Autophagy enhances antitumor immune responses induced by irradiated hepatocellular carcinoma cells engineered to express hepatitis B virus X protein

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Abstract. Hepatitis B virus X protein (HBx) plays a critical role in malignancy transformation of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). HBx sequence has been mapped with multi-epitopes which can elicit robust specific cytolytic T lymphocyte (CTL) responses. In our previous study, we developed an adenoviral vaccine against HBx oncoproteins to prevent growth of HBV-associated HCC. However, due to the weak immunogenicity of tumor antigen and pre-existing virus-neutralizing antibodies to the vaccine carrier preventing the vector from transducing target cells, the development of novel methods to enhance antigen presentation is urgently required. In the present study, we developed an adenoviral-mediated genetic engineering of hepatoma cell vaccine to express HBx and to evaluate if the novel vaccine could elicit specific immune responses. Our data showed that the irradiated tumor cells engineered to express HBx could significantly induce antitumor immune responses *in vivo*. The novel vaccine could induce a specific CTL response to recognize and lyse HBx-positive hepatoma cells *in vitro*. Both $CD8^+$ T and $CD4^+$ T lymphocytes are involved in the antitumor immune response induced by the novel vaccine. Furthermore, numerous autophagosomes and autolysosomes were found in the irradiated tumor cells engineered to express HBx. The results demonstrated that the irradiated HBx-modified tumor cell vaccine was a potent and promising therapeutic agent against HBx-positive HCC via induction of autophagy-enhanced $CD8^+$ T and $CD4^+$ T lymphocyte-mediated antitumor immune responses. The present findings have implications for the development of clinical immunotherapy against HBV-associated HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor in the world (1). Clinically, the majority of HCC cases are incompetently surgically resected and remain insensitive to radiation and chemotherapy treatments (2,3). The overall 5-year survival rate for patients with HCC worldwide is approximately 10% (4). Thus, the development of novel strategies against HCC is required.

Epidemiological studies have confirmed that the chronic infection with hepatitis B virus (HBV) is strongly associated with the development of HCC. HBV, belonging to the hepadnavirus family, has a relaxed partially double-strand circular DNA genome. This virus encodes four open reading frames with a 17-kDa non-structural multifunctional regulatory protein termed the hepatitis B virus X protein (HBx) (5). Numerous studies have suggested that HBx plays critical roles in regulating cell progression such as cell cycle, proliferation, or apoptosis, which mostly depend on impacting cellular signaling transduction. Meanwhile, HBx sequence has been mapped with multi-epitopes which can elicit robust specific cytolytic T lymphocyte (CTL) responses (6-8).

Studies has demonstrated that HBx expression in hepatoma tissues is positive in 80% of HCC cases and the long-time expression of HBx is considered to play a significant role in cellular malignant transformation which contributes to the development of HCC (2,3,9,10). Thus, it had been applied as a target for immunotherapy when HBx-specific CTL responses could be induced. It is well documented that CTL epitope peptides from HBx could induce HBx-specific $CD8^+$ T cells and eradicate tumor in xenografted nude mice (11). Ding *et al* (8) also reported that multi-epitope peptide-loaded virus-like particles as a vaccine could elicit an immune response to protect against HBV-related HCC. In addition, Wang *et al* (12) reported that 2 oral HBx vaccines delivered by live attenuated *Salmonella* could induce significantly specific

antitumor immunity. In our previous study, we demonstrated that HBx specific immunity against HCC was elicited by adenovirus vaccine encoding HBx (2).

Whole tumor cells are a source of tumor-associated antigens (TAA) for vaccination purposes. Vaccination with irradiated tumor cells has been studied in several animal models. However, due to the weak immunogenicity of tumor antigen, downregulated expression of MHC molecule and lacking costimulatory molecules on tumor cells, the original tumor cell vaccine is often unable to induce a strong immune response (9,13-15). Thus, the development of novel methods to enhance antigen presentation is urgently required. The genetic engineering of tumor cells to express cytokines, co-stimulatory molecules and tumor antigen has been used to improve the immunogenicity of tumor cell vaccines. Moreover, a plurality of genetically modified tumor cell vaccines has shown some promise in clinical trials (16-18).

In the present study, we developed an adenoviral-mediated genetic engineering of hepatoma cells vaccine to express HBx and to evaluate if the vaccine could elicit specific immune responses against HCC. Our results demonstrated that the irradiated HBx-modified hepatoma cell vaccines could elicit significant specific antitumor immunity.

Materials and methods

Cell culture. The human embryonic kidney cell line 293A and the murine HCC cell line Hepa1-6 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (both from Gibco-BRL, Carlsbad, CA, USA), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Stable Hepa1-6 cell line expressing HBx (Hepa1-6/HBx) was constructed as previously described and cultured in complete medium containing 200 μg/ml G418 (2). The cells mentioned above were propagated at 37˚C in humidified 5% CO_2 conditions.

Construction and amplification of recombinant adenovirus. The construction of recombinant adenovirus vectors encoding HBx protein (AdHBx), including vectors expressing no transgene (AdNull) was performed as previously described (19). Adenovirus vectors were amplified in 293A cells and viral titers were measured by a standard plague-forming assay. All viruses were stored in aliquot at -80˚C until use.

Infection of Hepa1-6 cells with adenovirus. Prior to adenovirus infection, the culture medium of Hepa1-6 seeded on the culture plates or dishes was decanted and washed with fresh medium without supplements. Viral dilution was added to cells at a multiplicity of infection (MOI) of 20 in a minimum culture volume and co-incubated with cells at 37˚C for 2 h. After a 2-h incubation, virus solution was replaced with complete medium and cells were incubated for 24 h at 37˚C. Then, the cells were harvested for experiments *in vivo* or *in vitro*.

Reverse transcription-polymerase chain reaction (RT-PCR) assay. The total mRNA of Hepa1-6 cells after 24 h-infection treatment was extracted by TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the protocol. The expression of HBx was assayed using PrimeScript RT-PCR kit (Takara Bio, Inc., Shiga, Japan) as previously described (20). Briefly, the RNA samples mentioned above were reverse transcribed to generate cDNA using random primers. Thereafter, the PCR was performed with primers for HBx gene as follows: forward, 5'-ATGGCTGCTAGGCTGTGCTG-3' and reverse, 5'-GGCAGAGGTGAAA AAGTTGC-3' designed on the basis of Gene Bank. And GAPDH was amplified as a control to validate accuracy and outcome of the products of RT-PCR measured by 1% agarose gel electrophoresis.

Vaccine preparation and immunization. The tumor cells infected with adenovirus for 24 h were digested, washed 3 times with fresh serum-free medium, irradiated with 50 Gy, and then adjusted to a concentration of $1x10^6$ cells in 100 μl prior to administration. C57BL/6 female mice 6-8 weeks old were purchased from Beijing Weitong Lihua Biological Technology Co., Ltd and maintained in pathogen-free and isothermal conditions. All procedures were reviewed and approved by the Animal Care and Use Committee of Sichuan University. Hepa1-6/HBx ($2.5x10^6$) tumor cells in 100 μl PBS were injected s.c. in the posterior flank of mice. When the tumor diameters were ~3 mm, mice were divided into 4 groups. The irradiated Hepa1-6 cells infected with AdHBx described above were vaccinated s.c. at a dose of $1x10^6$ cells each mouse in 100 μl PBS (day 0). Other groups received inoculation of PBS, irradiated Hepa1-6, or irradiated Hepa1-6 infected with AdNull in the same volume as the control experiments. Vaccination was boosted on day 14 and 21. The tumor size was measured using a sliding caliper every 3 days and the volume was calculated by the equation: 0.52 x (length x width^2).

Characterization of T-lymphocyte subsets. Three mice of each group were sacrificed a week after the last immunization. Spleens were aseptically removed and single-cell suspensions were isolated as previously described (2). Isolated cells (10^5) were stained with fluorochrome-coupled antibodies to CD4 and CD8 at 4˚C for 30 min. The cell suspensions were measured on FACSCalibur and the data were analyzed by cell quest software (both from BD Biosciences, Franklin Lakes, NJ, USA).

Cell proliferation assay. To verify the T-cell proliferative ability of immunized mice, lymphocytes were isolated from mice 1 week after the last vaccination and labeled by 0.5 μM CFSE for 10 min at 37˚C in 5% CO_2. Then an equal volume of fetal calf serum (FCS) was added to stop the reaction. The cells were centrifuged at 400 x g for 5 min and washed twice with complete medium. CFSE-labeled cells were resuspended in 10% RPMI medium, adjusted to $2x10^5$ cells/ml and stimulated by irradiated Hepa1-6/HBx at effector:target (E:T) ratios of 100:1. Cells were incubated in 24-well plates at 37˚C for 5 days. The labeled dilution was measured on FACSCalibur and the divisive generations were assayed via FlowJo software version 7.6.1 (Tree Star, Inc., San Carlos, CA, USA). Proliferation index was conveyed as the percentage of cells that had divided and the average number of cell divisions.

CFSE-based cytotoxicity assay. The cytotoxicity assay was performed as previously described with slight modifications (22,23). Isolated splenocytes from mice were as effector cells. After 48-72 h of stimulation with irradiated Hepa1-6/HBx, effector cells were washed twice and resuspended to a concentration of 10^7/ml. The Hepa1-6/HBx cells were used as target cells, labeled with CFSE (2.5 μM) as described above and adjusted to a concentration of 10^5/ml. Effector cells and target cells were mixed in total volume of 200 μl at E:T ratios of 100, 50, 25, 12.5, 6.25 in round-bottom polystyrene tubes, centrifuged at 400 x g for 45-60 sec and incubated at 37˚C in 5% CO_2 for 4-6 h. The target cells of each sample were 10^4 cells in 200 μl volume, and only target cells in the tube served as a negative control. Following incubation, each sample was supplemented with 20 μl PI (100 μg/ml) on the ice and the acquisition was performed by flow cytometry within 1 h. $CFSE^+/PI^+$ cells were considered as dead target cells. Percentage of specific lysis was then expressed as: % specific lysis = [dead targets in the sample (%) - spontaneously dead targets (%)/100 - spontaneously dead targets (%)] x 100.

Enzyme-linked immunospot assay. Specific IFN-γ-secreting T cells were measured using the Mouse IFN-γ ELISPOT kit (U-CyTech Biosciences, The Netherlands) according to directions of the manufacturer. The 96-well ELISPOT plates were coated with antibodies at 4˚C overnight before splenocytes were harvested. Plates were washed 3 times with PBS and blocked at 37˚C for 1 h. Splenocytes ($3x10^5$) co-cultured with irradiated Hepa1-6/HBx at 100:1 responder: stimulator ratios were added to wells in triplicate and incubated for 48 h at 37˚C. Subsequently, cells were decanted and plates were washed 6 times with wash buffer (PBS containing 0.05% Tween-20). Diluted biotinylated detection antibodies were added to each well and plates were incubated for 1 h at 37˚C. Then, antibodies dilution was removed by 6X wash using wash buffer. GABA (ϕ-labeled anti-biotin antibodies) solution was added to wells. After 1 h of incubation at 37˚C, plates were washed 6 times. Thereafter 35 μl AEC substrate solution was applied to every well and then plates were incubated for 10-20 min in the dark at room temperature. When distinct spots were developed, the reaction was stopped by the addition of demineralized water. Plates were dried at room temperature and spots were counted using a dissecting microscope. IFN-γ-producing cells were calculated by the numbers of spot-forming cells per 10^6 splenocytes.

Electron microscopy. The cellular ultrastructural analysis by electron microscopy was performed as previously described. Hepa1-6 cells were infected with AdHBx or AdNull and irradiated with X-rays. Infected cells were prepared without X-rays as controls. Cells were harvested and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer overnight. Then, cells were treated in 1% OsO_4, dehydrated in ethanol and embedded in Epon. Fixed cells were made into 0.1 mm sections, stained and then viewed with the electron microscope (Hitachi, H-7650, Japan).

Statistical analyses. Statistical significance of experimental groups was analyzed using Student's t-test and performed on GraphPad InStat version 3.06. The data were means ± SEM and P-values <0.05 were considered to indicate statistically significant differences, presented in figure captions.

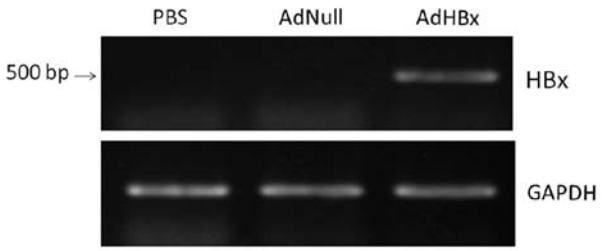

Figure 1. Gene expression of hepatitis B virus X protein (HBx) in Hepa1-6 after 24 h of adenoviral-mediated transient transfection. HBx expression was detected by RT-PCR in AdHBx-transfected Hepa1-6, compared with Hepa1-6 alone and AdNull-transfected Hepa1-6 as controls. GAPDH was utilized as an internal control to verify integrity of cDNA extracted from cells.

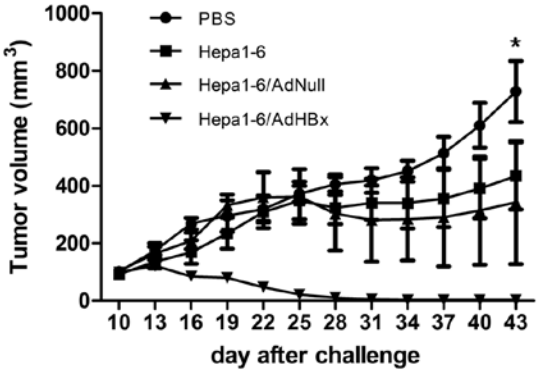

Figure 2. The therapeutic immunity against hepatocellular carcinoma (HCC) induced by Hepa1-6 /AdHBx vaccine *in vivo*. Mice bearing tumors were administered with vaccines at days 0, 14, 28 and the tumor size was measured using a sliding caliper every 3 days. The significant tumor regression of Hepa1-6/AdHBx-treated mice was observed in the growth curve (*P<0.001).

Results

Characterization of tumor cell vaccines. To prepare tumor cell vaccines engineered to express HBx, we infected Hepa1-6 cells with recombinant adenoviral vectors encoding HBx at an MOI of 20. We detected the expression of HBx in tumor cell vaccines by the means of RT-PCR. As shown in Fig. 1, only RNA extracts from Hepa1-6 cells infected with Ad-HBx, but not RNA extracts from cells infected with AdNull or uninfected, amplified a single distinct band.

Induction of therapeutic antitumor immunity. To investigate the therapeutic effect of this cellular vaccine *in vivo*, mice were challenged with $2.5x10^6$ Hepa-6/HBx cells. After 7 days, the first vaccination was administered (day 0), and then vaccination was boosted on day 14 and 21. We observed a significant delay in the growth of tumors in AdHBx-infected Hapa1-6 immunized mice with a statistically significant reduction (P<0.001) (Fig. 2). Thirty-four days after challenge, only 1 mouse bore a tumor, which was 18.1 mm^3 in size, while the tumors of the other 5 mice were completely eradicated. By contrast, the tumor regression was not observed in the mice administered PBS, Hepa1-6 or Hepa1-6/AdNull. The results

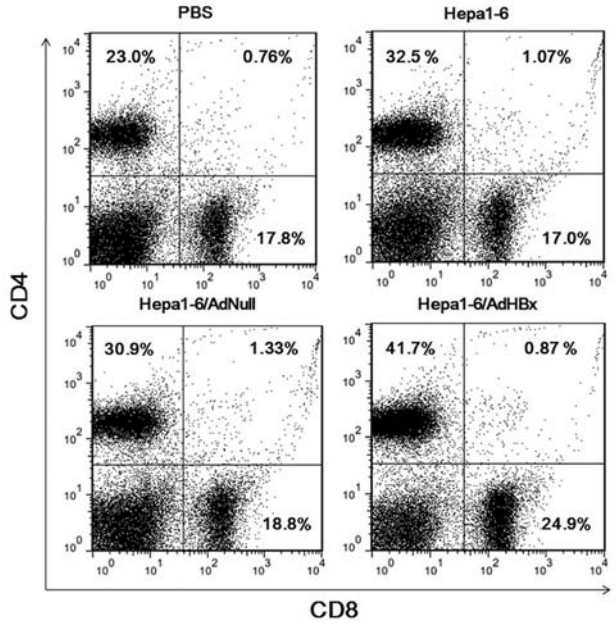

Figure 3. Frequency of activated T cells isolated from vaccinated mice. Splenocytes were isolated from mice 1 week after the last booster and stained by fluorochrome-conjugated anti-mouse CD4 and anti-mouse CD8 antibodies and measured by flow cytometry. The frequencies of $CD4^+$ and $CD8^+$ T cells were both significantly increased compared with controls ($P<0.05$ and $P<0.01$).

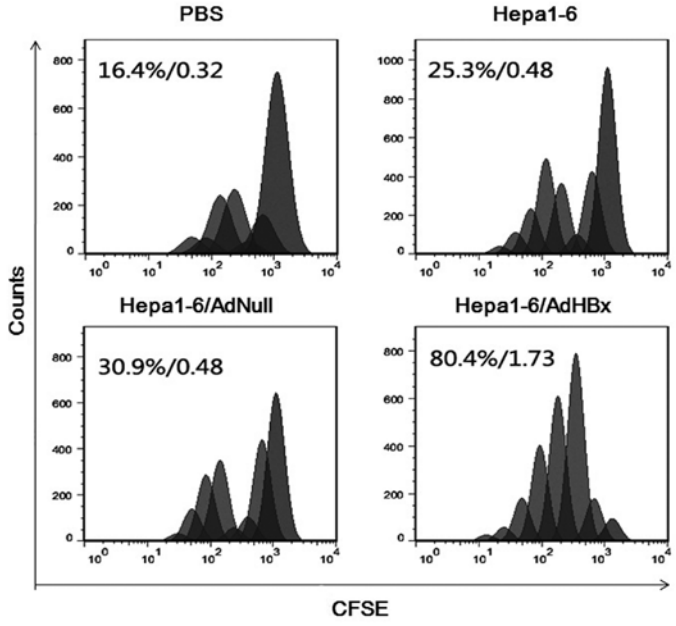

Figure 4. Proliferative capacity of T cells isolated from vaccinated mice. Mice were sacrificed after the last booster and spleen lymphocytes were isolated. Isolated cells were labeled by CFSE, and co-cultured with irradiated Hepa1-6/HBx as a stimulator for 5 days. Proliferative T cells were analyzed using flow cytometry. The percentage of cells that divided at least once (left) and the average number of divisions of the cells that underwent at least 1 division (right) are shown on the top left corner.

confirmed that the Hepa1-6/AdHBx tumor vaccine induced significant specific antitumor immunity to inhibit tumor growth and even eliminate tumors.

T-cell subsets following vaccination. Next, we characterized which type of T cells participated in the antitumor immunity. After 3 vaccinations, splenocytes were harvested and flow cytometry with 2-color staining for CD4 and CD8 was performed to characterize T-cell subsets. The component of $CD4^+$ and $CD8^+$ T cells of Hepa1-6/AdHBx treatment were 41.7 and 24.7% which were higher than those of the PBS, Hepa1-6, and Hepa1-6/AdNull groups (Fig. 3). The results demonstrated that $CD4^+$ and $CD8^+$ T cells both participated in the therapeutic immunological responses in mice vaccinated with Hepa1-6/AdHBx vaccines; i.e., MHC class I-and II-restricted immunity responses are involved in the antitumor therapy.

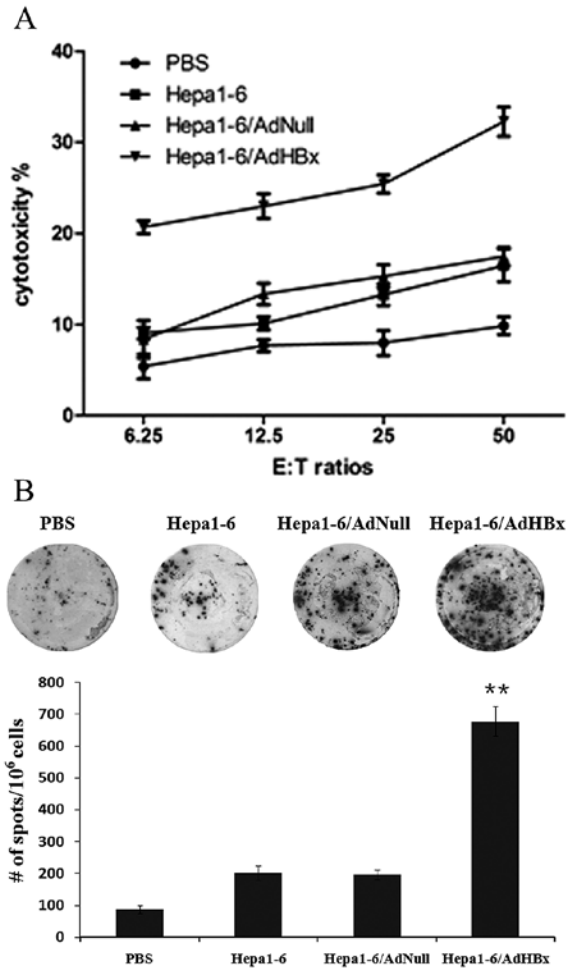

Figure 5. Detection of specific T responses in vaccinated mice. Lymphocytes were isolated from the spleens of vaccinated mice 1 week after the last vaccination, and cells were treated as follows: (A) lymphocytes stimulated with irradiated Hepa1-6/HBx cells for 72 h were added to fresh CFSE-labeled target cells at various E:T ratios for 4 h at 37˚C. Following incubation, PI was added to each sample. Then, specific cytotoxic activity was performed using flow cytometric analysis and the percentage of $CFSE^+PI^+$ cells was considered as specific T cell cytotoxic lysis when the E:T ratios ≥6.25, P<0.01. (B) Isolated lymphocytes were stimulated for 48 h as mentioned above, and IFN-γ production was measured by ELISPOT assay according to the manufacturer's instructions. Representative IFN-γ formation wells are presented by images and the average number of IFN-γ-secreted T cells is represented by the bars (**P<0.001).

Proliferative ability of lymphocytes. The lymphocyte proliferation assay was performed to evaluate the memory response in vaccinated mice. Spleen lymphocytes isolated were labeled by CFSE and incubated with irradiated Hepa1-6/HBx as a stimulator for 5 days. The assay was performed by flow cytometry and analyzed with FlowJo. As shown in Fig. 4, the percentage of cells that divided at least once and the average number of cell divisions were 80.4% and 1.73 respectively for lymphocytes from mice administered AdHBx-infected Hepa1-6 vaccines, which were higher than those from control mice. The results demonstrated that immunization with Hepa1-6/AdHBx vaccine was effective at inducing memory immune responses in animals.

Activation of HBx-specific CTLs. To determine whether vaccination of irradiated Hepa1-6 infected with AdHBx elicit HBx-specific CTL responses, spleen lymphocytes were isolated and stimulated with irradiated Hepa1-6/HBx cells for 72 h. Then, the reconstituted lymphocytes were co-cultured with CFSE-labeled Hepa1-6 stably expressing HBx as target cells for 4 h. As shown in Fig. 5A, the lymphocytes from mice administered AdHBx-infected Hepa-6 vaccines were able to lyse specific Hepa1-6 cells stably expressing HBx (target cells) in an E:T ratio-dependent manner with statistical significance at E:T ratios ≥6.25 as compared with controls (P<0.01). These findings suggested that irradiated HBx gene modified tumor cells could induce a specific CTL response to recognize and lyse HBx-positive Hepa1-6 cells.

Production of IFN-γ by lymphocytes. The frequency of IFN-γ secreting cells in splenocytes reflected the number of CTL and cellular immune response. To further evaluate HBx-specific CTL response, the IFN-γ-producing T cells relative to the number spleen cells were detected using IFN-γ ELISPOT assay. In these studies, mice were immunized with Hepa1-6 infected with AdHBx, AdNull or uninfected and PBS respectively. Splenocytes were harvested and stimulated with irradiated Hepa1-6/HBx cells and the spot-forming cells were measured after 48 h. As shown in Fig. 5B, the number of spot-forming cells produced by 10^6 splenocytes from the mice treated with Hepa1-6/AdHBx were ~8-fold compared with PBS treatment, and 4-fold compared with groups vaccinated with Hepa1-6 alone or Hapa1-6/AdNull. The results further indicated that vaccination with Hepa1-6/AdHBx could elicit specific CTL response.

HBx sensitizes cells to irradiation-induced autophagy. To explore the impact of HBx and X-ray irradiation on Hepa1-6 cells, we observed the cellular ultrastructure of tumor cells using transmission electron microscopy. Hepa1-6 cells were infected with AdHBx or AdNull and irradiated by X-rays. Cells were treated and analyzed by electron microscopy. The results indicated that autophagy was induced in the AdHBx-infected cells treated with X-ray irradiation. Numerous autophagosomes or autolysosomes are presented in Fig. 6D-F. However, no or few autophagic vacuoles were observed in the other groups (Fig. 6A-C).

Discussion

HCC is one of the most aggressive tumors worldwide. In China, the number of people diagnosed with advanced HCC increases annually. However, at present, HCC is often incompetently surgically resected and insensitive to radiation and chemotherapy treatments and the postsurgical recurrence and metastasis present further challenges to the treatments of HCC. A new efficacious therapy is required. In recent years, immunotherapy has emerged as a promising mode of treatment for cancer. HBx is closely associated with hepatic carcinogenesis (6,23-26). HBx expression is present in ~80% of the HCC cases including both carcinomas and pericancerous liver tissues. The multiple epitopes of HBx can elicit robust specific CTL responses and induce regress HBx-positive tumor (6-8).

In our previous study, we developed an adenoviral vaccine against HBx oncoproteins to prevent growth of HBV-associated HCC (2). Replication-deficient adenoviral vectors are widely utilized for transient transgene delivery due to their high

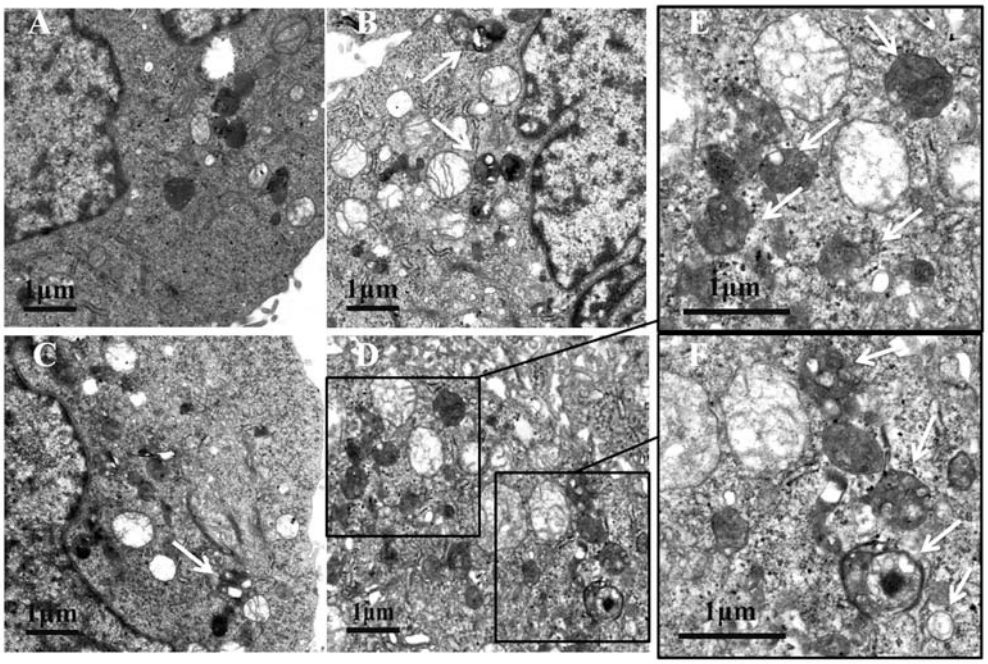

Figure 6. The representative electron graphs of the cellular ultrastructure of X-rays-treated cells. Hepa1-6 cells were transfected with AdNull (A,B) or AdHBx (C,D). After 24 h of incubation, cells were treated with or without X-rays. (A and C) Cells treated without X-rays; (B and D) cells irradiated with X-rays at 50 Gy (E and F) the same cells as (D) with different magnifications. Arrows point to autophagosomes or autolysosomes.

transfection efficiency and low toxicity (27,28); they have transitioned from tools for gene replacement therapy to vaccine delivery vehicles and are attractive vaccine vectors as they can induce both innate and adaptive immune responses in mammalian hosts (29). However, one major obstacle to the use of adenoviral vectors is the high prevalence of virus-neutralizing antibodies (VNAs) in humans. Pre-existing VNAs to the vaccine carrier prevent the vector from transducing target cells, which reduces the amount of vaccine antigen production and attenuates the adaptive immune responses (30).

In order to overcome the shortcoming of adenoviral vaccines, in the present study we infected hepatoma cells with adenovirus expressing HBx and developed irradiated tumor cells engineered to express HBx as a therapeutic vaccine against HCC. The irradiated tumor cells engineered to express HBx could significantly induce antitumor immune responses *in vivo*. While genetically unmodified tumor cells were also effective to a certain degree, the antitumor immunity was too weak to inhibit tumor growth significantly. The unmodified tumor cell vaccine could not induce strong and specific antitumor immunity, this was contributed to the poor immunogenicity of the tumor antigen, downregulated expression of MHC molecule and deficient co-stimulatory molecules on tumor cells (9, 13-15). In this study, tumor cells genetically engineered to express HBx may elicit significantly HBx-specific antitumor immunity besides faintly whole-cell antigen-mediated antitumor immune responses. The novel immunotherapy strategy may represent an advancement in the development of treatments for HCC.

It has been well demonstrated that $CD8^+$ T cells participate in antitumor immune response induced by various HBx-based vaccines (7,8). Antigen-specific $CD8^+$ CTLs play an important role in antitumor immune responses (31). In the present study, we also found that the frequency of $CD8^+$ T cells and the expression of T cytotoxic (Tc) 1-type cytokine (IFN-γ) markedly increased in mice immunized with irradiated HBx modified tumor cell vaccine compared with the other groups. Moreover, the vaccine could induce a specific CTL response to recognize and lyse HBx-positive hepatoma cells. However, aside from the frequency of $CD8^+$ T cells, that of $CD4^+$ T cells more markedly increased in mice immunized with irradiated HBx modified tumor cell vaccine in our present study. Accumulating evidence indicates $CD4^+$ T cells play important roles in inducing MHC class I-restricted tumor-specific immunity and in the delivery of help for priming of tumor-specific CTLs (32). Our study demonstrated that both $CD8^+$ and $CD4^+$ T lymphocytes participated in antitumor immune responses induced by the irradiated HBx-modified tumor cell vaccine.

A previous study found that HBx sensitized cells to starvation-induced autophagy via upregulation of beclin 1 expression (33). Another separate report indicated HBV could also induce autophagy in cell cultures, mouse liver and an infected patient via HBx (34). In this study, a considerable number of autophagosomes or autolysosomes was observed in irradiated HBx-modified tumor cells compared with control groups. Autophagy plays a physiological process that is important for maintaining intracellular homeostasis by promoting the transit of cytoplasmic materials, such as proteins, organelles and pathogens, for degradation within acidic organelles (35). In addition to maintaining cellular homeostasis, increasing studies have also revealed that autophagy plays an important role in innate and adaptive immunity. Autophagy participated in enhancing extracellular antigens for MHC class II presentation, regulating intracellular antigen processing for MHC class I presentation, and packaging antigens for optimal cross-presentation (36). Thus, autophagy may contribute to enhancing $CD8^+$ and $CD4^+$ T lymphocyte-mediated antitumor immune responses which were induced by the irradiated

HBx-modified tumor cell vaccine. The mechanism by which autophagy contributes to enhancing antitumor immune responses induced by the novel vaccine requires further study.

In conclusion, our results demonstrated that the irradiated HBx-modified tumor cell vaccine is a potent and promising therapeutic agent against HBx-positive HCC by inducing autophagy-enhanced $CD8^+$ and $CD4^+$ T lymphocyte-mediated antitumor immune responses. The present findings have implications for the development of clinical immunotherapy against HBV-associated HCC.

Acknowledgements

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References

1. Du Y, Kong G, You X, *et al*: Elevation of highly up-regulated in liver cancer (HULC) by hepatitis B virus X protein promotes hepatoma cell proliferation via down-regulating p18. J Biol Chem 287: 26302-26311, 2012.
2. Li Y, Cheng P, Wen Y, *et al*: T lymphocyte responses against hepatitis B virus-related hepatocellular carcinoma induced by adenovirus vaccine encoding HBx. Int J Mol Med 26: 869-876, 2010.
3. Dan Q, Sanchez R, Delgado C, *et al*: Non-immunogenic murine hepatocellular carcinoma Hepa1-6 cells expressing the membrane form of macrophage colony stimulating factor are rejected in vivo and lead to CD8+ T-cell immunity against the parental tumor. Mol Ther 4: 427-437, 2001.
4. Altekruse SF, McGlynn KA and Reichman ME: Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. J Clin Oncol 27: 1485-1491, 2009.
5. Rehermann B and Nascimbeni M: Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 5: 215-229, 2005.
6. Hsieh JL, Wu CL, Lee CH and Shiau AL: Hepatitis B virus X protein sensitizes hepatocellular carcinoma cells to cytolysis induced by E1B-deleted adenovirus through the disruption of p53 function. Clin Cancer Res 9: 338-345, 2003.
7. Malmassari S, Lone YC, Zhang M, Transy C and Michel ML: In vivo hierarchy of immunodominant and subdominant HLA-A*0201-restricted T-cell epitopes of HBx antigen of hepatitis B virus. Microbes Infect 7: 626-634, 2005.
8. Ding FX, Wang F, Lu YM, *et al*: Multiepitope peptide-loaded virus-like particles as a vaccine against hepatitis B virus-related hepatocellular carcinoma. Hepatology 49: 1492-1502, 2009.
9. Rosenthal FM, Zier KS and Gansbacher B: Human tumor vaccines and genetic engineering of tumors with cytokine and histocompatibility genes to enhance immunogenicity. Curr Opin Oncol 6: 611-615, 1994.
10. Yannelli JR and Wroblewski JM: On the road to a tumor cell vaccine: 20 years of cellular immunotherapy. Vaccine 23: 97-113, 2004.
11. Chun E, Lee J, Cheong HS and Lee KY: Tumor eradication by hepatitis B virus X antigen-specific $CD8^+$ T cells in xenografted nude mice. J Immunol 170: 1183-1190, 2003.
12. Wang YJ, Hou Y, Huang H, Liu GR, White AP and Liu SL: Two oral HBx vaccines delivered by live attenuated *Salmonella*: both eliciting effective anti-tumor immunity. Cancer Lett 263: 67-76, 2008.
13. Bailly M, Bertrand S and Doré JF: Increased spontaneous mutation rates and prevalence of karyotype abnormalities in highly metastatic human melanoma cell lines. Melanoma Res 3: 51-61, 1993.
14. Hicklin DJ, Marincola FM and Ferrone S: HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. Mol Med Today 5: 178-186, 1999.
15. Li MO, Wan YY, Sanjabi S, Robertson AK and Flavell RA: Transforming growth factor-β regulation of immune responses. Annu Rev Immunol 24: 99-146, 2006.
16. Nelson WG, Simons JW, Mikhak B, *et al*: Cancer cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer as vaccines for the treatment of genitourinary malignancies. Cancer Chemother Pharmacol 46 (Suppl): S67-S72, 2000.
17. Salgia R, Lynch T, Skarin A, *et al*: Vaccination with irradiated autologous tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor augments antitumor immunity in some patients with metastatic non-small-cell lung carcinoma. J Clin Oncol 21: 624-630, 2003.
18. Lutz E, Yeo CJ, Lillemoe KD, *et al*: A lethally irradiated allogeneic granulocyte-macrophage colony stimulating factor-secreting tumor vaccine for pancreatic adenocarcinoma. A Phase II trial of safety, efficacy, and immune activation. Ann Surg 253: 328-335, 2011.
19. Cheng P, Li Y, Yang L, *et al*: Hepatitis B virus X protein (HBx) induces G2/M arrest and apoptosis through sustained activation of cyclin B1-CDK1 kinase. Oncol Rep 22: 1101-1107, 2009.
20. Kong G, Zhang J, Zhang S, Shan C, Ye L and Zhang X: Hepatitis B virus X protein promotes hepatoma cell proliferation via upregulation of MEKK2. Acta Pharmacol Sin 32: 1173-1180, 2011.
21. Godoy-Ramirez K, Mäkitalo B, Thorstensson R, Sandström E, Biberfeld G and Gaines H: A novel assay for assessment of HIV-specific cytotoxicity by multiparameter flow cytometry. Cytometry A 68: 71-80, 2005.
22. Hao S, Li P, Zhao J, Hu Y and Hou Y: 17β-estradiol suppresses cytotoxicity and proliferative capacity of murine splenic NK1.1^+ cells. Cell Mol Immunol 5: 357-364, 2008.
23. Lee YI, Kang-Park S, Do SI and Lee YI: The hepatitis B virus-X protein activates a phosphatidylinositol 3-kinase-dependent survival signaling cascade. J Biol Chem 276: 16969-16977, 2001.
24. Shan C, Xu F, Zhang S, *et al*: Hepatitis B virus X protein promotes liver cell proliferation via a positive cascade loop involving arachidonic acid metabolism and p-ERK1/2. Cell Res 20: 563-575, 2010.
25. Ou DP, Tao YM, Tang FQ and Yang LY: The hepatitis B virus X protein promotes hepatocellular carcinoma metastasis by upregulation of matrix metalloproteinases. Int J Cancer 120: 1208-1214, 2007.
26. Gearhart TL and Bouchard MJ: The hepatitis B virus X protein modulates hepatocyte proliferation pathways to stimulate viral replication. J Virol 84: 2675-2686, 2010.
27. Neering SJ, Hardy SF, Minamoto D, Spratt SK and Jordan CT: Transduction of primitive human hematopoietic cells with recombinant adenovirus vectors. Blood 88: 1147-1155, 1996.
28. Nilsson M, Ljungberg J, Richter J, *et al*: Development of an adenoviral vector system with adenovirus serotype 35 tropism; efficient transient gene transfer into primary malignant hematopoietic cells. J Gene Med 6: 631-641, 2004.
29. Tatsis N and Ertl HC: Adenoviruses as vaccine vectors. Mol Ther 10: 616-629, 2004.
30. Pichla-Gollon SL, Lin SW, Hensley SE, *et al*: Effect of preexisting immunity on an adenovirus vaccine vector: in vitro neutralization assays fail to predict inhibition by antiviral antibody in vivo. J Virol 83: 5567-5573, 2009.
31. Vatakis DN, Koya RC, Nixon CC, *et al*: Antitumor activity from antigen-specific CD8 T cells generated in vivo from genetically engineered human hematopoietic stem cells. Proc Natl Acad Sci USA 108: E1408-E1416, 2011.
32. Martorelli D, Muraro E, Merlo A, Turrini R, Rosato A and Dolcetti R: Role of $CD4^+$ cytotoxic T lymphocytes in the control of viral diseases and cancer. Int Rev Immunol 29: 371-402, 2010.
33. Tang H, Da L, Mao Y, *et al*: Hepatitis B virus X protein sensitizes cells to starvation-induced autophagy via up-regulation of beclin 1 expression. Hepatology 49: 60-71, 2009.
34. Sir D, Tian Y, Chen WL, Ann DK, Yen TS and Ou JH: The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. Proc Natl Acad Sci USA 107: 4383-4388, 2010.
35. Crotzer VL and Blum JS: Autophagy and adaptive immunity. Immunology 131: 9-17, 2010.
36. Münz C: Antigen processing via autophagy - not only for MHC class II presentation anymore? Curr Opin Immunol 22: 89-93, 2010.