Anti-tumor effects of immunotherapeutic peptide on the treatment of hepatocellular carcinoma with HBc carrier

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Abstract. Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death. Tumor specific cellular and humoral immunotherapy may be a viable approach for the treatment of HCC. This study investigated specific inhibitory and cytotoxic effects on hepatocellular carcinoma (HCC) induced by the peptide, designated HBcΔ-5L, using HBc carrier with multiple T cell and B cell sequence insertions. We developed the HBcΔ carrier containing insertions of multiple CTL and T helper (Th) epitopes, which were selected from HCC tumor associated antigens (TAAs) including α fetoprotein (AFP), melanoma antigen gene (MAGE) and telomerase reverse transcriptase (TERT) antigen, and ligands for EGFR and IGFR, designated HBcΔ-5L. LDH release assay and IFN-γ ELISPOT assay were carried to determine whether HBcΔ-5L could induce specific cytotoxicity in peripheral blood mononuclear cells (PBMC) of HCC donors. The levels of antibodies and inhibitory effects of sera of immunized mice against HBcΔ-5L were also identified. LDH release assay revealed that PBMC from HCC donor group (n=8) stimulated with HBcΔ-5L could specifically kill target tumor cells and specific lysis was 62.7% (E:T=60:1). ELISPOT assay showed a significant increase in secretion of IFN-γ from PBMC of HCC donor group in response to HBcΔ-5L. Further, high specific antibody titers were elicited in immunized mice and revealed 42% inhibition of cell growth. These results indicated that inhibitory and cytotoxic effects could be efficiently induced by HBcΔ-5L recombinant particles using HBcΔ as carrier and suggested that it could be important in design of immunotherapeutic approaches.

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Introduction

With an estimated incidence of about one million cases, hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. The prevalence is >100 per million population in Southeast Asia, especially in China where hepatitis B virus infection is endemic, and some places in Africa, whereas in Europe and North America it is estimated as 2-4 per million population (1,2). Curative treatment can be offered only in a minority of cases and is limited to surgical resection or transplant, which are effective only in localized tumors (3). Alternative options such as percutaneous intratumoral ethanol injection, transarterial chemoembolization and radiofrequency thermal ablation are reserved for palliation (3,4). Therefore, identifying and establishing alternative approaches for treatment of HCC is quiet a challenge and of high interest. One approach that has shown promising results in other tumors is immunotherapy (5). Also in HCC, induction of specific cellular and humoral immunity are the focus of recent anti-tumor immunotherapy.

We investigated the possibility of truncated hepatitis B virus core (HBcΔ) (aa 1-149) as carrier for the construction of active peptide eliciting specific inhibitory and cytotoxic effects on HCC. It is well known that the carboxy-terminal region (aa 150-183) of the HBc responsible for nucleic acid binding can be deleted without disturbing the formation of HBc particles (6). In addition, we have successfully constructed a DNA vaccine against *Taenia solium* cysticercosis using HBcΔ as carrier (7). HBcΔ carrier is capable of ensuring a high level of B cell and T cell immunogenicity to foreign epitopes (8). In addition to the ability of the HBcΔ carrier moiety to provide T cell help to inserted sequences, the HBcΔ capsid mediates the T-cell-independent character of the humoral response to inserted epitopes, due to the high degree of repetitiveness of the epitopes and the proper spacing between them (9).

Recent progress in the molecular identification of tumor associated antigens (TAAs) and understanding the mechanisms of TAA presentation and their recognition by T lymphocytes provide a valuable and promising basis for immunotherapeutic interventions. Based on the above, we chose HBcΔ particles as carrier with internal and C-terminal insertions for engineering a synthetic multiple active peptide immunogen

including the following HCC TAA epitopes and growth factor receptor ligands.

α-fetoprotein (AFP) is found in ~80% of the HCC and is a potential target for immunotherapy in HCC. Both the murine and human T-cell repertoires can recognize AFP-derived epitopes in the context of the MHC I (10). We determined the identity of AFP-derived epitopes, presented in the context of HLA-A*0201, that could be recognized by the human T cell repertoire. The epitopes are $hAFP_{137-145}$ (PLFQVPEPV), $hAFP_{158-166}$ (FMNKFIYEI), $hAFP_{325-334}$ (GLSPNLNRFL) and $hAFP_{542-550}$ (GVALQTMKQ) which can consistently elicit cytokine production and cytotoxicity (11).

Melanoma antigen gene-A (MAGE-A) is a multi-gene family that consists of 12 homologous genes (MAGE-A1 to -A12). Individual MAGE-A expression varies from one tumor type to the other, but overall the large majority of tumors expresses at least one MAGE-A. Zerbini *et al* (12) reported the presence of tumor Ag-specific $CD8^+$ T cells (specific for MAGE-A1 and -A3) in HCC. So we chose a CTL epitope (p248V9:YLEYRQVPV) which could generate an HLA-A*0201-restricted CTL response and shared by MAGE-A1, -A2, -A3, -A4, -A6, -A10 and -A12 tumor antigens (13). To achieve a better result, we also chose another HLA-A1 restricted CTL epitope $MAGE_{161-169}$ (EADPTGHSY) (14).

Human telomerase reverse transcriptase (TERT) is a widely expressed TAA. Furthermore, its expression has been directly linked to tumor development such that inhibition of telomerase in telomerase-positive human tumors leads to growth arrest (15,16). So we chose DR4, DR11 and DR15 restricted $TERT_{766}$ (LTDLQPYMRQFVAHL) T help epitope (17) and TERT-derived HLA-A3 restricted CTL epitope (K973:KLFGVLRLK) (18).

It is evident that the level of growth factor expression is a determinant of tumor proliferation, invasiveness, and angiogenesis, and relates adversely to prognosis. Therefore, blockade of autocrine loops of growth factor is a rational strategy for the treatment of many malignancies (19,20). Ren *et al* (21,22), reported that E5 (EPFRS PDLAL ETYG) (the ligand for type I and II insulin-like growth factor receptors) and GE7 (NPVVG YIGER PQYRD L) (the ligand for epidermal growth factor receptor) and tested their roles in targeting tumor cells that overexpress them. In this report, we chose E5 and GE7 to induce the anti-tumor humoral immunity.

Therefore, to be effective, they should preferably be used in combination for the therapy of HCC. And because tumor cells might lose one or more TAA epitopes, we chose multiple epitopes of each antigen to increase the possibility of targeting. We described the engineering of a synthetic multiple active peptide immunogen and investigated its anti-tumor effects through induction of cellular and humoral immunity against HCC cells.

Materials and methods

Preparation of peripheral blood mononuclear cells from healthy volunteers and cancer patients. 8 healthy volunteers (6 male and 2 female, mean age 32.5-years) were enrolled in this study along with 8 HBV negative HCC patients (6 male and 2 female, mean age 49-years) were final diagnosis using operation pathology, cell puncture, symptom, physical signs, AFP, enhance CT, magnetic resonance imaging (MRI) and arteriography. Patients were classified as 2 stage I, 5 stage II and 1 stage III. Informed consent was obtained from all patients and donors. Peripheral blood mononuclear cells (PBMC) were isolated from donors by Ficoll-Hypaque density gradient centrifugation.

Animals. Six-week-old female BALB/c mice were used in the experiment. They were kept under specific pathogen-free conditions with free access to a standard commercial diet. And all animal procedures were performed according to approved protocols and in accordance with recommendations for proper care and use of laboratory animals.

Cell culture. Human HCC cell line SMMC-7721 cells (AFP^+, $MAGE\text{-}A^+$, $TERT^+$) (23-25) and human normal liver cells L-02 were purchased from Nanjing Keygentec Co. (China) and cultured at 37˚C in a humidified 5% CO_2 atmosphere in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/ml of penicillin and 100 μg/ml of streptomycin.

Construction, expression and purification of four recombinant proteins in E. coli cells. HBcΔ DNA was isolated from serum of one chronic active hepatitis patient. The primers used for PCR were designed according to the HBV sequence subtype adw2 (GenBank no. AY707087). P1: 5' GA TAT ACC ATG GAC ATT GAC C 3' and P2: 5' G TTG TTA AGC TTA AAC AAC AGT AGT TTC CGG AAG 3'. P1 and P2 and two other primers P3: 5' TGG GGA GAA TTC TGG ATC CTC CAA GTT ATT ACC C 3' and P4: 5' GAT CCA GAA TTC TCC CCA GCA TCT AGG GAT CTA G 3' were used to introduce *Bam*HI and *Eco*RI restricted sites between aa 78-79 of HBcΔ. The fragment of epitopes of MAGE and TERT and two ligands and another fragment of epitopes of AFP were inserted into the MIR and C-terminus of HBcΔ carrier, respectively, designated HBcΔ-5L. Then three fragments of epitopes of MAGE, TERT and AFP were inserted into the same sites of HBcΔ-5L, respectively, termed HBcΔ-MAGE, HBcΔ-TERT and HBcΔ-AFP. The schemes of four recombinant proteins are illustrated in Fig. 1. The expression and purification of recombinant proteins were generated as previously described (7).

Electron microscopy. To detect the particle formation ability of purified protein (HBcΔ-5L), 0.4 g/l HBcΔ-5L protein was adsorbed onto carbon-coated copper 400-mesh electron microscopy grids for 2-3 min, and negatively stained with 20 g/l uranyl acetate. Grids were examined with a Hitachi H 7650 transmission electron microscope (TEM) at 80 kV.

PBMC culture. Dendritic cells (DCs) were generated as described with some modifications (26). Briefly, PBMC were cultured in RPMI-1640 medium containing 10% FCS for 2 h at 37˚C. Adherent cells were cultured for 6 days in RPMI-1640 medium containing 10% FCS, 1000 U/ml human GM-CSF (Peprotech Inc., NJ, USA) and 500 U/ml human IL-4 (Peprotech Inc.). Culture medium and cytokines were refreshed every other day. On day 7 of culture, 10 ng/ml tumor necrosis factor-α (TNF-α, Peprotech Inc.) was added for the maturation of DCs.

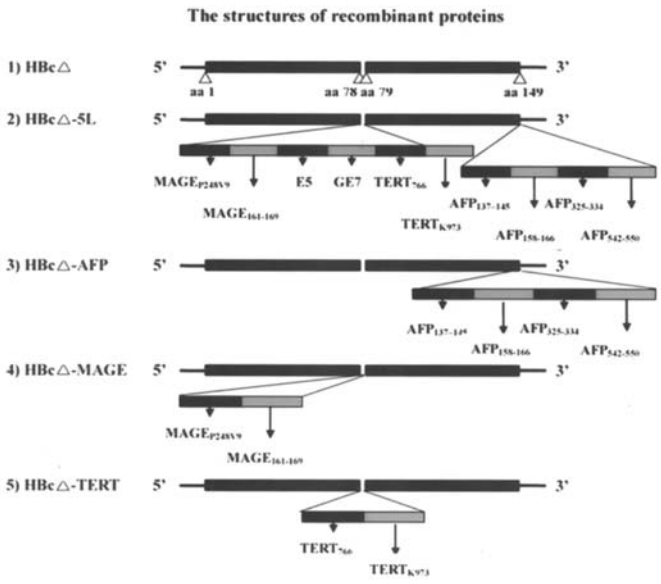

Figure 1. Scheme of representation of the structure of 4 recombinant proteins.

After 72 h maturation, autologous DCs were pulsed with peptide for 3 h. PBMC from donors were plated at a concentration of $5x10^6$ cells per well in a 24-well culture plate and stimulated with peptide at a concentration of 20 μg/ml on day 0 and with $1x10^7$ peptide-pulsed autologous DCs on day 7. rhIL-2 (100 IU/ml) (Chiron, Emeryville, CA, USA) was added to the culture every other day and the cells were cultured for 14 days.

Detection of peptide-specific CTL in vitro. Target cell lysis was determined using a lactate dehydrogenase (LDH)-release assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Two-week peptide-sensitized PBMC were used as effector cells and SMMC-7721 was used as target cells in the cytotoxicity assay. And L-02 cells were used as control. Briefly, target cells ($5x10^4$ cells/well) were cocultured with effector cells at different ratios of 1:15, 1:30 and 1:60 in a 96-well round-bottomed culture plate. Each experiment was carried out in triplicate and at least twice, and the average value was used to determine the percentage of viability. The percentage of target cell lysis was calculated by using the following formula: Percent cytotoxicity = (Experimental - Culture medium background)/(Maximum LDH release - Culture medium background) x 100. Maximum LDH release obtained by adding lysis solution (90 g/l Triton X-100) at the recommended dilution to target cells.

IFN-γ ELISPOT assay. Peptide-specific reactivity of PBMC was assessed by standard IFN-γ ELISPOT (MABtech, Nacha, Sweden). Briefly, a 96-well filter plate was precoated with 1.5 μg/well anti-human IFN-γ monoclonal antibody. Two-week peptide-sensitized PBMC ($1x10^4$/well) as effector cells and irradiated SMMC-7721 cells ($5x10^3$/well) as target cells were transferred to ELISPOT plate and incubated for 16 h at 37˚C. L-02 cells were used as control. The plates were then washed and incubated with 100 μl 1 μg/ml biotin labeling antibody and then conjugated with 100 μl second alkaline phosphatase labeled antibody (1:1000 dilution). NBT/BCIP-plus reagent (100 μl) was then used to colorize bound IFN-γ, forming visualized spots. The colored spots, representing cytokine producing cells, were counted under a dissecting microscope.

Serologic test. Six-week-old BALB/c mice were immunized subcutaneouly on week 0, 3 and 6, with 10 μg of HBcΔ-5L or 10 μg of HBcΔ or 100 μl PBS (n=10). On week 3, 5, 7 and 9, blood samples were collected from mice and stored at -20˚C for further assay. The levels of specific antibodies of sera of immunized mice against HBcΔ-5L were identified by sandwich ELISA. Briefly, 96-well plates were prepared with IGF-I (0.1 μg/well). Sera samples of mice were added and incubated on the plates at 37˚C for 1 h at 2-fold dilution. Goat anti-mouse HRP-labelled IgG (Sigma) were added at 37˚C for 1 h. And the plates were subsequently detected by TMB substrate. Absorbance was measured with a plate reader at 450 nm. An OD value ≥2.1 times that of negative control was considered positive. To detect the specificity of antibody, sera samples were incubated with HBcAb-HRP and HBeAb-HRP on HBc coated plates at 37˚C for 1 h and detected by TMB substrate by competitive ELISA. All assays were performed in duplicate. The wells with only added HBcAb-HRP or HBeAb-HRP were negative control. Inhibitory rate (%) = (negative control A_{450}-test A_{450})/negative control A_{450} x 100. Inhibitory rate ≥50% was considered positive.

MTT assay. SMMC-7721 cells were plated in 24-well plates with $2x10^4$/well in regular growth medium. Then cells were switched to serum-free media for 24 h, the incubation was continued in the absence (control) or presence of anti-HBcΔ-5L or anti-HBcΔ sera (1:200 dilution) at 37˚C for 12, 24, 48 and 72 h. In some experiments, growth was induced by IGF-I (10 ng/ml) in the absence or presence of sera at 37˚C for 24 h. L-02 cells were used as control. All treatments were done in triplicates. Growth was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (27). In all experiments, the reaction was terminated by adding a total of 60 μl of 5 mg/ml stock of MTT solution in PBS to each well. After incubation for 4 h at 37˚C, wells were aspirated and formazan crystals were lysed with 200 μl DMSO. Absorbance was measured with a plate reader at 570 nm using a 670-nm differential filter. Inhibitory rate (%) = (1-test A_{570}/control A_{570}) x 100.

Statistical analysis. All results were expressed as mean ± SD. Statistical differences between groups were determined by using Student's t-test. Differences were considered statistically when $P<0.05$.

Results

Expression and purification and identification of recombinant proteins in E. coli. Verification of pET-HBcΔ-5L, pET-HBcΔ-AFP, pET-HBcΔ-MAGE and pET-HBcΔ-TERT constructs was done by enzyme restricting and sequencing analyses. The schemes of four recombinant proteins are illustrated in Fig. 1. *E. coli* BL21 (DE3) cells harboring the plasmids expressing four recombinant proteins were stimulated by incubation with IPTG for 5 h. Cells were harvested, and lyzed in SDS sample buffer and examined for the production

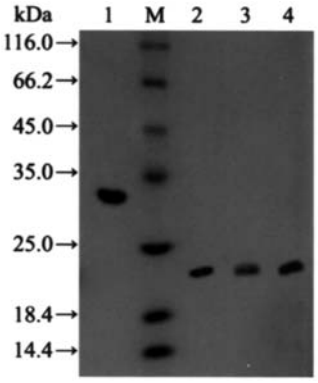

Figure 2. SDS-PAGE analysis of the purified 4 recombinant proteins. Proteins were separated by 12% SDS-PAGE gel and revealed by coomassie brilliant blue staining. Lane 1, purified protein HBcΔ-5L; lane 2, purified protein HBcΔ-AFP; lane 3, purified protein HBcΔ-MAGE; lane 4, purified protein HBcΔ-TERT; lane M, protein molecular weight markers.

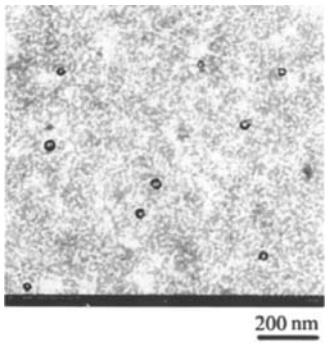

Figure 3. Electron microscopic image of particles formed by HBcΔ-5L. The particles were negatively stained with 20 g/l uranyl acetate. HBcΔ-5L particles appear as uniform particles with a diameter of 30 nm. Scale bar, 200 nm.

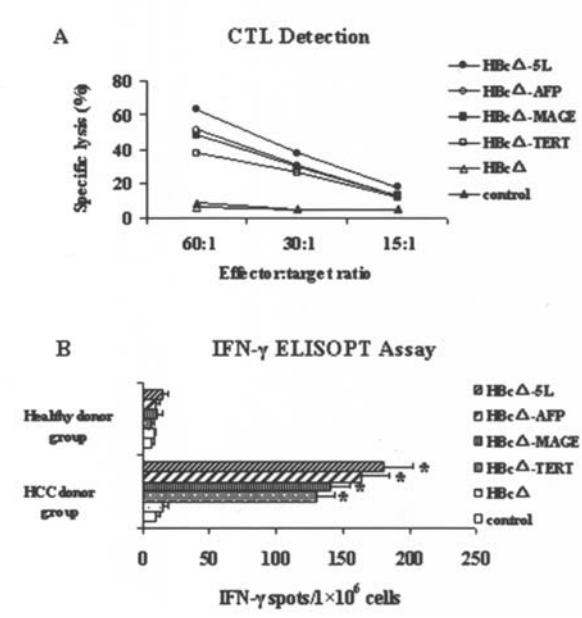

Figure 4. HBcΔ-5L induction of specific CTL responses and the production of IFN-γ. (A) PBMC from HCC donors group had cytotoxic activity against target cells (SMMC-7721) endogenously expressing the three antigens. Expanded effector PBMC were co-cultured at the indicated effector:target (E:T) cell ratio. Target cell lysis was quantitated using an LDH release assay. Entries represente mean values of three replicate cytotoxicity assay cultures. (B) IFN-γ ELISPOT assay. IFN-γ secreting PBMC from donors were measured using an ELISPOT assay after re-stimulation *in vitro* with target cells. The mean number of spot-forming cells per 10^6 cells is shown. Results are given as means ± SD. *P<0.05 vs. control.

of aimed proteins on SDS-PAGE (Fig. 2). Recombinant proteins were observed at 30 kDa (HBcΔ-5L), 22 kDa (HBcΔ-AFP), 21 kDa (HBcΔ-MAGE) and 21 kDa (HBcΔ-MAGE). After purification as described previously (7), the purified protein migrated as a single protein band on SDS-PAGE and possessed a high degree of purity.

The electron microscope image of HBcΔ-5L particles. The fine structures of HBcΔ particles which could accept long-length insertion and undergo correct folding were revealed by electron microscopy (Fig. 3). When HBcΔ-5L was analyzed by TEM, the presence of spherical particles with uniform morphology and size distribution were observed. The mean diameter of particles was 30 nm.

Detection of expression of three HCC TAAs of HBcΔ-5L. To detect the expression of three HCC TAAs (AFP, MAGE and TERT), PBMC from HCC patients (n=8) were stimulated *in vitro* with three recombinant proteins (HBcΔ-AFP, HBcΔ-MAGE, HBcΔ-TERT), respectively. Following stimulation, effector cells (PBMC) were co-cultured with target cells (SMMC-7721) (AFP^+, MAGE-A^+, $TERT^+$) by measurement of the release of LDH. LDH release assay indicated that these three proteins can induce PBMC from HCC donors of specific cytotoxicity on target cells (Fig. 4A). The ELISPOT assay revealed that the activated PBMC from HCC donor were markedly increased in contrast to control (P<0.05) (Fig. 4B).

Tumor-specific CTL activity and IFN-γ ELISPOT assay stimulated by HBcΔ-5L. To determine whether the CTL epitopes of protein HBcΔ-5L could induce a specific CTL response, PBMC from HCC patients (n=8) were stimulated *in vitro* with HBcΔ-5L protein. Following stimulation, cells were seeded at 60:1, 30:1 and 15:1 E/T ratios by measure of the release of LDH (Promega). The data in Fig. 4A show the cytotoxic activities generated in PBMC after restimulation with HBcΔ-5L. LDH release and target cell lysis by cytotoxic T cells demonstrated a significant correlation. This assay demonstrated only low level (5% or less) of PBMC of healthy donor CTL activation. This finding was likely due to a spontaneous leakage of granular contents from a minor population of activated PBMC. Taken together, these results established the HBcΔ-5L protein induced potent specific cytotoxic T cell responses in PBMC of the HCC donor group.

To determine the frequency of IFN-γ-secreting PBMC that were capable of responding to a TAA, we performed an IFN-γ ELISPOT assay (Fig. 4B). The number of IFN-γ positive spots of HCC patient group were 180±22 spots per 10^6 cells in the HBcΔ-5L group, compared to 15±4 spots per 10^6 cells in the healthy donor group (P<0.05).

HBcΔ-5L elicited mice generating specific antibodies. To identify the levels of antibodies against HBcΔ-5L after vacci-

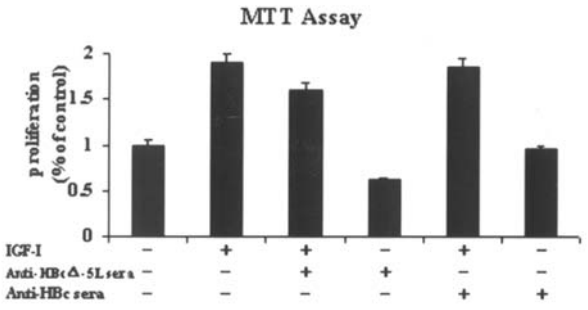

Figure 5. Effects of sera from HBcΔ-5L and HBcΔ carrier vaccination groups on the proliferation of SMMC-7721 cells. Cells were incubated with 10 ng/ ml IGF-I in the absence or presence of sera for 24 h. Control incubations contained neither IGF-I nor sera. Cellular growth was determined by MTT assay.

nation, sera from immunized mice were detected by ELISA. Results show that the positive rate of anti-IGF-I antibodies from sera of HBcΔ-5L vaccination group was 100%. Specific antibody titers increased along with vaccination, and the peak titer ($1:10^5$) was on week 7 then declined in week 9. The titer of sera of HBcΔ control group was 1:200 ($P<0.05$, contrast to HBcΔ-5L vaccination group). HBcAb and HBeAb titers (1:256-1:1024) were determined in sera of mice immunized with HBcΔ-5L by ELISA and were an order of magnitude lower than those obtained in mice immunized with HBcΔ carrier ($1:10^6$) ($P<0.05$). Sera from PBS control mice did not show reactivity against IGF-I and HBc (less 1:200).

Anti-HBcΔ-5L sera inhibited the proliferation of SMMC-7721 cells. To elucidate the mechanism of action of sera from HBcΔ-5L vaccination group on the induction of terminal differentiation, we examined, by MTT assay, the effect on cell growth without additional IGF-I (Fig. 5). Sera from HBcΔ-5L vaccination group (1:200 dilution) exhibited ~42% inhibition of SMMC-7721 proliferation at 24 h and remained at that level during the rest of the experimental period (72 h), and had little inhibitory effects on L-02 cells (data not shown). To determine whether sera from HBcΔ-5L vaccination group could inhibit IGF-I-induced growth of SMMC-7721 cells, 24 h serum-starved cells were incubated with sera in the presence of 10 ng/ml IGF-I for 24 h. As expected, IGF-I caused 2-fold stimulation of growth of SMMC-7721 cells over the controls. Interestingly, this stimulation was indeed attenuated by sera (1:200 dilution). In fact, in the presence of sera, there was ~60% proliferation of cell growth compared with the controls. Control sera against HBcΔ carrier had little inhibitory effect against SMMC-7721 or L-02 cells.

Discussion

In this study, we constructed the HBcΔ carrier, to which multiple CTL and Th epitopes of HCC TAAs and ligands for EGFR and IGFR was inserted, designed HBcΔ-5L, targeting cellular and humoral immune responses and inducing anti-tumor effect against HCC cells. The recombinant HBc particles as carrier have been used to display immuno-dominant epitopes of virus, bacterial as well as protozoan protein (28-30), but no data are available of TAAs. Anti-tumor immunotherapeutic applicability of the HBc particle as carrier has not been reported until now.

Herein, we showed for the first time the HBc particle as carrier to display multiple HCC TAAs eliciting robust cellular and humoral tumor-specific immune response. Enhancement of the natural immune response was demonstrated by *in vitro* cell-mediated cytotoxicity assays. Firstly, we tested specific responses against tumor cells of three recombinant proteins (HBcΔ-AFP, HBcΔ-MAGE, HBcΔ-TERT) inducing PBMC from HCC donor. Cytotoxic assays showed the ability of HBcΔ-5L to induce potent CTL reactions of PBMC of HCC donor group *in vitro* (E:T=60:1, 62.7% specific lysis). This was supported in our study by the observations that HBcΔ-5L significantly enhanced IFN-γ production and had the ability to induce potent anti-tumor responses. Sera from immunized mice were used to evaluate the immunogenicity of HBcΔ-5L, its specific antibody titer was extremely high (up to $1:10^5$). These results suggested that HBcΔ-5L induced specific anti-tumor cellular and humoral immune responses and supported its potential application in the clinical setting.

Our results pointed out special applications of the HBc particle as the TAAs epitope carrier. Important practical advantage of the HBc model consisted in the fact that chimeric HBc-derived particles were easy to purity. Our study used denatured acrylamide gel purification, making dissociation with subsequent re-association easier. Additionally, the use of HBc particle as carrier could prove capable of ensuring a high level of B cell and T cell foreign epitope immunogenicity. For example, our previous data indicated that HBc particle was effective in construction of the DNA vaccine against *Taenia solium* cysticercosis, the protective rate of this vaccine was exciting (7). The use of the HBc carrier may provide both T-cell-dependent and T-cell-independent character of the immune responses to inserted epitopes (31). It is important to note that the aimed protein HBcΔ-5L was successfully expressed in *E. coli* and re-associated mosaic particles (Fig. 3). Foreign peptides aligned with the selected sites of HBcΔ particles. High-density display of the foreign peptides played an extremely important role in inducing immune responses. In addition, insertion of foreign sequences internally within HBc, at MIR, which had previously been suggested to represent a dominant antibody binding region, drastically decreased the antigenicity and immunogenicity of HBc (32). Therefore, HBcΔ-5L could also induce promising effects in future clinical trials even in HBc positive HCC patients.

LDH release assay showed that the specific cytotoxic activities generated in PBMC after restimulation with HBcΔ-5L. LDH release assay is commonly used for testing cytotoxicity of various experimental compounds by measurement of the leakage of components from the cytoplasm into the surrounding culture medium. This technology has been demonstrated to be identical to values determined in parallel ^{51}Cr release assays (33,34). Because IFN-γ has been recognized as a critical cytokine involved in effective anti-tumor immune responses, the production of this cytokine in response to epitope-specific stimulation is critical for T cell-mediated tumor regression (35,36). We used IFN-γ ELISPOT assay to detect the activated PBMC. In

contrast to the HBc carrier, re-stimulated with HBcΔ-5L, IFN-γ secretion was significantly enhanced.

Because the combination of cellular and humoral immune responses has better protection from tumor challenge, where the absence of either the cellular or humoral arm leads to incomplete protection (37,38). We extended our studies to an analysis of humoral immune responses. Data demonstrated that protein HBcΔ-5L was capable of eliciting specific antibodies in immunized mice. For analysis of anti-HBcΔ-5L sera inhibitory effects on the proliferation of HCC cells, cells were incubated with sera in the presence of IGF-I. Interestingly, this stimulation was indeed attenuated by sera. The results confirmed that specific antibodies against ligands elicited by HBcΔ-5L had an inhibitory role on HCC cells.

In conclusion, our data indicate an application of HBc particle as carrier in an immunotherapeutic approache against HCC. We showed that the polyepitopic peptide HBcΔ-5L had the ability to induce tumor-specific humoral and cellular immune responses. The recombinant HBc particles may prove to be a suitable carrier on the display of TAAs. The clinical benefit remains to be established in future clinical trials. This study established the foundation for further optimization and refinements of the therapy strategy of cellular and humoral combination immunity with the ultimate goal of developing therapeutic methods for HCC patients.

Acknowledgements

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