Hepatitis B virus X protein (HBx) induces G2/M arrest and apoptosis through sustained activation of cyclin B1-CDK1 kinase

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Abstract. Hepatitis B virus X protein (HBx) is a multifunctional regulatory protein that is known to be involved in viral proliferation, transcriptional activation and cell growth control. However, the actual role of HBx in cell growth control remains controversial. In this study, the impact of HBx on cell growth *in vitro* and *in vivo* was further investigated. HBx was able to inhibit the growth of hepatocellular carcinoma (HCC) cells and induce G2/M arrest *in vitro*. Moreover, unlike many other G2/M arrest mechanisms, HBx did not inhibit cyclin B1-CDK1 kinase activity, but it persistently activated the cyclin B1-CDK1 kinase. *In vivo*, HBx inhibited tumor cell growth and induced apoptosis as well as inhibited the growth of vascular endothelial cells. In conclusion, HBx induced G2/M arrest and apoptosis through sustained activation of cyclin B1-CDK1 kinase, and negatively regulated cell growth *in vitro* and *in vivo*.

Introduction

Hepatitis B virus (HBV) is a small DNA virus with a 3.2-kilobase partially double-stranded DNA genome and is a causative agent of acute and chronic hepatitis (1). Epidemiological studies have shown that chronic HBV infection is closely associated with development of HCC (2). Among the four proteins translated from the HBV genome, the X gene product termed HBx has drawn much attention for its role in HBV-mediated carcinogenesis (1).

Numerous studies have shown that HBx is a pleiotropic protein involved in viral proliferation, transcriptional activation, apoptosis, and cell cycle control. Studies have been performed with primary mouse liver cells, established cell lines, and HBx transgenic mice. HBx has been reported to strongly mediate apoptosis, sensitize cells to proapoptotic stimuli, inhibit apoptosis, or have no impact on apoptosis (3-6). The effects of HBx on cell cycle progression are potentially more complicated. Some studies found that HBx stimulated cell cycle progression, accelerating transit through checkpoint controls at G0/G1 (7-9). Other reports showed that HBx could block G0/G1 and G1/S transit or had no effect on cell cycle profiles (6,10). Despite the extensive studies, the actual roles of HBx in cell growth control remains controversial.

In this study, to further investigate the influence of HBx on cell growth *in vitro* and *in vivo*, we constructed a recombinant HBx-expressing adenovirus and established a mouse model of chronic HBx-expressing adenovirus infection. The strategy mimics natural HBV infection and long-term HBx expression during HBV persistence in humans more accurately.

Materials and methods

Cell lines and cell culture. 293A (human embryonic kidney), Hepa1-6 (mouse HCC), HepG2 (human HCC) and MS1 (SV40 T antigen immortalized murine endothelial cell) cell lines were obtained from the American Type Culture Collection. Cells were cultured in humidified atmosphere with 5% CO_2 at 37˚C in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 units/ml streptomycin.

Adenovirus preparation and adenovirus infection. HBx-expressing adenovirus was prepared with a ViraPower adenovirus expression system (Invitrogen) according to the manufacturer's instructions. Briefly, cDNA encoding HBx was subcloned into pENTR11 cloning vector. The pENTR11-HBx plasmid was recombined with pAd vector

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using Gateway system (Invitrogen). As a control, pAd-lacZ vector was transfected into 293A cells to produce a control adenovirus. Cells were infected in DMEM supplemented with 2% FBS with adenovirus preparations diluted to the indicated multiplicity of infection (MOI). After 2 h, the media were removed and fresh media containing 10% FBS were added.

Soft agar colony forming assay. To investigate the effect of HBx on cell growth *in vitro*, soft agar colony forming assay was performed. Cells were seeded in 6-well plates and grew to ~95% confluence, then left untreated or infected with Ad-null or Ad-HBx (MOI 30). After 2 h, $5x10^3$ cells were resuspended in 0.375% agar-DMEM medium (1 ml) and poured on the top of solidified 0.5% agar-DMEM medium (2.0 ml/well) in 6-well plates. After 2 weeks, the colonies with >100 cells were counted and photographed.

Flow cytometry. Hepa1-6, HepG2 and MS1 cells were plated into 6-well plates at $5.0x10^5$, $2.0x10^5$ and $3.0x10^5$ cells/well, respectively, overnight. Then, cells were left untreated or infected with Ad-null or Ad-HBx (MOI 30). After 72 h, cell cycle profiles were analyzed by flow cytometer (Beckman Coulter) after propidium iodide (PI) staining.

Nuclear morphology. At 72-h postinfection, the cells were fixed with 4% formaldehyde for 15 min at 4˚C, followed by rinse with PBS twice. Then, the cells were stained with PI (50 μg/ml) in a dark chamber at room temperature for 10 min and rinsed twice with PBS again. Nuclear morphological changes were observed under inverted fluorescence microscope (Zeiss).

Western blotting. Western blotting was performed as previously described (11). Briefly, cells were lysed and equal proteins were separated on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). The blots were probed with mouse anti-HBx monoclonal antibody (3F6-G10 clone) (1:200; Serotec), mouse anti-cyclin B1 monoclonal antibody (ab72) (1:500; Abcam), rabbit anti-phospho-CDK1 (Tyr15) polyclonal antibody (1:1000; Cell Signaling Technology), rabbit anti-CDK1 polyclonal antibody (1:200; Santa Cruz Biotechnology), rabbit anti-ß-actin polyclonal antibody (1:1000; Sigma) overnight at 4˚C, followed by horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10000; Sigma) or horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10000; Sigma) for 1 h at room temperature, and detected using the enhanced chemiluminescence (ECL) detection system (Pierce).

In vivo studies. Female syngeneic C57BL/6 mice, 6-8-weeks old, were obtained from Laboratory Animal Center of Sichuan University and maintained in pathogen-free conditions. All procedures were reviewed and approved by the institute's Animal Care and Use Committee. Hepa1-6 cells ($1.5x10^6$) were injected s.c. into the right flank of each mouse. After 6 days, when the tumor diameters were about 6 mm, the mice were randomly divided into three groups and were given intratumoral injection of normal saline (NS) (100 μl
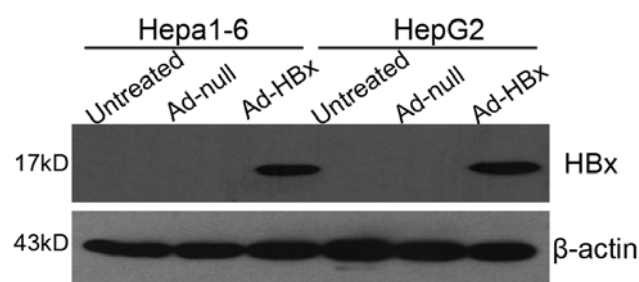

Figure 1. The expression of recombinant HBx in mammalian cells. Hepa1-6 and HepG2 were left untreated, or infected with Ad-null or Ad-HBx (MOI 30). After 72 h, the expression of HBx protein was detected using Western blotting.

each, n=8), Ad-null ($2x10^8$ pfu/100 μl each, n=7) or Ad-HBx ($2x10^8$ pfu/100 μl each, n=8) every three days, four times. Tumor dimensions were measured with calipers every 3 days and tumor volumes were calculated according to the following formula: V = 0.52 x length x width^2.

Histological analysis. Three days after the completion of intratumoral injection, the mice were sacrificed for histological analysis. Excised tumors were fixed in 4% formaldehyde, embedded in paraffin and cut into 3-5-μm sections. Apoptotic cells within tumor tissue were detected using the TUNEL kit (Promega), following the manufacturer's protocol. Apoptosis index was determined by counting the number of apoptotic cells and dividing by the total number of cells in the field (5 high power fields/slide). Angiogenic blood vessels in tumors was evaluated by CD31 immunohistochemistry as previously described (12). Microvessel density (MVD) was determined by counting the absolute number of the microvessel per high-power field in tumor tissue sections.

Statistical analysis. Statistical evaluations of numerical variables were performed using one-way ANOVA test in multiple groups. Significance was defined as P<0.05.

Results

Construction of recombinant HBx-expressing adenovirus. Positive clones were confirmed by restriction enzymes analysis and DNA sequencing. The expression of recombinant HBx was detected using Western blotting. As shown in Fig. 1, only protein extracts from Hepa1-6 and HepG2 cells infected with Ad-HBx, showed a single distinct band.

HBx inhibits the growth of HCC cells in vitro. The influence of HBx on the growth of HCC cells *in vitro* was evaluated by soft agar colony forming assay. Both untreated and Ad-null-infected Hepa1-6 and HepG2 cells formed many large colonies in soft agar after 2 weeks, whereas Ad-HBx-infected Hepa1-6 and HepG2 cells did not form any colonies or only formed several small colonies (Fig. 2A and B).

HBx induces G2/M arrest in HCC cells. Flow cytometric analysis showed that HBx induced a significant accumulation of cells in G2/M phase. Concomitant with this increase in
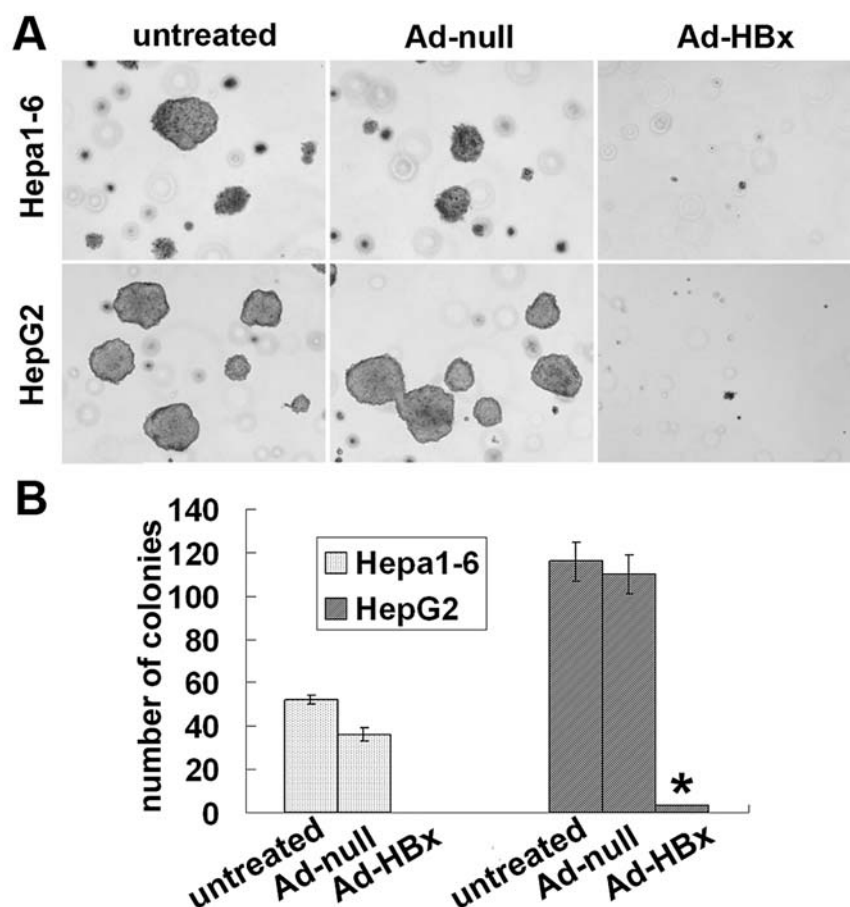
Figure 2. Effects of HBx on the growth of HCC cells *in vitro*. The effects of HBx on the growth of Hepa1-6 and HepG2 cells *in vitro* were evaludated by soft agar colony forming assays. (A) Morphology of clonies in soft agar assay (x50). (B) Quantitative analysis of soft agar colony formation of untreated, Ad-null-infected and Ad-HBx-infected cells. Columns, mean of three independent wells; bar ± SD. (*Three small colonies appeared in only 1 well).

the percentage of cells in G2/M phase was a decrease in the percentage of cells in G0/G1 phase and S phase (Fig. 3A). The percentages of Ad-HBx-infected Hepa1-6 and HepG2 cells in G2/M phase were 65.7±2.8% and 46.2±5.2% respectively, and higher than those of untreated and Ad-null-infected cells (P<0.01) (Fig. 3B and C).

HBx induces alterations of nuclear morphology in HCC cells. Microscopical examination showed that HBx induced an increased number of cells with gigantic and multiple nuclei (Fig. 3D). The results suggest that the cells infected with Ad-HBx can not complete the cell division and are blocked in G2/M phase.

HBx up-regulates the expression of cyclin B1 and down-regulates the expression of pCDK1. Because activation of the cyclin B1-CDK1 complex is a prerequisite for mitotic progression, we reasoned that the G2/M arrest induced by HBx might be due to an aberration in the pathways that regulate cyclin B1-CDK1 kinase activity. The activity of this complex is positively regulated by the fluctuating expression of cyclin B1 and negatively regulated by phosphorylation of CDK1 (Thr-14/Tyr-15) (13). Thus, the expressions of cyclin B1 and phospho-CDK1 in Hepa1-6 and HepG2 cells

following infection with Ad-HBx or Ad-null were detected using Western blotting. As shown in Fig. 4, increased levels of cyclin B1 and decreased levels of phospho-CDK1 were observed in both Ad-HBx infected cell lines, especially, in Ad-HBx infected Hepa1-6 cells. But total CDK1 was unaffected after Ad-HBx infection as judged by comparisons with ß-actin as a loading control.

HBx inhibits the growth of HCC in vivo. To further study the effect of HBx on the growth of HCC *in vivo*, we established a mouse tumor model and performed intratumoral injection of Ad-HBx. Our study showed that treatment with Ad-HBx significantly suppressed tumor growth in comparison with treatment with NS and Ad-null (P<0.01). Seven of eight mice administered with Ad-HBx showed complete tumor regression (Fig. 5A).

HBx induces apoptosis and inhibits vascular endothelial cell growth in vivo. Immunofluorescence microscopy of TUNEL staining revealed significantly more strongly positive nuclei in Ad-HBx-treated tumor tissues compared with control groups (Fig. 5B, upper panels). Quantification of apoptotic cells within tumor sections revealed a significant increase in the apoptosis index in the tumors treated with Ad-HBx
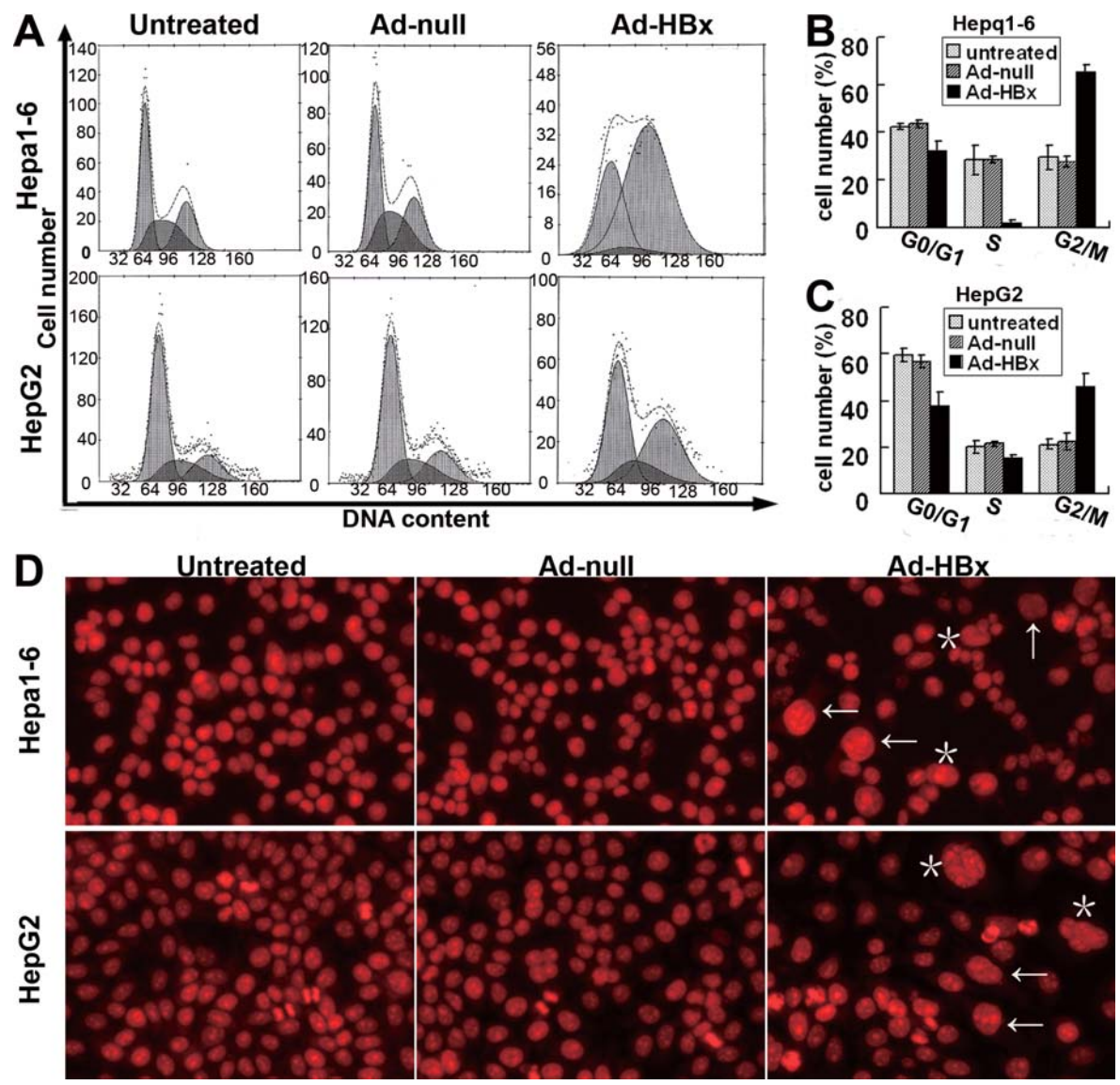
Figure 3. Effects of HBx on cell cycle distribution and nuclear morphology in HCC cells. (A) HBx induced a significant accumulation of cells in G2/M phase. (B and C) Quantification of cell cycle distribution. The quantification of cell cycle distribution was performed using CellQuest software. Data from 3 independent experiments are shown as mean ± SD. (D) HBx induced formation of giant cells with gigantic nuclei (arrows) and multiple nuclei (*), (x400).
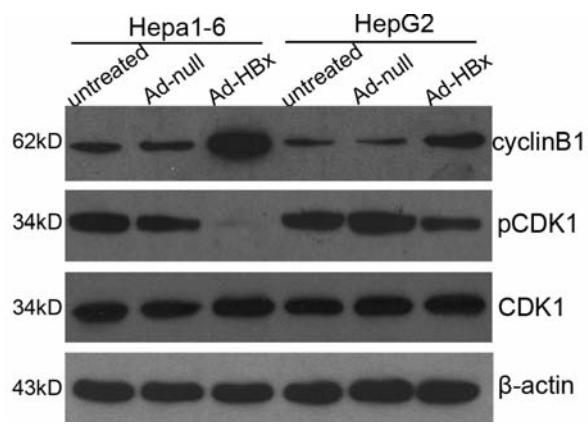

Figure 4. Effects of HBx on the expression of cyclin B1, pCDK1 and total CDK1. Hepa1-6 and HepG2 were left untreated, or infected with Ad-null or Ad-HBx (MOI 30). After 72 h, cells were lysed and the expression of cyclin B1, pCDK1 and total CDK1 was assessed by Western blotting. HBx induced up-regulation of cyclin B1 expression and down-regulation of pCDK1 expression, but did not affect the expression of total CDK1.

($33.1\pm7.8\%$) compared with tumors treated with either NS ($5.2\pm1.8\%$; $P<0.05$) or Ad-null ($8.1\pm2.2\%$; $P<0.05$) (Fig. 5C).

A previous study showed that HBx abrogated onco-protein-induced transformation ability in mouse fibroblasts (NIH3T3) (14). We explored whether HBx also had inhibitory effects on tumor-stromal cells besides HCC cells within Ad-HBx-treated tumor tissue. Angiogenic blood vessels within tumor tissue were detected by CD31 immuno-histochemistry. Treatment with Ad-HBx apparently reduced the number of vessels compared with treatment with NS and Ad-null ($P<0.05$) (Fig. 5B lower panels and D).

HBx induces G2/M arrest in MS1 murine endothelial cells. To further confirm the growth inhibitory effect of HBx on vascular endothelial cells and to investigate the possible mechanism, we tested the impact of HBx on murine endo-thelial cells *in vitro*. Flow cytometric analysis revealed that there was a higher percentage of Ad-HBx-infected cells in
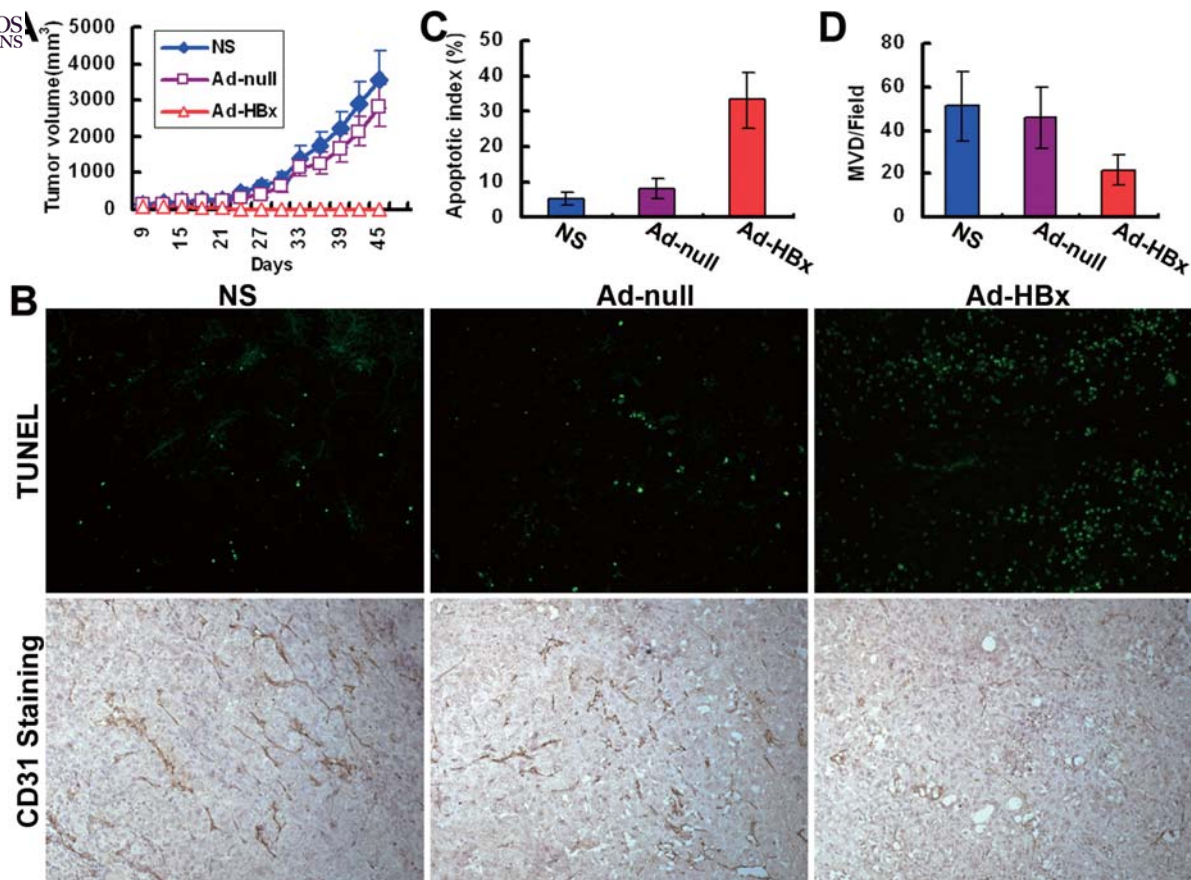
Figure 5. Effects of HBx on the growth of HCC *in vivo*. (A) Treatment with Ad-HBx significantly suppressed tumor growth (P<0.01). Point, average tumor volume; bar ± SD. (B) Apoptosis and microvessels within tumor tissue were deteced by TUNEL staining and CD31 immunohistochemical staining, respectively. Representative sections were taken from tumor tissue of NS-treated, Ad-null-treated and Ad-HBx-treated mice (x200). (C) Apoptotic index of Ad-HBx-treated tumors was higher than that of control groups (P<0.05). Columns, mean of apoptotic index; bar ± SD. (D) Treatment with Ad-HBx apparently reduced the number of microvessel compared with treatment with NS and Ad-null (P<0.05). Columns, mean of microvessel per high-power field; bar ± SD.
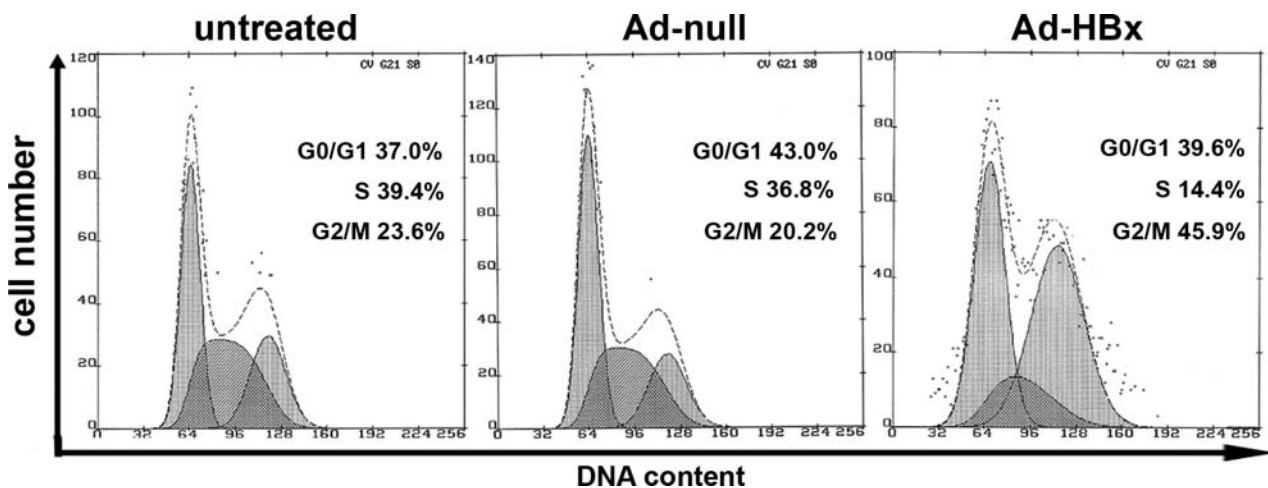

Figure 6. Effect of HBx on cell cycle distribution in MS1 murine endothelial cells. HBx induced a significant accumulation of cells in G2/M phase.

G2/M phase compared with untreated or Ad-null-infected cells (Fig. 6).

Discussion

In the present study, we demonstrated that HBx inhibited the growth of HCC cells and induce G2/M arrest *in vitro*. Previous studies reported that HBx disturbed multiple check-points in primary mouse liver cells, established cell lines, and HBx transgenic mice (6-10). Here, our study showed that HBx also induced cell cycle arrest at G2/M phase.

During late S-early G2 phase, cells prepare for mitosis by increasing the level of cyclin B1 and activating cdc25C, a phosphatase, which leads to dephosphorylation of phospho-

CDK1. Then, cyclin B1 forms a complex with CDK1 and the newly formed cyclin B1-CDK1 complexes are activated (15,16). Once DNA is damaged, cyclin B-CDK1 complexes are inactivated and the cells are blocked in G2 phase (17). In our study, Western blot analysis showed that HBx induced up-regulation of cyclin B1 expression and down-regulation of pCDK1 expression significantly. The results suggest that a failure in the inactivation of cyclin B1-CDK1 kinase complex may account for the observed G2/M block.

There is increasing evidence that many viral proteins can cause host G2/M cell cycle arrest. The G2/M arrest induced by some viral proteins is linked to inhibition of cyclin B1-CDK1 kinase activity, such as human immunodeficiency virus (HIV) Vpr protein and human papillomavirus type 1 (HPV1) E4 protein (18,19). However, our study showed that HBx could not inhibit cyclin B1-CDK1 kinase activity. Instead, HBx persistently activated the cyclin B1-CDK1 kinase. The mechanism of G2/M arrest induced by other viral proteins is similar to that of G2/M arrest induced by HBx, such as human parvovirus B19 NS1 protein and human papillomavirus type 16 (HPV16) E1^E4 protein (13,20).

Why can mitosis not be accomplished successfully even in the presence of the active cyclin B1-CDK1 complex? In normal cell cycle progression, successful entry into M phase is determined by activation of the cyclin B1-CDK1 kinase. Similarly, inactivation of cyclin B1-CDK1 kinase is required for completion of mitosis (11). During mitosis, cyclin B1 is degraded by anaphase-promoting complex (APC). APC can be inactivated by spindle-assembly checkpoint when microtubule organization is perturbed (21). Many reports have demonstrated that antimicrotubule agent can lead to inappropriate accumulation of cyclin B1 and activation of CDK1 and induce mitotic arrest (22). Therefore, a further study should be carried out to explore whether HBx is involved in perturbing microtubule dynamics and activating spindle checkpoint.

In vivo study, we established a mouse tumor model and performed intratumoral injection of Ad-HBx. The strategy mimics natural HBV infection and long-term HBx expression during HBV persistence in humans more closely. We found that HBx inhibited tumor growth and induced apoptosis *in vivo*. Thus, our findings support the view that HBx possesses proapoptotic activity.

Another interesting observation was that treatment with Ad-HBx apparently reduced the number of angiogenic blood vessels within tumor tissues. Moreover, we confirmed that HBx induced G2/M arrest in murine endothelial cells. These results suggest that HBx not only inhibits tumor cell growth but also inhibits stromal cell growth, such as vascular endothelial cells.

In this study, we have shown that HBx induced G2/M arrest and apoptosis. However, some studies had reported that HBx could positively regulate cell growth (5,7-9). The contradictory results might be due to the various different experimental systems. Firstly, the contradictory results may be caused by the use of different cell lines, each with a different differentiation status and different genetic background (23). Secondly, the discrepancy possibly originated from the difference in expression levels of HBx. High expression levels of HBx cause cell cycle block and apoptosis, whereas low expression levels of HBx show

adverse effects (24). Similarly, the normal physiological level of p53 displays an anti-apoptotic activity, whereas a high dose of p53 induces apoptosis (25,26).

Our results, obtained using different experimental systems, suggest that HBx can inibit the growth of HCC cells and vascular endothelial cells *in vitro* and *in vivo* through the induction of G2/M arrest and apoptosis. Furthermore, unlike many other viral proteins, the G2/M arrest induced by HBx is associated with the sustained activation of cyclin B1-CDK1 kinase.

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