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THE THERAPEUTIC EFFECT OF INTRATUMORAL INJECTION OF GM-CSF GENE-MODIFIED ALLOGENIC MACROPHAGES ON TUMOR-BEARING MICE*

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Both the antigen presenting ability and the cytotoxicity of macrophages can be enhanced by GM-CSF gene transfer. In the present study, the therapeutic effect of intratumoral injection with GM-CSF gene-modified allogenic macrophages on tumor-bearing mice observed. The peritoneal macrophages of C57BL/6 mice were transfected with GM-CSF gene mediated by recombinant adenovirus and the subcutaneous CT26 colon adenocarcinoma-bearing BALB/c mice were treated by intratumoral injection of the above macrophages. The survival time of the tumor-bearing mice were prolonged significantly and some tumor mass disappeared completely. The necroses of the tumor cells and massive infiltration of inflammatory cells were observed 6 days after treatment. 30 days after treatment, only the leftover of tumor cells and the inflammatory cells remained. The data indicated that introtumoral injection of GM-CSF gene-modified allogenic macrophages displayed more potent therapeutic effect on the preestablished tumor-bearing mice.

Key words: Gene therapy, GM-CSF, Allogenic macrophages, Colon adenocarcinoma, Adenovirus.

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Successful application of allo-BMT in leuke-mia treatment suggests that utilization of graft reaction and inflammatory reaction may be a new strategy in cancer treatment. In our previous study, we observed that the cytotoxicity and the antigen-presenting ability of GM-CSF gene-modified macro-phages were enhanced significantly.[1,2] In the present study, we further investigated the therapeutic effect of intratumoral injection with GM-CSF gene-modified allogenic macrophages on tumor-bearing mice.

MATERIALS AND METHODS

Cell Lines and Animals

CT26, a colonic adenocarcinoma cell line derived from BALB/c mice (H-2^d) was kindly provided by Prof. Zhen Yongsu from Chinese Academy of Medical Science, Beijing. 293, a human embryonic kidney cell line was kindly provided by Dr. Blankenstein from Max-Delbruck-Center of Molecular Medicine, Berlin, Germany. B16 melanoma cells and 293 cells were maintained in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 mmol/L 2- mercaptoethanol and 10% fetal calf serum (FCS). All the media were from Sigma and FCS from Shanghai Institute of Biological Products, Shanghai. Male, BALB/c mice and C57BL/6

mice, 6–8 weeks of age, obtained from Joint Ventures Sipper BK Experimental Animal Co. in Shanghai, were housed for at least 1 week in a specific-pathogen-free state before used in any experiment.

Preparations of Adenovirus and Gene Transfection of Macrophages

Virus Preparations and Gene Transfection of Macrophages were described previously.[1] In briefly, the recombinant adenoviruses Adex1CAmGM-CSF encoding murine GM-CSF gene (Ad-GM-CSF) and AdexlRxZ encoding LacZ gene (Ad-LacZ) were amplified to high titers in 293 cells. The titers determined by plaque assay on 293 cells were 5.0×10^9 pfu/ml and 3.6×10^9 pfu/ml respectively. Peritoneal macrophages were obtained from C57BL/ 6 mice and transfected with GM-CSF gene as following. The mice were killed and their peritoneal exudate cells were harvested and washed with RPMI-1640. The cells were cultured in 12-well culture plates in RPMI-1640 medium. All the nonadherent cells were completely removed from the cultures by allowing cells to adhere to the plastic for 4 h. For *in vitro* adenovirus infection, medium was discarded from the peritoneal macrophages seeded in 12-well culture plates, and 0.15 ml of viral stock was added to each well. After incubation for 1 h at 37°C, growth medium was added and the macrophages were then cultured. 24 h later, the GM-CSF gene-modified macrophages were harvested for further use. Our previous results demonstrated that the GM-CSF gene-modified macrophages could secrete high level of GM-CSF (38 ng/ml). The cytotoxicity and the antigen-presenting ability were also augmented significantly.

Treatment of Tumor-Bearing Mice by Intratumoral Injection with Allogenic Macrophages

BALB/c mice, subcutaneously inoculated with 2×10^5 CT-26 cells will develop obvious tumor mass 5 days later. The tumor-bearing mice were then divided into 8 groups with 8 mice in each group. The mice in each group received an intratumoral injection of one of the following preparations (50 μl):

A: RPMI-1640
B: 2×10^5 Mϕ from BALB/c mice.
C: 2×10^5 Mϕ from C57BL/6 mice.
D:2×10^5 LacZ gene-modified Mϕ from BALB/c mice.
E: 2×10^5 LacZ gene-modified Mϕ from C57BL/6 mice.
F: 2×10^5 GM-CSF cultured Mϕ from BALB/c mice.
G: 2×10^5 GM-CSF cultured Mϕ from C57BL/6 mice.
H: 2×10^5 GM-CSF gene-modified Mϕ from BALB/c mice.
I : 2×10^5 GM-CSF gene-modified Mϕ from C57BL/6 mice.

Assay for Cytotoxicity of CTL

Splenocytes from the mice after 3 weeks of treatment were separated by Ficoll-Hypaque centrifugation. The splenocytes were then cultured in the RPMI1640 complete medium containing 20 U/ml rIL-2 and 5000rad ^{60}Co irradiated wild-type B16 cells for 1 week. Four hours ^{51}Cr release assay was used to test the CTL activity. Briefly, target cells, wild-type B16 cells, in 1 ml RPMI1640 with 20% FCS were labeled with 200 μCi $Na_2{}^{51}CrO_4$(Amersham) for 4 hours. The labeled cells were washed three times in RPMI-1640 medium without FCS. $1X10^4$ targets cells were mixed with effector cells for 4 hours and the E/T ration was 100:1. 100 μl 10% SDS was added to the labeled targets cells for the maximal ^{51}Cr release control and 100 μl RPMI1640 medium was added to the target cells for spontaneous ^{51}Cr release control. γ count was determined on a 1275 MINGGAMA Counter (Wallac). Percentage of lysis was calculated as follows:

specific lysis%=
[cpm(experimental release) - cpm(spontaneous)]/
[cpm(maximal release) - cpm(spontaneous)]

Pathological Analysis

Pathological analysis of the tumor mass was performed after treatment. Briefly, The tissue blocks were fixed in 4% polyformalin in 0.1 mmol/L PBS, 4°C for 24–36 h. Then the tissue blocks were trimmed to be thin (1–1.5 mm) for following dehydration, hyaline, saturating and embedding with low melting point (52°C) paraffin, slice, HE staining and finally observation.

Statistical Analysis

The significance of differences was analyzed by Student's *t* test.

RESULTS

Antitumor Effect of Intratumoral Injection of Macrophages

In our previous study, we observed that the macrophages transfected with GM-CSF gene could express GM-CSF efficiently and their *in vitro* antitumor activity were also enhanced. We further investigated the therapeutic effect of the macrophages on tumor-bearing mice after intratumoral injection. As shown in Figure 1, there were no significant differences among the survival periods of the mice treated by RPMI-1640 or unactivated macrophages or the LacZ gene-modified macrophages from BALB/c mice. The survival period of the tumor-bearing mice treated by GM-CSF gene-modified macrophages from BALB/c mice was longer and there was 12.5% of tumor-bearing mice survived for more than 100 days. The survival time of the tumor-bearing mice treated by macrophages from C57BL/6 mice was prolonged markedly. Especially, 62.5% of the tumor-bearing mice treated by GM-CSF gene-modified macrophages from C57BL/6 mice survived for more than 100 days and the tumor masses in the survived mice disappeared. The results showed that the intratumoral injection of GM-CSF gene-modified allogenic macrophages displayed potent therapeutic effect on tumor-bearing mice.

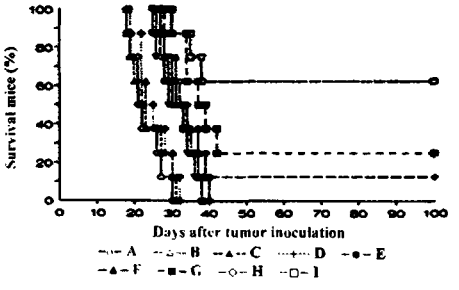

Fig.1. The survival periods of the tumor-bearing mice treated by intratumoral injection with GM-CSF gene-modified allogenic macrophages.

A: RPMI-1640
B: 2×10^5 Mϕ from BALB/c mice.
C: 2×10^5 Mϕ from C57BL/6 mice.
D: 2×10^5 LacZ gene-modified Mϕ from BALB/c mice.
E: 2×10^5 LacZ gene-modified Mϕ from C57BL/6 mice.
F: 2×10^5 GM-CSF cultured Mϕ from BALB/c mice.
G: 2×10^5 GM-CSF cultured Mϕ from C57BL/6 mice.
H: 2×10^5 GM-CSF gene-modified Mϕ from BALB/c mice.
I: 2×10^5 GM-CSF gene-modified Mϕ from C57BL/6 mice.

The Cytotoxicity of the Splenic CTL Induced from the Tumor-Bearing Mice Treated with Macrophages

The cytotoxicity of the CTL induced from the splenocytes in tumor-bearing mice treated with GM-CSF gene-modified allogenic macrophages increased significantly as compared with controls (Figure 2). The results demonstrated that the host specific antitumor immune responses was induced by intratumol injection with the GM-CSF gene-modified allogenic macrophages. However, the cytotoxicity of CTL was not so potent as expected. This suggested that other mechanisms might be involved in the above antitumor effect of GM-CSF gene-modified allogenic macrophages.

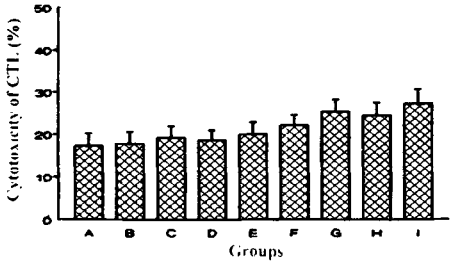

Fig. 2.Cytotoxicity of CTL induced from splenocytes in tumor-bearing mice treated with GM-CSF gene-modified allogenic macrophages.

A: RPMI-1640
B: 2×10^5 Mϕ from BALB/c mice.
C: 2×10^5 Mϕ from C57BL/6 mice.
D: 2×10^5 LacZ gene-modified Mϕ from BALB/c mice.
E: 2×10^5 LacZ gene-modified Mϕ from C57BL/6 mice.
F: 2×10^5 GM-CSF cultured Mϕ from BALB/c mice.
G: 2×10^5 GM-CSF cultured Mϕ from C57BL/6 mice.
H:2×10^5 GM-CSF gene-modified Mϕ from BALB/c mice.
I:2×10^5 GM-CSF gene-modified Mϕ from C57BL/6 mice.

The Pathological Characteristics of Tumor Masses after Treatment

We further observed pathological characteristics of the tumor masses of mice treated by macrophages. As shown in Figure 3, the necroses of the

tumor cells and a number of infiltrating infammatory cells were observed 6 days after treatment (Figure 3b). 30 days after treatment only the leftover of tumor cells and the infammatory cells remained (Figure 3c).

Fig. 3. The pathological analysis of the tumor mass in mice treated by intratumoral injection with GM-CSF gene-modified allogenic macrophages.

A: 6 days after treatment (control)
B: 6 days after treatment (experiment)
C: 30 days after treatment (experiment)

DISCUSSION

Experimental and clinical evidences have confirmed that inflammatory defects are often associated with cancer. A number of studies on cytokine gene therapy of cancer also demonstrated that similar inflammatory characteristics were observed in tumor when the tumor cells were transfected with different genes.[3] So it was suggested that inflammation induced in tumor site might display antitumor potential. Although graft reaction is the most serious side effect in transplantation, utilization of graft reaction may result in effective antitumor responses.

Macrophages are no only effector cells but also antigen-presenting calls (APCs). It is evident that macrophages are very important in host defences against malignant cells. It has been reported that GM-CSF can activate macrophages for tumoricidal activity and augment their antigen presentation capacity.[4,5] Our previous study demonstrated that the GM-CSF gene-modified autologous macrophages displayed potent antitumor effect after adoptive transfer. The potent graft reaction and inflammatory reaction might be induced by GM-CSF gene-modified allogenic macrophages because the activated macrophages could express high level of MHC class II molecules. After intratumoral injection of GM-CSF gene-modified allogenic macrophages, more potent therapeutic effect on preestablished tumor-bearing mice was observed because the survival time of the tumor-bearing mice were prolonged significantly and some of the tumor mass disappeared absolutely. The necroses of the tumor cells and a number of infiltrating inflammatory cells were also observed 6 days after treatment. The data indicated that the inhibition of tumor growth might come from the cytotoxicity of macrophages, the graft reaction, inflammatory reaction and the host specific antitumor immune responses induced by GM-CSF gene-modified allogenic macrophages through different mechanisms. The allogenic macrophages could phagocytize or inhibited the tumor cells directly. As the allogenic antigen, they could also induce the nonspecific immune responses which resulted in inflammation. The mechanisms how CTL was induced were under investigation.

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