Int. J. Cancer: **113,** 670–677 (2005)

Anti-angiogenic therapy subsequent to adeno-associated-virus-mediated immunotherapy eradicates lymphomas that disseminate to the liver

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Liver cancer has a very poor prognosis and lacks effective therapy. We have previously demonstrated that intraportal injection of adeno-associated-viral (AAV) particles that express angiostatin lead to long-term expression of angiostatin capable of suppressing the outgrowth of EL-4 tumors in the liver. Here we combine AAV-mediated angiostatin therapy with immunotherapy by employing an AAV vector encoding the T-cell costimulator B7.1. Incubation of EL-4 cells with AAV-B7.1 viruses resulted in the rapid expression of B7.1 on the surface of 80% of EL-4 cells. Mice that were vaccinated with B7.1-engineered tumor cells rejected the tumor cells and resisted a secondary challenge with unmodified parental cells. Splenocytes from the vaccinated mice were highly cytotoxic towards parental EL-4 cells *in vitro*. However, the vaccinated mice failed to resist the challenge of a heavy burden of EL-4 cells. Intraportal injection of AAV particles that express angiostatin into mice that had been vaccinated 1 month earlier with B7.1-engineered tumor cells protected mice against the challenge of a heavy burden of EL-4 cells and eradicated tumors that had disseminated to the liver. The combinational therapy increased the survival rate of mice with advanced liver cancer. These encouraging results warrant investigation of the employment of anti-angiogenic therapy subsequent to cancer immunotherapy for targeting unresectable disseminated liver metastases.

Key words: adeno-associated virus; liver cancer; immunotherapy; B7.1; angiostatin; gene therapy

The liver is the most frequent site of blood-borne metastases, being involved in about one third of all cancers, including the most frequent cancer types.[1] Despite extensive exploration for novel therapies, there is no effective treatment for liver metastases. A recent study suggests liver cancer may be susceptible to immunotherapy. Thus, there was a decreased frequency and risk of recurrence of disease for patients treated by infusion of activated autologous lymphocytes, and treated patients had a longer disease-specific survival.[2]

Vaccination with B7.1-transfected tumor cells induces specific anti-tumor immunity in models of relatively immunogenic tumors.[3–5] However, cell adhesion molecule (CAM)-mediated immunotherapy is problematical in that it is ineffective against large immune-resistant tumors and generates weak anti-tumor systemic immunity.[6] In searching for ways to more effectively harness and strengthen the anti-tumor activity of CAM-mediated immunotherapy, we reported that immunogene therapy employing the T-cell costimulator B7.1 could be vastly improved by combining it with anti-angiogenic agents such as angiostatin[7] and antisense hypoxia inducible factor (HIF)-1α.[8] Combination therapy overcame tumor immune-resistance and caused the complete and rapid eradication of large tumor burdens, which were refractory to monotherapy with either angiostatin, antisense HIF-1α or B7.1. This finding is highly relevant for the treatment of liver cancer, as Kim *et al.*[9] demonstrated that the HBV X-protein increases the transcriptional activity of HIF-1 under both normoxic and hypoxic conditions, and stimulates angiogenesis. Microvessels were found to be more abundant in dysplastic regions of the liver than in nonneoplastic regions, indicating that liver cancer may be critically dependent on angiogenesis.[10]

Adeno-associated-virus (AAV) is a nonpathogenic, helper-dependent member of the parvovirus family with several major advantages such as stable integration, low immunogenicity, long-term expression and the ability to infect both dividing and nondividing cells. We have established AAV as a system to rapidly induce persistent expression of anti-cancer agents. With this system, we have previously demonstrated that intraportal injection of AAV expression vector encoding angiostatin led to high-level, long-term (6 months) transgene expression localized to hepatocytes. AAV-generated angiostatin significantly suppressed the growth of both nodular EL-4 tumors, and EL-4 metastases that had disseminated to the liver.[11] However, angiostatin therapy, which indirectly targets tumors by inhibiting vascular endothelial cells, was unable to completely eradicate the liver tumors.

In our study, we investigate whether follow-up AAV-mediated, anti-angiogenic therapy can augment B7.1 immunotherapy to generate anti-tumor immunity sufficient to combat intractable liver cancer.

Materials and methods

Plasmid construction

The cytomegalovirus (CMV) enhancer/chicken β-actin promoter,[12] a 1.4 kb complementary DNA (cDNA) encoding full-length mouse angiostatin that consists of the signal peptide and first 4 kringle regions of mouse plasminogen, or a 1.2 kb cDNA encoding full-length mouse *B7.1*, and poly A sequences were inserted between the ITRs of an AAV vector, which has been described previously,[12] using appropriate restriction enzymes. For AAV empty vector controls, no insert was placed between the ITRs. A woodchuck hepatitis B virus posttranscriptional regulatory element (WPRE) was inserted into this construct to boost expression levels.[13,14] Plasmids were prepared using Qiagen plasmid purification kits.

Abbreviations: AAV, adeno-associated virus; APC, antigen presenting cell; CAM, costimulatory adhesion molecule; cDNA, complementary DNA; CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; HIF, hypoxia inducible factor; WPRE, woodchuck hepatitis B virus, post-transcriptional regulatory element.

Grant sponsor: HKU Foundation; Grant sponsor: National Natural Science Foundation of China; Grant number: 30100178; Grant sponsor: Shandong Provincial Scientific and Technological Committee of China; Grant number: 9836.

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Received 11 May 2004; Accepted 3 August 2004
DOI 10.1002/ijc.20624
Published online 7 October 2004 in Wiley InterScience (www.interscience.wiley.com).

Generation of AAV

AAV particles were generated by a 3 plasmid, helper-virus free packaging method[15] with slight modification. The method has been described previously.[11] Briefly, AAV, AAV-angiostatin, AAV-B7.1 and the helper pFd, H22 vectors were transfected into 293 cells using calcium phosphate precipitation. Cells were harvested 70 hr after transfection and lysed. After centrifugation at 5,000*g*, they were filtered to remove any particulate matter prior to fractionation on a heparin column. The AAV particles were isolated by heparin affinity column chromatography. Peak virus fraction was dialyzed. A portion of the samples was subjected to quantitative PCR analysis using the AB Applied Biosystem Tagman PCR assay to quantify the genomic titer. The assay was a modified dot-blot protocol whereby AAV was serially diluted and sequentially digested with DNAse I and Proteinase K. Viral DNA was extracted with phenol-chloroform and precipitated with ethanol. A standard amplification curve was set up at a range from 10^2 to 10^7 copies, and the amplification curve corresponding to each initial template copy number was obtained. The AAV, AAV-angiostatin and AAV-B7.1 viral particles had similar genomic titers. Viral particles were reconfirmed using a commercial analysis kit (Progen, Frankfurt, Germany) and were stored at −80°C prior to animal experiments.

Mice, cell lines and antibodies

Male C57BL/6 mice (H-2b), 6–8 weeks old, were obtained from the Laboratory Animal Unit of the University of Hong Kong. The syngeneic (H-2b) EL-4 thymic lymphoma cell line was purchased from the American Type Culture Collection (Rockville, MD). It was cultured at 37°C in DMEM medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine and 1 mM pyruvate. Anti-angiostatin and anti-CD4 (clone Gk1.5) antibodies were purchased from Calbiochem-Novabiochem Corporation (Temecula, CA) and Chemicon International, Inc., (Boston, MA), respectively. Anti-B7.1, anti-CD8 (clone 53-6.7), anti-NK (clone PK136) and anti-CD31 (MEC13.3) antibodies were purchased from Pharmingen (San Diego, CA).

Transfection of EL-4 cells, and analysis of transgene expression

Primary EL-4 cells (5×10^5/well in 96-well plates) were incubated in a total volume of 50 μl of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and infectious AAV was added resulting in a multiplicity of infection (MOI) between 1 and 500. Cells were harvested 2, 6, 12, 24 and 48 hr following infection, fixed with 4% paraformaldehyde solution, blocked with 3% bovine serum albumin and incubated with anti-B7.1 Abs. They were then incubated with fluorescein-isothiocyanate (FITC)-conjugated secondary Abs and observed by fluorescence microscopy. Cells transfected with empty AAV vector alone served as controls.

Flow cytometry

After AAV transduction, EL-4 tumor cells were harvested, purified by Ficoll density gradient centrifugation to remove extraneous virus and washed. Cells were incubated with specific Abs for 30 min in PBS, 4% FCS, 0.1% sodium azide, 20 mM HEPES (N-2-hydroxyethylpiperazine- N′-2 ethanesulfonic acid) and 5 mM EDTA, pH 7.3, on ice and washed. Nonspecific binding was controlled by incubation with an isotypic control rat IgG1 MAb (BD Pharmingen, San Diego, CA). Cells transfected with empty AAV vector alone served as controls. The level of expression of the transgene was assessed by FACScan analysis. Cells were then used as CTL targets as described below and for animal experiments.

Animal experiments

All surgical procedures and care administered to the animals were approved by the University Ethics Committee and performed according to institutional guidelines. Animals were randomly assigned to treatment. Each group contained 10 mice. Each experiment was repeated at least once. The disseminated liver metastasis models consistently yielded tumors in at least 90–95% animals. Equal numbers of parental EL-4 cells and equal numbers of empty AAV virus particles served as controls.

Vaccination of mice with AAV-B7.1-transfected EL-4 tumor cells, and analysis of liver tumors

C57BL/6 mice were anesthetized with 10% ketamine/xylazine solution by intraperitoneal injection, and their abdomens were prepared with betadine solution. A right subcostal incision was used to open the abdominal cavity. After the hilar of the liver was surgically exposed, 2×10^5 AAV-B7.1-transfected EL-4 tumor cells were slowly injected into the portal vein with a 30-gauge needle, and pressure was applied with a sterile cotton tip applicator until the injection site was hemostatic. Hemostasis was performed and the abdominal cavity was closed. The mice were laparotomized under anesthetization to observe tumors on the surface of livers. Four weeks later, the mice with visible tumors were killed, and their livers excised. The livers were then frozen and cryostated to prepare transverse 10 μm sections, which were made at 5 different levels to cover the entire liver. The sections were mounted and stained with haematoxylin and eosin. The area occupied by each tumor in the liver was measured under microscopy using the Sigma Scan program and then the areas were combined to obtain the total tumor area. The relative areas occupied by the tumors were calculated in accordance with the following formula: total tumor areas/liver area × 100.

Challenge of the vaccinated mice with parental tumor cells

The mice without visible tumors on the surface of livers from the experiments above were intraportally injected with 2×10^5 or 2×10^6 parental EL-4 tumor cells to detect whether systemic anti-tumor immunity had been generated. Four weeks later the mice were killed and hepatomized. The relative areas occupied by tumors in the livers were analyzed as above.

AAV-angiostatin therapy to combat disseminated liver cancers in mice vaccinated with AAV-B7.1-transfected EL-4 tumor cells

Mice vaccinated with AAV-B7.1-transfected EL-4 tumor cells and found to be free of liver tumors were intraportally injected with 2×10^6 parental EL-4 tumor cells with a 30-gauge needle, followed by intraportal transfusion of 3×10^{11} particles of AAV-angiostatin. Pressure was applied with a sterile cotton tip applicator until the injection site was hemostatic. Homeostasis was performed and the abdominal cavity was closed. Unvaccinated mice and empty AAV virus were used as controls. Four weeks after the operation, the mice were killed and their livers excised. The relative areas occupied by tumors in the livers were analyzed as above.

Survival studies

Mice vaccinated with AAV-B7.1-transfected EL-4 tumor cells that were found to be free of tumors and subsequently challenged with 2×10^6 parental EL-4 tumor cells, and intraportally transfused with AAV-angiostatin, were weighed thrice weekly and assessed together with controls. Moribund mice were euthanized according to preestablished criteria as described previously.[11]

Immunohistochemistry of tissue sections

Cryosections (10 μm thickness) prepared from livers following intraportal delivery of therapeutic agents were incubated overnight with specific Abs. They were subsequently incubated for 30 min with appropriate secondary antibodies (VECTASTAIN® Universal Quick kit, Vector Laboratories, Burlingame, CA), and developed with Sigma FAST™ DAB (3,3′-diaminobenzidine tetrahydrochloride) and $CoCl_2$ enhancer tablets (Sigma Chemical Co., St. Louis, MO). Sections were counterstained with Mayer's hematoxylin.

Western blot analysis

Tissues from mice were excised, minced, and homogenized in protein lysate buffer. AAV-transfected cells were lysed in the same

buffer. Debris was removed by centrifugation at 10,000 *g* for 10 min at 4°C. Lysates from each group of mice were pooled, and the protein content determined. Protein samples (100 μg) were resolved on 10% polyacrylamide SDS gels and electrophoretically transferred to nitrocellulose Hybond C extra membranes (Amersham Life Science, Buckinghamshire, England). After the membranes were blocked with 5% BSA, blots were incubated with specific primary Abs, followed by horseradish peroxidase-conjugated secondary antibodies and developed by enhanced chemiluminescence (Amersham International plc, Buckinghamshire, England) and exposure to X-Ray film. Band density was quantified using the Sigma Scan Program.

Cytotoxicity assays

Splenocytes harvested from mice vaccinated with AAV-B7.1-transfected EL-4 tumor cells and found to be free of liver tumors, and incubated at 37°C with EL-4 target cells in graded E:T ratios in 96-well round-bottom plates. After a 4 hr incubation, 50 μl of supernatant was collected, and lysis was measured using the Cyto Tox 96 Assay kit (Promega, Madison, WI). Background controls for nonspecific target and effector cell lysis were included. After background subtraction, the percentage of cell lysis was calculated using the formula: 100 × (experimental-spontaneous effector-spontaneous target/maximum target-spontaneous target).

Statistical analysis

For the tumor volume and relative areas occupied by tumors, Kruskal-Wallis tests were performed to test the significance of the treatment effect, whereas log rank tests were performed for survival data. For other data, results were expressed as mean values ± standard deviation (s.d.), and a Student's *t*-test was used for evaluating statistical significance; *p* values were considered to be statistically significant when less than 0.05. Each experiment was repeated at least once.

Results

Rapid and efficient transfection of EL-4 tumor cells with AAV-B7.1

The efficiency of transfection of parental EL-4 tumor cells by AAV-B7.1 viruses *in vitro* was analyzed by measuring the expression of B7.1 on the surface of transfectants by flow cytometry (Fig. 1*a*), and was confirmed by immunohistochemistry (Fig. 1*b*) and Western blot analysis (Fig. 1*c*). EL-4 cells transfected with AAV-B7.1 viruses expressed higher levels of B7.1 compared to untransfected parental EL-4 cells. Increased levels of B7.1 could be detected in over 80% of the EL-4 tumor cells transfected with AAV-B7.1 after only 24 hr of infection (data not shown). Similar high levels of transfection of dendritic cells by AAV have been reported previously, demonstrating that AAV can be highly efficacious in transducing cells.[16] The rapid expression of virally expressed B7.1 presumably relates to the high metabolic activity of tumor cells.

Vaccination with AAV-B7.1-transfected tumor cells leads to infiltration of tumors by CD8$^+$ T cells and NK cells, and inhibition of the formation and growth of tumors in the liver

EL-4 cells (2×10^5) that had either been transfected with either empty AAV or AAV-B7.1 were intraportally injected into the livers of mice ($n = 10$) to compare the formation and growth of disseminated hepatic metastatic tumors infected with AAV *vs.* those infected with AAV engineered to express *B7.1*. Four weeks later, all mice underwent laparotomy, and mice with visible tumors were hepatectomized. Livers were sectioned, and the relative areas occupied by tumors were measured with a Sigma Scan program as illustrated in Figure 2*a*. The mean relative areas were 3.2% and 22.9%, respectively, after treatment with 2×10^5 EL-4 cells transfected with either AAV-B7.1 or empty AAV. Thus, vaccination with AAV-B7.1-transfected EL-4 cells led to statistically significant ($p < 0.001$) reductions (86%) in the relative areas occupied by tumors. Furthermore, 60% of the mice were completely free of liver tumors, whereas in contrast only 5 to 10% of mice vaccinated with AAV-transfected tumors were free of liver tumors.

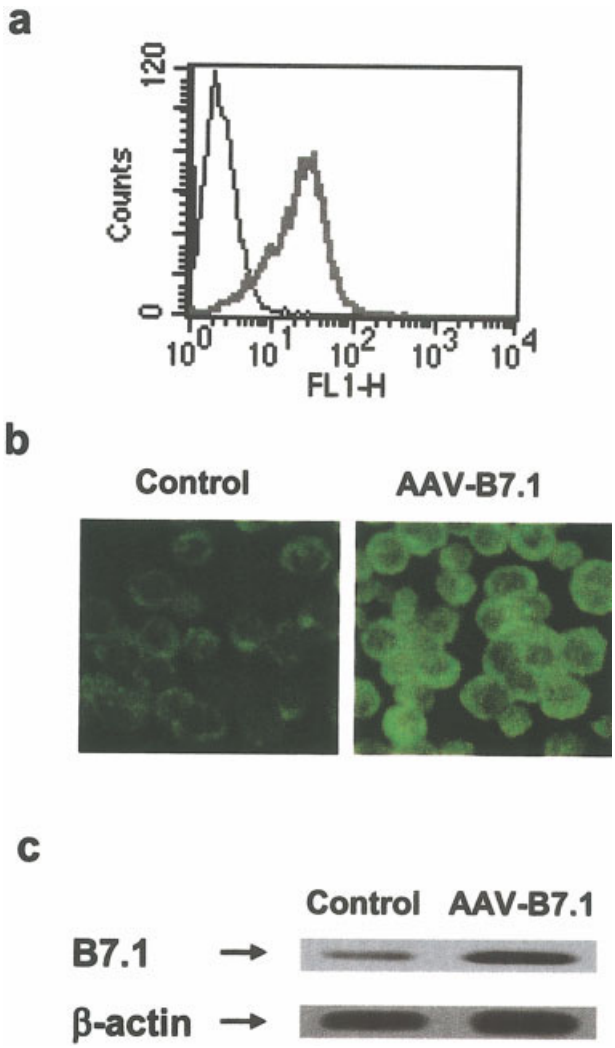

Figure 1 – AAV delivery of B7.1. (*a*) FACscan analysis of B7.1 expression. Parental EL-4 cells were incubated with AAV-B7.1 particles for 24 hr, stained with anti-B7.1 MAb and subjected to flow cytometry. B7.1 protein expression on the surface of AAV-B7.1-infected EL-4 cells is shown by thick lines and background staining of AAV-B7.1-infected EL-4 cells with secondary Abs by light lines. (*b*) Fluorescence microscopy analysis of the *in vitro* transfection efficiency of AAV-B7.1. B7.1 expression on EL-4 cells transfected with AAV-B7.1 was visible following immunostaining with specific MAb and FITC-labeled secondary Ab. EL-4 cells incubated with empty AAV and stained with antibody served as controls. (*c*) Western blot analysis of B7.1 expressed by EL-4 cells. B7.1 expression in AAV-B7.1-transfected and control AAV-transfected EL-4 cells was further confirmed by Western blot analysis. Blots were stained with an anti-β-actin MAb to confirm that gel lanes contained identical amounts of protein.

To further investigate the anti-tumor immunity generated by AAV-B7.1 mediated vaccination, liver tumors were immunostained with antibodies specific for different lymphocyte subsets in order to measure leukocyte infiltration. Disseminated tumors from the control group contained few CD8$^+$ and CD4$^+$ lymphocytes, and sparse numbers of NK cells (Fig. 2*b*). In contrast, highly elevated numbers of CD8$^+$ T cells and NK cells, and conversely small numbers of CD4$^+$ T cells infiltrated the tumors of mice immunized with AAV-B7.1-transfected tumor cells (Fig. 2*b*). This

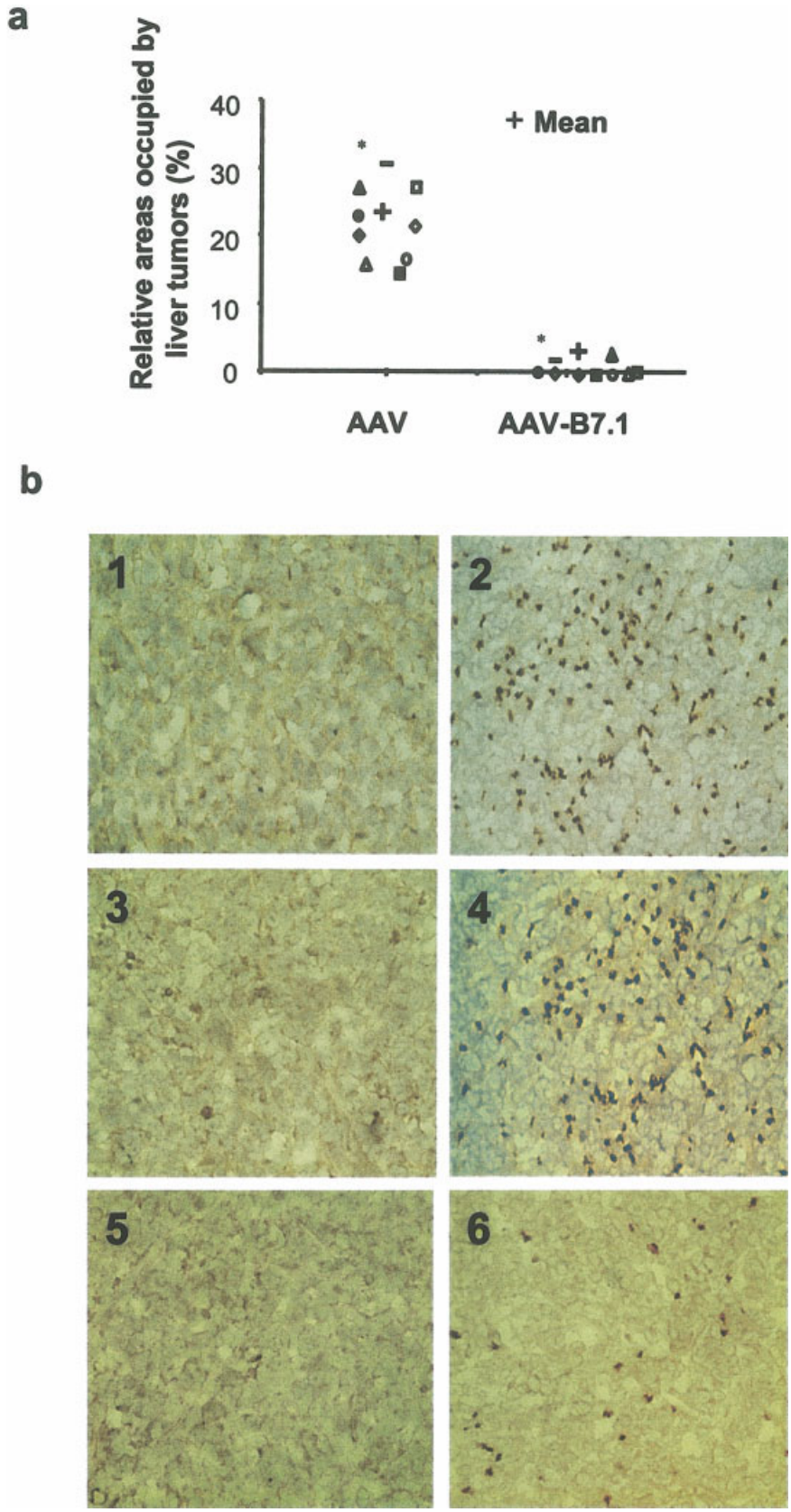

FIGURE 2 – Vaccination with AAV-B7.1-transfected EL-4 cells leads to the infiltration of tumors by $CD8^+$ T cells and NK cells, and inhibits growth of tumors that disseminate to the liver. Tumors disseminated to the liver were established by intraportal injection of 2×10^5 EL-4 cells that had been transfected with either AAV-B7.1 or empty AAV particles. Four weeks later, all the mice underwent laparotomy to observe visible tumors on the surface of livers. (*a*) Livers were sectioned, and relative areas of tumors were measured with a Sigma Scan program. Mean relative areas occupied by tumors are indicated by the large crosses. (*b*) Liver tumors from vaccinated mice (2, 4 and 6) and unvaccinated mice (1, 3 and 5) were immunostained with leukocyte-type specific Abs to show infiltrating $CD8^+$ T cells (1 and 2), NK cells (3 and 4) and $CD4^+$ T cells (5 and 6).

result indicates that AAV-B7.1-mediated anti-tumor immunity mainly relies on $CD8^+$ T cells and NK cells, in accordance with previous reports.[6,8]

Memorized immunity induced by AAV-B7.1-mediated vaccination is prolonged, tumor-specific and protects against subsequent tumor challenge

The anti-tumor cytolytic activity against parental EL-4 cells displayed by splenocytes from tumor-free cured mice, which had been intraportally injected 28 days earlier with AAV-B7.1-transfected EL-4 cells, was significantly ($p < 0.01$) augmented *vs.* splenocytes from mice that had received empty AAV transfected EL-4 cells (Fig. 3*a*). Anti-tumor cytolytic activity against the original modified EL-4 cells engineered to express B7.1 that were used to vaccinate mice was even greater indicating that expression of B7.1 by EL-4 cells facilitates tumor cell lysis by cytolytic splenocytes (data not shown) as reported previously.[6] The anti-tumor cytolytic activity of splenocytes from naïve mice is presumably due to NK cell activity. A proportion of the anti-tumor cytolytic activity of splenocytes from mice vaccinated with AAV-B7.1-transfected EL-4 cells is also probably due to NK cell activity, as we have previously reported that anti-tumor cytolytic activity generated in response to B7.1-mediated immunotherapy is both NK cell, and $CD8^+$ T-cell-dependent,[6] and as above, NK cells infiltrate the tumors in increased numbers. The mice cured of their tumors by intraportal injection of AAV-B7.1- transfected EL-4 cells were rechallenged by intraportal injection of 2×10^5 parental EL-4 tumor cells. Tumors reappeared in only 10% (1/10) of mice, indicating that systemic anti-tumor immunity had been established. In contrast, disseminated hepatic tumors appeared in 100% (10/10) of the unvaccinated mice with significantly large areas (up to 30%) occupied by tumors (data not shown). However, the memorized anti-tumor immunity failed to protect against a challenge with a much larger number (2×10^6) of parental EL-4 tumor cells. In this situation, tumors that disseminated to the liver were observed in all the mice, though the average relative areas occupied by tumors were significantly smaller ($p < 0.01$) in vaccinated mice (30% of liver) than in unvaccinated (51% of liver) mice (Fig. 3*b*). Note that naïve animals injected with 2×10^5 control EL4 cells in Figure 2*a* showed a lower percentage (22.9%) of tumor areas as compared to mice already injected and rechallenged with B7.1-expressing cells (30%, Fig. 3*b*), presumably because the number (2×10^6) of cells used in the latter challenge was increased by 10-fold. In the latter case, when the same number (2×10^5) of cells was used in the challenge only 10% of mice developed tumors, whereas 100% of mice vaccinated with EL-4 cells infected with empty AAV developed tumours as described above.

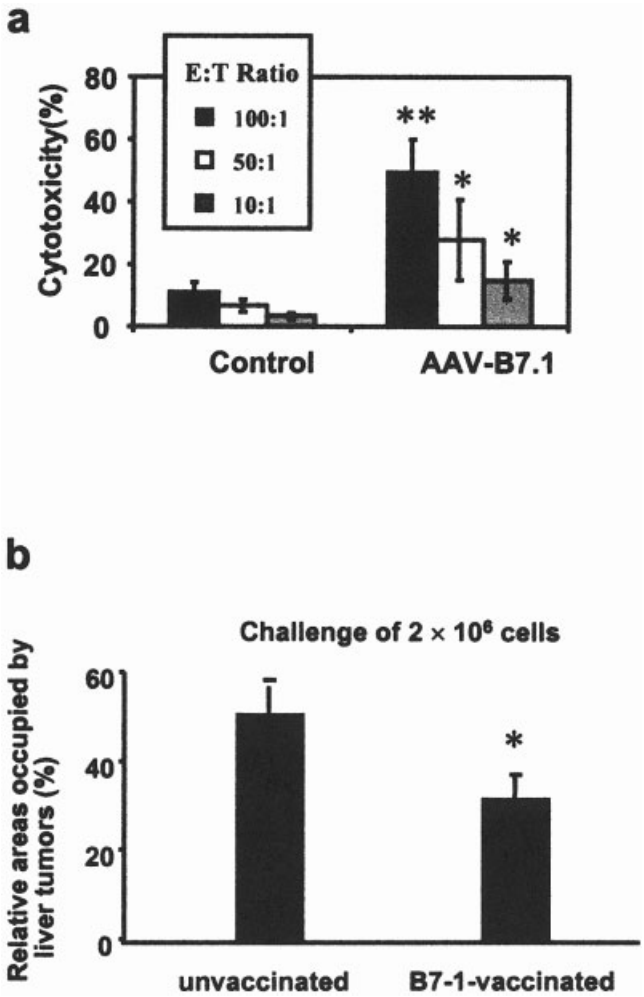

FIGURE 3 – Anti-tumor immunity generated by vaccination with AAV-B7.1-transfected EL-4 cells is memorized. (*a*) Splenocytes from mice vaccinated with AAV-B7.1-transfected tumor cells display enhanced anti-tumor cytolytic activity against parental EL-4 cells. Spleens were removed from C57BL/6 mice that were free of liver tumors following intraportal injection of AAV-B7.1-transfected EL-4 cells. Splenocytes were prepared and tested for cytotoxic activity against parental EL-4 tumor cells. Their activity was compared to that of splenocytes from control animals that received empty AAV-transfected EL-4 cells. The percentage cytotoxicity is plotted against various effector to target (E:T) ratios. (*b*) Vaccinated mice inhibit an intraportal rechallenge of a heavy burden of 2×10^6 parental EL-4 cells. The growth of tumors disseminated to the liver was suppressed compared to the growth of tumors in control mice injected with empty AAV transfected EL-4 cells. * and ** indicate a significant and highly significant difference from control groups of mice at $p < 0.01$ and $p < 0.001$, respectively.

Subsequent antiangiogenic therapy with AAV-angiostatin augments the therapeutic efficacy of AAV-B7.1-mediated vaccination to protect mice against a heavy tumor burden

We previously reported that injection of a recombinant AAV-angiostatin vector *via* a portal vein led to long-term expression of exogenous angiostatin in the liver, which inhibited tumor vascularization.[11] Similar results were achieved in our study in that the expression of transgenic angiostatin in hepatocytes rose rapidly to a high level within 2 weeks, increased to a peak level within 2 months and then was stably expressed at a constant level for at least 6 months after injection of AAV-angiostatin. AAV-angiostatin therapy resulted in a statistically significant reduction in tumor vessel density and increased the median distance to the nearest anti-CD31 MAb-labeled vessel from an array of points within the tumor (data not shown; for details please refer to ref. 11).

We investigated whether intraportal delivery of AAV-angiostatin 1 month following vaccination with AAV-B7.1-transfected tumor cells would augment the therapeutic efficacy of B7.1-mediated immunotherapy. AAV-B7.1-transfected EL-4 tumor cells (2×10^5) were intraportally injected into the livers of mice. Four weeks later, these vaccinated mice underwent laparotomy, and mice with visible liver tumors were excluded from the following experiments. The mice without tumors were intraportally injected with 2×10^6 EL-4 parental cells, followed by intraportal injection of either empty AAV ($n = 10$) or AAV-angiostatin ($n = 10$). Unvaccinated mice used as controls were intraportally injected with 2×10^6 parental EL-4 cells, followed by either empty AAV ($n = 10$), or AAV-angiostatin ($n = 10$). The mice were sacrificed 4 weeks later, hepatectomized and the livers transversely sectioned. The relative areas occupied by tumors in the livers are illustrated in Figure 4*a*. The mean relative areas of tumors in unvaccinated mice receiving either empty AAV or AAV-angiostatin were 42.3% and 17.7%, respectively. Thus, AAV-angiostatin significantly suppressed the growth of tumors that had dissemi-

nated to the liver by 56%, in accord with our previous report.[11] Vaccination with AAV-B7.1-transfected EL-4 cells also significantly suppressed the growth of tumors by 38% such that 26.2% of the liver was occupied by tumors, compared to 42.3% of the liver in the unvaccinated mice. The mean relative area occupied by liver tumors in mice vaccinated with AAV-B7.1-transfected EL-4 cells and treated with AAV-angiostatin was only 5.6%, and only 50% of mice had visible tumors on the surface of the liver. The reduction in the relative areas occupied by liver tumors was decreased by 87% compared to unvaccinated mice treated with empty AAV, by 79% compared to mice vaccinated with AAV-B7.1-transfected EL-4 cells and treated with empty AAV and by 68% compared to unvaccinated mice treated with AAV-angiostatin. Thus, the sizes and numbers of tumors on the surface of livers of mice vaccinated with AAV-B7.1-transfected EL-4 cells and subsequently treated with AAV-angiostatin were greatly reduced, compared to unvaccinated mice treated with empty AAV, vaccinated mice treated with empty AAV and unvaccinated mice treated with AAV-angiostatin (Fig. 4*b*).

Vaccination with AAV-B7.1-transfected tumor cells synergizes with intraportal delivery of AAV-angiostatin in improving the survival rate of mice bearing liver tumors

We further investigated whether the synergy obtained by vaccination with AAV-B7.1-transfected EL-4 cells followed by AAV-angiostatin therapy would offer a survival benefit for mice. C57BL/6 mice were intraportally injected with 2×10^5 AAV-B7.1-transfected EL-4 tumor cells. Four weeks later, all the mice underwent laparotomy. Mice with visible tumors in their livers were excluded from the experiments. All mice without tumors were intraportally injected with 2×10^6 parental EL-4 cells, followed by intraportal injection of either 3×10^{11} particles of empty AAV ($n = 10$) or 3×10^{11} particles of AAV-angiostatin ($n = 10$). Unvaccinated mice used as controls were intraportally injected with 2×10^6 parental EL-4 cells, followed by intraportal injection of either 3×10^{11} particles of empty AAV ($n = 10$) or 3×10^{11} particles of AAV-angiostatin ($n = 10$). Both vaccination with AAV-B7.1-transfected EL-4 cells and AAV-angiostatin therapy resulted in significant improvement in the survival rate of mice, compared to unvaccinated mice treated with empty AAV. Median survival times for mice vaccinated with AAV-B7.1-transfected EL-4 cells and treated with empty AAV, and for unvaccinated mice treated with AAV-angiostatin was 33 days and 42 days, respectively, which are significantly ($p < 0.05$ and $p < 0.01$, respectively) different from the median survival time of 25 days for unvaccinated control mice treated with empty AAV (Fig. 4*c*). Combinational therapy by vaccination with AAV-B7.1-transfected EL-4 cells and intraportal delivery of AAV-angiostatin led to a statistically longer survival rate. Six of 10 mice in the combination therapy group survived for more than 100 days after tumor cell inoculation (Fig. 4*c*).

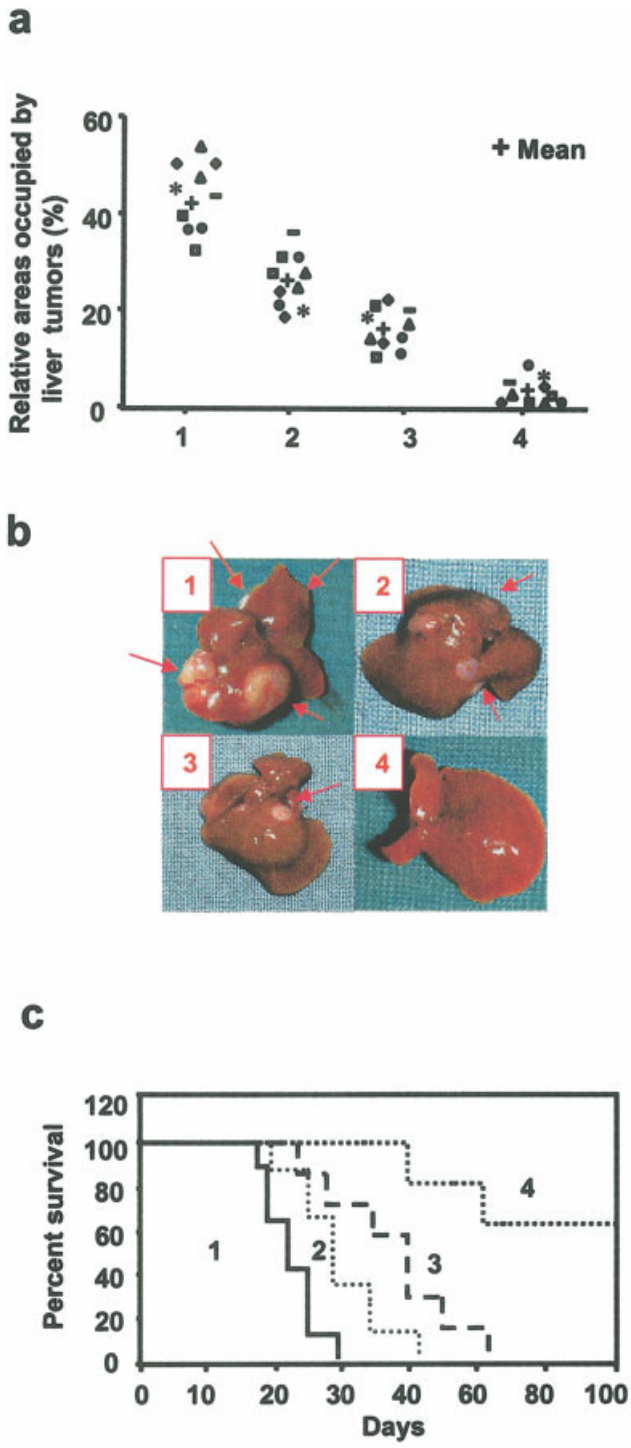

Figure 4 – Vaccination with AAV-B7.1-transfected tumor cells augments AAV-angiostatin therapy to eradicate disseminated liver tumors and improve the survival of mice. (*a*) Combination therapy inhibits tumorigenesis. C57BL mice were vaccinated by intraportal injection of 2×10^5 AAV-B7.1-transfected EL-4 cells, and 4 weeks later mice that were free of tumors were intraportally injected with 2×10^6 parental EL-4 cells and either 3×10^{11} particles of AAV-angiostatin (data 4) or empty AAV virus (data 2). Unvaccinated mice treated with AAV-angiostatin (data 3) or empty AAV virus (data 1) were included as controls. The mice were hepatectomized 4 weeks later, the livers were removed and sectioned and relative areas of tumors were measured. Mean relative areas occupied by tumors are indicated by the large crosses. (*b*) Visualization of tumors on the surface of livers. Representative photographs of livers with metastatic tumors from unvaccinated mice treated with empty AAV (photo 1), vaccinated mice treated with empty AAV (photo 2), unvaccinated mice treated with AAV-angiostatin (photo 3) and vaccinated mice treated with AAV-angiostatin (photo 4). The arrows point to tumors in the livers. (*c*) Combinational therapy increases survival rates and times. Mice that had been vaccinated by intraportal injection of AAV-B7.1-transfected EL-4 cells 4 weeks earlier were intraportally injected with 2×10^6 parental EL-4 cells and either 3×10^{11} particles of AAV-angiostatin (curve 4) or empty AAV viruses (curve 2). Unvaccinated mice treated with AAV-angiostatin (curve 3) or empty AAV virus (curve 1) were included as controls. Mice were observed thrice weekly and were sacrificed when they became moribund by preestablished criteria. A plot of survival curves is illustrated.

Discussion

Metastatic liver cancer is common and remains problematic as conventional therapies, such as surgery, chemotherapy or radiation therapy, have little impact. To have a major clinical impact, systemic or regional therapies must be capable of eradicating all liver metastases.[17] Anti-angiogenic therapy is widely regarded as a promising treatment strategy to combat cancers including metastases based on the fact that most tumors are dependent on angiogenesis for survival. Angiogenesis is required to supply of nutrients, growth factors, hormones and oxygen for tumor growth and metastatic development.[18] However, despite many successful experiments with antiangiogenic therapies in suppressing the growth of tumors and their metastases,[19–21] disappointing reports have emerged recently.[22,23] To optimize anti-angiogenic therapy, we established a novel AAV gene transfer system. Intraportal transfusion of AAV-angiostatin led to long-term and persistent expression of transgenic angiostatin in the liver, resulting in significant suppression of tumor cells that had disseminated to the liver.[11] However, anti-angiogenic therapy using AAV-angiostatin could not completely eradicate disseminated liver tumors, presumably because while anti-angiogenic proteins are effective at inducing tumor regression, they are not directly tumoricidal, and hence tumor regrowth frequently recurs once treatment is suspended.

T-cell costimulation is one of the most important factors in the induction of T-cell responses and antitumor immunity.[24,25] At least 2 signals are required for activation of naïve T cells, namely, an antigen specific signal delivered through the T-cell receptor and an antigen-independent or costimulatory signal. Intratumoral gene transfer of the T-cell costimulator B7.1 has been shown to induce the eradication of established tumors. B7.1-induced anti-tumor immunity was largely dependent on $CD8^+$ T cells and NK cells, accompanied by augmented tumor-specific cytolytic T-cell activity involving both the perforin and fas-ligand pathways.[6–8,26] In accord, we revealed in the present study that B7.1-mediated immunotherapy induced the infiltration of large numbers of $CD8^+$ T cells, and NK cells into liver tumors, whereas there was only a small increase in the numbers of $CD4^+$ T cells. In another approach, *ex vivo* transfection of the *B7.1* gene into immunogenic tumor cells attributes such cells with an ability to generate anti-tumor CTLs and prevent tumorigenesis when transfectants are injected into animals.[3–5] Therefore, immunization with B7.1-transfected tumor cells provides another useful method for stimulating immune responses in combating multiple metastases after surgery. The AAV-mediated transfection system used in the present study is advantageous as it can be employed to rapidly and easily transfect tumor cells, which might otherwise be difficult to transfect *ex vivo*. Our study demonstrates for the first time that localized intraportal delivery of AAV-B7.1-transfected EL-4 cells induces memorized anti-tumor immunity, which renders vaccinated mice with the ability to resist a challenge of parental EL-4 cells. However, the key finding of our study is that angiostatin and B7.1-immunotherapy synergize in causing the eradication of tumors that disseminate to the liver. In contrast, neither vaccination with AAV-B7.1-transfected EL-4 cells nor AAV-angiostatin monotherapy was effective. The therapeutic efficacy of B7.1-immunotherapy is long-lived, such that synergy with anti-angiogenic treatment was achieved when AAV-angiostatin treatment was initiated 1 month following immunotherapy. The results contrast with those obtained in a study,[27] in which the interaction between cyclophosphamide (CPA) and angiostatin on the growth of primary Lewis lung carcinoma (LLC) tumors was examined. Angiostatin enhanced the antimetastatic effects of CPA, but without significantly inffuencing the effects of CPA on primary tumor growth. CPA plus angiostatin inhibited endothelial tube formation. It was argued that interrupting specific steps in the angiogenesis process might be an effective approach to the treatment of subclinical distant metastases.[27] The interaction between angiostatin and B7.1 differs from that between angiostatin and CPA as we have previously shown that the former combination completely eradicates established primary tumors, whereas monotherapies are ineffective.[7] We suggest that B7.1 inhibits tumor metastasis by eradicating single tumor cells and microfoci, whereas angiostatin inhibits endothelial tube formation required for the growth and establishment of metastases, perhaps in combination with an immune factor derived from B7.1 immunotherapy. The combination of the 2 approaches, targeting different facets of the process of metastasis, yields a synergistic outcome.

Localized vector delivery has been used to specifically target transgene expression within tumors.[28–30] Although systemic vector delivery may be the best option in many clinical settings, the unique anatomic features of the liver facilitate regional gene therapy approaches for unresectable hepatic metastases.[29] The advantages of localized vector delivery are obvious, namely, it can induce high level expression of transgenic proteins *in situ* to achieve effective anti-tumor activity and reduce the possibility of side-effects compared to a systemic delivery approach.

The combinational gene therapy approach described herein, using initial treatment with the costimulatory molecule B7.1 and subsequential treatment with the angiogenesis inhibitor angiostatin, led to persistent over-expression of exogenous angiostatin in hepatocytes for up to 6 months and suppressed the growth of lymphomas that had disseminated to the liver. The results have important implications for the treatment of cancers of the liver that are intractable to treatment.

Acknowledgements

The work was supported in part by a seed grant from the HKU Foundation to RA Xu, a grant from the National Natural Science Foundation of China (30100178) and a Young and Middle-aged Excellent Scientists' Prize Fund from Shandong Provincial Scientific and Technological Committee of China (9836) to X Sun. X Sun is a recipient of a Wellcome Trust Research Leave Fellowship, UK.

References

1. Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell 1994;79:185–8.
2. Takayama T, Sekine T, Makuuchi M, Yamasaki, S, Kosuge T, Yamamoto J, Shimada K, Sakamoto M, Hirohashi S, Ohashi Y, Kakizoe T. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. Lancet 2000; 356:802–7.
3. Baskar S, Ostrand-Rosenberg S, Nabavi N, Nadler LM, Freeman GJ, Glimcher LH. Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. Proc Natl Acad Sci USA 1993;90:5687–90.
4. Chen L, Ashe S, Brady WA, Hellstrom I, Hellstrom KE, Ledbetter JA, McGowan P, Linsley PS. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. Cell 1992;71:1093–102.
5. Townsend SE, Allison JP. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. Science 1993; 259:368–70.
6. Kanwar JR, Berg RW, Lehnert K, Krissansen GW. Taking lessons from dendritic cells: multiple xenogeneic ligands for leukocyte integrins have the potential to stimulate anti-tumor immunity. Gene Ther 1999;6:1835–44.
7. Sun X, Kanwar JR, Leung E, Lehnert K, Wang D, Krissansen GW. Angiostatin enhances B7.1-mediated cancer immunotherapy independently of effects on vascular endothelial growth factor expression. Cancer Gene Ther 2001;8:719–27.
8. Sun X, Leung E, Kanwar JR, Lehnert K, Wang D, Krissansen GW. Gene transfer of antisense hypoxia inducible factor-1α enhances the therapeutic efficacy of cancer immunotherapy. Gene Ther 2001;8: 638–45.
9. Kim K-R, Moon H-E, Kim K-W. Hypoxia-induced angiogenesis in human hepatocellular carcinoma. J Mol Med 2002;80:703–14.
10. Lovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet 2003;362:1907–17.

11. Xu R, Sun X, Chan D, Tse L-Y, Xu S, Xiao W, Kung H-F, Krissansen GW, Fan S-T. Long-term expression of angiostatin suppresses liver metastatic cancer in mice. Hepatology 2003;47:1451–60.
12. Xu L, Daly T, Gao C, Flotte TR, Song S, Byrne BJ, Sands MS, Parker PK. CMV-beta-actin promoter directs higher expression from an adeno-associated viral vector in the liver than the cytomegalovirus or elongation factor 1 alpha promoter and results in therapeutic levels of human factor X in mice. Hum Gene Ther 2001;12:563–7.
13. Donello J, Loeb JE, Hope TJ. Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. J Virol 1998;72: 5085–92.
14. Xu R, Janson CG, Mastakov M, Lawlor P, Young D, Mouravlev A, Fitzsimons H, Choi KL, Ma H, Dragunow M, Leone P, Chen Q, et al. Quantitative comparison of expression with adeno-associated virus (AAV-2) brain-specific gene cassettes. Gene Ther 2001;8:1323–32.
15. Xiao W, Chirmule N, Schnell MA, Tazelaar J, Hughes JV, Wilson JM. Route of administration determines induction of T cell independent humoral response to adeno-associated virus vectors. Mol Ther 2000;1:323–9.
16. Liu Y, Chiriva-Internati M, Grizzi F, Salati E, Roman JJ, Lim S, Hermonat PL. Rapid induction of cytotoxic T-cell response against cervical cancer cells by human papillomavirus type 16 E6 antigen gene delivery into human dendritic cells by an adeno-associated virus vector. Cancer Gene Ther 2001;8:948–57.
17. Tada H, Maron DJ, Choi EA, Barsoum J, Lei H, Xie Q, Liu W, Ellis L, Moscioni AD, Tazelaar J, Fawell S, Qin X, et al. Systemic IFN-β gene therapy results in long-term survival in mice with established colorectal liver metastases. J Clin Invest 2001;108:83–95.
18. Folkman J. Role of angiogenesis in tumor growth and metastasis. Semin Oncol 2002;29:15–8.
19. Bouquet C, Frau E, Opolon P, Connault E, Abitobl M, Griscelli F, Yeh P, Perricaudet M. Systemic administration of a recombinant adenovirus encoding a HAS-angiostatin Kringle 1-3 conjugate inhibits MDA-MB-231 tumor growth and metastasis in a transgenic model of spontaneous eye cancer. Molec Therapy J Am Soc Gene Therapy 2003;7:174–84.
20. O'Reilly MS, Holmgren L, Chen C, Folkman J. Angiostatin induces and sustains dormancy of human primary tumours in mice. Nat Med 1996;2:689–92.
21. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, et al. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 1994;79:315–28.
22. Pawliuk R, Bachelot T, Zurkiya O, Eriksson A, Cao Y, Leboulch P. Continuous intravascular secretion of endostatin in mice from transduced hematopoietic stem cells. Molec Therapy J Am Soc Gene Therapy 2002;5:345–51.
23. Eisterer W, Jiang X, Bachelot T, Pawliuk R, Abramovich C, Leboulch P, Hogge D, Eaves C. Unfulfilled promise of endostatin in a gene therapy-xenotransplant model of human acute lymphocytic leukemia. Molec Therapy J Am Soc Gene Therapy 2002;5:352–9.
24. Ostrand-Rosenberg S, Baskar S, Patterson N, Clements VK. Expression of MHC class II and B7-1 and B7-2 costimulatory molecules accompanies tumor rejection and reduces the metastatic potential of tumor cells. Tissue Antigens 1996;47:414–21.
25. Pulaski BA, Term DS, Khan S, Muller E, Ostrand-Rosenberg S. Cooperativity of Straphylococcal aureus enterotoxin B superantigen, Major Histocompatibility Complex Class II, and CD80 for immunotherapy of advanced spontaneous metastases in a clinically relevant postoperative mouse breast cancer model. Cancer Res 2000;60: 2710–5.
26. Kanwar JR, Shen WP, Berg R, Krissansen GW. Effect of survivin antagonists on the growth of established tumors and B7.1 immunogene therapy. J Natl Cancer Inst 2001;93:1541–52.
27. Mauceri HJ, Seetharam S, Beckett MA, Schumm LP, Koons A, Gupta VK, Park JO, Manan A, Lee JY, Montag AG, Kufe DW, Weichselbaum RR. Angiostatin potentiates cyclophosphamide treatment of metastatic disease. Cancer Chemother Pharmacol 2002;50:412–8.
28. Bass C, Cabrera G, Elgavish A, Robert B, Siegal GP, Anderson SC, Maneval DC, Curiel DT. Recombinant adenovirus-mediated gene transfer to genitourinary epithelium in vitro and in vivo. Cancer Gene Ther 1995;2:97–104.
29. de Roos WK, Fallaux FJ, Marinelli AW, Lazaris-Karatzas A, von Geusau AB, van der Eb MM, Cramer SJ, Terpstra OT, Hoeben RC. Isolated-organ perfusion for local gene delivery: efficient adenovirus-mediated gene transfer into the liver. Gene Ther 1997;4:55–62.
30. Lee SS, Einsenlohr LC, McCue PA, Mastrangelo MJ, Lattime EC. Intravesical gene therapy: in vivo gene transfer using recombinant vaccine virus vectors. Cancer Res 1994;54:3325–8.