Enhanced antitumoral effect of adenovirus-mediated cytosine deaminase gene therapy by induction of antigen-presenting cells through stem cell factor/granulocyte-macrophage colony-stimulating factor gene transfer

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Suicide gene therapy has been studied intensively for the treatment of cancer. A limited antitumoral effect was obtained by intratumoral injection of adenovirus harboring *Escherichia coli* cytosine deaminase gene (AdCD) in tumor-bearing mice followed by continuous administration of 5-fluorocytosine (5FC). To address the drawbacks of the limited potential for the induction of antitumoral immunity by CD suicide gene therapy, we hypothesized that antigen-presenting cells (APCs) might contribute to the efficient induction of an antitumoral immune response in tumor-bearing mice undergoing suicide gene therapy. We preinjected the mice with murine stem cell factor (SCF)-encoding adenovirus (AdSCF) and murine granulocyte-macrophage colony-stimulating factor (GM-CSF)-encoding adenovirus (AdGM-CSF); after 7 days, the mice were inoculated with CT26 colon adenocarcinoma. AdCD was injected intratumorally into tumor-bearing mice followed by 5FC administration. The results showed that AdSCF/AdGM-CSF treatment could increase the number, surface molecule expression, and function of APCs efficiently. A more significant growth inhibition of established tumors and a prolongation of the survival period were observed in tumor-bearing mice after AdSCF/AdGM-CSF pretreatment in combination with AdCD/5FC therapy when compared with mice treated with AdSCF or AdGM-CSF in combination with AdCD/5FC, or AdCD/5FC alone ($P < .01$). Cytotoxic T-lymphocyte activity was induced efficiently after the combined therapy, and mRNA of tumor necrosis factor- α , interleukin-4, interferon- γ , and interleukin-2 was present in the tumor mass after combined therapy, suggesting that a more potent antitumoral response was induced by enhanced APCs. Our results demonstrated that AdSCF/AdGM-CSF pretreatment could activate APCs, and that these APCs could present the tumor antigens released from AdCD/5FC-killed tumor cells and activate the antitumoral response of the host, thus increasing the therapeutic efficiency of suicide gene therapy. **Cancer Gene Therapy (2000) 7, 177–186**

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Several suicide genes, such as herpes simplex thymidine kinase gene (HSV-tk), *Escherichia coli* cytosine deaminase gene (CD), and *Varicella zoster* thymidine kinase gene, have been developed for the treatment of tumors both in animal models and in patients.^{1–3} Approximately 10% of the patients undergoing clinical trials with gene therapy received suicide gene therapy.⁴ Transfer of a suicide gene into tumor cells could result in the sensitization of tumor cells to the nontoxic prodrugs. HSV-tk/ganciclovir (GCV) is one of the most extensively

studied suicide gene systems. It has been found to be especially effective in the treatment of brain tumors, and satisfactory therapeutic effects have been achieved in various tumor models.^{5,6} The CD enzyme exists in many bacteria and fungi but not in mammalian cells. 5-Fluorocytosine (5FC), which is widely used for the treatment of fungal and bacterial infections, is metabolized by CD enzyme to 5-fluorouracil, a highly toxic agent for the therapy of various kinds of tumors.^{7–12} 5-Fluorouracil is one of the most commonly used chemotherapeutic agents for the treatment of gastrointestinal tumors such as colon adenocarcinoma, pancreatic cancer, etc. The growth of tumor cells modified to express CD could be inhibited significantly by 5FC both *in vitro* and *in vivo*. Therapy with suicide gene transfer was sometimes effective, but in other instances it was reported to be not efficient in the induction of antitumoral immunity, so

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tumor recurrence or metastasis often occurred after suicide gene therapy.¹³⁻¹⁶

Antigen (Ag)-presenting cells (APCs), especially dendritic cells (DCs) and macrophages, can efficiently present tumor Ag during a T-cell-mediated immune response. T cells can recognize tumor Ags presented by major histocompatibility complex (MHC) molecules on the surface of APCs. Cytokines, such as stem cell factor (SCF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), play important roles in the recruitment of APCs. GM-CSF is one of the most important growth factors in the differentiation of APCs. A large quantity of DCs can be propagated through GM-CSF/tumor necrosis factor- α (TNF- α) or GM-CSF/interleukin-4 (IL-4) supplements. SCF has been found to function synergistically with GM-CSF to promote a substantial expansion of APCs, because it can stimulate earlier progenitor hematopoietic cells. In our previous report, we have illustrated that tumor Ag-pulsed, GM-CSF gene-modified APCs could elicit potent antitumoral effects *in vivo*.¹⁷ Combined treatment with adenovirus-mediated SCF and GM-CSF gene transfer might provide a potent approach to the production of APCs *in vivo*.

We hypothesized that pretreatment of mice with SCF/GM-CSF gene transfer in combination with CD suicide gene therapy might have more significant antitumoral effects than SCF and/or GM-CSF gene therapy or CD suicide gene therapy. Therefore, in this study AdSCF and AdGM-CSF were preinjected into mice to increase the number, surface molecule expression, and function of APCs for more efficient tumor Ag presentation. AdCD/5FC was administered for the eradication of pre-established tumors, and the APCs could process and present the tumor Ags released by AdCD/5FC gene therapy to the immune system and augment the antitumoral response of the host.

MATERIALS AND METHODS

Animals and cell lines

Male or female BALB/c and C57BL/6 mice of 6 weeks of age (provided by SIPPR BK Experimental Animal Company, Shanghai, People's Republic of China) were housed in a specific pathogen-free environment for all experiments. The following cell lines were used: CT26, a colon adenocarcinoma cell line derived from BALB/c mice; C8, a FBL-3-specific CD4⁺ T helper (Th) cell line derived from C57BL/6 mice; and 293, a continuous cell line derived from human embryonic kidney cells. All were maintained in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 mM 2-mercaptoethanol, and 10% fetal calf sera (FCS). All culture media were purchased from Life Technologies (Gaithersburg, Md); FCS was provided by the Shanghai Institute of Biological Products (Shanghai, People's Republic of China).

Recombinant adenovirus preparation

The following adenoviruses were constructed from human adenovirus serotype 5 using homologous recombination: The replication-defective recombinant adenovirus AdLacZ, which

encodes β -galactosidase, AdCD, which encodes *E. coli* CD, AdSCF, which encodes murine SCF, or AdGM-CSF, which encodes murine GM-CSF.^{18,19} The expression of these genes was driven by the CAG promoter. These adenoviruses were propagated in 293 cells, and the titers of the adenovirus were determined with a plaque-forming unit assay.

Ag presentation and phenotype analysis of APCs after induction by AdSCF/AdGM-CSF

C57BL/6 mice were preinjected with 10⁷ AdSCF/AdGM-CSF, and the mice were sacrificed after 9 days for the collection of APCs. Resident macrophages were washed from the peritoneal cavity with RPMI 1640 medium, and the cell pellet was resuspended in complete media after centrifugation at 1000 rpm for 10 minutes at 4°C. The adherent macrophages were detached, and the number of macrophages from each mouse was enumerated under microscopy. The macrophages were then suspended in phosphate-buffered saline (PBS) at a density of 2.5 $\times$ 10⁶/mL. A total of 5 μ g/mL lipopolysaccharide (*E. coli* 055B5) was added to the macrophages and cultured for 24 hours for IL-1 induction. The IL-1 β content in the supernatants was determined using an ELISA kit (R&D Systems, Minneapolis, Minn).

Spleen cells were isolated from sacrificed mice, added on 14.5% metrizamine, and subsequently centrifuged at 600 $\times$ g for 15 minutes. The DCs were collected, enumerated, washed three times, and subsequently suspended in PBS at a density of 5 $\times$ 10⁶/mL. The DCs were inactivated with mitomycin C and cultured with FBL-3-specific CD4⁺ Th (C8) cells in the presence of FBL-3 Ag peptide (CCLCLTVFL, purity >95%, synthesized by CyberSyn). Proliferation of the CD4⁺ Th (C8) cells was determined by [³H]thymidine deoxyribose (Amersham, Arlington Heights, Ill) incorporation as reported elsewhere.²⁰

The isolated macrophages and DCs were washed three times with PBS, incubated with rat anti-mouse monoclonal antibodies against Ia and B7 (PharMingen, San Diego, Calif) for 30 minutes at 4°C, and washed an additional three times with PBS. Next, cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulin G (PharMingen) for 30 minutes at 4°C. The cells were then washed with PBS and resuspended in PBS containing 1% formaldehyde. Control incubations used to determine levels of background staining with the second antibody used irrelevant normal rat anti-mouse immunoglobulin G. Flow cytometry was analyzed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, Calif).

In vivo experimental procedures

BALB/c mice were divided into four groups and were injected intraperitoneally (i.p.) with the following adenoviruses: 10⁷ murine AdGM-CSF, 10⁷ AdSCF, 10⁷ AdSCF/10⁷ AdGM-CSF, or PBS. After 7 days, all of the mice in the four groups were inoculated subcutaneously (s.c.) with 5 $\times$ 10⁴ CT26 colon adenocarcinoma cells. After the tumor nodule grew to $\sim$ 5 $\times$ 5 mm², 10⁸ AdCD was administered intratumorally (i.t.) in tumor-bearing mice once a day for 3 consecutive days. Administration of 5FC 500 mg/kg/day began at 24 hours after the first injection of AdCD and was repeated twice a day for 7 consecutive days. The length and width of the tumor mass were measured with calipers after tumor inoculation, and the tumor sizes were expressed as length $\times$ width (mm²). A total of 10 tumor-bearing mice in each group were housed for 2 months for observation of survival time.

Cytokine mRNA assay

Tumor nodules were obtained from the sacrificed mice 2 weeks after the combined therapy of AdSCF/AdGM-CSF and AdCD/5FC. The tumor mass was minced, and total RNA was isolated. The reverse-transcriptase polymerase chain reaction (RT-PCR) method was used for TNF- α , IL-4, interferon- γ (IFN- γ), and IL-2 mRNA assays. The primers for the PCR analyses were as follows: IL-2 sense, TCC ACT TCA AGC TCT ACA G; IL-2 antisense, GAG TCA AAT CCA GAA CAT DCC; TNF- α sense, GCA GGT CTA CTT TGG AGT CA; TNF- α antisense, ACA TTC GAG GCT CCA GTG AA; IFN- γ sense, CAT GAA AAT CCT GCA GAG CC; IFN- γ antisense, GGA CAA TCT CTT CCC CAC CC; IL-4 sense, ATT ATG CAT ATC CAC GGA TGC GAC AA; IL-4 antisense, AAG GAT CCT TAC GAG TAA TCC ATT TGC ATG A.

First-strand cDNA was synthesized from total RNA by reverse transcription and PCRs were carried out according to the method of Ramesh et al.¹³ Amplification was carried out for 35 cycles (95°C for 20 seconds, 54–57°C for 30 seconds, and 72°C for 30 seconds) depending upon the template to be amplified, followed by a final extension for 15 minutes. The amplified PCR products were detected by agarose gel electrophoresis.

Cytotoxicity assay

A 4-hour ⁵¹Cr release assay was performed for cytotoxic T-lymphocyte (CTL) cytotoxicity determination.²¹ Splenic lymphocytes were isolated from sacrificed tumor-bearing mice after the last injection of 5FC. The lymphocytes were cocultured with inactivated CT26 cells in the presence of 200 U/mL recombinant IL-2 (Genzyme, Cambridge, Mass) for 7 days and subsequently collected as CTL effector cells. CTL activity was determined with a standard 4-hour ⁵¹Cr release assay. A total of 2×10^6 CT26 cells in 0.5 mL RPMI 1640 with 20% FCS were labeled with 200 μ Ci Na⁵¹CrO₄ (Amersham) for 2 hours. The labeled cells were washed three times in serum-free medium. A total of 10^4 target cells were then mixed with effector cells for 4 hours at 37°C at the ratio indicated. For the maximal ⁵¹Cr release control, 0.1 mL of 0.1N HCl was added to the target cells; for the spontaneous ⁵¹Cr control, 0.1 mL of medium was added to the labeled cells. The amount of ⁵¹Cr released was determined by γ -counting on a 1275 Minigamma Counter (LKB-Wallac, Turku, Finland), and the percentage of specific lysis was calculated as follows: $[(\text{experimental cpm} - \text{spontaneous cpm})/(\text{maximal cpm} - \text{spontaneous cpm})]$.

Statistics

All experiments were run in triplicate; the results are means $\pm$ SD of triplicate determinations. Statistical analysis was performed using the Student's *t* test. A *P* value of $<.05$ was considered to be statistically significant.

RESULTS

Increased number, surface molecule expression, and function of APCs by i.p. injection of AdSCF/AdGM-CSF

To augment the antitumoral effects of AdCD/5FC through the induction of APCs, we first examined the effects of AdSCF/AdGM-CSF injection on the number and function of APCs. The data in Table 1 demonstrate

Table 1. Effects of AdSCF/AdGM-CSF Pretreatment on the Recruitment of APCs

Treatment	Number of APCs/mouse	
	Macrophages	DCs
PBS	1.8×10^6	9×10^4
AdLacZ	2.2×10^6	1.1×10^5
AdSCF	$4.5 \times 10^{6*}$	$4.7 \times 10^{5*}$
AdGM-CSF	$8.5 \times 10^{6*}$	$5.4 \times 10^{6*}$
AdSCF/AdGM-CSF	$1.2 \times 10^{7*†}$	$5.7 \times 10^{6*}$

A total of 10^7 adenoviruses encoding murine SCF or GM-CSF were injected i.p. into C57BL/6 mice; after 9 days, peritoneal macrophages or splenic DCs were isolated. *, *P* $<.01$ versus PBS or AdLacZ groups; †, *P* $<.01$ versus AdSCF or AdGM-CSF groups.

that preinjection of the mice with AdSCF or AdGM-CSF can significantly increase the number of macrophages in the peritoneum (*P* $<.01$), and more macrophages were found in the mice with the combined injection of AdSCF and AdGM-CSF (*P* $<.01$). The counts of DCs derived from the spleens of the mice increased markedly after the pretreatment of AdSCF, AdGM-CSF, or AdSCF/AdGM-CSF when compared with those derived from PBS- or AdLacZ-treated mice (*P* $<.01$). AdGM-CSF, either alone or in the presence of AdSCF, was able to elicit more potent stimulating effects on the production of DCs than AdSCF alone (*P* $<.01$).

Flow cytometry was used to determine MHC class II (Ia^b) and B7 expression on the peritoneal macrophages and splenic DCs. The results in Figure 1 demonstrate that pretreatment of the mice with AdSCF increased the expression of MHC class II on macrophages compared with AdLacZ pretreatment. When AdGM-CSF and AdSCF were combined for the pretreatment of mice, the peritoneal macrophages expressed higher levels of Ia and B7 than those in mice that received other pretreatments. AdGM-CSF preinjection was able to increase the expression of Ia and B7 on DCs. A greater expression of these molecules on DCs was observed when AdSCF was combined for pretreatment of the mice, whereas AdSCF alone elicited only slightly increased effects on the expression of these molecules.

The supernatants of the macrophages were collected for IL-1 assay with enzyme-linked immunosorbent assay kits (R&D Systems). The macrophages of AdSCF/AdGM-CSF-treated mice were able to produce 388 pg/mL/48 hours/ 10^6 cells, which was significantly higher than the amount produced by macrophages of AdSCF-, AdGM-CSF-, AdLacZ-, or PBS-treated mice (*P* $<.01$, <200 pg/mL/48 hours/ 10^6 cells of IL-1 were produced by macrophages from mice in the other four groups).

The DCs were inactivated and used as stimulators to CD4⁺ Th (C8) cells in the presence or absence of specific Ag. The results in Figure 2 show that DCs derived from mice treated with AdGM-CSF alone or in combination with AdSCF can markedly stimulate the

Figure 1. Expression of Ia and B7 molecules on macrophages and DCs. Peritoneal macrophages and splenic DCs were prepared from mice that had been preinjected with AdSCF and/or AdGM-CSF 9 days previously. Molecule expressions were determined on a FACScalibur flow cytometer (Becton Dickinson). **A:** Ia on macrophages; **B:** B7 on macrophages; **C:** Ia on DCs; **D:** B7 on DCs.

proliferation of C8 cells compared with the DCs from AdSCF- or PBS-treated mice ($P < .01$). C8 cells stimulated with DCs in the absence of FBL-3 Ag proliferated very poorly. These data indicate that pretreating mice

with AdSCF/AdGM-CSF can markedly increase the number, surface molecule expression, and function of APCs; and these cells might be important for the induction of antitumoral immunity.

Figure 2. Proliferation of Th cells stimulated by inactivated DCs. C57BL/6 mice were injected i.p. with AdSCF/AdGM-CSF; after 9 days, splenic DCs were isolated. The DCs were inactivated by mitomycin C and cultured with FBL-3-specific CD4⁺ Th (C8) cells in the presence or absence of the FBL-3 Ag peptide CCLCLTVFL. Proliferation of the C8 cells was determined by [³H]thymidine deoxyribose incorporation assay.

More potent antitumoral effect of AdCD/5FC suicide gene therapy in combination with AdSCF/AdGM-CSF pretreatment

BALB/c mice were inoculated s.c. with 5×10^4 CT26 colon adenocarcinoma cells; when the tumor nodule grew to $\sim 5 \times 5$ mm² after tumor inoculation, the tumor-bearing mice were injected i.t. with PBS,

AdLacZ, or AdCD followed by 5FC administration. As shown in Figure 3, obvious inhibition of CT26 growth and a prolongation of the survival time of the tumor-bearing mice were found in mice treated with AdCD/5FC compared with the mice treated with PBS, AdLacZ, or 5FC ($P < .01$). A total of 4 of 10 mice with AdCD/5FC suicide gene therapy lived up to 60 days after tumor inoculation. All tumor-bearing animals in control groups died within 40 days after tumor inoculation.

After we observed the antitumoral effects of AdCD/5FC on an established colon adenocarcinoma, we found that AdCD/5FC therapy was not very satisfactory for the treatment of established tumors *in vivo*. Immune responses play important roles in the antitumoral process of the host. We hypothesized that the antitumoral effects of suicide gene therapy might be increased by the efficient induction of an immune response through the generation of APCs. Therefore, we pretreated the mice with an i.p. injection of AdSCF/AdGM-CSF for the induction of APCs. The data in Figure 4A demonstrate that preinjection of the mice with AdSCF/AdGM-CSF or AdGM-CSF in combination with AdCD/5FC can significantly inhibit the growth of established tumors and prolong the survival period of the tumor-bearing mice when compared with the mice treated with AdSCF/AdCD/5FC or AdCD/5FC alone ($P < .05$). AdSCF and/or AdGM-CSF were injected in the absence of suicide gene therapy to test their direct antitumoral effects, and the data in Figure 4B indicate that AdSCF and/or AdGM-CSF treatment without CD/5FC therapy does not prolong the survival period of tumor-bearing mice. We also used this approach for the treatment of established CT26 tumors, and the results showed that when AdGM-CSF and Ad SCF were administered at the same time as AdCD for the treatment of established tumors, potent antitumoral effects were also observed

Figure 3. A: Suppression of established CT26-derived s.c. tumors by AdCD/5FC suicide gene therapy. The i.t. injection of AdCD began when the established tumors grew to $\sim 5 \times 5$ mm²; i.p. injection of 5FC began 24 hours after AdCD injection. Tumor sizes were expressed as length $\times$ width (mm²). **B:** Survival period of CT26-bearing mice after treatment with AdCD/5FC.

Figure 4. A: Potent growth inhibition of an established CT26 colon adenocarcinoma by AdCD/5FC suicide gene therapy in combination with AdSCF/AdGM-CSF preinjection. Pretreatment with AdSCF/AdGM-CSF was performed 1 week before tumor inoculation. The i.t. injection of AdCD began when the established tumors grew to $\sim 5 \times 5$ mm², i.p. injection of 5FC began 24 hours after AdCD injection. Tumor sizes were expressed as length $\times$ width (mm²). **B:** Survival period of CT26-bearing mice after combined therapy.

(data not shown). These data indicated that treating mice with AdGM-CSF and AdSCF, both for prevention and therapy of experimental tumors, could augment the antitumoral effects of suicide gene therapy efficiently.

Increased expression of cytokines in microenvironment of the tumor after combined therapy

Next, to determine whether the local suppression of an immune response in the microenvironment of a tumor could be reverted after combined therapy, we determined the expression of the cytokines in the tumor mass. RT-PCR was performed for the determination of the cytokine mRNA in tumor masses after various treatments. In the mice treated with PBS or LacZ, no IL-2, IL-4, TNF- α , and IFN- γ mRNA were detected. IL-4 mRNA was present in the tumor masses from tumor-bearing mice treated with AdCD/5FC. Treating mice with AdSCF in combination with AdCD/5FC could lead to the presence of IL-4, TNF- α , and IFN- γ mRNA in the tumor mass. AdGM-CSF administration, with or without AdSCF and in combination with AdCD/5FC treatment, was found to induce the expression of IL-2, IL-4, TNF- α , and IFN- γ mRNA in tumor nodules (Fig 5). No cytokine mRNA was detected in the tumor mass derived from the mice treated with AdSCF and/or AdGM-CSF, AdCD/5FC, or AdLacZ. These data suggest that the locally produced cytokines might contribute to the enhanced antitumoral effects of combined therapy with AdSCF/AdGM-CSF/AdCD/5FC.

Induction of specific antitumoral immunity in tumor-bearing mice after combined therapy

CTLs induced from spleen cells obtained from the sacrificed tumor-bearing mice were used in cytotoxic assays against CT26 cells according to the 4-hour ⁵¹Cr release method. As shown in Figure 6, the lymphocytes from AdSCF/AdGM-CSF/AdCD/5FC-treated mice showed a marked increase of cytotoxic activity against CT26 cells, but not against Renca cells derived from BALB/c mice, when compared with lymphocytes from AdCD/5FC alone, AdSCF/AdCD/5FC-treated mice, or AdGM-CSF/AdCD/5FC-treated mice ($P < .01$). The cytotoxic effects could be inhibited when an anti-CD8 antibody was added before the cytotoxicity assay (data not shown), suggesting that the augmentation of tumor-specific immunity plays a important role in the enhanced antitumoral response.

DISCUSSION

Suicide gene therapy has developed rapidly for the treatment of cancer, for the killing of virus-positive lymphocytes, for the killing of *Leishmania*, and for the treatment of cardiovascular smooth muscle cells.²²⁻²⁷ A CD suicide gene system has been used successfully for the treatment of experimental colon adenocarcinoma, pancreatic cancer, glioma, breast cancer, etc.²⁸⁻³⁰ Clinical trials with adenovirus-mediated CD gene therapy for the treatment of metastatic colon carcinoma of the liver have been initiated recently.³¹ The main drawback to the use of suicide gene therapy is its limited efficacy in targeted gene transfer and in the induction of antitumoral immunity; thus, tumor recurrence often occurs

Figure 5. mRNA analysis of IL-2, IL-4, TNF- α , and IFN- γ in tumor masses of tumor-bearing mice after AdCD/5FC suicide gene therapy in combination with AdSCF/AdGM-CSF pretreatment. Total RNA was isolated from the tumor mass; RT-PCR was used for the assay of mRNA.

because the resistant or metastatic tumor cells survive the therapy. A recent study has demonstrated that glioma cells transduced with an CD/HSV-tk fusion gene can exhibit enhanced metabolic suicide and radiosensitivity.³² Tissue-specific promoters such as α -fetoprotein, carcinoembryonic Ag, or ERBB2 transcriptional regulators were used for selected delivery of suicide genes into tumors.^{19,33–35} A phase I-II clinical study of the treatment of metastatic malignant melanoma using suicide gene therapy suggested that the localized tumoricidal activity may trigger a more general antineoplastic action by facilitating a specific antitumoral immune response.³⁶

One of the mechanisms of the “bystander effect” in the HSV-tk/GCV system is the phagocytosis of apoptotic vesicles of dying transduced tumor cells by neighboring nontransduced tumor cells.^{37–40} Recently, Denning and Pitts⁴¹ demonstrated that the HSV-tk/GCV bystander effect requires gap junctional intercellular communication. When cultures lack this communication, no bystander effect is observed. Another important mechanism involved in the bystander effect in both the HSV-tk and CD/5FC systems is the induction of an immune response.^{13,42–45} The inducing efficacy of the antitumoral

Figure 6. Cytotoxicity of CTLs derived from splenic lymphocytes derived from tumor-bearing mice after treatment with AdCD/5FC and/or AdSCF/AdGM-CSF. Cytotoxicity was determined by a standard 4-hour ⁵¹Cr release assay using CT26 cells as target cells.

immunity of the host has been shown in many studies, but this effect was usually not strong enough to completely eradicate the remaining tumor cells; tumor recurrence often occurred. To augment the immune response of the host, tumor Ag or cytokine genes were combined with the HSV-tk suicide gene for tumor treatment.^{37,46–48}

In the CD/5FC system, we found that combined transfer of the CD gene and IL-2 or of the GM-CSF gene followed by 5FC injections could synergistically elicit antitumoral effects in part through the efficient induction of a specific antitumoral immune response.^{49,50} When poorly immunogenic tumor cells were genetically transduced to simultaneously express the cytokine IL-6 and CD, the tumor cells (205-IL-6-CD) became highly immunogenic and were able to be rejected by normal mice without 5FC treatment. Mice with preexisting wild-type pulmonary micrometastases exhibit prolonged survival and an increased rate of cure when treated with live 205-IL-6-CD cells as a therapeutic vaccine.⁵¹ In this study, the CTL activity from AdCD/5FC-treated mice was very weak, and was significantly increased after the combination of AdSCF/AdGM-CSF treatment. These data demonstrate that the combination of cytokine and suicide gene therapy might be more efficient in inducing the antitumoral immunity of the host.

APCs play a key role in T-cell-mediated antitumoral responses. APCs actively participate in the processing and presentation of tumor Ags. T cells stimulated by DCs can elicit potent antitumoral responses. Recombinant cytokines such as GM-CSF, IL-4, and TNF- α allow for the generation of large numbers of mature DCs, and these cells could be used as adjuvants in active immunotherapy strategies for malignancies.⁵² In GM-CSF- and IL-4-propagated APCs, DCs were considerably more potent at stimulating allogeneic T cells than adherent macrophages.⁵³ Both Ag-pulsed, cytokine-generated DCs and macrophages were able to efficiently immunize mice against cancer; these APCs did not differ in their ability to activate human leukocyte Ag class I-restricted T-cell responses by exogenous peptide presentation.⁵⁴ Cotransfection of tumor cells with two synergistic cytokine genes was able to increase the immunogenicity and decrease the tumorigenicity more efficiently. Enhanced efficacy in the treatment of established metastatic tumors was achieved by a combination of IL-2 gene and the IL-6 gene-transfected tumor vaccine.⁵⁵ Although SCF alone does not effect on colony formation, it enhances both the numbers and size of DC colonies generated *in vitro* by GM-CSF and TNF. SCF appears to recruit very early progenitors of a high proliferative potential with the capacity to differentiate into APCs.^{56,57} SCF and GM-CSF were reported to synergistically stimulate the production of APCs, so SCF in combination with other cytokines could be useful for the *ex vivo* generation of large numbers of APCs. In this study, we have shown that preinjecting the mice with AdSCF or/and AdGM-CSF could significantly increase the number of macrophages in the peritoneum and the counts of DCs derived from the spleen. These data

suggest that AdSCF/AdGM-CSF can efficiently augment the antitumoral effects of AdCD/5FC through the recruitment of APCs and the elevated Ag-presenting ability of APCs.

Within the tumor mass, IL-2, IL-4, TNF- α , and IFN- γ gene expression were detected after combined therapy with AdSCF/AdGM-CSF/CD/5FC. IL-4 and TNF- α are important cytokines for the induction of DCs in combination with GM-CSF. IFN- γ is a potent stimulating molecule for macrophage activation. IL-2 is essential for the induction and activation of tumor-specific CTLs. Adenovirus-mediated GM-CSF gene transfer *in vivo* could induce DCs to infiltrate into the tumor mass. A large number of DCs were also reported at the site of inoculation with GM-CSF-transfected tumor cells. The APCs may be derived from the differentiation of hematopoietic precursors induced by GM-CSF or SCF, and the presence of GM-CSF and SCF at the site of the tumor may attract DCs from surrounding tissues.^{58–61}

In conclusion, we have found that i.p. preinjection of mice with AdSCF/AdGM-CSF followed by treatment of the tumor-bearing mice with AdCD/5FC could suppress tumor growth and prolong the survival period of the tumor-bearing mice more significantly. Spleen CTL activity was induced more significantly, and TNF- α , IL-4, IFN- γ , and IL-2 mRNA was found to be present at the tumor site after combined therapy. These AdSCF/AdGM-CSF-primed DCs showed increased Ag presentation as measured by the Th cell proliferation assay. Our results demonstrate that pretreating mice with AdSCF/AdGM-CSF can activate APCs, and that these APCs can present the tumor Ags released from AdCD/5FC-killed tumor cells and activate the antitumoral response of the host, thus increasing the therapeutic efficiency of AdCD/5FC suicide gene therapy.

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