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

YINGYING YAN1,2*, NING LIU1*, LIAN LU1, CHUAN-MOU ZANG1, BIN SHAO1, YUHUA LI1, YANJUN WEN1, YUQUAN WEI1 and PING CHENG1

1State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School; 2School of Life Sciences, Sichuan University, Chengdu, Sichuan 610041, P.R. China

Received February 11, 2013; Accepted April 24, 2013

DOI: 10.3892/or.2013.2531

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 cytolitic 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.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor in the world (1). Clinically, the majority of HCC cases are incompletely 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 hepatavirus 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 have 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. 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

Correspondence to: Dr Ping Cheng, State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, 17 People’s South Road, Chengdu, Sichuan 610041, P.R. China
E-mail: ping.cheng@foxmail.com

*Contributed equally

Key words: hepatocellular carcinoma, hepatitis B virus X protein, vaccine, autophagy
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% CO2 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 plaque-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'-ATGGGTGTCTAGGCTGTGCTG-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 1x10⁶ 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 (2.5x10⁶) 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 1x10⁶ 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 (1x10⁶) 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% CO2. 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 2x10⁵ 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% CO₂ 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.

**Electron microscopy.** The cellular ultrastructural analysis by electron microscopy was performed as previously described. Hepa-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% OsO₄, 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.

**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 Hepa-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 Hepa1-6 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 mm² 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 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 Hepa-6/AdHBx-treated mice was observed in the growth curve (P<0.001).*
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.
elicit HBx-specific CTL responses, spleen lymphocytes were vaccinated of irradiated Hepa1-6 infected with AdHBx 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 or 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 10⁶ 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 Hepa1-6/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. These results suggested that autophagy was induced in the AdHBx-infected cells treated with X-ray irradiation.

**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

**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 cells that divided at least once and the average number of cell divisions were 80.4% and 1.73 respectively for lymphocytes of cells that divided at least once and the average number of IFN-γ-secreting T cells is represented by the bars (’P<0.001).

**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 Hepa1-6 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.
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 vaccine.
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

This study was supported by the National Natural Science Foundation of China (grant no. 81101728), the National Science and Technology Major Projects of New Drugs (grant no. 2012ZX09103301-036), the National Science and Technology Major Project for Infectious Diseases Control (grant no. 2012ZX10002014-004) and the Research Fund for the Doctoral Program of Higher Education of China (grant no. 20101018120086).

References