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Gene therapy by membrane-expressed superantigen for α-fetoprotein-producing hepatocellular carcinoma

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Staphylococcus *enterotoxin A (SEA) is a powerful immunostimulant, which can stimulate T cells bearing certain T-cell receptor β-chain variable regions, when bound to major histocompatibility complex II molecules.* In vivo *administration of intact superantigen in sufficient therapeutic amounts risks unwanted cytotoxicity against normal cells. In this study, we used SEA fused with CD80 transmembrane region (named as SEAtm) driven by α-fetoprotein (AFP) enhancer/promoter to reduce toxicity and to improve safety and efficiency in the application of SEA. We demonstrated that SEAtm by adenovirus from the AFP enhancer/promoter (AdAFPSEA) could be expressed on the surface of AFP-producing cell line Hepa1-6 instead of non-AFP-producing cell lines. Hepa1-6 infected by recombinant adenovirus stimulated proliferation of splenocytes and activated CD4+ and CD8+ T cells* in vitro. *After AdAFPSEA was injected into the subcutaneously established hepatoma* in vivo, *the expression of SEA was detected in tumor tissues, which subsequently induced tumor-specific cytotoxic T cells in spleen. Moreover, hepatocellular carcinoma (HCC) xenografts were suppressed by treatment with AdAFPSEA and the survival time of treated mice was prolonged. These findings suggest that membrane-expressed SEA by adenovirus from AdAFPSEA can generate stronger local and systemic antitumor responses against HCC.*
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Keywords: *hepatocellular carcinoma; superantigen;* Staphylococcus *enterotoxin A; α-fetoprotein; enhancer/promoter; immunogenetherapy*

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide with over a million new cases annually. Most of the therapeutic strategies in current use (surgery, transplantation, irradiation or chemotherapy) are either palliative or only beneficial to a small percentage of patients. The latest developed biotherapy provides potential for treatment of HCC. Among the various strategies used in cancer biotherapy, activation of the immune system by vector-mediated transfer of immunomodulatory genes is a promising approach because tumor cells lack or downregulate stimulatory surface antigens to escape protective immune responses.[1] Introduction of immunostimulatory antigens such as major histocompatibility complex (MHC) I, MHC II or B7-1 onto surfaces of tumor cells can induce antitumor immunity as demonstrated by rejection of parental tumor *in vivo*.[2,3] Superantigens are potent immunomodulatory molecules, each of which stimulates T cells bearing certain T-cell receptor v β-elements and is capable of activating more than 10–25% of the T-cell population when bound as an unprocessed protein outside the antigenic groove of MHC II molecules.[4] Superantigen activation of lymphocyte results in cytokines production, proliferation and cytotoxicity and can elicit systemic antitumor immunity.[5–7] Superantigen-based antitumor strategies may offer therapeutic promise.[8,9] However, *in vivo* administration of intact superantigen in sufficient therapeutic amounts risks unwanted cytotoxicity against normal cells because superantigens preferentially direct cytotoxicity against MHC II-positive cells. Therefore, successful employment of superantigen in tumor immunotherapy requires targeting superantigen to the site of tumor. Wahlsten *et al*.[10] facilitated association of superantigen TSST1 with cell membranes by fusing its coding region to the transmembrane region (TM) sequence of the proto-oncogene c-*erb-B-2* and elicited antitumor response *in vitro* and *in vivo*. Similarly, antibody-targeted superantigen targeted superantigenicity to the site of the tumor and induced local and systemic immunity with reduced cytotoxicity.[11] *Staphylococcus* enterotoxin A (SEA) is a powerful immunostimulant. To target SEA to the site of a tumor, TM of CD80 has been fused genetically with SEA (named as SEAtm) in our lab to make it express on the surface of the tumor cells.[12]

Recombinant adenovirus is widely used for the transfer of foreign genes into various mammalian cells.

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However, the utilization of these vectors for cancer gene therapy requires the specific and efficient expression of the transferred gene in tumor cells because infections of adenovirus to cells lack tissue specificity. One targeting strategy most often applied is that expression of the transgene is controlled by a tumor-specific promoter, like α-fetoprotein (AFP) promoter. Recently, adenovirus vectors containing transcriptional control elements which express the suicide gene preferentially to tumor cells *in vitro* and *in vivo* have been reported.[13,14] In mice, five separate regulatory elements have been defined, a promoter, a repressor and three distinct enhancer elements in the upstream region of AFP gene. Each enhancer element is 200–300 bp in length. Several studies have indicated that the activity of enhancer I is the highest in transgenic mice.[15]

To target therapeutic gene to hepatoma cells, we constructed replication-defective adenovirus vector containing SEAtm gene under the control of the AFP enhancer I/promoter, then evaluated the specific expression of SEA gene in AFP-producing cells *in vitro* and *in vivo*, and assessed the activity of SEA expressed on hepatoma cells infected by AdAFPSEA to induce proliferation of splenocyte *in vitro* and antitumor responses after intratumoral treatment *in vivo* in this study.

Results

Expression of SEA on the surface of Hepa1-6 cells after infection in vitro

Hepa1-6, B16 and NIH3T3 cells were infected by varying amounts of recombinant adenovirus AdAFPSEA. After 48 h of infection, the expression of SEA was analyzed by flow cytometry (FCM). Non-infected Hepa1-6 cells were used as control. With the rise of AdAFPSEA amounts, the SEA-positive cell rates of Hepa1-6 increased significantly but changed slightly for B16 and NIH3T3. Moreover, the positive rates of B16 and NIH3T3 were very low (Figure 1a and b). SEA was expressed on the surface of Hepa1-6 cells infected with AdAFPSEA but not in Hepa1-6 cells infected with Ad (empty), AdAFP or AdSEA by indirect immunofluorescence (Figure 1c).

Hepa1-6 cells after infection with AdAFPSEA stimulate spleen lymphocyte proliferation

The biological activity of SEA expressed on the surface of Hepa1-6 cells *in vitro* was determined by lymphocyte proliferation assays and lymphocyte sub-population analysis (in triplicate). Hepa1-6 cells infected with AdAFPSEA stimulated spleen lymphocyte proliferation VS Hepa1-6 cells infected with Ad (empty), AdAFP,

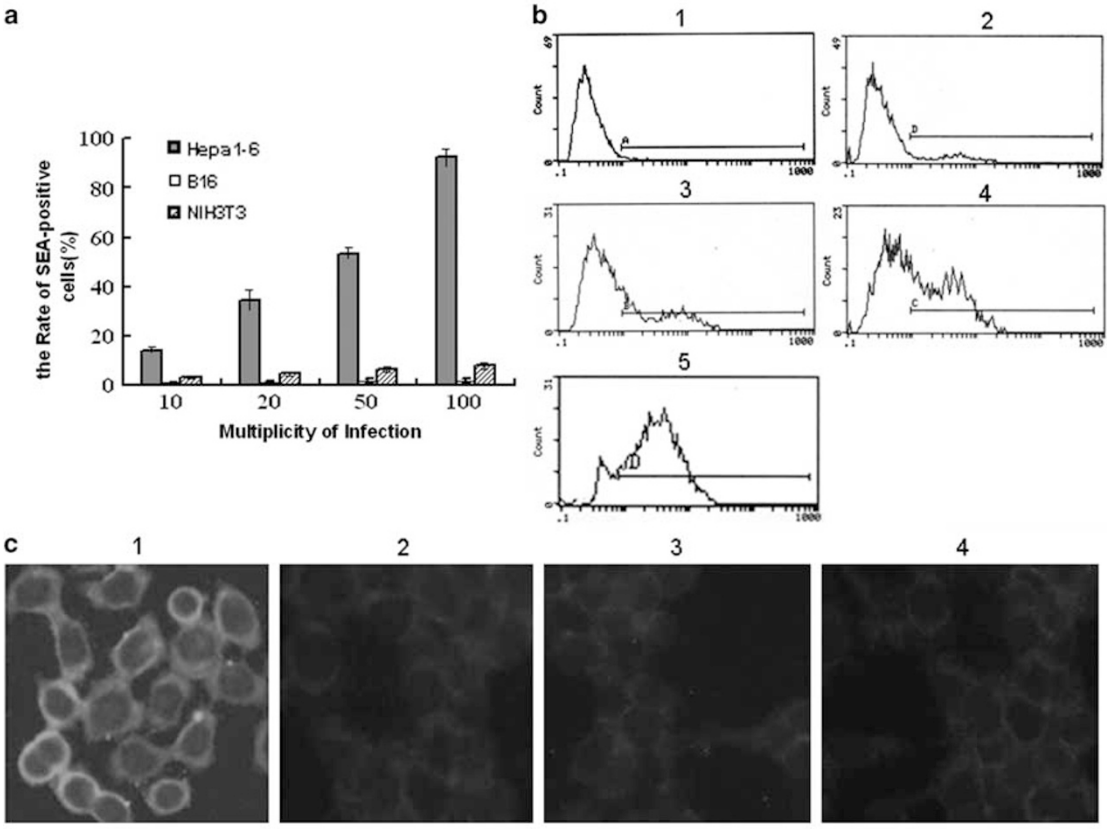

Figure 1 Expression of SEA in different cell lines was analyzed by FCM and indirect immunofluorescence after infection by recombinant adenovirus AdAFPSEA. (**a**) The SEA-positive cell rates of infected B16, NIH3T3 or Hepa1-6 at MOI 10, 20, 50 and 100. The results are means ± s.d. of three experiments. (**b**) (1-5) The SEA-positive cell rates of Hepa1-6 infected at MOI 0, 10, 20, 50 and 100. The expression rates are 1.6, 13.5, 32, 51 and 91.5%, respectively. (**c**) Visualizing SEA on the surface of Hepa1-6 cells by indirect immunofluorescence. Hepa1-6 cells attached to glass coverslips were infected by AdAFPSEA (c1), AdAFP (c2), AdSEA (c3) or Ad (empty) (c4) at MOI 100 for 48 h. Cells were stained with a rabbit anti-SEA polyclonal antibody and FITC goat anti-rabbit IgG. SEA was expressed on the surfaces of Hepa1-6 cells infected by AdAFPSEA, instead of Hepa1-6 cells infected by Ad (empty), AdAFP and AdSEA.

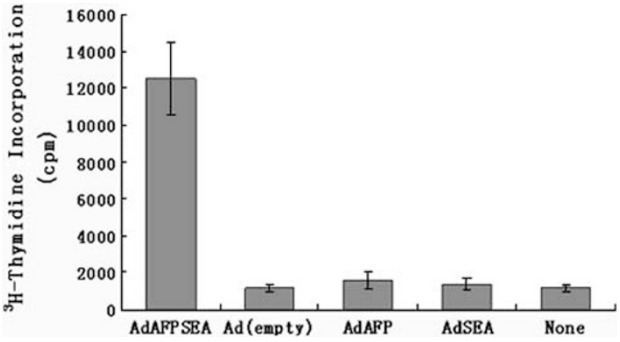

Figure 2 Hepa1-6 infected by AdAFPSEA stimulated proliferation of splenocytes. Hepa1-6 cells were infected by AdAFPSEA, AdAFP, AdSEA or Ad (empty) at MOI 100, irradiated and added at a 1:2 tumor/lymphocyte (T:L) ratio to 2×10^5 lymphocytes/well in a 96-well plate. Values on the *y* axis represent the average c.p.m. $\pm$ s.d. of triplicate cultures. Background values of lymphocytes alone were subtracted. The c.p.m. of splenocytes stimulated with Hepa1-6 infected by AdAFPSEA was significantly higher than the other groups ($P<0.05$). There was no statistical difference among splenocytes stimulated with Hepa1-6 infected by AdAFP, AdSEA, Ad (empty) and non-infected Hepa1-6.

Table 1 Sub-population of T lymphocytes in splenocytes after stimulation with Hepa1-6 infected by different adenovirus

Group	*CD4⁺ T cells (%)*	*CD8⁺ T cells (%)*	*CD69⁺ T cells (%)*	*CD4/CD8*
AdAFPSEA	27.4 $\pm$ 1.3[a]	19.2 $\pm$ 0.8[a]	34.2 $\pm$ 1.1[a]	1.43 $\pm$ 0.2[a]
AdAFP	19.8 $\pm$ 0.8	15.7 $\pm$ 0.5	2.3 $\pm$ 0.4	1.26 $\pm$ 0.1
AdSEA	18.6 $\pm$ 0.5	15.4 $\pm$ 0.6	3.4 $\pm$ 0.5	1.21 $\pm$ 0.1
Ad (empty)	19.5 $\pm$ 0.5	16.7 $\pm$ 0.3	1.8 $\pm$ 0.3	1.17 $\pm$ 0.1
None	20.1 $\pm$ 0.6	16.3 $\pm$ 0.6	2.5 $\pm$ 0.5	1.23 $\pm$ 0.1

Abbreviations: AFP, α-fetoprotein; SEA, *Staphylococcus* enterotoxin A.
[a]Indicates $P<0.05$. The numerical values were the mean $\pm$ s.d. in each group (in triplicate). The number of CD4⁺, CD8⁺ and CD69⁺ T cells and ratio of CD4⁺ to CD8⁺ T cells in splenocytes after stimulation with Hepa1-6 infected AdAFPSEA were higher than other groups ($P<0.05$). There was no statistical difference among mice treated with Ad (empty), AdAFP, AdSEA and PBS.

AdSEA or non-infected Hepa1-6 cells ($P<0.05$) (Figure 2). The numbers of CD4⁺, CD8⁺ and CD69⁺ T cells and the ratio of CD4⁺ to CD8⁺ T cells in splenocytes stimulated with Hepa1-6 cells infected by AdAFPSEA were higher than those in splenocytes stimulated with Hepa1-6 cells infected by AdSEA, AdAFP, Ad(empty) or non-infected ($P<0.05$) (Table 1).

Expression of SEA in tumor and normal hepatic tissue after infection in vivo

After tumors were injected with AdAFPSEA, AdSEA or Ad (empty) and normal liver was injected with AdAFP-SEA, expression of SEA in tumor tissue and hepatic tissue was detected by Western blotting. The molecular weight of amino acids of SEAtm was 36 361 Da. The results showed that a protein with a size of approximately 37 kDa was detected in tumor tissues treated with AdAFPSEA, which was consistent with the expected size of the SEAtm fusion protein. However, there was no protein of expected size in tumor tissues treated with AdSEA or Ad (empty) and normal hepatic tissue injected with AdAFPSEA (Figure 3).

Figure 3 Western blot analysis of tumor tissues injected with AdAFPSEA, AdSEA or Ad (empty) and hepatic tissue injected with AdAFPSEA. Cell lysates containing 100 μg protein were subjected to Western blot analysis using SEA antibody. M: marker; 1–3: from hepatoma injected with AdAFPSEA, AdSEA, Ad (empty), respectively; 4: from hepatic tissue injected with AdAFPSEA. Cell lysates from AdAFPSEA had specific expected band. There were no bands in other groups.

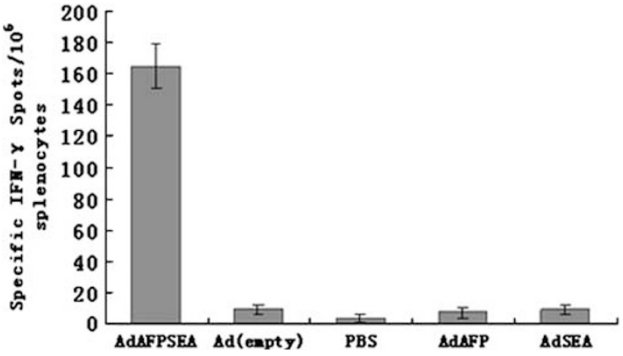

Figure 4 ELISpot assays of tumor-specific T-cell precursors from the splenocytes of treated mice restimulated with inactivated Hepa1-6. C57BL/6 mice were intratumorally treated with Ad (empty), AdAFP, AdSEA or AdAFPSEA. The control group received only PBS. The number of INF-γ-producing tumor-specific T-cell precursors was determined by using the ELISpot assay. The spot numbers were the mean $\pm$ s.e. in each group. Statistical analysis by two-tailed Student's *t*-test revealed that mice treated with AdAFP-SEA generated higher IFN-γ⁺ spot number than AdAFP ($P<0.05$, $n=6$), AdSEA ($P<0.05$, $n=6$), Ad (empty) ($P<0.05$, $n=6$) or PBS ($P<0.05$, $n=6$). There was no statistical difference among mice treated with Ad (empty), AdAFP, AdSEA and PBS.

Intratumoral treatment with recombinant adenovirus induces systemic antitumor immunity

Tumor-bearing mice received intratumoral treatment for four times. Splenocytes were isolated from the treated mice for enzyme-linked immunosorbent spot (ELISpot) and cytotoxicity assay (in triplicate). Following 24 h of splenocytes coculture with irradiated Hepa1-6 cells, cells were removed and interferon-γ (IFN-γ)-producing cell frequency was determined for each group of mice with different treatments. As shown in Figure 4, the number of IFN-γ-producing cell frequency in the splenocytes from mice treated with AdAFPSEA was greater than that from mice treated with AdAFP, AdSEA, Ad (empty) or phosphate-buffered saline (PBS) ($P<0.05$). There were no significant differences among mice treated with AdAFP, AdSEA, Ad (empty) and PBS.

Splenocytes were cocultured with irradiated Hepa1-6 cells for 3 days in the presence of recombinant human interleukin-2 (IL-2) (40 U/ml), collected as cytotoxic T-lymphocyte (CTL) effector cells, and tested against Hepa1-6 cells as target cells. CTL activity was determined at effector:target (E:T) ratios of 5:1, 10:1 and 50:1 by a standard CytoTox 96 non-radioactive cytotoxicity

assay. As shown in Figure 5, lymphocytes derived from the mice treated with the AdAFPSEA showed the highest CTL activity compared with lymphocytes derived from all other groups ($P<0.05$).

Tumor regression and survival time

Six tumor-bearing mice in each group were monitored for their survival period. The results in Figure 6a show that hepatoma-bearing C57BL/6 mice treated with AdAFPSEA survived for a longer time than those treated with AdAFP, AdSEA, Ad (empty) or PBS ($P<0.05$), but there was no significant difference in the survival period among the mice treated with AdAFP, AdSEA, Ad (empty) or PBS. The results in Figure 6b show that tumor growth in mice treated with AdAFPSEA was markedly inhibited relative to that in mice treated with AdAFP, AdSEA, Ad (empty) or PBS. The antitumor effect of AdAFPSEA treatment was signicantly stronger than other groups ($P<0.05$). There were no significant differences in tumor growth inhibition among the groups treated with AdAFP, AdSEA, Ad (empty) or PBS.

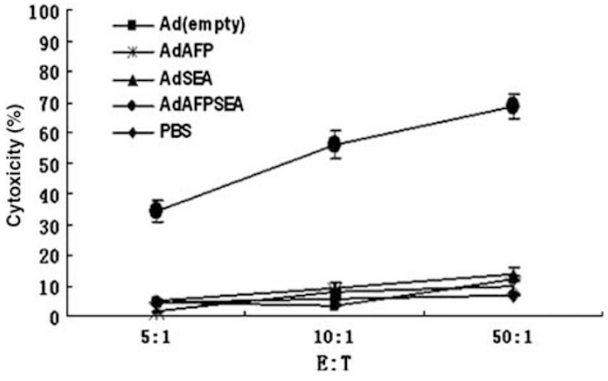

Figure 5 Tumor-specific lysis against Hepa1-6 cells by CTLs induced by intratumoral treatment with recombinant adenovirus. The splenocytes of mice with different treatments were harvested and restimulated with irratiated Hepa1-6 cells for 3 days. The percentage of specific lysis of splenocytes on tumor cells was determined by a non-radioactive cytotoxicity assay. The tumor-specific lysis of splenocytes from mice treated with AdAFPSEA showed a higher level than the other groups. There was no statistical difference among mice treated with Ad (empty), AdAFP, AdSEA and PBS.

Discussion

In the present study, we show that SEAtm by adenovirus from the AFP enhancer/promoter (AdAFPSEA) can be expressed on the surface of AFP-producing cell line Hepa1-6 instead of non-AFP-producing cell lines. Hepa1-6 infected by recombinant adenovirus stimulates proliferation of splenocytes *in vitro* and intratumoral treatment with AdAFPSEA generates stronger local and systemic antitumor responses against HCC.

Recombinant adenoviruses have been used as highly efficient vectors for *in vitro* and *in vivo* gene deliveries.[16,17] Adenovirus-mediated gene transduction is demonstrated in a broad spectrum of eucaryotic cells independent of cell replication. However, this vector presents one problem that is the nonspecific transduction of therapeutic genes into cells other than target cells. Therefore, one potential strategy is to design an adenoviruses vector that can express therapeutic genes selectively in tumor cells. In this study, the expression of heterologous gene is limited to AFP-producing cells by using the AFP promoter/enhancer element.

Treatment exploiting the therapeutic potential of SAgs requires modifications to decrease systemic activation as a consequence of SEA–MHC class II interaction with monocytes and B cells and to localize the cytotoxic capabilities of SAg-activated T cells to tumor sites. As a superantigen, SEA is a powerful immunostimulant. To achieve these aims, we used membrane-expressed SEAtm driven by AFP enhancer/promoter to target SEA expression to the tumor sites. SEA was efficiently and specifically expressed on the surface of AFP-producing Hepa1-6 cells but not in non-AFP-producing cell lines *in vitro*, as shown by FCM assay and indirect immunofluorescence. Furthermore, SEA expressed on the surface of Hepa1-6 was biologically active *in vitro*, as shown by the ability of Hepa1-6 infected by AdAFPSEA to elicit proliferation of splenocytes. A hallmark of early T-cell activation is the surface expression of the CD69 molecule. Increase of $CD69^+$ cells in splenocytes stimulated by Hepa1-6 infected by AdAFPSEA indicated that

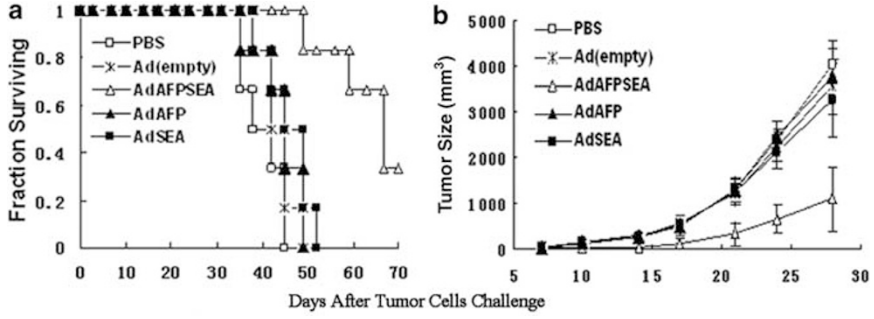

Figure 6 Immunogenetherapy of pre-established Hepa1-6 hepatoma with recombinant adenovirus. Mice bearing tumors were treated with 10^9 PFU of adenovirus or PBS once a week for 4 weeks. (**a**) Kaplan–Meier curves were generated from survival data ($n=6$ mice/group). The overall survival rates of mice treated with AdAFPSEA were significantly ($P<0.05$) higher than those of the other groups. There were no statistical differences among mice treated with Ad (empty), AdAFP, AdSEA and PBS. (**b**) Tumor growth kinetics was followed over time by caliper measurements, and mean tumor volumes (mm^3) were calculated. Error bars depict s.e. ($n=6$ mice/group). Treatment with AdAFPSEA gave significantly better results than treatment with AdAFP, AdSEA, Ad (empty) or PBS ($P<0.05$). There was no statistical difference among mice treated with Ad (empty), AdAFP, AdSEA and PBS.

activation of T lymphocytes was induced by SEA expressed on the surface of Hepa1-6 cells. After stimulation with Hepa1-6 infected by AdAFPSEA, the proportion of $CD4^+$ and $CD8^+$ T cells in splenocytes increased, but the ratio $CD4^+$ T cells to $CD8^+$ T cells decreased. The results indicated that SEA expressed on the surface of Hepa1-6 activated both $CD4^+$ T cells and $CD8^+$ T cells, but stimulated $CD4^+$ T cells more strongly than $CD8^+$ T cells. Western blotting assay indicated that SEAtm was specifically expressed in tumor tissues *in vivo*.

An antitumor strategy that anchors a superantigen on MHC II-negative tumor cells assumes T-cell stimulation, but it circumvents conventionally defined MHC 'presentation'. Numerous studies have demonstrated that fusion proteins of SEA and Fab regions of monoclonal antibodies (mAbs) showed a greater than 10-fold reduction in MHC class II binding compared to native SEA. Superantigen elicited MHC II-independent T-cell stimulation *in vitro* as long as co-stimulatory signals were provided.[18,19] Furthermore, it has been demonstrated in several reports that anchoring SEA onto MHC II-negative tumor cells through antibodies directs T-cell-mediated cytotoxicity against these tumors with reduced toxicity.[20,21] Wahlsten *et al*.[10] facilitated association of TSST1 with cell membranes by fusing its coding region to the TM sequence of the proto-oncogene c-*erb-B-2* to construct TSST1-TM and $TSST_{88–194}$-TM lacking TSST1 MHC II-binding domain. Tumor cells precoated with TSST1-TM or $TSST_{88–194}$-TM stimulated proliferation of human peripheral blood lymphocytes *in vitro*, whereas uncoated tumor cells did not. Mice preimmunized with TSST1-TM- or $TSST_{88–194}$-TM-coated tumor cells mounted a systemic response that resulted in significant antitumor immunity as measured by regression of a parental tumor challenge.[10] These results suggested that superantigen can induce immunostimulation in the absence of MHC II molecules, and that artificially anchoring a superantigen onto a cell surface can substitute for MHC II presentation.

Endogenously produced IFN-γ was shown to protect the host against the growth of transplanted tumors and also the formation of primary chemically induced and spontaneous tumors[22–26] and play a crucial role in the eradication of tumors *in vivo*.[27] Injection of neutralizing mAbs for IFN-γ into mice bearing transplanted, established Meth A tumors blocked lipopolysaccharide-induced tumor rejection.[22] In addition, transplanted fibrosarcomas grew faster and more efficiently in mice treated with IFN-γ-specific mAbs. Moreover, IFN-γ was involved in the antitumor effects of antibody-targeted superantigens.[28] ELISpot is a sensitive functional assay used to measure INF-γ production at the single-cell level. The ELISpot result that showed intratumoral treatment with AdAFPSEA increased the frequency of heptoma-specific INF-γ-producing cells in spleen. $CD8^+$ CTLs are one of the most crucial components among antitumor effectors.[29] To determine the tumor-specific CTLs frequency in splenocytes induced by intratumoral treatment with AdAFPSEA, cytotoxicity assays were performed. In this study, cytotoxicity assays showed that intratumoral treatment with AdAFPSEA induced heptoma-specific CTLs in the splenocytes. The ELISpot and cytotoxicity assays indicated that intratumoral treatment with AdAFPSEA induced systemic antitumor immunity. These tumor-specific immune cells may be recycled in the tumor to eliminate it.

SEA was chemically conjugated or genetically fused to mAbs directed against tumor-associated antigens. Mice bearing relevant antigen-transfected B16 melanoma cells treated with mAb-SEA experienced 85–99% inhibition of tumor growth and long-term survival. The MHC-independent superantigen-mediated antibody-dependent cell cytotoxicity (SADCC) following tumor targeting of the fusion protein has the potential to mediate lysis (inhibit growth) and promote a cascade of events. Initially, T-cell activation stimulates the production of perforins for direct cell lysis[30] and induces cytokine production by both the antigen-presenting cells and T cells. Cytokines can suppress tumor growth both directly and synergistically.[31] In addition, cytokines can induce LAK activity, activate natural killer cells and macrophages,[32–34] facilitate penetration of high molecular weight proteins and upregulate cell adhesion molecules and MHC class II expression on tumor cells. These factors favor additional fusion protein interactions with subsequent SADCC[30,35] and recruit peripheral blood cells into this inflammatory milieu.[35] In this study, the results of intratumoral treatment of recombinant virus induced systemic and local antitumor immunity. The antitumor immune mechanisms may be similar to mAb-SEA fusion protein. More research is needed to understand the mechanism in greater details.

In summary, our findings indicate that adenovirus-mediated delivery of SEAtm driven by AFP enhancer/promoter can generate stronger local and systemic antitumor responses against HCC. The local expression of SEA possibly will not induce systemic toxicity or organ dysfunction, which will be observed in next steps. The underlying mechanism for its antitumor immunity needs more research. This approach will make the application of superantigen safer and more specific in targeting, leading to novel strategies for tumor treatment with superantigen.

Materials and methods

Cell lines

Murine AFP-producing hepatoma cell line Hepa1-6 (kindly provided by Professor Lixin Wei from the Second Military Medical University, Shanghai, China), human embryonic 293 cell, mouse melanoma cell line B16 and mouse fiberblast cell line NIH3T3 were conserved in the lab. All the cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 (Gibco-BRL, Gaithersburg, MD, USA) or high-glucose Dulbecco's modified Eagle's medium (DMEM), which were supplemented with 10% fetal bovine serum and penicillin/streptomycin in an atmosphere of 5% CO_2 chamber at 37°C.

Cloning of AFP promoter and enhancer I and SEAtm

The murine AFP promoter was amplified by polymerase chain reaction (PCR) from total DNA of hepatic tissue of C57BL/6 with the primers (5′-GCCGAATTCTCTGAAG TGGTCTTTGT-3′ containing an *Eco*RI site and 5′-GCC GGATCCGGCTGCTTGTTCCTTCACCG-3′ containing a *Bam*HI site) and cloned into pBluscriptIIKS+. The resulting plasmid was named pKS-P. The murine AFP enhancer I was amplified by PCR from total DNA of

hepatic tissue of C57BL/6 with the primers (5′-GCCGTC GACATAAAGCTGGTTTGAGTTTTTGTC-3′ containing a *Sal*I site and 5′-GCCGAATTCGGCGACTGGGTTTG AATTTTGC-3′ containing an *Eco*RI site) and cloned into pKS-P to construct the plasmid pKS-EP. The construction of pLXSNSEAtm containing SEAtm was performed in our lab as described previously.[12] The DNA of SEAtm was amplified by PCR from pLXSNSEAtm with the primers (5′-GCGAGATCTCCATGAAAAAAACAGCAT TTACA-3′ containing a *Bgl*II site and 5′-GCGTCTAGATT ATACAGGGCGTACACTTTC-3′ containing an *Xba*I site) and subcloned into pMD18-T vector to construct the plasmid pMD18-SEA. All constructs were confirmed by DNA sequencing.

Recombinant adenovirous preparation

We used the AdEasy vector system (Qbiogene Inc., Morgan, Irvine, CA, USA), that is, human adenovirus serotype 5 and rendered replication defective by the deletion of the E1 and E3 gene. Preparation of adenovirus was performed as described in the manufacturer's protocol with minor modifications. We digested the transfer plasmid pShuttle and pShuttle-CMV (CMV, cytomegalovirus) with *Kpn*I and *Eco*RI, respectively. The longer fragment of pShuttle and the shorter fragment of pShuttle-CMV were linked by T_4 DNA ligase. The resulting new transfer plasmid was named as pShuttle2 with the simian virus 40 (SV40) polyA signal sequence and without CMV promoter. SEAtm digested by *Sal*I and *Xba*I from pMD18-SEA was subcloned into the *Xho*I and *Xba*I site of pShuttle2 upstream of SV40 polyA signal sequence and was named as pShuttle2-SEA. AFP enhancer/promoter digested by *Sal*I and *Bam*HI from pKS-EP was subcloned into the *Sal*I and *Bgl*II site of pShuttle2-SEA upstream of SEAtm to construct pShuttle2-EPSEA. AFP enhancer/promoter was subcloned into the *Sal*I and *Xba*I site of pShuttle2 to construct pShuttle2-EP. The plasmid pShuttle2, pShuttle2-EP, pShuttle2-SEA or pShuttle2-EPSEA was linearized by *Pme*I and subsequently co-transformed into *Escherichia coli*. BJ5183 cells with an adenoviral backbone plasmid pAdEasy-1. Recombinants were selected by kanamycin resistance and confirmed by *Pac*I restriction endonuclease analysis and named as Ad (empty), AdAFP, AdSEA or AdAFPSEA, respectively. Finally, the recombinant plasmid linearized by *Pac*I was transfected into HEK293 cells using the Lipofectin regent (Invitrogen Corporation, Carlsbad, CA, USA). Recombinant adenoviruses AdAFPSEA were purified by single plaque isolation. The optimal clone was selected at the level of protein expression. Proliferation, purification and tittering of Ad (empty), AdAFP, AdSEA and AdAFPSEA were performed as described in the manufacturer's protocol. None of the stocks of virus used in the experiments contained detectable replication-competent viruses as evaluated by PCR assay, which used two pairs of primers to detect adenoviral E1A DNA.

Adenovirus-mediated SEA-specific expression in vitro

Hepa1-6, B16 and NIH3T3 cells were plated at a density of 5×10^5 cells/well in six-well culture plates 24 h before AdAFPSEA infection. Immediately before infection, culture medium was aspirated and varying amounts of adenovirus were distributed over the cell monolayer. The ratio of the number of adenovirus per cell was expressed as multiplicity of infection (MOI). After 48 h of culture, the cells were digested with 0.02% ethylenediaminetetraacetic acid, washed in PBS containing 5% calf serum, incubated with a rabbit anti-SEA polyclonal antibody (ViroStat Inc., Portland, ME, USA) and detected with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G (IgG). The analysis was performed by FCM.

The expression of SEA on the surface of Hepa1-6 cells was also visualized by *in situ* indirect immunofluorescent staining. Hepa1-6 cells were seeded at a low density onto glass coverslips and grown for 48 h after infection with AdAFPSEA, AdAFP, AdSEA or Ad (empty) at MOI 100. The cells were then rinsed with PBS before the detection of SEA was performed using the Abs as described above. Coverslips were mounted onto glass slides. Images were captured with a $\times 10$ neofluor objective on a Nikon E1000 microscope connected to a photometrics PXL cooled CCD camera using IPlab Spectrum (Signal Analytics, Vienna, VA, USA).

Biological activity of SEA expressed on Hepa1-6 cells in vitro

The biological activity of SEA expressed on Hepa1-6 cells *in vitro* was measured using a lymphocyte proliferation assay. Splenocytes were isolated from the spleens of healthy C57BL/6 mice over a Ficoll–Hypaque gradient and aliquoted to 2×10^5 cells/well in 96-well U-bottom plates (Costar). The splenocytes were cocultured with irradiated (10 000 cGy) Hepa1-6 infected by Ad (empty), AdAFP, AdSEA or AdAFPSEA of MOI 100 at the ratio of 1:2 (tumor cell to lymphocyte) in 0.2 ml RPMI-1640 medium containing 10% fetal calf serum. After 72 h of incubation (37°C, 5% CO_2), cells were harvested for proliferation and subset analysis. Cells were labeled with [^{3}H]thymidine (1 μCi/well) for 18 h before harvesting. Incorporation of [^{3}H]thymidine was determined using a liquid-scintillation counter. At the same time, cells were stained with PE-conjugated anti-mouse CD4 or CD8 for FCM to detecct $CD4^+$ T cells or $CD8^+$ T cells, respectively, or with PE/Cy5-conjugated anti-mouse CD69 for FCM to analyze T-cell activation.

Mice tumor preparation

Female C57BL/6 mice, 6–8 weeks of age, were obtained from the Experimental Animal Center (Fourth Military Medical University, Xi'an, China) under strictly controlled specific-pathogen-free conditions at the Cancer Institute Animal Facility (Xi'an, China). Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed, as well as the current version of the Chinese Law on the Protection of Animals. Mice were held in accordance with the permission of the responsible authority. Tumors were generated by subcutaneous injection of 10^6 Hepa1-6 cells in 0.1 ml of PBS into the flank of C57BL/6 (per mouse). Visible tumors had developed at 8–9 days after tumor cell inoculation. Treatment was performed when the largest diameter of tumor exceeded 0.5 cm.

Tumor treatment in vivo

When the largest diameter of tumor exceeded 0.5 cm, mice were injected intratumorally with 1×10^9 PFU/mouse of Ad (empty), AdAFP, AdSEA or AdAFPSEA

in 100 μl PBS. Mice intratumorally injected with 100 μl PBS/mouse were used as the control. Each group contained 12 mice. The mice were treated once a week for 4 weeks. Tumor sizes were measured before virus injection and subsequently twice a week. Linear calipers were used to measure the longest diameter (a) and width (b). The tumor volume was calculated using the formula: ($ab^2/2$) and was plotted as the mean tumor volume of the group ($\pm$standard error (s.e.)) versus days post-tumor challenge. The survival times of mice were recorded and Kaplan–Meier curves were generated. Seven days after the last injection, six mice of each group were killed and spleens were removed for cytotoxicity of CTLs and IFN-γ ELISpot.

Western blotting analysis of SEA gene-specific expression in vivo

AdAFPSEA was injected into normal liver of C57BL/6 mouse. The hepatic tissue and tumor biopsies were obtained 48 h after infection for Western blotting analysis. A rabbit anti-SEA polyclonal antibody (ViroStat Inc.) was used for detection of SEA expression. Western blotting was performed as described previously,[36] but with minor modifications. Aliqots of the hepotoma cell extracts were subjected to 12% SDS-polyacrylamide gel electrophoresis, and proteins were electrotransferred to polyvinylidene fluoride (PVDF) (Immobin P, Millipore, Bedford, MA, USA) at 100 V for 1 h on ice, using transfer buffer (15% v/v methanol, 25 mM Tris, 200 mM glycine, pH 8.6). The membrane was stained with Ponceau S (0.5% Ponceau S, 1% acetic acid in water) to assess the quality of transfer and then destained in water. Then, the membrane was blocked with PBS solution containing 0.1% Tween 20 and 5% bovine serum albumin (BSA) for 1 h at 4°C. After overnight incubation in antibody (diluted 1:200 in PBS, 0.1% Tween 20 and 1% BSA), the membrane was washed for 30 min in PBS-Tween 20, and then incubated at 37°C for 1 h with biotin-conjugated goat anti-rabbit. After 30 min PBS wash, the membrane was incubated in the avidin/horseradish peroxidase solution (ABC kit) for 3 h and then in diaminobenzidine detection reagent (Sigma, St Louis, MO, USA) covered with plastic wrap. A low-range protein marker (Bio-Rad, Hercules, CA, USA) was used to estimate the molecular weight of the fragment.

Systemic antitumor cytotoxicity assays of CTLs

The CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, WI, USA) was performed to measure the cytotoxic activity of the splenocytes in mice bearing tumors treated with PBS, Ad (empty), AdAFP, AdSEA or AdAFPSEA, according to the manufacturer's protocol with minor modifications. Briefly, splenocytes of mice 7 days after the last treatment were cultured in the presence of human IL-2 (40 U/ml) and irradiated (10 000 cGy) Hepa1-6 cells. After 3 days, Hepa1-6 targets cells were plated at 1×10^4 cells/well on 96-well U-bottom plates (Costar), then the splenocytes (effector cells) were added in a final volume of 100 μl at 5:1, 10:1 and 50:1 ratios (effector cells to target cells). The plates were incubated for 45 min in a humidified chamber at 37°C, 5% CO_2, and centrifuged at 500 g for 5 min. Aliquots (50 μl) were transferred from all wells to fresh 96-well flat-bottom plates, and an equal volume of reconstituted substrate mix was added to each well. The plates were incubated at room temperature for 30 min and protected from light. Then, 50 μl of stop solution was added, and the absorbance values were measured at 492 nm. The percentages of cytotoxicity for each effector to target cell ratio was calculated as ((A (experimental)−A (effector spontaneous)−A (target spontaneous)) $\times$ 100)/(A (target maximum)−A (target spontaneous)).

IFN-γ ELISpot

Mouse IFN-γ ELISpot assay was performed in PVDF-bottomed 96-well plates (Millipore, Bedford, MA, USA) by using a murine IFN-γ ELISpot kit (Diaclone, Besancone, France) according to the manufacturer's instructions with minor modifications. Briefly, plates were coated overnight at 4°C with anti-IFN-γ capture antibody and washed three times with PBST (PBS+0.05% Tween 20). Plates were blocked for 2 h with 2% skimmed dry milk. Splenocytes (1×10^6 cells/well) of mice after treatment were then added together with the indicated number of lethally irradiated (10 000 cGy) Hepa1-6 cells (5×10^4/well) and incubated for 24 h at 37°C. Only splenocytes were added into wells as negative control. Cells were then removed and a biotinylated IFN-γ detection antibody was added for 1 h. Following extensive wash with PBST and PBS, the plates were incubated with streptavidin-alkaline phosphatase for 1 h at 37°C. Spots were visualized by the addition of the alkaline phosphatase substrate BCIP/NBT. The number of dots in each well was counted by two separate investigators in a blinded manner using a dissecting microscope.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to determine differences of immune response among the various treatment groups. Newman–Keuls tests were performed as *post hoc* analysis for one-way ANOVA. The antitumor effects were considered statistically significant when the P-value was less than 0.05.

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