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Cooperative stimulation of cisplatin-mediated apoptosis by hepatitis B virus X protein and hepatitis C virus core protein

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The co-infection with hepatitis B virus (HBV) and hepatitis C Virus (HCV) is associated with a more severe liver disease and increased frequency in the development of hepatocellular carcinoma compared to those with single infection. Here, we demonstrated that HBV X protein (HBx) and HCV Core cooperatively up-regulated the level of p53 in human hepatoma HepG2 cells. The elevated p53 subsequently stimulated the expression of proapoptotic Bax whereas it repressed the expression of anti-apoptotic Bcl2. These effects, however, were not observed in p53-negative Hep3B cells. Consistently to their cooperative regulation of apoptotic effectors, HBx and HCV Core additively stimulated cisplatin-mediated apoptotic cell death of HepG2 but not of Hep3B cells. These results may help to explain the development of a more severe liver disease in patients co-infected with HBV and HCV as well as some contradictory results on the roles of HBx and Core in apoptosis.

Key words – Apoptosis, Cisplatin, Core, HBV, HBx, HCV

Introduction

The tumor suppressor p53 is known to play a key role in growth arrest, DNA repair, and apoptosis after cell stress, primarily through its ability to regulate the transcription of downstream target genes in the cell [1]. The p53 apoptotic target genes can be divided into two groups, the first group encodes proteins that act through receptor-mediated signaling and the second group encodes proteins that regulate apoptotic effector proteins [2]. The latter group includes members of Bcl2 family such as Bcl-2 and Bax proteins. Whether apoptosis is induced or blocked is mostly determined by the relative ratios and the dimerisation status of Bcl2 and Bax [3]. Homodimers of Bcl2 block apoptosis whereas Bax homodimers elicit the opposite effect; Bcl2-Bax heterodimers are inactive. As p53 activates the transcription from the Bax gene and inhibits the transcription from the Bcl2 gene, it alters the balance of Bcl2 and Bax such that Bax facilitates the release of cytochrome *c* from the mitochondria, thus activating the caspase cascade that ultimately leads to apoptosis [4].

Hepatitis B (HBV) and C (HCV) viruses are two major etiologic agents of chronic hepatitis, which is closely related to the development of hepatocellular carcinoma. HBV X protein (HBx) and HCV Core have been considered to play important roles during development of hepatic diseases by HBV and HCV, respectively [5,6]. Both proteins are multifunctional viral regulators that modulate transcription, cell responses to genotoxic stress, protein degradation, and signaling pathways, which may ultimately affect cell growth, cell death, and carcinogenesis. Some of these processes might involve the alteration of p53 functions because both HBx and HCV Core are known to affect the level of p53 [7-9]. In this study, we coexpressed HBx and HCV Core in hepatoma HepG2 cells to mimic the situation of coinfection in human liver, and compared the level of p53, Bax and Bcl2 in these cells with that of single expression. Cisplatin, a widely used anticancer agent, facilitated the study as it elevates the basal level of p53 and triggers an apoptotic response [10]. In addition, we investigated whether the altered expression of Bax and Bcl2 in the presence of HCV Core and/or HBx subsequently affects on the apoptotic cell death induced by cisplatin. To demonstrate the importance of p53 in these processes, we compared the results with those obtained with p53-negative Hep3B cells.

Materials and methods

Plasmids

pCI-neo-core K that encodes HCV core protein under the human cytomegalovirus immediate-early promoter was

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described previously [11]. Plasmid pCI-neo-IRES-HBx which encodes X region (nt. 1374-1839, accession number D23677) of the HBV DNA was constructed by subcloning the PCR amplified product into the *Xba*I and *Not*I sites of pCI-neo (Promega) and inserting the encephalomyocarditis virus internal ribosome entry site (IRES) in front of HBx [12]. The bicistronic expression vector pCI-neo-core-IRES-HBx was constructed by subcloning the HCV core gene in front of IRES in pCI-neo-IRES-HBx, thus to translate HCV core protein cap-dependently whereas HBx in an IRES-dependent manner [12].

Transfection and Western blotting analysis

Hepatoma cell lines, HepG2 and Hep3B (2×10^5 cells per 60-mm diameter plate) were transfected with a calcium phosphate-DNA precipitate containing 5 μg each of HCV Core and/or HBx expressing plasmid DNA as previously described [13]. After 48 hr, cells were harvested and lysed in buffer [50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% SDS, 1% NP-40] supplemented with protease inhibitors. 10 μg of cell extracts was separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF; Amersham). Western blotting was performed with anti-p53 monoclonal antibody (Santa Cruz), anti-Bax monoclonal antibody (Santa Cruz), anti-Bcl2 monoclonal antibody (Santa Cruz), or anti-actin monoclonal IgG (Santa Cruz), and subsequently detected by chemiluminescent ECL kit (Amesham) as recommended by the manufacturer.

Generation of stable cell lines expressing HBx and/or HCV Core

Stable hepatoma cell lines expressing either HBx and/or HCV Core were generated as described previously [12]. Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 500 μg/ml G418 (Gibco-BRL). To detect the expression of transfected genes, semi-quantitative RT-PCR was performed. 3 μg of RNA was reverse transcribed with the corresponding antisense primer. One quarter of the reverse transcribed RNA was amplified with *Taq* polymerase (95℃, 5 min; 30 cycles of 95℃, 1 min-56℃, 1 min-72℃, 30 sec; 72℃, 5 min) using the appropriate primer pairs. The primers for p53, HBx, and glyceraldehyde 3-phosphate dehydrogenase were described previously [11]. For the detection of HCV core transcripts, sense primer 5′ TCC GGA TCC CTG TCA TCT TCT GTC CCT 3′, and antisense primer 5′ TCG CTT AGT GGA TCC TGG GGG CAG 3′ were used.

MTT assay

The MTT assay is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to blue formazan crystals. The cells were seeded onto 96-well plates, and appropriate concentrations of cisplatin ranging from 2.5 to 10 μM were then added. After 24-48 h, MTT dye (Sigma), at a concentration of 5 mg/ml, was added and the plates were incubated for 12 hr at 37℃. Optical density was determined by eluting the dye with DMSO (Sigma), and the absorbance was measured at 560 nm. At least three independent experiments were performed.

Fluorescence activated cell sorter (FACS) Analysis

Cell cycle profile was analyzed using flow cytometry. Briefly, 2×10^6 cells were trypsinized, washed twice with PBS and fixed in 80% ethanol. Fixed cells were washed with PBS and resuspended in 50 μg/ml propidium iodide containing 125 U/ml RNase A. DNA contents were analyzed by flow cytometer using the Cell-FIT software (Becton-Dickinson Instruments).

Results

Cooperative regulation of p53, Bax and Bcl2 by HBx and HCV Core

Initially, we investigated the effect of HBx and HCV Core, either individually or in a combination, on the level of p53 (Fig. 1). As demonstrated previously [7,8], the level of p53 was increased approximately 3 and 2 fold in the presence of HCV Core (lane 2) and HBx (lane 3), respectively. The activation of p53 was additive when the two proteins were co-expressed (lane 4), suggesting the presence of co-operation between HCV Core and HBx in the activation of p53. Consequently, the expression of Bcl2 was decreased (Fig. 1) whereas that of Bax was activated (Fig. 1) in the presence of HCV Core and/or HBx. The effects of HCV Core and HBx on the expression of p53, Bcl2, and Bax were more clearly demonstrated when the basal level of p53 was increased by cisplatin treatment (Fig. 1, lanes 5 to 8). This result also indicates that HCV Core and HBx stimulated cisplatin-mediated activation of p53 in hepatoma cells in an additive manner. In contrast, according to a similar experiment in p53-negative Hep3B cell, both

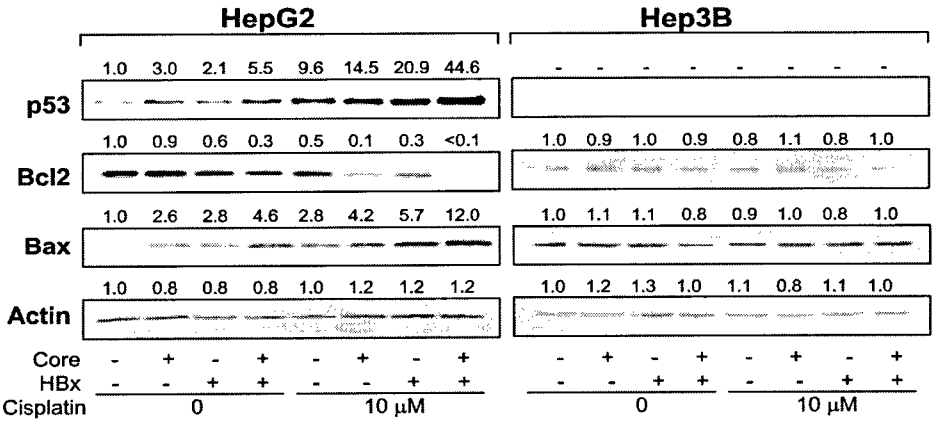

Fig. 1. Additive effect of HCV Core and HBx on the expression of apoptotic effectors. Levels of p53, Bcl2, Bax, and actin was determined in either HepG2 (lanes 1 to 8) or Hep3B cells (lanes 9 to 16) transiently transfected with an empty vector (lanes 1, 5, 9 and 13), HCV Core- (lane 2, 6, 10 and 14), HBx- (lane 3, 7, 11 and 15), or HCV Core and HBx- (lane 4, 8, 12 and 16) expression vector. For lanes 5 to 8 and 13 to 16, 10 μM of cisplatin was treated for 24 hr before harvesting. The intensity of bands on the filters was quantified using BIO-PROFIL image analysis software (Vilber Lourmat, France) and indicated above the corresponding one as a relative value compared to the untreated control (lanes 1 and 9).

proteins hardly affected the expression of Bcl2 and Bax both in the absence (Fig. 1, lanes 9 to 12) or presence (Fig. 1, lanes 13 to 16) of cisplatin. Therefore, we concluded that both HBx and HCV Core alter the expression of Bax and Bcl2 in a p53-dependent pathway.

Cooperative induction of apoptotic cell death by HBx and HCV Core

Having established that HBx and HCV Core up-regulate the level of p53 in an additive manner and subsequently alter the balance between Bax and Bcl2, we tested whether the cell death is actually increased in the presence of viral proteins. For this purpose, we first prepared HepG2 and Hep3B cell lines expressing HBx and/or HCV Core. Five different cell lines expressing HCV Core and/or HBx were tested to show that differences in their response to cisplatin are not just due to the chance selection of cell clones that survive at different rates. According to RT-PCR analysis, Core and HBx are expressed almost equally in the corresponding cell lines but not in the parent cells (Fig. 2, A and C). According to the MTT assay, expression of the viral proteins itself was not sufficient to induce a detectable change in cell death compared to the parent cell lines (data not shown). This result indicates that the altered expression of Bax and Bcl2 by HBx and HCV Core in the absence of cisplatin was not sufficient to change apoptotic cell death. Upon treatment with cisplatin, the cell death was increased in the presence of HCV Core and HBx in an additive manner (Fig. 2B). In contrast, cisplatin was not so effective to induce cell death of p53-negative Hep3B cell lines, indicating that cisplatin acts mainly via activation of p53 as demonstrated previously by Qin and Ng [10]. In addition, HCV Core either alone or in combination with HBx hardly affected the cell death by cisplatin in Hep3B cells, which indicates that p53 is required for the efficient induction of cell death by HBx and HCV Core.

To further explore the effect of HBx and HCV Core on cell cycle progress and apoptotic cell death, we performed flow cytometry experiments. As shown in Fig. 3A, a similar proportion of sub G1 phase that represents apoptotic cells with fragmented DNA was observed in all of the HepG2 cell lines except HBx-expressing ones. In the presence of cisplatin, the apoptotic event of HepG2 cells was clearly increased by HCV Core and HBx in an additive manner. Considering that HBx also increased the level of p53 and Bax, the inhibition of apoptosis by HBx might occur via a p53-independent pathway. Interestingly, the proportion of G1 phase in HBx-expressing cells was considerably higher compared to that of other cells, suggesting that most of the HBx-expressing cells seemed to be arrested at G1 phase. Similarly to the result with MTT assay, no clear differences in the induction of apoptosis were observed among Hep3B cell lines although the proportion of subG1 phase was slightly increased in the presence of cisplatin.

Discussion

According to the present study, both HBx and HCV

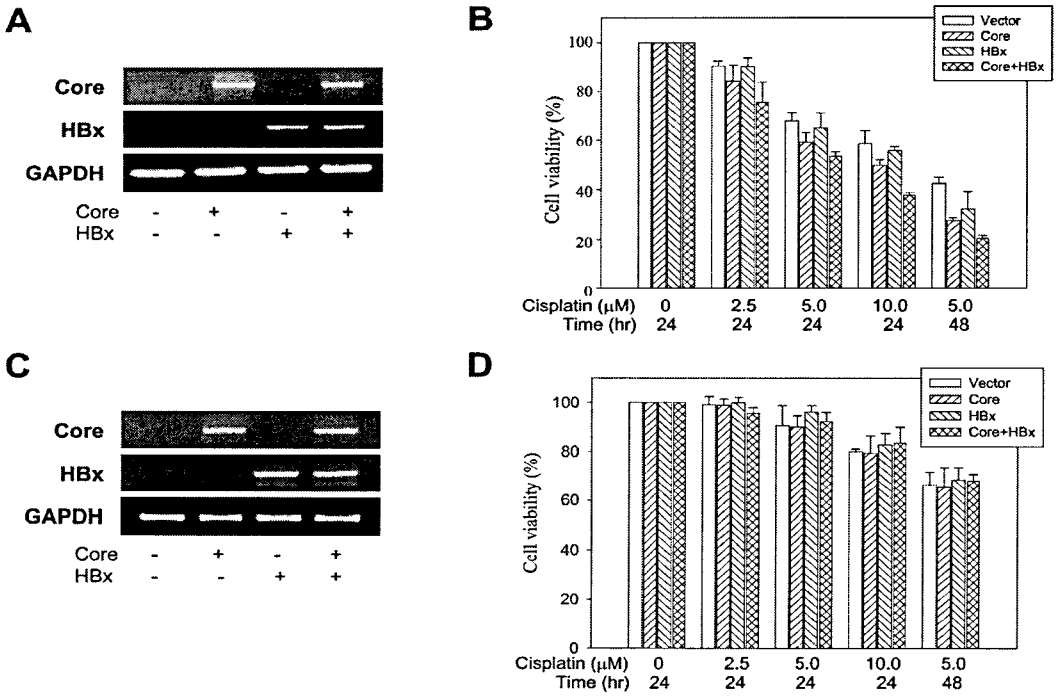

Fig. 2. Additive effect of HCV Core and HBx on the cisplatin-mediated cell death. Either HepG2 (A) or Hep3B (B) cells were stably transfected with an empty vector (lanes 1), HCV Core- (lane 2), HBx- (lane 3), or HCV Core and HBx- (lane 4) expression vector. RT-PCR using an appropriate set of primers was performed to detect the expression of Core, HBx and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). MTT assay was performed to measure cell death of HepG2 (B) and Hep3B (D) cell lines under the indicated conditions. The data represent the averages of five different clones for each group and the standard deviations (error bars) from three independent experiments.

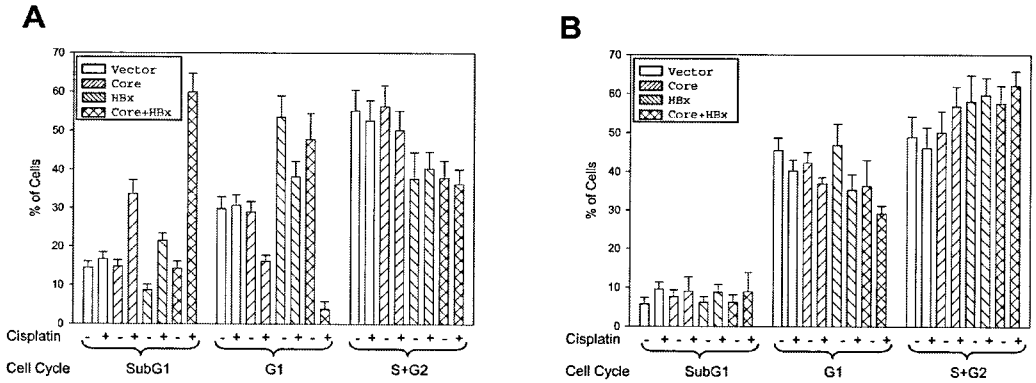

Fig. 3. The effect of HCV Core and HBx on the cell cycle progression and apoptosis. A representative set of HepG2 (A) and Hep3B (B) cell lines were either untreated or treated with 10 μM of cisplatin for 24 hr, and then were stained with propidium iodide and analyzed by flow cytometry to determine DNA contents. The percentage of the subG1, G1, S/G2 population from two independent experiments is presented.

Core additively activate p53, which results in activation of Bax and repression of Bcl2. However, it was possible to detect clear phenotypic changes in cell growth after treatment with cisplatin. Under this condition, both HCV Core and HBx additively stimulated apoptotic cell death of p53-positive HepG2 cells but not of Hep3B cells. Therefore, the effects of HCV Core and HBx on the apoptotic cell death could be different depending on the status of p53 as well as on the presence of other apoptotic stimuli such as cisplatin. Therefore, the present study may explain, at least in part, the contradictory effects of HCV Core and HBx on the apoptotic cell death reported so far [14,15].

Interestingly, HBx seemed to inhibit apoptotic cell death when acted alone in the absence of cisplatin. It is rather

complex to understand the decreased apoptotic cell death by HBx if considering the stimulatory effect of HBx on the level of p53. It is possible that some unknown p53-independent survival signals might be also induced by HBx and effectively countervailed the apoptotic signal generated by HBx via a p53-dependent pathway. Actually, HBx is known to activate survival signals via p53-independent pathways [16]. Under this situation, the cells are arrested by the elevated p53 at a certain cell cycle stage but are prevented to undergo apoptotic cell death in the presence of survival signals. Actually, according to the present study, HBx-expressing HepG2 cells exhibited an extraordinary high proportion of G1 phase. Most of the G1 arrested cells might eventually undergo apoptosis when other apoptotic agents such as cisplatin and HCV Core are involved and thus the balance is moved to the apoptotic side. Actually, apoptotic cell death was increased when HBx and HCV Core were expressed together in the same cell, compared to those with single expression. Such a cooperative induction of apoptotic cell death might be important to understand more severe liver diseases in patients who coinfected with HBV and HCV compared to those with single infection [17,18].

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초록 : B형 간염 바이러스 X 단백질과 C형 간염 바이러스의 코어 단백질에 의한 cisplatin-매개성 세포예정사의 협조적 촉진

권현진 · 장경립*
(부산대학교 생명과학부)

B형 간염 바이러스(HBV)와 C형 간염 바이러스(HCV)에 함께 감염되면 단독 감염의 경우보다 더 심각한 간질환이 유발되고 간암으로의 발전 가능성도 높아진다. 본 연구에서는 HBV의 X 단백질(HBx)과 HCV의 코어 단백질이 인간 간암세포주인 HepG2 세포에서 p53의 양을 협조적으로 증가시킨다는 것을 보여 주었다. 이로 인하여 세포예정사를 촉진하는 Bax 단백질의 발현이 더 증가하는 반면에 세포예정사를 억제하는 Bcl2의 발현은 더 억제됨이 관찰되었다. 그러나 이러한 효과들은 p53-음성인 Hep3B 세포에서는 관찰되지 않았다. 나아가 HBx와 코어 단백질은 HepG2의 cisplatin-매개성 세포예정사를 협조적으로 증가시키는 반면에 Hep3B에서는 이러한 효과가 나타나지 않았다. 이러한 연구 결과들은 HBV와 HCV가 동시에 감염되었을 경우에 나타나는 임상적인 소견을 이해하고 세포예정사에 미치는 HBx와 코어 단백질의 영향에 대한 기존의 상충적인 연구결과들을 해석하는데 도움을 줄 수 있다.