis, and pro-apoptosis [4,7,10]. So far, the mechanism balancing or favoring these different outcomes is not known but the level of HBx in liver might be one of important determinants. It was shown that high level of HBx expression in cells induced a high rate of transformation [11] and HBx transgenic mice with high HBx level showed a high incidence of hepatocellular carcinoma formation [12,13].

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The expression of HBx gene in human liver is low both in HBV-replicating cells and in the HBV-infected liver specimens with cirrhosis or with hepatocellular carcinoma [14,15]. In HBV genome, expression of HBx gene is basically controlled by the enhancer I, which consists of multiple protein binding sites harboring an AP-1-related site and NF-IL2 binding sites [16,17]. The molecular analysis on HBV promoters revealed that the innate promoter activity of HBx gene was actually high but the expression of HBx gene in human liver tissues appeared to be repressed [16,18]. Consistently, the frequency of immunoreactive HBx in HBV-related liver specimens was also much lower than other HBV gene products, HBc and HBs and its expression was only limited to a small number of hepatocytes [14,15]. It is known that HBx protein degrades with a short half-life of 15–30 min [19,20], attributing to the low intracellular level of HBx protein. Interestingly, several lines of evidence demonstrated that the intracellular level of HBx protein could be regulated in different cellular environments. Interaction of the damaged DNA-binding complex (DDB1–DDB2) with HBx was shown to increase the stability of HBx protein [21]. Under the hypoxic condition, expression of HBx gene was augmented with concomitant increase of angiogenic activity mediated through the increase of vascular endothelial growth factor (VEGF), the downstream target gene of HBx [22]. We have also reported that HBx expression was predominantly localized in human liver specimens with high necroinflammatory activity [14] and its expression in vivo could be increased by treatment with adriamycin [23,24], one of the commonly used anticancer drugs. Taken together, these results strongly suggested that the expression of HBx gene could be altered in some hepatocytes under different cellular microenvironments, especially where HBV-infected liver cells encounter high intracellular stress under high immune response or genotoxic attack.

In the present study, we elucidated that the genotoxic stress by adriamycin, but not by cisplatin and 5-fluorouracil, could increase the intracellular HBx level by enhancing intracellular reactive oxygen species (ROS) level, demonstrating that ROS exerts a profound effect on HBx expression.

Materials and methods

Cell culture. The HBx-transfected ChangX-34 cells [23,24] and PLC/PRF/5 and HepG2.2.15 human hepatoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5–10% fetal bovine serum (GIBCO) in a humidified CO_2 incubator. ChangX-34 cells were derived from human Chang liver cells (CCL-13 and ATCC) and express HBx in a doxycycline-inducible manner [23]. HepG2.2.15 cells were originated from HepG2 cells after transfection with a vector that contains two head-to-tail dimers of HBV [25]. HBV DNA in these cells was chromosomally integrated and episomal HBV particles were produced [25]. PLC/PRF/5 cells were originally derived from a human hepatoma biopsy and contained multiple HBV integrants that retained the innate enhancer of HBV [26,27]. For the increase of HBx protein by adriamycin, ChangX-34 cells were treated with 1 μg/ml of adriamycin for 1 day and PLC/PRF/5 and HepG2.2.15 cells were treated for 2–3 days. Hydrogen peroxide (H_2O_2, Merck) at the concentration of 1 mM was determined to be optimal for the increase of HBx level without reduction of α-tubulin level. For the cells treated with H_2O_2, Dulbecco's modified Eagle's medium without sodium pyruvate (Gibco) was used to suppress the free radical scavenging effect of pyruvate [28].

Immunoblotting. After ChangX-34 cells were washed with phosphate-buffered saline, the cells were directly lysed in the presence of RIPA buffer. The cell extracts containing 30–50 μg/ml of total protein were separated on a 12–15% SDS–polyacrylamide gel followed by blotting to nitrocellulose membrane. The membrane was blocked in Tris-buffered saline with 5% non-fat dried milk and incubated with polyclonal anti-HA (Hemagglutin, Santa Cruz Biotechnology) antibody to detect HA-tagged HBx protein [23]. The complexes were detected with horseradish peroxidase-conjugated secondary antibody and visualized using the ECL system (Amersham Pharmacia Biotech).

Immunofluorescence staining and flow cytometric analysis. After cells were seeded on a coverslip for 1 day, the cells were treated with adriamycin or H_2O_2 for 1–3 days and directly fixed in a cold mixture of methanol/acetone (50:50) for 10 min. Cells were incubated with polyclonal anti-HA antibody or polyclonal anti-HBx antibody [14] and then incubated with fluorescence isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). The polyclonal rabbit anti-HBx antibody used in this study was raised against synthetic peptides spanning 81–95 and 144–154 amino acid residues of HBx protein [14]. Expression and localization of HBx protein were observed under a fluorescence microscope. For the quantification of HBx level, the cells were fixed with 4% paraformaldehyde, permeabilized with permeabilization buffer (phosphate-buffered saline wihout Mg^{++} and Ca^{++}, 1% FBS, 0.1% saponin), and then incubated with anti-HBx antibody and subsequently with FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). The intensities of fluorescence in these cells were analyzed using FACS Vantage (Beckton–Dickinson Immunocytometry Systems).

Measurement of ROS level. Intracellular ROS levels were determined by using an oxidative-sensitive fluorescence dye, dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probe). Cells ($\sim 3 \times 10^5$) in a 100-mm plate were treated with different chemotherapeutic agents for 0.5–24 h depending on the purpose of experiment. Cells were treated with 10 μM of DCF-DA for 15 min, harvested after addition of 0.25% trypsin-EDTA, and washed with phosphate-buffered saline. The intensities of fluorescence in cells were analyzed using FACS Vantage at the wavelength of 526 nm.

Results

Prolonged treatment of adriamycin increases the cellular level of HBx protein

We have recently reported that adriamycin (doxorubicin), one of the commonly used chemotherapeutic drugs, caused a marked accumulation of cellular HBx protein in HBx-expressing ChangX-34 cells [23,24]. ChangX-34 cells were previously established in our laboratory by stably expressing HBx gene tagged with an epitope of influenza hemagglutinin (HA) under Tet-on promoter [23]. In these cells, HBx protein can be detected by either anti-HBx antibody or anti-HA

antibody, resulting in the same patterns after doxycycline treatment [23]. Western blot analysis using anti-HA antibody showed that adriamycin treatment at early time-points of 3–6 h rather reduced the basal level of HBx protein in ChangX-34 cells, but at later time-points of 18–24 h HBx abruptly rebound high to the level of more than ten times increase (Fig. 1A). These findings well correlate with the previous observation that HBx mRNA upon adriamycin treatment also showed a dramatic increase at 18 h [24]. Adriamycin seemed to compete for the binding to TetR-activator with doxycycline, suppressing the transcription on the Tet-on promoter, explaining the decrease of HBx protein level at early time-points. However, prolonged treatment of adriamycin inhibited the rapid turnover of HBx mRNA and protein [24] and thereby overrode the transcriptional repression, resulting in an abrupt increase of HBx protein level at 18–24 h. Interestingly, this increase in HBx level was rather abrupt because it was not prominent at 15 h upon adriamycin treatment (data not shown). On the other hand, addition of 2 μg/ml of doxycycline into ChangX-34 cells induced HBx protein as early as 3 h and gradually further increased it until 24 h (Fig. 1B). We then further extended the effect of adriamycin in other HBV-related hepatoma cells, PLC/PRF/5 and HepG2.2.15 cells. HBV DNA with the innate HBV enhancer was chromosomally integrated in these cells [25–27]. To determine the level of HBx protein in these cells, we employed immunofluorescence staining method and flow cytometric analysis using the anti-HBx antibody. It is known that HBx protein in these cells was hardly detected by western blot analysis due to its low expression level [25–27] as well as poor immunogenicity of HBx protein. We had shown that the anti-HBx antibody available in our laboratory recognized HBx better in cells fixed in methanol/acetate [14] but not the denatured form of HBx on the SDS–PAGE (data not shown). The specificity of anti-HBx antibody in immunohistochemical staining has been shown by performing absorption experiment using HBx peptide [14]. We observed that HBx protein in ChangX-34 cells predominantly localized in the cytoplasm and at the nuclear periphery, which became stronger in the presence of doxycycline as we previously reported [23]. Treatment of ChangX-34 cells with adriamycin for 24 h also significantly increased the intensity of FITC-labeled HBx protein (Fig. 2A). When HepG2.2.15 and PLC/PRF/5 cells were treated with adriamycin for 24 h, there was no visible increase of HBx protein (data not shown). However, an increase of HBx protein was apparent at 48 h (Fig. 2A). Flow cytometric analysis further elucidated a progressive increase of HBx protein upon adriamycin treatment in HepG2.2.15 cells as well as in PLC/PRF/5 cells as shown in fluorescence shift to the right at 48 and 72 h (Fig. 2B). These results clearly demonstrate that adriamycin enhances the intracellular level of HBx protein in different HBV-related liver cell lines that express HBx gene under different promoters.

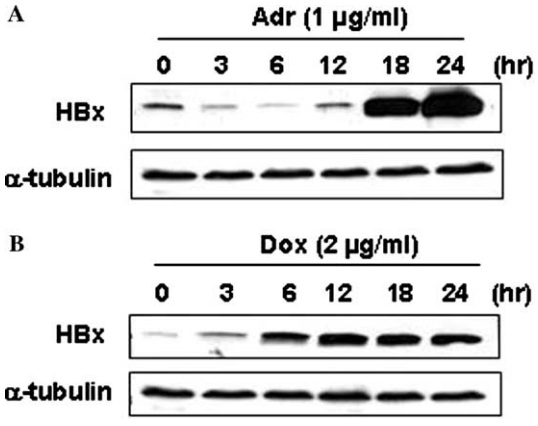

Fig. 1. The time course of HBx protein expression upon adriamycin treatment in ChangX-34 cells. ChangX-34 cells were treated with 1 μg/ml of adriamycin (A) or 2 μg/ml of doxycycline (B) for 0–24 h. Expression levels of HBx protein in each cell lysate were analyzed by immunoblotting. The level of α-tubulin content in each cell lysate normalized the protein concentration.

A close correlation between accumulation of HBx protein and generation of ROS

Adriamycin has been widely used as an anti-cancer drug in different types of tumor. In addition, in transcatheter arterial chemoembolization (TACE) for unresectable hepatocellular carcinoma adriamycin has been commonly used as an adjuvant chemotherapeutic agent [29]. We asked whether other chemotherapeutic drugs also modulated the cellular level of HBx, therefore three commonly used chemotherapeutic agents were tested. Interestingly, we observed that only adriamycin increased cellular HBx level, whereas two different concentrations of cisplatin and 5-flurouracil did not cause an enhancement of HBx protein but rather appeared to show some suppressive effects (Fig. 3A). At these concentrations, adriamycin and cisplatin exerted similar apoptotic effect and 5-fluorouracil showed less in these cells (data not shown). These chemotherapeutic agents have different anti-cancer mechanisms causing DNA strand breakage and cross-linking or by interfering DNA synthesis. We noticed the observation that administration of adriamycin to rats caused an oxidative stress [30]. Therefore, we determined the pattern of ROS generation upon adriamycin treatment in ChangX-34 cells using DCF-DA fluorescence dye at different time-points. Evident increase of ROS level was observed as early as 30 min upon adriamycin treatment, continued to accumulate until 18 h (Fig. 3B). In contrast, cisplatin and 5-fluororacil did not increase ROS level when these cells were treated for 2, 10 or 24 h. Differences in accumulated ROS levels at 24 h were compared in Fig. 3C.

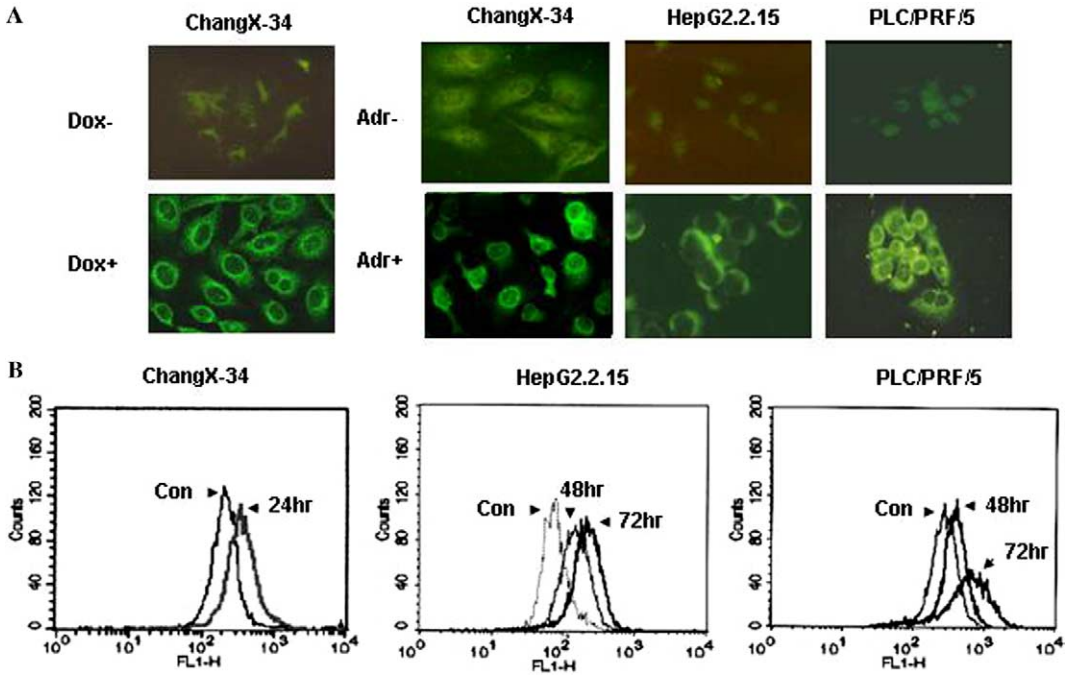

Fig. 2. Adriamycin increases the intracellular HBx level in HBV-related hepatoma cells. ChangX-34 and two other HBV-related hepatoma cells, HepG2.2.15 and PLC/PRF/5, seeded on a coverslip were treated with 2 μg/ml of doxycycline or 1 μg/ml of adriamycin for 24–48 h. Cells were fixed with a mixture of methanol/acetone, reacted with rabbit anti-HBx antibody, and visualized with FITC-conjugated secondary antibody under fluorescence microscope (A). For the quantification of HBx level, the fluorescence intensities were analyzed by flow cytometric analysis (B).

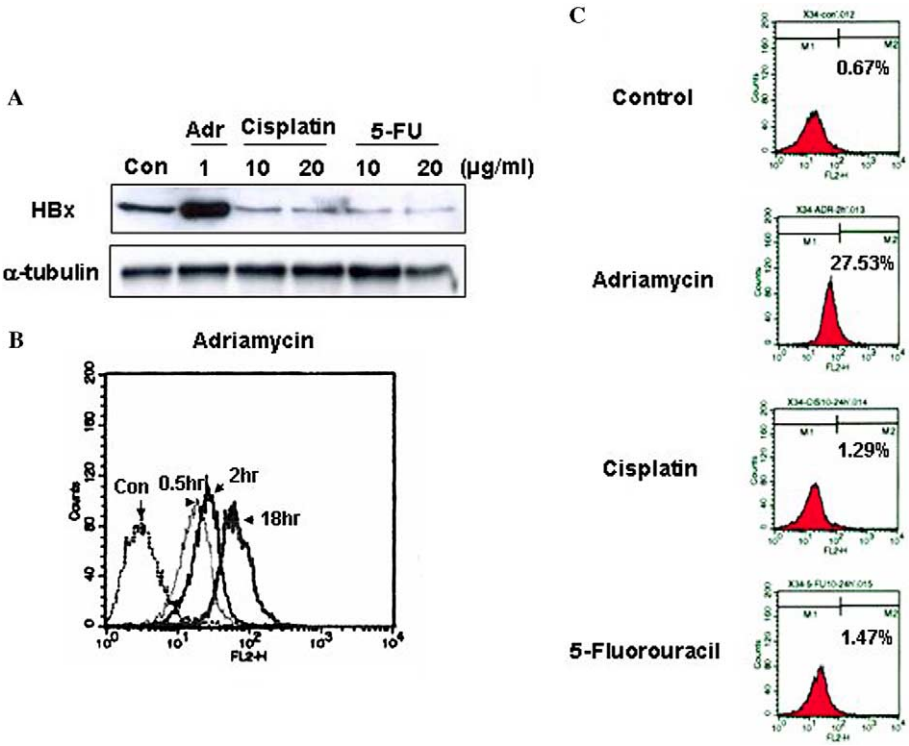

Fig. 3. A close correlation between increase of HBx level and generation of ROS. ChangX-34 cells were treated with adriamycin (Adr; 1 μg/ml), cisplatin (10 and 20 μg/ml), and 5-flurouracil (5-FU; 10 and 20 μg/ml) for 24 h and expression of HBx protein in each cell lysate was analyzed by immunoblotting (A). ChangX-34 cells were treated with adriamycin for 0.5–18 h and further incubated with 10 μM of DCF-DA for 15 min to measure the intracellular ROS levels. The cells were harvested and the intensities of DCF-DA fluorescence were analyzed by using FACS Vantage (B). After treatment with different chemotherapeutic drugs as described in (A), the intracellular ROS levels were determined using DCF-DA (C). The percentage indicates the relative intensity at the gated channel of M2.

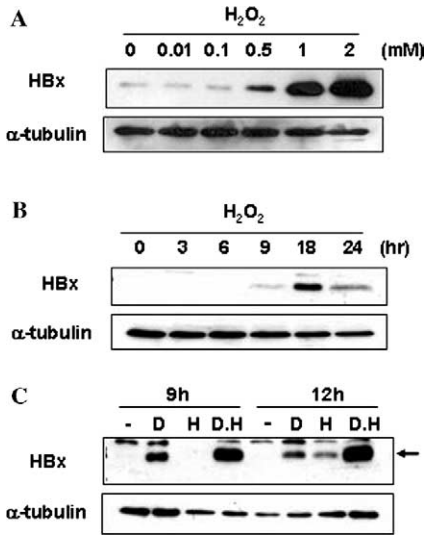

Fig. 4. Direct addition of H_2O_2 increases HBx protein level. ChangX-34 cells were treated with different concentrations of H_2O_2 for 24 h (A) or 1 mM of H_2O_2 was added to the cells for 0–24 h (B). ChangX-34 cells were treated with either doxycycline (D; 2 μg/ml) or H_2O_2 (H; 1 mM) and together (D.H) for 9–12 h. Total lysates were subjected to SDS–PAGE and immunoblotted with anti-HA antibody and anti-tubulin antibody.

Measuring mean values after DCF-DA staining indicated that the ROS level after adriamycin treatment was significantly higher than those induced by cisplatin and 5-fluorouracil (Fig. 3C). Thus, these data elucidated a close correlation between accumulation of HBx protein and generation of ROS upon treatment of chemotherapeutic agents.

ROS increases the cellular level of HBx protein

To verify the role of ROS on the regulation of HBx, we directly treated ChangX-34 cells with hydrogen peroxide and examined the level of HBx protein. When different concentrations of H_2O_2 were added to ChangX-34 cells for 20 h, 0.5 mM of H_2O_2 did show a visible increase of HBx protein and higher concentrations of H_2O_2 showed a dose-dependent increase of HBx protein (Fig. 4A). Addition of 1 mM of H_2O_2 into ChangX-34 cells induced a visible increase of HBx protein as relatively early as 9 h and further increased it at 18 h (Fig. 4B). Furthermore, when ChangX-34 cells were co-treated with 2 μg/ml of doxycycline and 1 mM of H_2O_2, a synergistic or an additive increase of HBx protein level was clearly observed at 9 and 12 h (Fig. 4C), unlike our previous findings that there was no synergistic increase of HBx protein after co-treatment of adriamycin and doxycycline [24]. We also further treated whether the same modulatory effect of H_2O_2 on HBx level could be observed in other cells. FACS analysis elucidated a progressive increase of HBx protein upon H_2O_2 treatment in HepG2.2.15 cells as shown in fluorescence shift to the right at 24 and 48 h (Fig. 5, left panel). In PLC/PRF/5 cells, a fluorescence shift upon H_2O_2 treatment was also observed at 24 and 48 h (Fig. 5, right panel). Thus, this is the first to provide the evidence that cellular level of HBx protein can be significantly increased at the environments generating high level of ROS.

Antioxidant abolishes the adriamycin-induced accumulation of HBx protein

Given that enhancement of HBx protein by adriamycin is mediated through the generation of ROS, we expected that antioxidant would abolish it. We first determined whether an antioxidant, *N*-acetylcysteine (NAC), prevented the induction of ROS. ChangX-34 cells were pretreated with 10 mM of NAC for 1 h and further incubated along with 1 μg/ml of adriamycin for 24 h. We observed the increase of ROS levels by adriamycin, which was partly inhibited by NAC (Fig. 6A). The inhibitory effect with 10 mM of NAC on the HBx

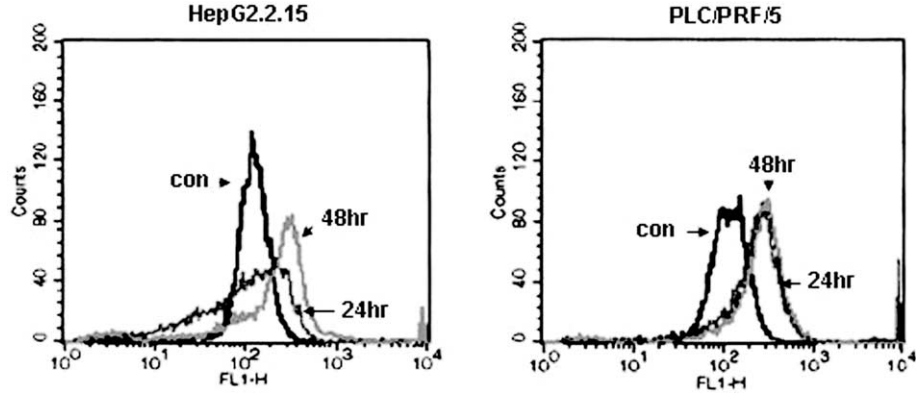

Fig. 5. ROS increases HBx level in HepG2.2.15 and PLC/PRF/5 cells. HepG2.2.15 and PLC/PRF/5 cells were treated with 1 mM of H_2O_2 for 24–72 h. Cells were fixed and reacted with rabbit anti-HBx antibody and subsequently with FITC-conjugated secondary antibody. The fluorescence intensities were compared using FACS analysis.

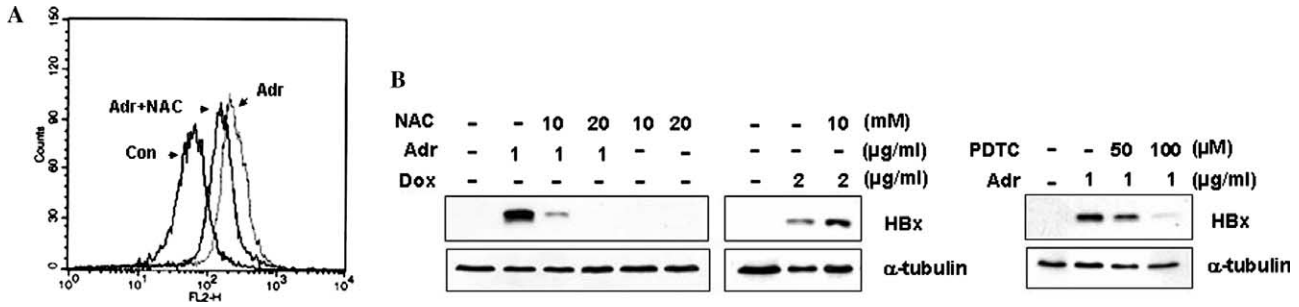

Fig. 6. Antioxidant abolishes the adriamycin-induced accumulation of HBx protein. ChangX-34 cells were pre-tre1ated with 10 mM of *N*-acetylcysteine (NAC) and further incubated with adriamycin (1 μg/ml) for 24 h. The changes in intracellular ROS level were determined using DCF-DA fluorescence dye (A). ChangX-34 cells were pre-treated with 10–20 mM of *N*-acetylcysteine or 50–100 μM of pyrrolidinedithiocarbamate (PDTC) and further incubated with adriamycin (1 μg/ml) or doxycycline (2 μg/ml) for 24 h. Total lysates were subjected to SDS–PAGE and immunoblotted with anti-HA antibody and anti-tubulin antibody (B).

protein level was evident and 20 mM of NAC completely abolished the adriamycin-induced HBx protein (Fig. 6B). In contrast, enhancement of HBx protein level upon doxycycline treatment was not suppressed by the presence of 10 mM of NAC. To ascertain the modulatory effect of antioxidant, we employed another antioxidant, pyrrolidinedithiocarbamate (PDTC). We again observed a significant inhibitory effect of PDTC on adriamycin-induced HBx protein. Thus, the data clearly demonstrated that ROS could significantly alter the cellular level of HBx protein.

Discussion

In this report, we demonstrated that the level of HBx protein can be drastically increased at the environment in which the cellular ROS level is augmented. It is currently recognized that the expression rate of HBx gene was much lower than other HBV gene products [14,15] and HBx protein in human liver was localized in only a small number of hepatocytes during the progression of chronic hepatitis to hepatocellular carcinoma [14,15]. One of the reasons could be low production of HBx mRNA transcript itself [31]. It has been shown that HBx mRNA transcript in human liver with hepatitis or hepatocellular carcinoma was almost never detectable using a typical Northern blot analysis method [14,31]. The other reason could be that HBx protein decays with a short half-life of 15–30 min as we [24] and other groups [19,20,32] previously reported. Thus, it seems that the level of HBx protein in human liver remains low. We recently found that intracellular HBx protein can be significantly enhanced by adriamycin in HBx-expressing ChangX-34 cells [23,24]. Here, we further confirmed the modulatory effect of adriamycin on HBx expression in both PLC/PRF/5 cells and HepG2.2.15 cells (Fig. 2). A significant increase of HBx protein in these cells was observed at 48–72 h after adriamycin treatment determined by both immunofluorescence staining (Fig. 2A) and FACS analysis (Fig. 2B). Since adriamycin significantly reduced the turnover of HBx protein as we previously demonstrated [24], the modulatory effect of adriamycin on HBx expression would be applied to human liver specimens that are related to HBV infection. However, the mechanism involved in the regulation of HBx protein stability is not known. It is of interest to notify that HBx directly binds to the components of proteasome complex, such as PSMA7 and PSMC1 [32–35]. Earlier studies showed that binding of HBx to the proteasome complex interfered with degradative processes of some transcription factors but proteasome inhibitors did not modify the turnover of HBx protein itself [33,34]. However, others showed that proteasome inhibitors retarded the degradation of HBx protein [32], causing a significant accumulation of HBx protein. Therefore, major portion of HBx protein might be degraded in proteasome-dependent manner. It was also reported that physical interaction of HBx to the DDB1 subunit of the damaged DNA-binding complex was shown to increase the stability of HBx protein [21]. Since we observed that adriamycin significantly increased the half-life of HBx protein up to 6-fold [24], adriamycin might interfere with the proteasome-dependent degradation of HBx protein. It is also possible that adriamycin as a DNA-damaging agent may alter the kinetic profiles of HBx and DDB1 interaction.

An interesting discovery for involvement of ROS in the regulation of HBx expression comes from the observation that other chemotherapeutic drugs such as cisplatin and 5-fluorouracil did not have any increasing effects on HBx expression (Fig. 3A), which correlated with their inability in generating ROS (Fig. 3C). In fact, direct addition of H_2O_2 to ChangX-34 cells did increase the HBx level in a dose-dependent manner (Fig. 4A). An apparent increase of HBx level also appeared in PLC/PRF/5 cells and HepG2.2.15 cells upon H_2O_2 treatment by FACS analysis (Fig. 5). Thus, cellular ROS level is an important regulator of HBx expression. It has been reported that a fraction of cytoplasmic HBx associates with

mitochondria [36–38]. Mitochondrial targeting of HBx has been shown to lead to cell death [37], while others reported that this association led to a decrease of the mitochondrial membrane potential and induced ROS [36,38]. Therefore, it seemed that HBx triggered ROS generation, which in turn could increase HBx. However, we speculated that a certain threshold of ROS level would be required because transient increase of ROS by single cytokine treatment did not stabilize HBx (data not shown). This idea was of a great interest for us because we previously reported that HBx expression in human liver was low but showed a significant correlation with high necroinflammatory activity determined by pathological evaluation as well as by Fas expression [14]. Cytokines play an important role in inflammatory process and ROS and NO are pivotal mediators [39,40]. Inflammatory cytokines mainly include interleukin-1 (IL-1), IL-6, tumor necrosis factor α (TNF-α), and interferon-γ (INF-γ) depending on the duration of inflammation [39].

Noticeably, IL-6 and TNF-α are known to be up-regulated by HBx [41,42]. Therefore, it can be postulated that in certain microenvironments in liver, a strong positive feedback between HBx and ROS generation can be developed [38,43,44]. We would like to also mention that the basal ROS level in HBx-expressing Chagng-34 cells was similar to that of the parental Chang cells (data not shown), probably due to the compensation of antioxidant balance in ChangX-34 cells during long-term culture of these cells. Anyhow, we think that these observations provide an insight by which we may not only understand the expression patterns of HBx protein in human liver, but also prevent or delay the pathogenesis of liver diseases by controlling the HBx level.

It is of note that H_2O_2 triggered earlier induction of HBx protein, whereas it took longer time to accumulate HBx protein by adriamycin (Figs. 2 and 5). Therefore, adriamycin and H_2O_2 may share some common mechanism in accumulating cellular HBx but may not share all. Moreover, there was a synergistic increase of HBx level by co-treatment with doxycycline and H_2O_2 as we expected. Doxycycline is expected to turn on the transcription process at the Tet-on promoter, thereby increasing both HBx mRNA and HBx protein levels [23,24]. H_2O_2 appeared to further accumulate HBx level at the post-transcriptional level by slowing down the turnover rate of HBx protein. Therefore, both adriamycin and H_2O_2 share the mechanism in the stabilization of HBx protein. This was strongly supported by the observation that the adriamycin-induced HBx protein was abolished by antioxidants, NAC and PDTC (Fig. 6), indicating that adriamycin-induced ROS is mainly responsible for increase of HBx. In contrast, enhancement of HBx protein level upon doxycycline treatment was not suppressed by the presence of NAC. These results clearly demonstrated that doxycycline and adriamycin increased the HBx level in separate pathways, thereby being able to exert a synergistic effect.

In summary, adriamycin significantly increased the cellular ROS level, thereby increasing HBx level. Therefore, cellular microenvironments generating ROS such as severe inflammation may aggravate the pathogenesis of liver disease by accumulating the cellular HBx level.

Acknowledgments

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