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Basic Investigations

ENHANCED ANTITUMOR EFFECTS OF SUICIDE GENE THERAPY BY SIMULTANEOUS TRANSFER OF GM-CSF GENE IN LEUKEMIA-BEARING MICE[1]

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In the present report, antitumor effect of combined transfer of suicide gene and cytokine gene was studied. Adenovirus engineered to express E. Coli. cytosine deaminase (AdCD) and/or adenovirus engineered to express murine granulocyte-macrophage colony-stimulating factor (AdGMCSF) were used for the treatment of leukemia-bearing mice. The mice were inoculated s.c. with FBL-3 erythroleukemia cells and 3 days later received intratumoral injection of AdCD in the presence or absence of AdGMCSF followed by intraperitoneal 5-fluorocytosine (5FC) treatment. The results demonstrated that mice received combined therapy of AdCD/5FC and AdGMCSF developed tumors most slowly and survived much longer when compared with mice treated with AdCD/5FC alone, AdGMCSF alone, AdlacZ/5FC or PBS. Combined transfer of CD gene and GM-CSF gene achieved higher specific CTL activity than control therapies. Pathological examination illustrated that the tumor mass showed obvious necrosis and inflammatory cell infiltration in mice after combined therapy. The results demonstrated that combined transfer of suicide gene and cytokine gene could synergistically inhibit the growth of leukemia in mice and induce antitumor immunity of the host. The combination therapy might be a potential approach for cancer gene therapy.

Key words: Cytosine deaminase, Suicide gene, Gene therapy, Adenovirus, Granulocyte macrophage colony-stimulating factor, Leukemia

Transfer of a suicide gene, such as HSV tk gene or cytosine deamanase (CD) gene, into tumor cells could result in the sensitization of tumor cells to nontoxic prodrugs. About 10% of the patients undergoing clinical trials with gene therapy received suicide gene therapy.[1,2] Cytosine deaminase (CD) exists in many bacteria and fungi but not in mammalian cells. 5-fluorocytosine (5FC), being widely used for the treatment of fungi and bacteria infections, could be metabolized by CD enzyme to 5-fluorouracil (5-FU), a highly toxic agent for the therapy of various kinds of tumors.[3-5] Tumor cells transduced with cytokine genes showed increased immunogenecity and decreased tumorigenecity, and tumor vaccine prepared with tumor cells transfected with cytokine genes can induce potent antitumor cellular immunity.[6]

We wonder whether the combined use of CD suicide gene and granulocyte-macrophage colony-stimulating factor (GM-CSF) gene therapy might have more significant antitumor effects than CD suicide gene therapy or GM-CSF gene therapy which were proved to be not satisfactory when used alone. In this study, we found that the antitumor effects of CD gene

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therapy could be enhanced by simultaneous transfer of GM-CSF gene in an established murine tumor model with leukemia.

MATERIALS AND METHODS

Animals and Cell Lines

Male or female C57BL/6 mice, 6-8 weeks of age, were purchased from Joint Ventures Sipper BK Experimental Animal Co., Shanghai. FBL-3, an erythroleukemia cell line derived from C57BL/6, and 293, a continuous cell line derived from human embryonic kidney were maintained in RPMI-1640 medium supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml, 2-mercaptoethanol 50 mmol/L and 10% fetal calf serum (FCS). All culture media were purchased from Gibco-BRL, USA, and fetal calf serum (FCS) provided by Shanghai Institute of Biological Products, Shanghai.

Preparation of Recombinant Adenoviruses

Replication-defective recombinant adenoviruses, AdlacZ (harboring β-galactosidase gene), AdCD (harboring E. coli. CD gene) and AdGMCSF (harboring murine GM-CSF gene) were constructed from human adenovirus serotype 5 using homologous recombination. The recombinant adenoviruses were propagated with 293 cells and the titers of the adenoviral preparations were determined by plaque-forming assay on 293 cells. The produced recombinant adenoviruses were stored at -70 ℃ for in vivo experiments.[7]

Experimental Regimen

FBL-3 leukemia cells were taken from continuous culture, washed for three time and resuspended in PBS with a density of 1×10^6/ml. C57BL/6 mice were inoculated s.c. with 2×10^5 FBL-3 cells. The tumor-bearing mice were divided into 5 groups and the mice in each group were injected intratumoraly with any of the following preparations: PBS, AdlacZ, AdCD, AdGMCSF, or AdCD in combination with AdGMCSF. Injections of 10^8 PFU virus in 0.1 ml PBS was performed 3 days after the inoculation of tumor cells and 5FC (Sigma Chemical Co. USA) 300 mg/kg was injected i.p. into AdlacZ-, AdCD-treated mice daily for 10 consecutive days. 10 days after tumor inoculation, the length and width of the tumor mass were measured with caliber every other day and the tumor volume was expressed as length × width mm^2.

Histologic Examination

Subcutaneous tumor nodules were taken from sacrificed tumor-bearing mice 3 days after the last injection of 5FC. The tumor samples were fixed in 10% formalin solution, dehydrated and embedded in paraffin. Thin-sliced sections were stained with hematoxylin and eosin.

Cytotoxic Assay

Splenic lymphocytes were isolated from sacrificed tumor-bearing mice 3 days after the last injection of 5FC and co-cultured with ^{60}C0-inactivated FBL-3 cells and 50 IU IL-2 (purified in our department, 3×10^7 IU/mg) for 6 days, and then collected as CTL effector cells. The CTL activity was determined by a standard 4-hour ^{51}Cr release assay. Briefly, 2×10^6 FBL-3 cells in 0.5 ml RPMI-1640 with 20% FCS were labeled with 200 μCi $Na^{51}CrO_4$ (Amersham, Arlington Heights, USA) for 2 hours. The labeled cells were washed three times in PBS. 10^4 target cells cells were then mixed with effector cells for 4 h at 37℃ at the ratio indicated. For the maximal ^{51}Cr release control, 0.1 ml of NP40 (Sigma) was added to the target cells, and for the spontaneous ^{51}Cr control, 0.1 ml PBS was added to the labeled cells. The amount of ^{51}Cr released was determined by γ counting, and CTL activity was calculated as: CTL activity (%)=(experimental cpm−spontaneous cpm) (maximal cpm−spontaneous cpm) × 100

Statistics

Statistical analysis was performed using the Student's t test. A $P<0.05$ was considered to be statistically significant.

RESULTS

More Potent Inhibition of Tumor Growth in Leukemia-Bearing Mice by Combined Therapy

C57BL/6 mice will develop palpable tumors 3-7

days after subcutaneous inoculation with FBL-3 cells. The data in Figure 1 demonstrated that the tumors in mice treated with PBS or AdLacZ/5FC grew rapidly. Treatment of the tumor-bearing mice with either AdCD/5FC or AdGMCSF significantly suppressed the growth of the tumors ($P<0.05$). Combined treatment of the tumor-bearing mice with AdCD/5FC and AdGMCSF more significantly inhibited the growth of the subcutaneous tumors when compared with AdCD/5FC or AdGMCSF therapy ($P<0.05$).

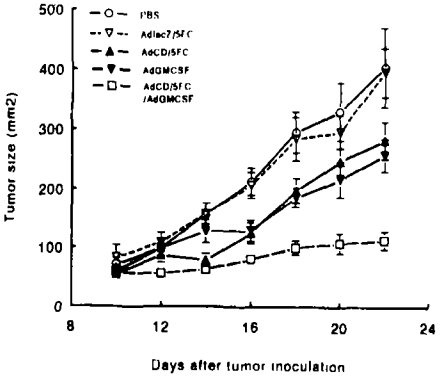

Fig. 1. Inhibition of established subcutaneous tumor growth by administration of adenovirus engineered to express E. coli CD gene followed by 5FC treatment in the presence or absence of adenovirus engineered to express murine GM-CSF.

More Significant Prolongation of Survival Period of Leukemia-bearing Mice by Combined Therapy

Five mice in each group were observed for their survival period. The results in Figure 2 demonstrated that mice treated with either AdlacZ/5FC or AdGMCSF were able to survive much longer than those mice treated with PBS or AdCD/5FC ($P<0.05$). The survival time could be further prolonged when both AdCD/5FC and AdGMCSF were administrated in tumor-bearing mice, and 2 of 5 mice lived more than 90 days ($P<0.05$).

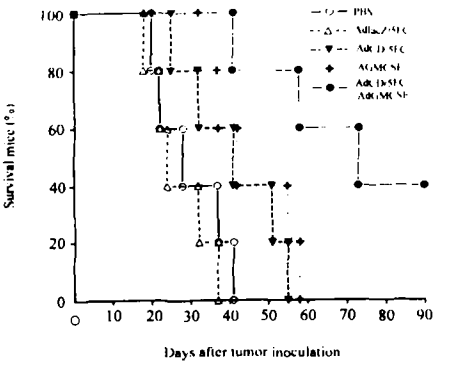

Fig. 2. Survival period of leukemia-bearing mice after combined therapy with AdCD/5FC and/or AdGMCSF.

More Massive Necrosis and Inflammatory Cell Infiltration of Tumor Mass in Leukemia-bearing Mice after Combined Therapy

The results in Table 1 illustrated that large area of tumor necrosis and a great number of inflammatory cells (mainly neutrophils, monocytes and lymphocytes) were present inside and around the tumors in mice treated with AdCD/5FC in combination with AdGMCSF. Large area of tumor necrosis was present in AdCD/5FC treated mice. Medium tumor necrosis and inflammatory cell infiltration could be observed in AdGMCSF treated mice. However, few tumor necrosis and infiltration of inflammatory cells was found in mice treated with PBS or AdlacZ/5FC.

Table 1. Pathological analysis of tumor mass in leukemia-bearing mice after combined therapy with AdCD/5FC and AdGMCSF

Groups	Tumor necrosis	Infiltration of inflammatory cells in necrosis area	Infiltration of inflammatory cells inside the tumors	Infiltration of inflammatory cells around the tumors
PBS	–	–	–	–
AdLacZ/5F	–	–	–	+
AdCD/5FC	+++	+	+	+
AdGMCSF	++	++	+++	++
AdCD/5FC/AdGMCSF	+++	+++	+++	+++

1. Tumor necrosis: – no necrosis, + less than 1/3 of the tumor size, ++ 1/3~2/3 of the tumor size, +++ more than 2/3 of the tumor size; 2. Infiltration of inflammatory cells: – no, + minimal, ++ medium, +++ intense

Higher CTL Activity after Combined Gene Therapy

As shown in Figure 3, AdCD/5FC treatment of the tumor-bearing mice did not elevate the splenic CTL activity significantly when compared with PBS or AdLacZ/5FC treatments. Combination of AdCD/5FC and AdGMCSF therapy could increase the CTL activity more markedly as compared with AdCD/5FC, AdLacZ/5FC, or PBS ($P>0.05$), suggesting that the tumor-specific immunity could be augmented after combined therapy with AdCD/5FC and AdGMCSF.

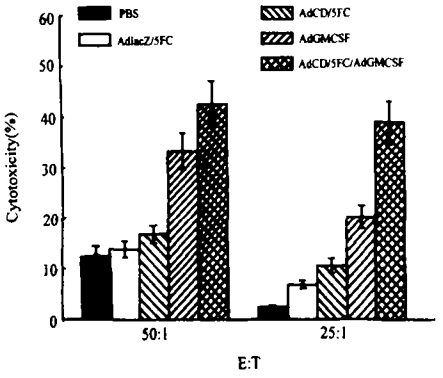

Fig. 3. Induction of splenic CTL from leukemia-bearing mice after combined therapy with AdCD/5FC and AdGMCSF.

DISCUSSION

Suicide gene therapy has been extensively studied for the treatment of malignant tumors, killing of virus-positive lymphocytes, killing of parasites, and treatment of cardiovascular smooth muscles. CD suicide gene system has been successfully utilized for the treatment of experimental colon adenocarcinoma, pancreatic cancer, glioma, breast cancer etc. One of the drawbacks utilizing suicide gene therapy is its limited efficacy in the induction of antitumor immunity, so tumor metastasis or recurrence often happens after the therapy.[8] In this study, antitumor effects were observed in tumor-bearing mice after treatment with adenovirus-mediated CD gene transfer followed by 5FC administration. It is showed that this effect was not satisfactory and the tumor-specific antitumor immunity was not efficiently induced.

GM-CSF gene transfer into tumor cells could render the tumor cells to stimulate potent, specific and long lasting antitumor immunity. It was reported that GM-CSF could stimulate the proliferation and differentiation of antigen-presenting cells, thus augmenting the antitumor response of the host.[7,9,10] We simultaneously transdued tumor model with CD suicide gene and GM-CSF gene, and our results illustrated that the combined therapy could suppress tumor growth and prolong the survival period of the tumor-bearing mice most efficiently. Obvious tumor necrosis and inflammatory cell infiltration were present in the tumors, and marked augmentation of splenic CTL activity was observed in tumor-bearing mice after the combined therapy. These data suggested that co-transfection of the tumors with CD suicide gene and GM-CSF gene could efficiently kill the tumor cells and induce the antitumor immunity of the host.

In conclusion, combined therapy of AdCD/5FC and AdGMCSF, addressing the drawbacks of cytokine gene therapy or suicide gene therapy which were both proved to be not satisfactory when used alone, might be a potential approach for gene therapy of cancer.

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