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Immunotherapy of hepatocellular carcinoma with a vaccine based on xenogeneic homologous α fetoprotein in mice

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ABSTRACT

α-Fetoprotein (AFP) is a diagnostic marker for the presence of hepatocellular carcinoma, and a potential target for immunotherapy. Unfortunately, the immunity to AFP is presumably difficult to elicit because of immune tolerance acquired during the development of immune system. In the present study, we used AFP as a model antigen to explore the feasibility of the immunotherapy of AFP-positive liver cancer by the breaking of immune tolerance against AFP in a cross-reaction between the xenogeneic homologues and self molecules. Recombinant rat AFP was prepared as a vaccine, and mouse AFP was prepared as a control. Immunized with rat AFP was effective at protective and therapeutic antitumor immunity in hepatocellular carcinoma model in mice. Both humoral and cellular immune responses may be responsible for the antitumor activity against AFP-positive tumor cells, and no marked side effects were observed in the immunized mice. Thus, our study may provide an effective vaccine strategy for the treatment of AFP-positive hepatocellular carcinoma, and may be of importance to further exploration of the breaking of immune tolerance to self molecules.

Hepatocellular carcinoma (HCC) is one of the most common malignances worldwide with more than 1.2 million global annual incidences [1,2]. Liver resection, transplantation and percutaneous ablation are potentially curative treatments and resulted in 5-year survival rates higher than 50%. In the West and Asia, these treatments can be applied to 30% of patients with early stage and well-preserved liver function. However, most HCC patients are diagnosed at advanced stages that preclude the optimum use of those radical treatments, who can only receive palliative treatments which have very poor survival advantages [1,2]. Clearly, there is a pressing need for developing novel strategies for the non-surgical HCC population.

A hopeful non-surgical approach against HCC is the immune-based therapy. Serum α-fetoprotein (AFP) is a diagnostic marker for the presence of HCC, and an obvious choice of a protein target made by HCC cells [2,3]. Although AFP is secreted, its peptides are processed by the cell and can be presented to both $CD8^+$ and $CD4^+$ T cells [2–4]. It is conceivable that the breaking of immune tolerance against AFP should be a useful approach for HCC therapy with active immunity. Unfortunately, the immunity to AFP is presumably difficult to elicit because of immune tolerance acquired during the development of the immune system [5].

Many genes were highly conserved during the evolutionary process, which is characterized by the gene similarity among different species [6,7]. Sequence comparison analysis by searching Swissport database in the National Center for Biotechnology Information (NCBI) shows that the primary sequence of rat AFP (rAFP) at the amino acid level is 88% identical with mouse homologue (mAFP). The current study explores the feasibility of immunotherapy of AFP-positive HCC with the xenogeneic AFP protein as a vaccine by the breaking of the immune tolerance against AFP in a cross-reaction between the xenogeneic homologues and self molecules. We found that the xenogeneic protein vaccine was effective in both protective and therapeutic antitumor activity, in which autoreactive immunity may be directed against the AFP-positive tumor cells in both humoral and cellular immunity. These results may provide a new vaccine strategy for active immunotherapy by the breaking of the immune tolerance against AFP in a cross-reaction way.

Materials and methods

Cell lines. Hepa1-6 mouse HCC cells and McArdle RH-7777 (McA) rat HCC cells were obtained from American Type Culture Collection (ATCC) and grew in RPMI medium 1640 with 10% FBS.

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These tumor cell cultures were maintained in a 37 °C incubator with a humidified 5% CO_2 atmosphere.

Vaccine preparing. Cultured cells including Hepa1-6 and McA were harvested and total RNA was isolated by using TRIzol reagent (Invitrogen), respectively. Each of the RNA was subjected to reverse transcription-polymerase chain reaction for amplification of the full length of mouse and rat AFP. The primers for the amplified fragment of mAFP were 5′-CGCGAGCTCAAAGCATTGCACGAAAAT GAG-3′ and 5′-GACGGTCGACGAGATGTTTAAACGCCCAA-3′. The primers for that of rAFP were 5′-CGGGTACCAGAGTACTGCACACAA ATGAG-3′ and 5′-AACTGTCGACTCCTGGAGATGTTTAAACCCC-3′. Amplified products were then subcloned into pET-32a(+) (Novagen) and were named pET-mAFP and pET-rAFP, respectively. The pET-mAFP and pET-rAFP were then used to transform *Escherichia coli* BL21DE(3) and RossetaDE(3) strains (Novagen), respectively. Transformants were cultured for recombinant protein expression by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. The proteins were purified from the bacterial lysates by affinity chromatography on a nickel-nitrilotriacetic acid gel matrix (Qiagen) under denaturing conditions, and elution was achieved with the buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, and 180 mM imidazole, pH 8). After refolding by extensively dialysis, the proteins were further purified by ion exchange and gel filtration chromatography to eliminate contaminants, and the fusion Trx-tag were removed by enterokinase (Novagen). Proteins were confirmed by SDS–PAGE and Western blot analysis, and endotoxin was removed by using ProteoSpin™ Endotoxin Removal Micro Kit (Norgenbiotek). Finally, the purified proteins were dissolved in pyrogen-free 0.9% saline before use for vaccination and were mixed with complete or incomplete Freund's adjuvant, as described [5].

Tumor models and immunization. Hepa1-6 HCC tumor model was established in C57BL/6 mice. Proteins (75 μg per injection) were emulsified 1:1 (volume/volume) with complete Freund's adjuvant (CFA) for the first immunization, followed by a boost in incomplete Freund's adjuvant (IFA) at 2 weeks and weekly thereafter, as described previously [6]. Tumor volume was determined by the following formula: tumor volume (mm^3) = $\pi/6 \times$ length (mm) × width (mm) × width (mm). All studies involving mice were approved by the West China Hospital Cancer Center's Animal Care and Use Committee.

Purification of immunoglobulin, its inhibition of cell proliferation in vitro, and its adoptive transfer in vivo. Immunoglobulins were purified from the pooled sera derived from the mice on day 7 after the fourth immunization or from control mice, by affinity chromatography (CM Affi-Gel Blue Gel Kit, Bio-Rad). For determination of the effects of purified immunoglobulins on cell proliferation, tumor cells (2×10^5/ml) were exposed to various concentrations (1–150 μg/ml) of the immunoglobulin for 72 h of culture. The number of viable cells was determined by a trypan blue dye exclusion test, and the percentage inhibition was calculated [6]. For assessment of the efficacy of immunoglobulin in anti-tumor in vivo, purified immunoglobulins (10–300 mg/kg) were adoptively transferred intravenously 1 day before mice were challenged with 2.5×10^6 tumor cells, and then mice were treated twice per week for 3 weeks.

In vitro cytotoxicity assay. A 4-h ^{51}Cr release assay was performed for the determination of the AFP-specific cytotoxicity mediated by CTLs, as described [7]. Briefly, splenocytes obtained from the immunized mice were treated with ammonium chloride-potassium lysis buffer to deplete erythrocytes. A total of 100 μl of effector cells and ^{51}Cr-labeled target cells were assigned at different Effect–Target ratios to each well of microtiter plates and incubated for 4 h at 37 °C. Samples were then harvested, and the activity was calculated by the formula: cytotoxicity% = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

Purification of $CD4^+$ or $CD8^+$ T cells in vitro and their adoptive transfer in vivo. Purified T lymphocytes were obtained by the treatment with specific Abs plus complement, as described [8]. Briefly, nylon wool-purified splenic T cells (10^7 cells/ml) in complete medium were incubated on ice for 45 min with purified anti-CD4 (10 μg/ml, clone GK 1.5; ATCC) or anti-CD8 (10 μg/ml, clone 2.43; ATCC), and washed with complete medium and incubated at 37 °C for 1 h in the presence of complement (rabbit Low-Tox-M; Cedarlane Laboratories, Canada). γδ T cells and NK cells were also depleted using anti-TCR-γδ (clone UC7-13D5; BD PharMingen) and anti-NK1.1 (clone DX5; BD PharMingen) plus complement. Furthermore, Abs plus complement-treated cells were removed of B cells and adherent cells by panning on anti-mouse Ig-coated dishes. Depletion of immune cell subsets was confirmed by flow cytometry (BD PharMingen). One day after the adoptive transfer of 2×10^6–2×10^7 cells, the mice were challenged with 2.5×10^6 tumor cells.

Statistical analysis. Data are expressed as means ± SEM of at least 10 mice. For comparison of individual time points, ANOVA and an unpaired Student's *t-test* were used. Survival curves were constructed according to the Kaplan–Meier method.

Results

Induction of protective and therapeutic antitumor immunity

To investigate the protective antitumor immunity, the mice were immunized by i.p. injection of protein vaccines (mAFP+CFA/IFA, rAFP+CFA/IFA), vaccine vehicle (CFA/IFA), or saline alone (non-immunized) once a week for 4 weeks as described in Materials and methods, and then challenged with 2.5×10^6 live Hepa1-6 cells. There was an apparent protection from tumor growth in mice immunized with rAFP compared with untreated mice or CFA/IFA alone or mAFP-immunized mice (Fig. 1A). The survival rate of the

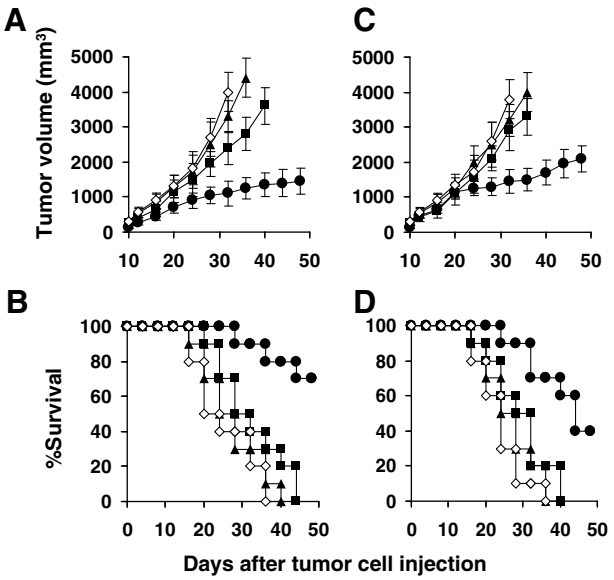

Fig. 1. Induction of protective and therapeutic antitumor immunity. (A,B) Mice were immunized with 75 μg rAFP (●), mAFP (■), vehicle alone (CFA, ▲) or saline alone (non-immunized,◇) for 4 weeks and then were challenged with Hepa1-6 tumor cells as described in Materials and methods. There was an apparent difference in tumor volume (A) and mice survival (B) between rAFP-immunized and control groups. Results are expressed as means ± SEM. (C,D) When tumor was palpable (day 7 after the injection of Hepa1-6 cells), mice were treated by s.c. injection of 75 μg rAFP (●), mAFP (■), vehicle alone (CFA, ▲) or 0.9% saline (non-immunized, ◇) once a week for 4 weeks. Treatment with rAFP resulted in the inhibition of tumor growth (C) and longer life span (D).

mice injected with rAFP was also significantly greater than that of the controls ($p < 0.005$, by log-rank test) (Fig. 1B).

The therapeutic efficacy of the protein vaccine rAFP was next tested in the established tumors. The mice were treated starting at day 7 after the injection of 2.5×10^6 Hepa1-6 cells, when tumor was palpable. Treatment with rAFP once weekly for 4 continuous weeks resulted in significant antitumor activity in Hepa1-6 HCC model. The survival rate of the tumor-bearing mice treated with rAFP was also significantly greater than that of the controls ($p < 0.005$) (Fig. 1C and D). In addition, the antitumor effect with the immunization of rAFP was not found in AFP-negative tumors such as lewis lung cancer (LL/2) and B16 melanoma cells (Supplementary Fig. 1A and B).

The mice immunized with these vaccines have been, in particular, investigated for the potential toxicity. No adverse consequences were indicated in gross measures such as life span (Supplementary Fig. 1C), weight loss (Supplementary Fig. 1D), ruffling of fur, behavior, and feeding. Furthermore, no pathologic changes of liver, kidney, lung, spleens, and brain were found by the microscopic examination.

Characterization of antibodies against AFP and the possible antitumor efficacy

In an attempt to explore the mechanism by which the antitumor response was induced with rAFP, we identified antibodies against AFP in the immunized mice. Sera from rAFP-immunized mice recognized not only recombinant protein rAFP, they also recognized recombinant protein mAFP in Western blot analysis (Fig. 2A). In contrast, the sera isolated from the controls showed negative staining (Fig. 2B and C). Sera from mice immunized with rAFP recognized a single 70-kDa band in AFP-positive Hepa1-6 cells, with the same size recognized by commercially available anti-AFP antibodies, but not in AFP-negative tumor cells (Fig. 2D).

To identify the possible deposition of autoantibodies within tumor or normal tissues, we investigated the tissues by immunofluorescence staining. As shown in Fig. 3E–G, there was the deposition of IgG on the tumor tissues from rAFP-immunized mice, but not from the controls. Furthermore, the mice depleted of $CD4^+$ T lymphocytes were immunized with rAFP and did not develop detectable IgG-specific fluorescence on the tumor cells (Fig. 2H). In contrast, the depletion of $CD8^+$ lymphocytes or NK cells showed no effect (Fig. 2I,J). Also, no IgM- or IgA-specific fluorescence was found. In addition, no detectable deposition of autoantibodies was found within the major organs such as liver, kidney, spleen and brain in the immunized or control mice.

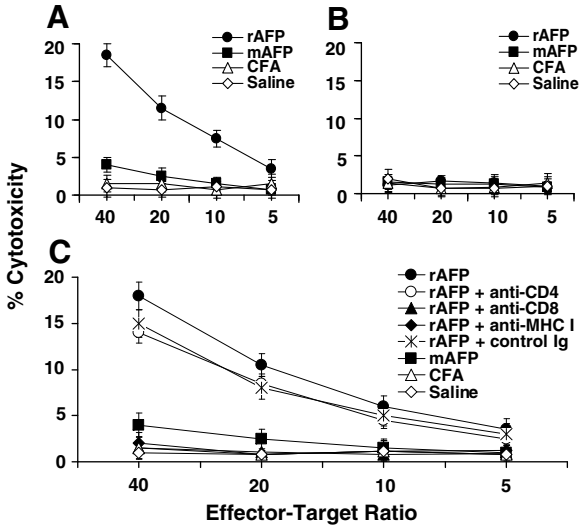

Fig. 3. Representative experiment of CTL-mediated cytotoxicity in vitro. T cells derived from the spleens of rAFP-immunized mice were tested against Hepa1-6 (A) or B16 (B) tumor cells at different Effector-Target ratios by a standard 4-h ^{51}Cr release assay. T cells derived from rAFP-immunized mice (●) showed increased cytotoxicity against AFP-positive target cells but not against AFP-negative cells, compared with control groups, including mAFP (■), CFA (△), or non-immunized (◇) mice. In addition, rAFP-induced tumor killing activity can be blocked by anti-CD8 or anti-MHC class I (anti-H-2Kb/H-2Db) mAb (C).

Immunoglobulin subclass response to the recombinant protein of mouse or rat AFP was determined by ELISA and found to be elevated significantly in IgG1, IgG2a, and Ig2b with little increase in IgM or IgA level in sera obtained from the mice at day 7 after the fourth immunization, compared with the controls (Supplementary Fig. 2A). To explore the role of immune cell subsets in Ig subclass response, we treated mice by i.p. injection of either the anti-CD4, anti-CD8, anti-NK mAb or isotype controls at 7 day before the first immunization, and then twice per week for 4 weeks. The mice depleted of $CD4^+$ T lymphocytes did not develop detectable Abs against mouse or rat AFP. In contrast, the depletion of $CD8^+$ lymphocytes or NK cells showed no effect (Supplementary Fig. 2A).

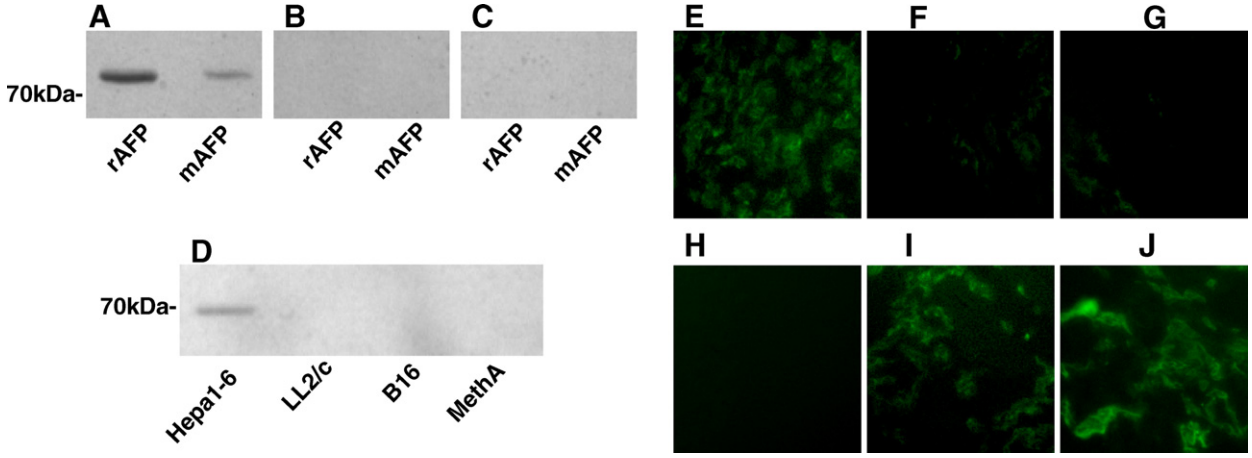

Fig. 2. Characterization of the autoantibodies. Recombinant rAFP and mAFP can be recognized by the sera isolated from mice immunized with rAFP (A) but negative staining from mice immunized with mAFP (B) or CFA (C) in Western blot analysis. Sera from mice immunized with rAFP also recognized a single 70-kDa band in AFP-positive tumor cells (Hepa1-6), but not in AFP-negative tumor cells (LL2/c, B16, and MethA cells) (D). There was the deposition of autoantibodies within tumor tissue from mice immunized with rAFP (E), but not from mice immunized with mAFP (F) or CFA (G). The mice depleted of $CD4^+$ T lymphocytes were immunized with rAFP and did not develop detectable IgG-specific fluorescence, but the depletion of $CD8^+$ lymphocytes (I) or NK cells (J) showed no effect.

These data suggest that the induction of antibodies against AFP may be involved in $CD4^+$ T lymphocytes. However, the adoptive transfer of purified immunoglobulins isolated from rAFP immunized mice did not show apparent protection against tumor growth (Supplementary Fig. 2B). These results suggest that although AFP-specific antibodies were induced by immunized with rAFP, they may not be involved in a role of the direct tumor killing.

Cellular immune response in rAFP-induced antitumor activity

We found that there was no antitumor activity induced by rAFP in nude mice, suggesting that T-cell subsets may be required for the antitumor response. To explore the role of the immune cell subsets in antitumor activity elicited by rAFP, the tumor killing activity of CTLs in vitro was examined. T cells isolated from spleens of mice immunized with rAFP exhibited increased cytotoxicity against AFP-positive syngeneic tumor cells (Hepa1-6) (Fig. 3A) but not against AFP-negative cells (B16) (Fig. 3B). This cytotoxicity could be blocked by anti-CD8 or anti-MHC class I mAb, but not by anti-CD4 in vitro (Fig. 3C), suggesting that the killing activity observed may result from MHC class I-dependent $CD8^+$ CTL activity.

Next, we depleted the $CD4^+$ or $CD8^+$ T lymphocytes or NK cells by using the corresponding monoclonal antibodies. Depletion of $CD8^+$ T lymphocytes could completely abrogate the antitumor activity with the immunization of rAFP, whereas the depletion of $CD4^+$ lymphocytes showed partial abrogation of the antitumor activity in vivo. In addition, treatment with mAb against NK cells or isotype controls failed to abrogate the antitumor activity (Fig. 4A). Furthermore, adoptive transfer of CD4-depleted ($CD8^+$)T lymphocytes isolated from rAFP-immunized mice exhibited significant antitumor activity against AFP-positive tumor cells (Hepa1-6) but not against the other syngeneic AFP-negative tumors, whereas the transfer of $CD4^+$ T lymphocytes and $CD8^+$ T lymphocyte subsets from control mice had no effect (Fig. 4B–E).

Mice depleted of $CD4^+$ T lymphocytes did not develop detectable CTL activity (Supplementary Fig. 2C) and autoantibodies (Supplementary Fig. 2A), whereas mice depleted of $CD8^+$ T lymphocytes showed detectable autoantibodies (Supplementary Fig. 2A), but without CTL activity (Supplementary Fig. 2C). These data suggest that $CD4^+$ T lymphocytes may be required for the induction of both autoantibodies and CTL response to the immunization with rAFP vaccine, and $CD8^+$T cells may be involved in a role of the direct tumor killing.

Discussion

Several observations have been made in the present study. The vaccine rAFP could induce both protective and therapeutic antitumor immunity against AFP-positive HCC in mice. Our finding indicate that autoreactive immune response against the AFP-positive tumor cells may be provoked in a cross-reaction with the xenogeneic AFP, and that the antitumor activity following xenogeneic AFP vaccination may be involved in both T cell effectors and autoantibodies. MHC class I-dependent $CD8^+$ CTL activity was found in cytotoxicity assay in vitro, and the antitumor response was completely abrogated by depletion of $CD8^+$ lymphocytes in vivo. More importantly, the antitumor activity can be acquired with adoptive transfer of $CD8^+$ T lymphocytes. These results suggest that $CD8^+$ CTL-mediated immune response may be responsible for the antitumor activity by vaccination with xenogeneic vaccine. Furthermore, autoantibodies in sera and on the tumor tissues were identified. IgG subclasses were substantially increased in response to xenogeneic AFP vaccine, which suggests that humoral immunity may also be responsible for the antitumor activity. The role of humoral immunity in the antitumor response is further supported by the finding that the lack of both CTL activity and autoantibodies by the depletion of $CD4^+$ T lymphocytes were associated with the abrogation of the antitumor activity. These results suggest that both humoral and cellular immune response, including antibody and CTL response, may be required for the antitumor immune response against AFP-positive tumor with the xenogeneic vaccine rAFP.

It is known that $CD4^+$ T lymphocytes are essential for the generation of both humoral and cellular immune responses [9–11], and required for the generation and maintenance of CTLs [12,13], and $CD8^+$ T cells could induce tumor cell cytolysis and killing by cell cycle inhibition, apoptosis, and induction of macrophage activity [14]. The finding that the depletion of $CD4^+$ T lymphocytes leads to the abrogation of antitumor activity may result from the failure in the induction of CTLs. Thus, both of $CD4^+$ and $CD8^+$ T lymphocytes may be responsible for the antitumor activity against AFP-positive tumor cells. The mechanism by which autoimmune

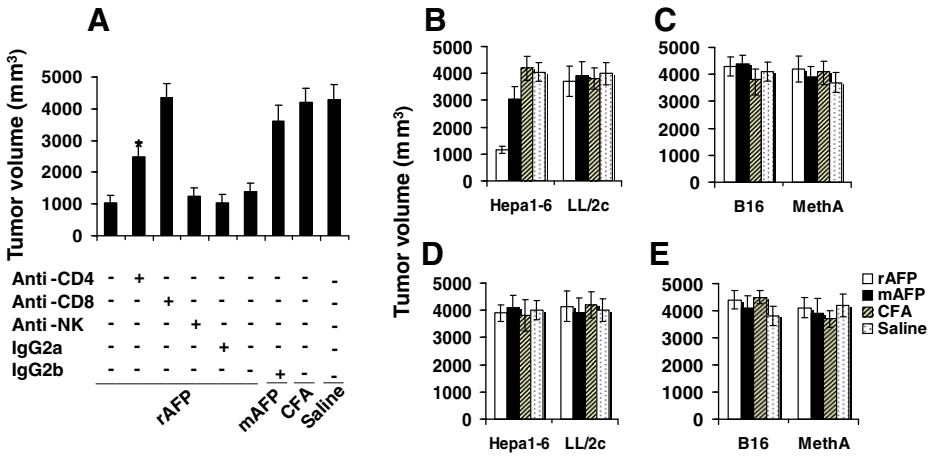

Fig. 4. Abrogation of antitumor activity by depletion of the immune cell subsets and adoptive transfer of T cell subsets in vivo. (A) Depletion of $CD8^+$ T lymphocytes showed complete abrogation of the antitumor activity with the immunization of rAFP, whereas the depletion of $CD4^+$ T lymphocytes showed the partial abrogation. Data represent day 35 after tumor cell injection. (B–E) T cells were isolated from mice immunized with rAFP+CFA, mAFP+CFA, CFA or 0.9% saline, and were depleted of $CD4^+$ or $CD8^+$ lymphocytes as described in Materials and methods. The adoptive transfer of 2×10^7 CD4-depleted ($CD8^+$) (B,C) T cells from mice immunized with rAFP showed the antitumor activity only against AFP-positive Hepa1-6 cells but not against AFP-negative LL/2c, B16 or MethA. In contrast, adoptive transfer of CD8-depleted ($CD4^+$) T cells (D,E) showed no antitumor activity.

response induced with xenogeneic homologous protein may be involved in the antigen-specific cross-reactivity, in other words, molecular mimicry. The mimics of tumor-associated antigens could induce antitumor cellular responses mediated by T lymphocytes [15–17]. If a molecular codes for a peptide that is closely related to a peptide of the host, provided that adjuvants were used at the same time, the molecular might generate vigorous immune responses and then induce T lymphocytes which can react effectively against cells bearing the cross-reacting self antigen [15–17,18].

The immunity to self-antigens is difficult to elicit because of immune tolerance acquired during the development of immune system [5]. In our study, the induction of antitumor immunity by overcoming immune tolerance to self-molecules with xenogeneic counterparts may circumvent the facts that few tumor-specific antigens have been identified in human solid tumors and that the host usually shows immune tolerance to self-molecules. Considering these efforts represent the generation of autoimmune response to a self-molecular, we wished to determine whether the induction of AFP-specific immune responses was accompanied by toxicity. No marked side effects of autoimmunity were observed in the immunized mice. Thus, the finding in our study may be of importance to further exploration of the breaking of immune tolerance to self molecules in cancer therapy, and may provide a new vaccine strategy for the treatment of AFP-positive hepatocellular carcinoma.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.08.061.

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