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***Basic Study***

**Activation of AMPK/MnSOD signaling mediates anti-apoptotic effect of hepatitis B virus in hepatoma cell**

Li L *et al*. MnSOD protects hepatoma cell against apoptosis

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**Abstract**

**AIM:** To investigate the anti-apoptotic capability of the hepatitis B virus (HBV) on the HepG2 hepatoma cell line and its mechanisms.

**METHODS:** Cell viability and apoptosis were measured by MTT assay and flow cytometry, respectively. Targeted knockdown of manganese superoxide dismutase (MnSOD), AMP-activated protein kinase (AMPK) and hepatitis B virus X protein (HBx) genes as well as AMPK agonist AICAR and antagonist compound C were employed todetermine the correlations of these genes expression.

**RESULTS:** HBV markedly protected the hepatoma cells from growth suppression and cell death in condition of serum deprivation. Decrease of superoxide anion production accompanied with increase of MnSOD expression and activity were found in HepG2.215 cells. Moreover, AMPK activation contributed to the up-regulation of MnSOD. And HBx protein was identified to induce the expression of AMPK and MnSOD.

**CONCLUSION:** Our results suggest that HBV suppresses mitochondrial superoxide level and exerts an anti-apoptotic effect by activating AMPK/MnSOD signaling pathway, which may provide a novel pharmacological strategy to prevent HCC.

**Key words:** Hepatitis B virus; Reactive oxygen species; Apoptosis; Manganese superoxide dismutase; AMP-activated protein kinase

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**Core tip:** Hepatitis B virus markedly protected the cells from growth suppression and cell death in condition of serum deprivation. Decrease of superoxide anion production accompanied with increase of manganese superoxide dismutase (MnSOD) expression and activity were found in HepG2.215 cells. Moreover, AMP-activated protein kinase activation contributed to the up-regulation of MnSOD. And hepatitis B virus X protein was identified to promote the expression of AMPK and MnSOD.

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**INTRODUCTION**

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed malignant cancers worldwide, while 50% cases and deaths occurred in China[1]. The chronic hepatitis B virus (HBV) infection has been internationally recognized as one of the major risk factors for the development of hepatocellular carcinoma (HCC)[2]. An estimated 350 million people chronically infected and 600000 hepatitis B-related deaths occurred every year all over the world[3]. Accumulated evidences have shown that HBV proteins, particularly hepatitis B virus X protein (HBx) and surface protein (HBs), are implicated in hepatocyte carcinogenesis[4]. However, the mechanisms underlying HBV-induced malignant transformation remain ambiguous.

Apoptosis, also named programmed cell death, plays a crucial role in the development and homeostasis in normal tissue[5]. Recently, the studies have indicated that defect or insufficient apoptosis may contribute to carcinogenesis, tumor progression and resistance of tumor cells to chemo-radiotherapy[6-8]. For that reason, escape of apoptosis has been identified as one of prominent hallmarks of cancer[9]. Reactive oxygen species (ROS), as toxic products of cell metabolism, can cause cell apoptosis by leading to cellular DNA damage and subsequent activating apoptotic signaling pathways[10]. In cancer, tumor niches characterized with poor nutrient and oxygen usually possess oxidative stress with excessive ROS formation[11,12]. Mitochondrial ROS (mtROS) especially superoxide anion, a natural by-product of electron transport chain activity, is the main source of cellular ROS[13].Thus, decreasing mtROS production to relieve oxidative stress is very important for tumor survive and progression.

Manganese superoxide dismutase (MnSOD), a key antioxidant enzyme, is responsible for scavenging superoxide anion. Liver malignant tumors have been shown to express higher protein level and activity of MnSOD than their benign counterparts[14]. Aggressive tumors possessing invasive phenotype also have a high level of MnSOD, which can facilitate them to reach distant organs[15]. Therefore, increased MnSOD expression and activity may protect cells against apoptosis and offer a growth advantage, thereby acquiring a more aggressive phenotype.

The expression of MnSOD can be modulated by many molecular factors at transcription, translation and posttranslational modifications levels, for example p53, Sp1, and NF-κB[16-18]. AMP-activated protein kinase (AMPK) is also reported to act as a new regulator of MnSOD expression in endothelial cells[19]. Moreover, AMPK activation is associated with protection of hepatocytes against oxidative stress[20].

Based on the aforementioned studies, we investigated the effect of HBV on the growth and survival of HepG2 cells, and explored the underline molecular mechanism. Herein, we demonstrated that HBV protected HepG2 cells from growth suppression and apoptosis in the conditions of serum deprivation. Furthermore, AMPK activation-induced up-regulation of MnSOD contributed to the resistant of HBV-integrated HepG2 cells to apoptosis caused by superoxide, which could explain in part HBV-induced hepatocellular cells malignant transformation in the context of growth factors withdrawal.

**MATERIALS AND METHODS**

***Cell culture***

The human hepatoma cell line HepG2 was obtained from Cell Bank of Chinese Academy of Sciences where it was authenticated. HepG2.215 cell line, which was derived from HepG2 cells by integrating HBV genome and persistently produced hepatitis B virus, was kindly provided by Prof. Erwei Song (Sun Yat-sen memorial hospital of Sun Yat-sen university, China). All of the cell lines were maintained in DMEM (Gibco, Gaithersburg, MD, USA) supplied with 10% FBS and 1% penicillin/streptomycin, and incubated at 37 ℃ in a humidiﬁed incubator at 5% CO2.

***Regents***

The HiPerFect transfection reagent was obtained from QIAGEN (QIAGEN, Carson City, CA). Antibodies of AMPKα and phospho-AMPKα (Thr172) were purchased from Cell Signaling (Cell Signaling Technology, MA). Anti-MnSOD antibody was from BD (BD Pharmingen, San Diego, CA, USA). Antibody of HBx (anti-HBx) was obtained from Abcam (Abcam, Cambridge, UK). AICAR, Compound C and anti-β-actin was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

***Cell viability assay***

Cells were seeded in 24-well plates in quadruplicate. After indicated treatments, cells viability was determined by the 3-[4, 5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) (Sigma, MO) following the manufacture’s protocol. Absorbance was measured at 570 nm wavelength.

***Cell apoptosis assay***

Cells were prepared as described elsewhere[21]. AnnexinV and propidium iodide (KeyGEN BioTECH, Nanjing, China) were added for incubation in the dark for 15 min at 4 °C, and then cells were analyzed by a flow cytometer (Gallios, Beckman).

***Mitochondrial superoxide anion detection***

Measurements of mitochondrial superoxide anion formation in cells were performed as previously described[22]. In brief, HepG2 and HepG2.215 cells were incubated with 5 μM MitoSOX (Invitrogen, Carlsbad. CA) for 20 min at 37 °C. Cells were digested using EDTA (Invitrogen, Carlsbad. CA), and then washed three times using HBSS with Ca/Mg (Invitrogen, Carlsbad. CA). Mean ﬂuorescent intensity was measured by ﬂow cytometry (Gallios, Beckman).

***MnSOD activity measurement***

MnSOD activity was measured with a commercial SOD kit (Cayman Chemical) according to the manufacturer’s protocol. Briefly, 1 mmol/L potassium cyanide was added in order to inhibit Cu/Zn-SOD and extracellular SOD, thus only MnSOD activity was detected. O2- was generated by adding Hypoxanthine/xanthine oxidase and detected by tetrazolium salt through reading the absorbance at 450 nm wavelength.

***RNA interference***

The siRNAs for silencing AMPK, MnSOD and HBx genes as well as scrambled siRNA were purchased from Ribobio (Guangzhou, China). Transfection of synthetic siRNAs was performed with HiPerFect (QIAGEN, Carson City, CA) according to the manufacturer’s instructions. The sense sequences of double-strand siRNA were as follows: siAMPK,5'-UGCCUACCAUCUCAUAAUATT-3'; siMnSOD,5’-GGAGAAUGUAACUGAAAGATT-3’;

siHBx-1,5’- CCGACCUUGAGGCAUACUUdTdT-3’;

siHBx-2, 5’-UGUGCACUUCGCUUCACCUTT-3’.

***Western blotting analysis***

Western blotting analysis was performed as described previously[23]. Antibodies for MnSOD, AMPK, Phospho-AMPK and HBx were used at 1: 1000 dilution. Antibodies for β-actin were used at 1:10000 dilution. Bound antibody was visualized using HRP-conjugated secondary antibodies.

***Statistical analysis***

All data were expressed as mean ± SD. SPSS 13.0 software was used for the one-way analysis of variance (ANOVA) and *t*-test in all statistical analyses (SPSS, Chicago, IL, USA). A value of *P* less than 0.05 was considered statistically signiﬁcant.

**RESULTS**

***HepG2.215 is more tolerant to serum-deprivation environment***

In order to assess the effect of HBV virus on the proliferation of HepG2 cells, we employed the HepG2.215 cell line which was derived from the HepG2 cell line and persistently produced HBV virus. We found that the HepG2.215 cell line showed faster growth kinetics compared with the HepG2 cell line at day 4 and day 6 after serum depletion (Figure 1A). Moreover, the number of apoptotic cells of HepG2 cell line was significantly increased at day 4 and day 6 compared with that of HepG2.215 cell line. In contrast, the number of apoptotic HepG2.215 cells stayed at a much lower level at all testing times (Figure 1B and C). These data suggest that HBV proteins may protect HepG2.215 cells against apoptosis by serum depletion.

***Decreased mitochondrial superoxide level maybe due to increased MnSOD expression and activity***

To explain the different anti-apoptotic ability of the two cell lines, we investigated the production of mitochondrial superoxide which is a well-known killer of cells[10]. Decreased mitochondrial superoxide level was found in the HepG2.215 cell line (Figure 2A). Since MnSOD is the regulator of mitochondrial superoxide, we therefore detected the expression and activity of MnSOD in the two cell lines. As shown in Figure 2B and C, both the expression and activity of MnSOD in HepG2.215 cells were higher than that of HepG2 cells.

***MnSOD mediates the apoptotic resistance of HepG2.215 cells***

To further verify the role of MnSOD in the apoptotic resistance of HepG2.215 cells, the MnSOD siRNA was synthesized. Western blotting analysis revealed that MnSOD siRNA specifically knocked down MnSOD in HepG2.215 cells (Figure 3A). Knockdown of MnSOD decreased cell viability and increased mitochondrial superoxide formation and the number of apoptotic HepG2.215 cells (Figure 3B-D), which suggested that MnSOD played a critical role in apoptotic resistance of HepG2.215 cells.

***AMPK activation contributes to up-regulation of MnSOD in HepG2.215 cells***

To figure out the upstream factor involving the modulation of MnSOD, AMPK was investigated. We showed the protein levels of p-AMPK and AMPK were increased in HepG2.215 cells (Figure 4A). Both knockdown of AMPK and treatment of AMPK inhibitor Compound C reduced the expression of MnSOD (Figure 4B and C). Conversely, AMPK activator AICAR increased the expression of MnSOD (Figure 4C). Furthermore, the expression of p-AMPK, AMPK and MnSOD were inhibited by HBx knockdown (Figure 4D). These results suggest that HBV up-regulates MnSOD *via* AMPK.

**DISCUSSION**

As a major cause for HCC development, HBV can promote HCC in many ways, including enhancing host chromosomal stability, inducing inflammation-mediated immune escapes, regulating epigenetic modification or altering the expression of oncogenes and tumor-suppressor genes[24]. Due to these internal changes, hepatoma cells acquire the capacity of fast-growing, anti-apoptosis and metastasis[25,26]. In this study, we confirmed that HBV-integrated HepG2 cells exerted survival benefit compared with its parent cell line HepG2 in the serum-deprivation condition which can to some extent mimicked the tumor cell adapation to adverse growth conditions. In line with previous studies, we also found that HBV conferred HepG2 cells resistance to apoptosis[26,27]. Our data suggest that HBV apparently acts to promote the growth and viability of hepatoma cells in growth-factor-restricted conditions.

Increased level of ROS by creating a potentially toxic environment to the cells represents one critical mechanism underlying cell death[28]. Superoxide anion is the precursor of other ROS such as H2O2 and peroxynitrite, and because of that the organelles most vulnerable to oxidative stress are the mitochondria[29].  MnSOD is an essential antioxidant enzyme in the mitochondrion that acts on superoxide anion[30]. Here, we showed that HBV reduced the level of superoxide anion. Consistently, the expression and activity of MnSOD were up-regulated in HBV-integrated HepG2 cells. This result was supported by the study that in patients with HBV infection, there was an average 5-fold rise of serum MnSOD[31].

The expression and activity of MnSOD are not static in different tumorgenesis stages. For transformed phenotype, MnSOD levels were maintained at a low level that it could directly potentiate mitochondrial defects, leading to the gene mutations. For acquiring a more aggressive phenotype, enhanced MnSOD activity may protect cells against mitochondrial injury, thereby conferring a growth advantage to the cancer cells[16]. The present study demonstrated that knockdown of MnSOD increased the production of superoxide anion and the apoptosis of HepG2.215 cells, which indicated that MnSOD protected hepatoma cells against apoptosis by detoxing superoxide anion, and conferred a growth advantage to those cells. However, since the function of MnSOD is to convert diffusion-restricted and mild-toxicant superoxide anion to freely diffuse and strong-toxicant H2O2, which means increased MnSOD may enhance the production of more toxicant H2O2, the mechanism of modulation of tumor cell survival by MnSOD seems confusing. It has been reported that HBx expressing cell line showed significant reduced sensitivity to H2O2-induced cell death, and the level of intracellular ROS did not elevate in HBx expressing cell line after exposure to H2O2 in the medium[32]. Based on these findings, we speculate that HBV-infected cells may express relatively high amounts of catalase, therefore they would be able to counteract the cytotoxic effects of peroxide, thus the outcome of increased MnSOD activity would more likely reflect the capacity of MnSOD to reduce levels of oxygen radicals. Unexpectedly, the level of catalase in HBV-related hepatocellular carcinoma specimens was lower than those of surrounding non-tumor tissues[33]. Thus, further investigation is required to explain the tolerance of HBV-infected cells to H2O2-induced cell apoptosis, which will be helpful for us to understand the mechanism of MnSOD-modulated tumor cell survival.

AMPK, a serine/threonine protein kinase, is well known for its role in controlling energy metabolism. Recently, it comes into focus because of its potential roles in regulating other signaling pathways, such as in regulating oxidative stress[34]. Studies have reported that activation of AMPK by AICAR, or overexpression of constitutively activated AMPK suppressed O2− production in human neutrophils or HUVECs[35,36]. A similar observation was also found in HepG2 cells, which showed that AA+ iron-induced reactive oxygen species generation was inhibited by isorhamnetin through AMPK activation[20]. These studies indicate AMPK appears to be the key factor for cellular function protection in the presence of oxidative stress. Emerging evidences suggest that AMPK inhibits oxidant production by decreasing the expression of NADPH oxidases or increasing the expression of UCP-2 as well as MnSOD[19,35,36]. In the present study, HBV-integrated HepG2 cells displayed elevated AMPK protein level, which remains consistent with the expression of MnSOD. By utilizing a specific siRNA, or a selective agonist (AICAR) and antagonist (compound C) of AMPK, we observed that knockdown of AMPK and compound C resulted in the reduction of MnSOD protein level. Moreover, activation of AMPK by AICAR up-regulated the expression of MnSOD. Taken together, these findings demonstrate that AMPK is responsible to of the up-regulation ofMnSOD expression in HBV-integrated HepG2 cells.

Additionally, numerous studies have shown that HBx protein serves as a transactivator in the pathogenesis of HCC through regulating cell transformation, apoptosis and cellular immune system[37-39]. In our study, HBx was identified as the active ingredient of HBV proteins to promote the expression of AMPK and MnSOD. This is consistent with previous investigation reported by Severi *et al*[32] that HBx expressing cell line is more resistant to ROS-induced cell apoptosis than HBsAg expressing cell line. These data suggest that HBx may alleviates oxidative stress by upregulating AMPK/MnSOD axis to maintain “normal” live cancer cell functions.

In summary, our current study demonstrates that HBV suppresses mitochondrial superoxide level and exerts an anti-apoptotic effect by activating AMPK/MnSOD signaling parthway in HBV-infected HepG2 cells. These ﬁndings may provide a novel mechanism involved in HBV-triggered carcinogenesis, could therefore might be useful in the design of new pharmacological approaches to prevent HCC.

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**COMMNETS**

***Background***

The chronic hepatitis B virus (HBV) infection is one of the major risk factors for the development of hepatocellular carcinoma (HCC). However, the mechanisms underlying HBV-induced HCC remain ambiguous. Recently, accumulated evidence has shown that escape of apoptosis may contribute to carcinogenesis.

***Research frontiers***

Previous experiments have revealed that liver malignant tumors and patients with HBV-infection express higher protein level of manganese superoxide dismutase (MnSOD) than their counterparts. Here, the authors showed that high expression of MnSOD protected hepatoma cells against apoptosis by detoxing superoxide anion, and conferred a growth advantage to those cells. These results explain how HBV offers a survival benefit to hepatoma cells.

***Innovations and breakthroughs***

This is the first study to demonstrate that HBV protects hepatoma cells against apoptosis *via* AMPK/MnSOD signaling pathway. HBV markedly protected the cells from growth suppression and cell death in condition of serum deprivation. Decrease of superoxide anion production accompanied with increase of MnSOD expression and activity were found in HepG2.215 cells. Moreover, AMPK activation contributed to the up-regulation of MnSOD. And HBx protein was identified to promote the expression of AMPK and MnSOD. These results provide further evidence for the role of HBV as a major cause of HCC development *via* an anti-apoptosis mechanism involving activation of AMPK/MnSOD signaling pathway.

***Applications***

The present results suggest that HBV suppresses mitochondrial superoxide level and exerts an anti-apoptotic effect by activating AMPK/MnSOD signaling pathway, which may be useful in the design of new pharmacological approaches to prevent HCC.

***Peer-review***

In this paper, Li *et al* aim to investigate the anti-apoptotic capability of the hepatitis B virus on the Hep G2 hepatoma cell line by suppressing mitochondrial superoxide levels. Generally, their findings seem to be interesting, anyway it should be validated in different cell lines, such as HepG2.117.

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**Figure 1** **Resistance of HepG2.215 cells to apoptosis.** A: Typical photographs of the HepG2 and HepG2.215 cells cultured for 6 d after serum depletion. The data are representative of three independent experiments; B: MTT assay for the viability of cells cultured for 2, 4 and 6 d after serum depletion. Data represents absorbance at 570 nm and are shown as mean ± SD of quadruplicate. a*P* < 0.05 and c*P* < 0.01 *vs* HepG2 cells group. The data are representative of three independent experiments; C: In parallel experiments, samples were subjected to FITC-Annexin V/ propidium iodide staining and the quantitative analysis of apoptotic cells were performed using flow cytometry. Quantification of apoptotic cells are shown as mean ± SD of triplicates.

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**Figure 2** **Decreased mitochondrial superoxide production is accompanied by increased MnSOD expression and activity in HepG2.215 cells.** A: Mitochondrial superoxide level was decreased in HepG2.215 cells. Cells were cultured in serum-free medium for 6 d. Mitochondrial superoxide anion formation was measured by flow cytometry and MitoSOX. Quantification of mitochondrial superoxide anion is shown as mean ± SD of triplicates. a*P* < 0.05 *vs* HepG2 cells group; B: Cell lysates were objected to Western blotting analysis. The protein level of MnSOD was detected; C: MnSOD activity was measured by the commercial SOD Assay Kit. Quantification of MnSOD activity are shown as mean ± SD of triplicates. a*P* < 0.05 *vs* HepG2 cells group.

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**Figure 3** **MnSOD contributes to decreased mitochondrial superoxide and apoptotic cells in HepG2.215.** A: After 6 d, the interference effect of MnSOD siRNA (siMnSOD) was analyzed with western blotting analysis. MnSOD siRNA (siMnSOD) or non-specific siRNA (siCTRL) was transfected into HepG2.215 cells for 12 h before serum depletion. B: After 6 d, cells were harvested for quantification of mitochondrial superoxide anion formation by flow cytometry. In parallel, C cell viability and D apoptotic cells were separately determined by MTT assay and flow cytometry. a*P* < 0.05 and c*P* < 0.01 *vs* siCTRL group.

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**Figure 4 AMPK activation is required for MnSOD expression in HepG2.215.** A: Cell lysates were subjected to western blotting analysis. The protein levels of AMPK and p-AMPK were detected; B: Cells were transfected with AMPK siRNAs (siAMPK) or a nonspecific control siRNA (siCTRL) for 6 d. The protein levels of AMPK and MnSOD were detected by western blotting analysis; C: Cells were subjected to 1 mM AMPK activator AICAR or inhibitor compound C for 6 d. The protein levels of AMPK, p-AMPK and MnSOD were detected by western blotting analysis; D: Cells were transfected with HBx siRNAs (siHBx) or a nonspecific control siRNA (siCTRL) for 6 d. The protein levels of AMPK and MnSOD were detected by western blotting analysis.