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Up-regulation of the expression of costimulatory molecule CD40 in hepatocytes by hepatitis B virus X antigen

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ABSTRACT

Hepatitis B virus (HBV) is a major causative agent of hepatocellular carcinoma (HCC) but the pathogenesis remains poorly understood. To provide novel insights into the pathogenesis of HBV, we examined the expression profile of HBV-positive HepG2.2.15 and -negative HepG2 cells. Genes that were markedly up- or down-regulated in the presence of HBV are involved in signal transduction, apoptosis, transcriptional regulation, protein degradation and oncogenesis. Among the analyzed co-signaling molecules CD40, CD80, CD86, B7-H1, B7-DC, OX40, and B7RP-1, CD40 was the only one up-regulated. Following establishment of stable HepG2 cell lines transfected with HBV genes, we found that HBxAg up-regulated the expression of CD40. We also found that CD40 activation by CD40L could promote the expression of negative co-signaling molecule B7-H1, rather than induce the apoptosis of HepG2HBx cell as expected. These results suggest that CD40 up-regulation by HBxAg may play a facilitating role in the pathogenesis causing HCC.

Hepatitis B virus (HBV) is a major causative agent of hepatocellular carcinoma (HCC) and constitutes a major public health problem worldwide [1,2]. The HBV genome is composed of a 3.2-kb circular DNA and encodes four overlapping open-reading frames, including PreS/S, PreC/C, P, and HBx [3]. HBV is often integrated in various truncated forms into the host genome during HCC development, and HBx appears to be the most frequent viral polypeptide found in HCC [4–6]. HBx has been shown to function as a transcriptional *trans*-activator of numerous cellular transcription elements [7]. Despite the successes of these scientific efforts, the mechanisms whereby HBV contributes to the development of HCC have not been adequately analyzed.

Co-signaling molecules are cell-surface glycoproteins that can direct, modulate and fine-tune T-cell receptor (TCR) signals [8,9]. Co-signaling molecules are essential for the communication of T-cells with virtually all other host cells [10]. Accumulating evidence suggests that the reason why the host immune system cannot eradicate tumors is not the absence of recognizable tumor antigens, but rather the inability of these antigens to stimulate an effective immune response [11]. HCC is generally considered to be one of the most poorly immunogenic cancers. Though some studies have demonstrated that co-signaling molecules are involved in the immune regulation of HCC [12,13], little is known about the precise role of those co-signaling molecules throughout HCC progression.

In this study, we utilized microarray technology to examine the expression profile, especially of co-signaling molecules, in HBV-positive and -negative hepatocyte cell lines, followed by focused investigation of the potential role of the co-signaling molecules up- or down-regulated by HBV.

Materials and methods

Plasmids. Mammalian expression plasmids with the HBV X, S, C, P, e, PreS1, or PreS2 gene were constructed by inserting the corresponding gene fragments into pcDNA3.1(+) (Invitrogen), generating the recombinants pcDNA3.1(+)/HBx, pcDNA3.1(+)/HBs, pcDNA3.1(+)/HBc, pcDNA3.1(+)/HBp, pcDNA3.1(+)/HBe, pcDNA3.1(+)/HB-preS1, and pcDNA3.1(+)/HB-preS2, respectively.

Cell culture and transfection. HepG2 cells (Human hepatocellular liver carcinoma cell line) and HepG2.2.15 cells (HepG2 cells transfected with hepatitis B virus DNA) (both from ATCC) were cultured in RPMI 1640 (GIBCO) and 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator. Cells at 80% confluence were transfected with the recombinant plasmids using Lipofectamine 2000 transfection reagents (Invitrogen) according to the manufacturer's instructions. Following incubation for the indicated time period, cells were harvested and screened for G418 antibiotic (Sigma) resistance to establish stable transfected cell lines. PLC and C4 cell lines, derived from human HCC cells infected with HBV, or with *X* gene interfered, respectively (ATCC), were cultured under the same

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conditions. Healthy human adult liver epithelial cell line QSG7701 (Sciencell) was cultured in DMEM (GIBCO) and 10% fetal bovine serum at 37 °C in a 5% CO_2 incubator.

Microarray analysis. The microarray experiment was commissioned to CapitalBio Co. Ltd. (China, Beijing). The genechip contained 54,614 human genes. Briefly, control and test RNA samples were reverse-transcribed to the cDNA probe, labeled with Cy3 (control) or Cy5 (experimental) and subjected to competitive hybridization. Quantification and normalization of intensity signals were performed using LuxScan 3.0 software (CapitalBio, Co. Ltd.). Three independent experiments were performed, and for each test and control sample, two hybridizations were performed by using a reversal luorescent strategy. Only genes whose alteration tendency kept consistency in both microarray assays were selected as differentially expressed genes.

Quantitative RT-PCR. Total RNA was isolated by RNeasy Midi kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was prepared from total cellular RNA using random hexanucleotide primers and PrimeScript™RT Reagent Kit (TaKaRa). Quantitative (q) PCR experiments were performed using Premix Ex Taq™ reagents (TaKaRa)and amplification carried out according to the manufacturer's instructions. Primers were designed based on the sequence data obtained from the NCBI database using Primer5.0 software. The 5′ and 3′ primer pairs designed for CD40 were 5′-ATCTCGCTATGGTTCGTC-3′ (forward) and 5′-AGGCATTCCGTTTCAG-3′ (reverse), respectively. The thermal cycling conditions consisted of 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 65 °C for 30 s. The emission intensity from SYBR Green binding to double-stranded DNA was detected by the iCycler detection system (BioRad Laboratories, Hercules, CA, USA). To adjust for variations in starting template concentrations, gene expression was normalized with an internal reference gene (beta-actin).

Flow cytometry. HepG2 and HepG2.2.15 cells were incubated with anti-CD40 antibody (eBioscience) for 30 min in PBS at 4 °C. Cells were washed three times with PBS, and then stained with FITC-labeled anti-mouse antibodies at 4 °C for 30 min. After washing, cells were analyzed on a FACScan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences). Additional co-signaling molecules were detected by the same method.

Western blot analysis. Cells were lysed in a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and 2 mM phenylmethylsulfonyl fluoride. Total protein concentration was determined by BioRad protein assay kit (BioRad). Thirty micrograms total protein was subjected to 12% SDS–PAGE and blotted onto a polyvinylidene fluoride membrane. The membrane was blocked (Roche) and incubated (4 °C overnight with shaking) with anti-human CD40 antibody (Abcam) (1:100) or anti-beta-actin antibody (Abcam) (1:200). The blots were then incubated with HRP-conjugated anti-rabbit antibody (1:200) at room temperature for 2 h. The labeled bands were detected by chemiluminescence using ECL Western blotting detection reagents (Roche) and exposure to X-ray film.

Immunofluorescence staining. Cells cultured on a coated slide were fixed with 4% methanol at room temperature for 30 min. They were then incubated with 1% bovine serum albumin (BSA) blocking solution in PBS and then with a 1:100 diluted anti-human HBc antibody (eBioscience) and anti-human HBx, HBe, or HBs monoclonal antibody (Abcam) for 1.5 h. Following washing in PBS, the cells were incubated with a 1:200 diluted HRP-conjugated anti-mouse antibody for 40 min at 37 °C and visualized with DAB and DAPI (Sigma) reagents.

Assessment of apoptosis. The extent of apoptosis in HepG2 and HepG2HBx after rshCD40 (eBioscience) activation was assessed according to the Annexin V-FITC apoptosis detection kit's protocol (Merck). After washing with PBS, cells (1×10^6) were collected and

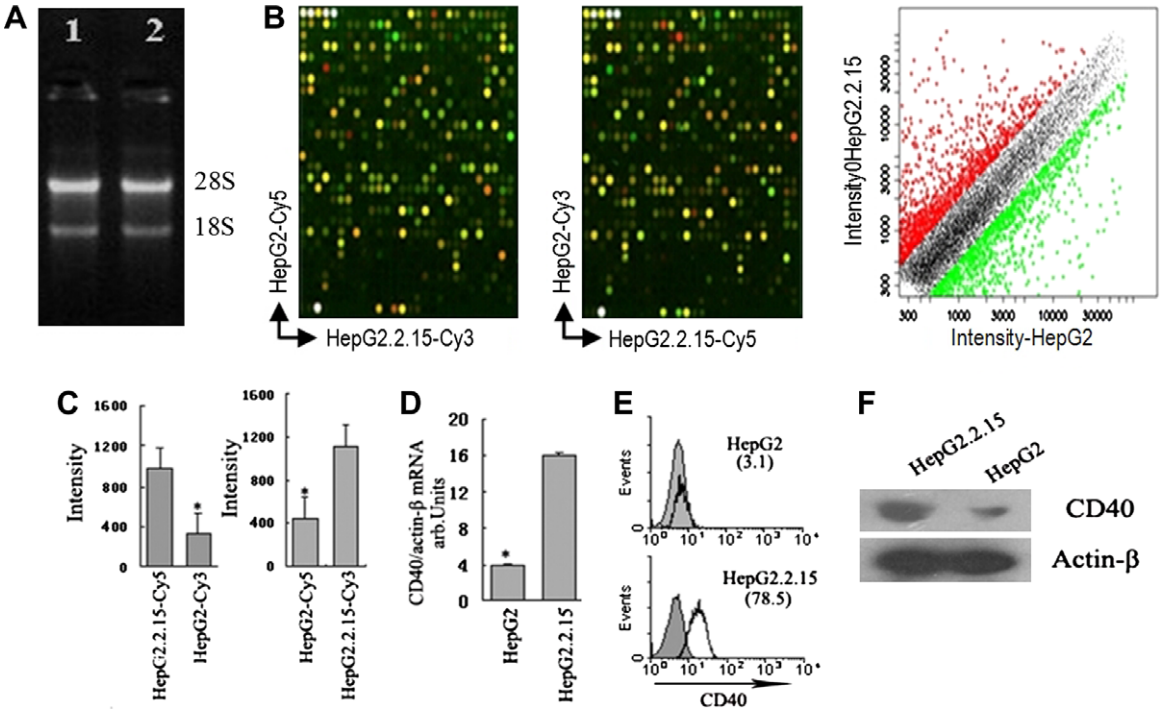

Fig. 1. Microarray analysis. (A) the formaldehyde gel electrophoresis of mRNA from HBV-infected HepG2.2.15 cells or control HepG2 cells. (B) Exchanged fluorescent light Cy3/Cy5 and scatterplot derived from the sample HepG2 and HepG2.2.15 analyzed by genechip. (C) Exchanged fluorescent light Cy3/Cy5 of CD40 derived from HepG2 and HepG2.2.15 cells analyzed by genechip. CD40 expression in HepG2.2.15 and HepG2 cell lines was analyzed by quantitative RT-PCR (D), FACS (E), or Western-blot (F), respectively. A significant difference ($P < 0.05$) is denoted by ∗.

resuspended in binding buffer, and Annexin V-FITC and propidium iodide were added to each sample and incubated in the dark for 5 min. Annexin V-FITC binding of the sample was determined by flow cytometry.

Results and discussion

Differential expression of CD40 in HepG2.2.15 and HepG2 cell lines

Previous studies demonstrated that HBV altered the expression of a wide array of cellular genes in infected HCC cells [14,15]. In order to determine the expression profiles that are affected by HBV, we performed microarray analysis with HCC cell lines HepG2 and HepG2.2.15 (HepG2 cells transfected with HBV DNA). Total RNA was isolated and the quality of RNA was examined by formaldehyde gel electrophoresis (Fig. 1A). The 28S/18S ratio was about 2:1, measured by densitometric tracing analysis (data not shown).

mRNA isolated from HepG2.2.15 cells was compared with HepG2 cells by using a genechip printed with 54,614 human genes. Genes that were altered consistently with expression greater than two fold were selected and partially summarized in Table 1.The profile of differentially expressed genes altered by HBV following signal normalization is shown in (Fig. 1B). Of the total 54,614 genes, 4462 genes were induced and 2592 genes were repressed by HBV. The differentially expressed genes functioned in a variety of cellular processes including signal transduction, apoptosis, transcriptional regulation, protein degradation and oncogenesis regulation (Table 1). As shown in Table 1, the expression of CD40, an important co-stimulatory molecule of antigen processing cells (APC), was up-regulated 4- to 7-fold, indicating co-stimulatory molecules might involve the HBV-related HCC pathogenesis.

In order to more fully define genes of co-signaling molecules that are affected by HBV, we detected the expression of CD40, CD80, CD86, B7-H1, B7-DC, OX40, B7RP-1 and found only CD40 was apparently up-regulated in HepG2.2.15 in comparison to HepG2 cells (Fig. 1C). The up-regulated CD40 expression, as indicated in our genechip analysis, was confirmed by quantitative RT-PCR, flow cytometry and Western blot analyses (Fig. 1D–F, respectively). These results indicated that HBV infection induced the expression of CD40 in hepatocytes.

HBx up-regulated the expression of CD40 in hepatocytes

Previous studies demonstrated that HBxAg could *trans*-activate a wide range of genes that promote growth, inhibit apoptosis, and/or stimulate tumorigenesis [16,17]; accordingly, we addressed the question of which HBV protein was responsible for the up-regulation of CD40 expression as observed in hepatocytes. We constructed serial recombinant plasmids expressing candidate HBV genes and subsequently established stably transfected HepG2 cell lines. The expression of HBV encoded proteins were then detected by flow cytometry (Fig. 2A) and immunofluorescent staining (Fig. 2B). Results also showed that the expression of CD40 was up-regulated in HepG2 cell line in the presence of HbxAg (Fig. 2C), while other HBV genes did not affect CD40 expression in a detectable manner (data not shown). We also detected the expression level of CD40 in other hepatoma cell lines. First, we used PLC cells which were derived from

Table 1
Positive and negative transcriptional regulation in HepG2 and HepG2.2.15 cells.

Gene classification	GenBank Accession No.	Name of gene encoding mRNA	
Protein degradation machinery	NM006263	Proteasome activator subunit 1 (PA28 alpha)	+
	BF679700	Ubiquitin-conjugating enzyme E2D3	++++
	T87178	Ubiquitin specific peptidase 40	++++
	AU157008	Proteasome (prosome, macropain) 26S subunit	−−−−
	NM003334	Ubiquitin-activating enzyme E1	−−−−
Apoptosis response protein	NM001196	BH3 interacting domain death agonist	+
	NM001250	CD40 molecule TNF receptor superfamily member 5	++
	AI760495	Cytochrome *c*, somatic	++++
	AB015653	Caspase 9, apoptosis-related cysteine peptidase	−−
	NM001065	Tumor necrosis factor receptor superfamily, member 1A	−−−
	NM003806	Harakiri, BCL2 interacting protein	−−−
	U60521	Caspase 9, apoptosis-related cysteine peptides	−−−−
Modulators and signaling transducers	BC005035	RAB3B, member RAS oncogene family	+
	AI636647	Forkhead box H1	++
	N29327	Zinc finger protein 439	+++
	AI198212	COX11 homolog, cytochrome *c* oxidase assembly protein	++++
	BQ894022	Phosphodiesterase 1A, calmodulin-dependent	−
	BC005373	Mitochondrial ribosomal protein S18B	−−−
Transcriptional regulators	AB018284	Eukaryotic translation initiation factor 5B	+
	BF793888	CD2 (cytoplasmic tail) binding protein 2	+
	NM005758	Heterogeneous nuclear ribonucleoprotein A3 pseudogene 1	+
	AL569326	Protein kinase (cAMP-dependent, catalytic) inhibitor beta	++
	BE466160	Transcribed locus	+++
	CA442932	Polymerase (RNA) I polypeptide B	++++
	NM006560	CUG triplet repeat, RNA binding protein 1	−−−−
	NM006561	CUG triplet repeat, RNA binding protein 2	−−−−
Oncogenes and tumor suppressor genes	NM005245	FAT tumor suppressor homolog 1	+
	NM007275	Tumor suppressor candidate 2	+
	AW292657	Tumor suppressor candidate 3	++++
	AI184027	Dachsous 2 (*Drosophila*)	++++
	AA479016	Tumor suppressor candidate 1	−
	BC029491	Prenyl(decaprenyl)diphosphate synthase, subunit2	−

Note: Increase (+, 2- to 4-folds; ++, 4- to 7-folds; +++, 7- to 10-folds; ++++, >10-folds) or decrease (−, 2- to 4-folds; −−, 4- to 7-folds; −−−, 7- to 10-folds; −−−−, >10-folds).

a patient infected with HBV and C4 cells in which the *X* gene was interfered. The results showed that the expression of CD40 was up-regulated in the PLC cell line, while there was no significant change detected in the C4 cell line (Fig. 2D). Then, we used the HBx transiently-transfected QSG7701 cell line and observed similar results (Fig. 2E). Collectively, these findings suggest that HBxAg up-regulated the expression of CD40 in hepatocytes.

Functional consequences of CD40 activation on hepatocytes

CD40 was first identified and functionally characterized as a membrane protein on B cells [18]. Recently, the immune cell-specific expression pattern of CD40 was disputed when it was found to be expressed by some carcinoma cell lines [19–22]. An equivocal function effect was found in these particular carcinoma cell lines when treated with a soluble trimeric form of CD40 Ligand (CD40L); this evidence suggested that CD40 signal might have dual effects of both cell survival and death, depending on cellular characteristics or conditions [23–25]. To examine whether recombinant soluble human CD40L (rshCD40L) can induce apoptosis in human hepatocarcinoma cell lines, we cultured HepG2 and HepG2HBx cells in the presence of rshCD40. The resultant proportion of apoptotic tumor cells was determined by Annexin V staining. As shown in Fig. 3A, there was no significant increase in the number of apoptotic cells detected of rshCD40L-treated HepG2HBx or HepG2 cells, indicating that CD40/CD40L activation did not contribute to hepatocyte programmed cell death.

Co-signaling molecules are essential for the communication of T-cells with virtually all other host cells. There have been several reports on anti-tumor immunotherapy based upon induction of co-signaling molecules expression on tumor cells to stimulate more effective anti-tumor immune response [12,26,27]. In our study, FACS assay revealed that activation of CD40 by a soluble CD40L produced no significant effect on surface expression levels of co-stimulatory molecules B7-DC, B7RP-1, CD80, and CD86, each considered capable of enhancing the antigen-presenting capacity of tumor cells [10,28–30]. However B7-H1, a negative co-stimulatory molecule for tumor immunity [31–33], was up-regulated (Fig. 3B), suggesting HCC cells could utilize this inhibitory signal to facilitate its immune-escape and proliferation.

In summary, this study demonstrated that CD40 was up-regulated by the HBxAg in HCC cells. Furthermore, functional studies suggest up-regulated expression of CD40 may play a prominent role in immune-escape and proliferation of tumor cells by imparting resistance to apoptosis in conjunction with up-regulation of negative co-stimulatory molecule B7-H1.

Declaration

The authors declare no competing interests exist in publication of this article.

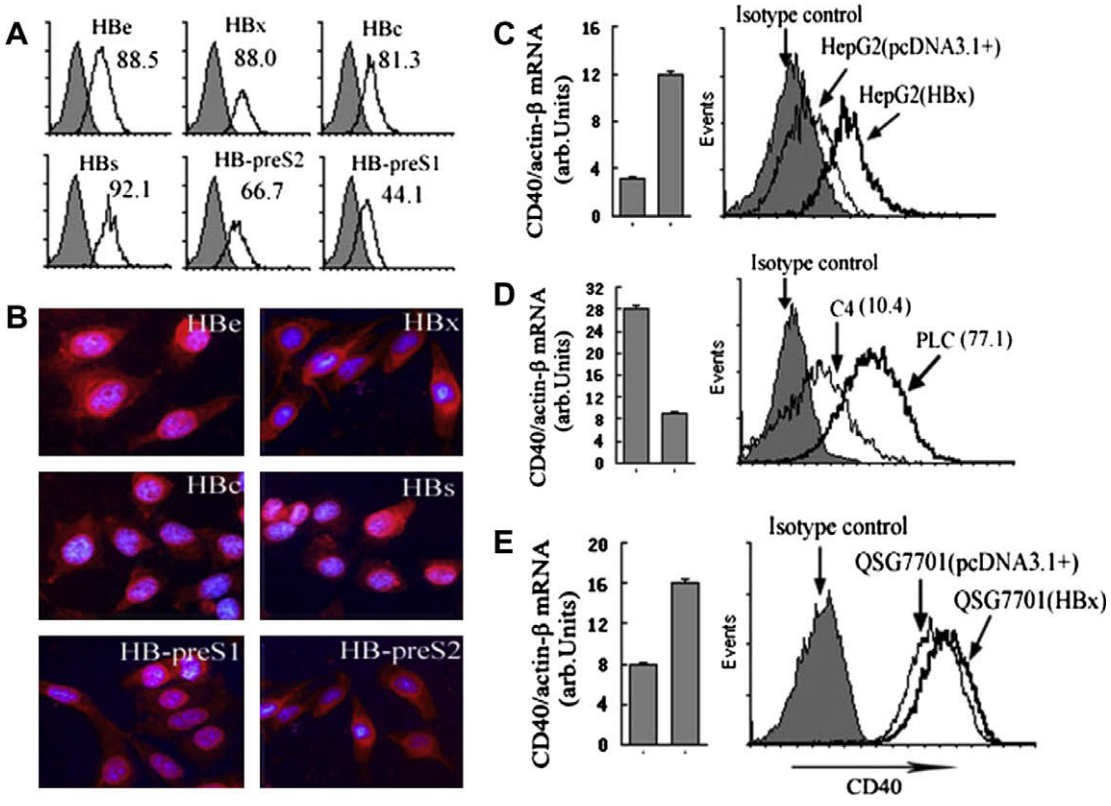

Fig. 2. Up-regulated expression of CD40 by HBxAg. After establishment of stable HepG2 cell lines transfected with serial recombinant plasmids expressing HBV genes, the expression levels of HBV genes were analyzed by flow cytometry (A) and immunofluorescent staining (B, 400×) in HepG2HBe, HepG2HBx, HepG2HBc, HepG2HBs, HepG2HB-PreS2, HepG2HB-PreS1 cell lines, colors red represents HBV gene products and blue represents cell nucleus. (C) CD40 expression was analyzed by qRT-PCR and FACS in HepG2HBx and HepG2pcDNA3.1^{+} control cells, respectively. (D) CD40 expression was analyzed by qRT-PCR and FACS in PLC and C4 cell lines, respectively. (E) CD40 expression was analyzed by qRT-PCR and FACS in HBx transiently transfect QSG7701 cell line and control cells, respectively. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

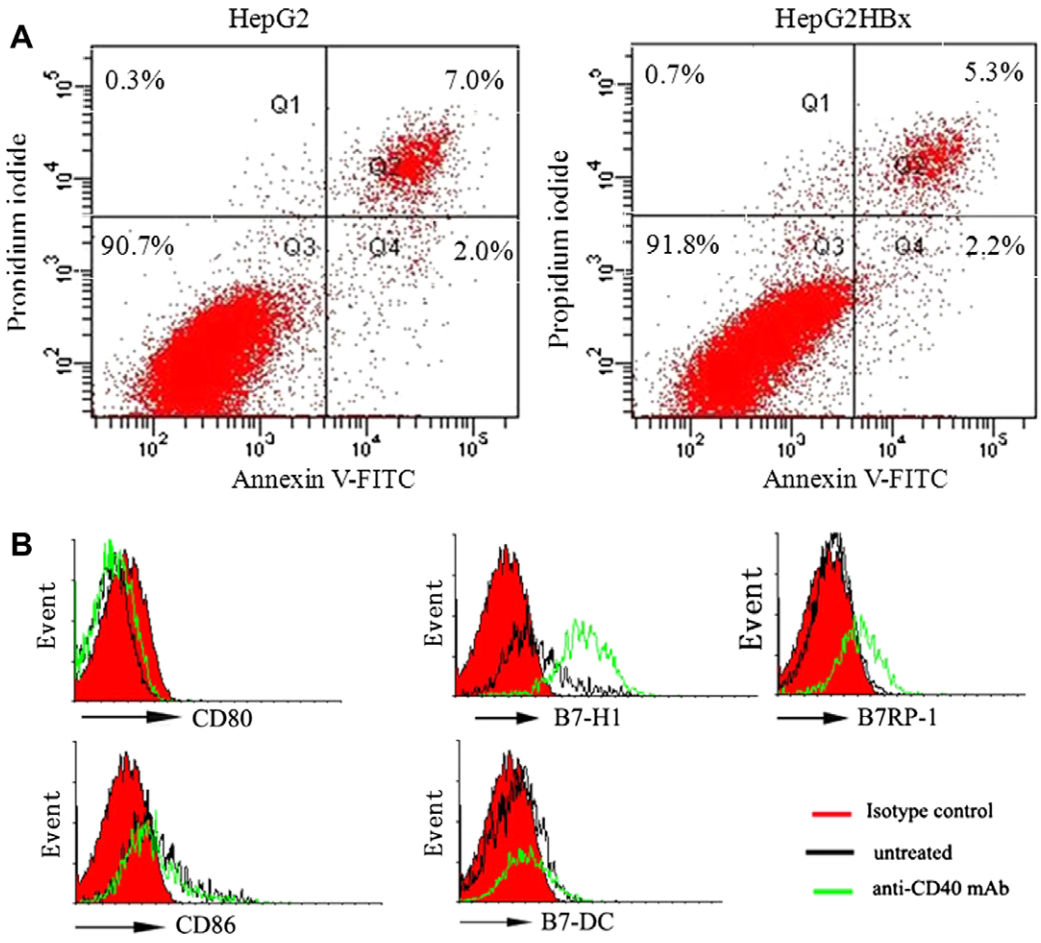

Fig. 3. Consequences of CD40 activation on hepatocytes. (A) FACS analysis of the apoptotic state of HepG2HBx and control cells after CD40 activation by rshCD40L. Cells in the lower and upper right quadrants represent early and late apoptotic cells, respectively. (B) The expression of co-stimulatory molecules after CD40 activation on HepG2HBx, analyzed by flow cytometry.

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