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ANTITUMOR EFFECT OF INTRATUMORAL INJECTION OF LIPOSOME-ENCAPSULATED G-CSF GENE AND *IN SITU* BIOLOGICAL CHARACTERISTICS OF THE TREATED TUMOR CELLS*

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In order to investigate the antitumor effects of the *in vivo* G-CSF gene therapy mediated by liposome and its mechanisms, human G-CSF gene was encapsulated into liposome and was directly injected into tumor mass of C-26 colon adenocarcinoma-bearing mice. After direct intratumoral injection of liposome encapsulated G-CSF DNA, the subcutaneous tumor growth was dramatically inhibited and the survival time was prolonged significantly. Tumor regression could be observed in about 30% of C-26-bearing mice. By the analysis of the antitumor mechanisms, we found that anti-G_{418} (600ug/ml) clone could be selected from the tumor cells freshly separated from the treated C-26 tumor mass, and secretion of G-CSF in the supernatant could be detected. Northern-blot also confirmed the expression of hG-CSF by the tumor cells. Higher expressions of MHC class I(H-2k^d) molecule and ICAM-1 on the tumor cells could be observed. The results demonstrated that liposome can effectively transfect G-CSF gene into tumor cells *in situ*, and then increase the immunogenicity of the tumor cells which may contribute to the activation of the local antitumor immune responses effectively.

Key words: Granulocyte colony-stimulating factor, Gene therapy, Colon neoplasma, Liposome, MHC class I molecule, Adhesion molecule.

Gastric-intestinal tumor is one of the most common kind malignant tumors to cause human death. Though the classic operation combined with chemotherapy can achieve a certain therapeutic effect on it, recurrence after surgical excision of the primary tumor remained a difficult problem to be solved for cancer patients couldn't receive a completely series of chemotherapy to eliminate the tumor cells because of hematopoietic or immune injury. Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor that is responsible for the differentiation and proliferation of hematopoietic progenitor cells to mature granulocyte, and can increase the number of peripheral neutrophils to make it possible receiving next chemotherapy to improve the therapeutic effects. Recent reports has been demonstrated that it can reduce its tumorigenicity by G-CSF gene transfection into tumor cells, and showed to be effective to active immune response.[1,2] we also proved that fibroblast-mediated G-CSF gene therapy has exert anti-metastasic effect, and better results could be observed in C-26-bearing mice received high dose chemotherapy

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after G-CSF gene therapy.[3] In view that the direct *in vivo* gene therapy is much more comfit for the future application, we investigated the antitumor effect of intratumoral injection of liposome encapsulated G-CSF gene, and analyzed the change of the biological characteristics of the tumor cells.

MATERIALS

Reagents

rhG-CSF(Filgrastim, 300 μg) was purchased from Kirin Brewery Co. 5-Fu, G418 were supplied by Sigma Chemicals. α -^{32}PATP, CM was products of Amersham. Standard recombinant human G-CSF was provided by Boehringer with the specific activity of 1×10^7 U/mg. The plasmid expressing human G-CSF cDNA was constructed as described previously.[4] Lipofectin was purchased from Gibco, FITC-labed anti-H-2K^d (major histocompatibility complex class I, MHC-I) MoAb were supplied by ParMingen, anti-ICAM-1 was purified from the culture supernatants of hybridoma B21-2(ATCC).

Mice and Cell Lines

Murine C-26 colon adenocarcinoma cell line (H-2K^d) was kindly provided by Prof. Zheng Yongshu (Medical and Drug biotechnical institute of Chinese Medical academy). NSF60,G-CSF dependent cell line, was cultured in RPMI1640 medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin at 37℃, 5% CO_2. Male BALB/c mice, 6–8 weeks old, were purchased from Shanghai Sipper BK Experimental Animal Co.

Detection of G-CSF Activity

The biological activity of G-CSF was determined using an G-CSF-dependent NSF60 cell line by the MTS/PMS method. The unit of G-CSF was defined relative to standard recombinant human G-CSF (Boehringer).

Treatment of C-26 Colon Adenocarcinoma-bearing Mice

To establish C-26 colon adenocarcinoma-bearing mice model, normal BALB/c mice were s.c injected with 1×10^5/mouse live C-26 cells in a volume of 0.1 ml in the right hip. After 5 days, the tumor-bearing mice were randomly divided into seven groups to receive different treatments as following. Each group consisted of ten mice. The mice were monitored for survival or sacrificed 15 days after the treatment to assay immune activities. In group A (PBS), mice were given an intratumoral injection of 0.1 ml liposome which encapsulated PBS. In group B (plasmid DNA), mice were administered intratumoral injection of 0.1 ml liposome which encapsulated 10 μg plasmid DNA. In group C (5-Fu), mice were given an i.p injection of 0.5 ml 5-Fu (150 mg/kg). In group D (rhG-CSF), mice were administered intratumoral injection of 0.1 ml rhG-CSF (2 μg every day for 14 days). In group E (G-CSF DNA), mice were administered intratumoral injection of 0.1 ml liposome which encapsulated 10 μg G-CSF DNA(repeated once after 7 days). In group F (5-Fu+rhG-CSF), mice were received 5-Fu as in group C, then were administered intratumoral injection of 0.1 ml rhG-CSF (2 μg every day for 14 days). In group G (5-Fu+G-CSF DNA), mice were received 5-Fu as in group C, then were administered intratumoral injection of 0.1 ml liposome which encapsulated 10 μg G-CSF DNA (repeated once after 7 days).

Identification of hG-CSF mRNA Expression in Tumor Cells *in situ*

To identify the effect of liposome mediated gene transfer *in situ*, tumor cells which were freshly isolated from the treated mice were used to extract total RNA, and the expression of G-CSF transcripts in the tumor cells was determined by Northern blot analysis.[5]

Determination of Surface Molecules Expressed on Freshly Isolated Tumor Cells with Monoclonal Antibodies

Cell surface molecules expression were determined by flow cytometry using monoclonal antibody. 1×10^6 freshly isolated tumor cells per experimental condition were suspended in 0.5 ml of phosphate buffered saline (PBS), and 10 μl of FITC-labeled anti-H-2K^d or anti-ICAM-1 monoclonal antibody was added, respectively. The cells were incubated for 45 min at 4℃, and were washed twice with PBS, then the cells were analyzed on a FACScan (Becton Dickinson).

Statistical Analysis

Statistical analysis was performed using unpaired *t* test.

Results

Inhibition of C-26 Colon Adenocarcinoma Growth after Intratumoral Injection of Liposome Encapsulated G-CSF Gene

In our previous study, we had demonstrated that fibroblast-mediated G-CSF gene therapy could inhibit metastasis of colon carcinoma to liver effectively. In this research, we determined whether liposome mediated G-CSF gene therapy *in situ* could exert a therapeutic effect on subcutaneous C-26 colon adenocarcinoma *in vivo*. As shown in Figure 1, the growth of subcutaneous C-26 was significantly suppressed when rhG-CSF was intratumorally injected. The growth of C-26 could also be inhibited markedly after intratumoral injection of liposome encapsulated G-CSF gene. 3 weeks after treatment, there was significant difference in the tumor growth of mice between groups given with G-CSF gene and rhG-CSF, whereas the tumor growth was not inhibited in the tumor-bearing mice treated with control plasmid DNA or PBS.

Antitumor Effect of G-CSF Gene Therapy Mediated by Liposome *in situ* Combined with High-dose Chemotherapy

Then, we determined whether the therapeutic efficacy of G-CSF gene therapy mediated by liposome *in situ* could be improved by the combination with high-dose chemotherapy. As illustrated in Figure 1, the tumor growth was significantly inhibited after the C-26 colon adenocarcinoma-bearing mice received high-dose 5-Fu ($P<0.05$), but the survival time of the tumor-bearing mice was not prolonged or even shorten because of infectious diseases. When the C-26-bearing mice received high-dose chemotherapy was administered with intratumoral injection of rhG-CSF or liposome encapsulated G-CSF gene, the tumor growth were inhibited significantly, and more effective results could be observed in C-26-bearing mice given G-CSF gene therapy. Both of the mean survival time of C-26-bearing mice which were administrated with rhG-CSF or G-CSF gene therapy was also prolonged significantly, but G-CSF gene therapy could prolong the survival time of C-26-bearing mice more effectively than rhG-CSF did *in vivo*, and there was 30% of the mice survived more than 90 days, as demonstrated in Figure 2.

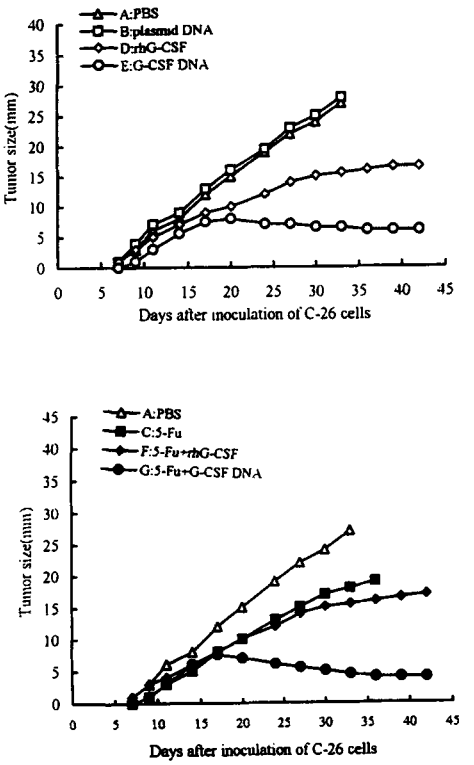

Fig. 1. Inhibition of C-26 colon adenocarcinoma growth after G-CSF gene therapy *in situ* or combined with high-dose chemotherapy.

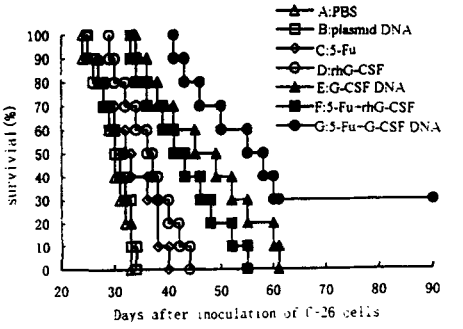

Fig. 2. The survival of C-26 colon adenocarcinoma-bearing mice after G-CSF gene therapy *in situ* or combined with high-dose chemotherapy.

G-CSF mRNA Expression in Solid Tumor Cells

after G-CSF Gene Therapy *in situ*

In order to investigate the antitumor mechanisms of G-CSF gene therapy mediated by liposome *in situ*, northern blot analysis was exert to determine hG-CSF mRNA expression in freshly isolated tumor cells. In this study, there could be determined a hybridization signals in tumor cells freshly isolated from the mice administrated with intratumoral injection of liposome encapsulated G-CSF gene, whereas there couldn't be detected in the mice after intratumoral injection of liposome encapsulated control plasmid DNA. The result demonstrated that liposome can deliver G-CSF gene into tumor cells *in situ* effectively.

Determination of G-CSF Secretion by Tumor Cells *in situ* Transfected with G-CSF Gene

For further proven the efficacy of G-CSF gene transfection into tumor cells, freshly isolated tumor cells were selected with G418, and high level of G-CSF could be detected in the supernatant of G418-resistant clones(28–150 U/10^5 cell/ml/24 h). The result also suggested that liposome can transfect G-CSF gene into tumor cells effectively.

Expression of H-2K^d and ICAM-1 Molecules on Tumor Cell after G-CSF Gene Transfection

Major histocompatibility complex (MHC) class I antigen and ICAM-1 are important molecules participating in tumor antigen presentation and recognition, and tumor cells usually present lower expression. In this research, there were no obvious changes in H-2K^d and ICAM-1 molecule expression on the tumor cells after the C-26-bearing mice administrated with PBS, plasmid DNA or high-dose chemotherapy. After the C-26-bearing mice were intratumorally given with G-CSF gene, higher expression of H-2K^d and ICAM-1 molecule on the freshly isolated tumor cells could be detected by FACS analysis (as demonstrated in Figure 3). Those

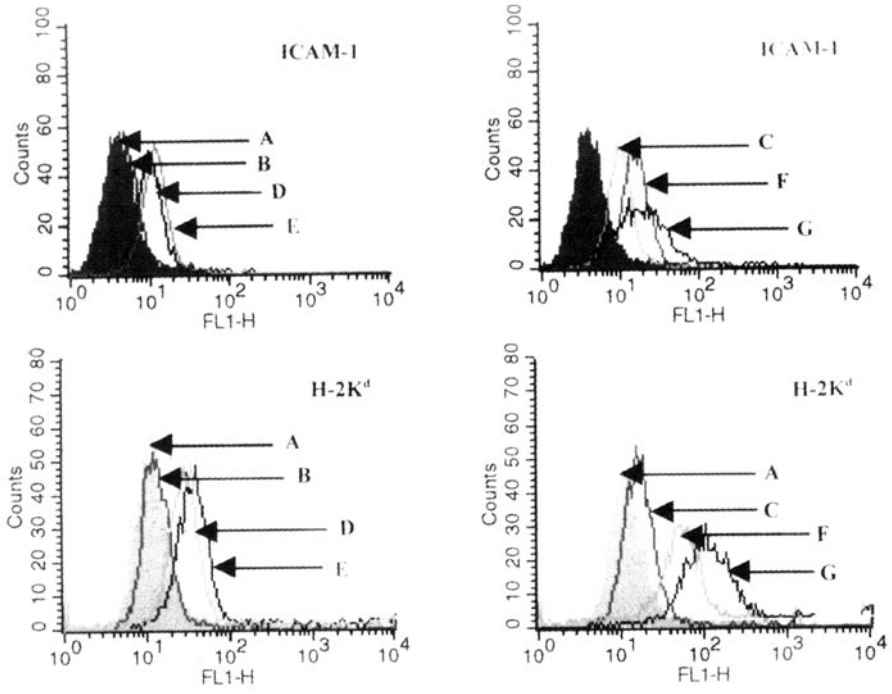

Fig. 3. Expression of H-2K^d and ICAM-1 molecules on the freshly isolated tumor cells after G-CSF gene therapy *in situ* or combined with high chemotherapy. A:PBS, B: plasmid DNA, C:5-Fu, D:rhg-CSF, E:G-CSF DNA, F:5-Fu+rhG-CSF, G:5-Fu+G-CSF DNA.

results showed that rhG-CSF intratumoral injection do not increase the expression of H-2K^d and ICAM-1 molecules, but G-CSF gene transfection into tumor cells can change the immunological characteristics of the tumor cells, enhance the immunogenicity of the tumor cells to activate local and systemic antitumor immune responses.

DISCUSSION

It has been recognized that tumor cells are usually lack of specific antigen to contribute to stimulate host's immune response. Different protocol are carrying out to explore an effective immunotherapy for cancer, including *ex vivo* cytokine gene therapy mediated by fibroblast or other cells.[6,7] Another interesting way is to develop cytokine gene-modified tumor cells as potentially effective tumor vaccines, which by local cytokine secretion and enhancement in immunogenicity to stimulate potent active tumor-specific immune response. In view of that, one of the major efforts focused on tumor immunotherapy is to develop a way which could either break immune supression by cytokine secretion or induce active immune response by enhancement in immunogenicity. So new strategies to improve the therapeutic effects for tumor are worthwhile to explore. In recent years, there has been a great surge of interest in liposome mediated gene therapy, it has been exhibiting many advantages in gene therapy research because of its simple manipulation and high efficacy in gene transfection.[8] In this research, we investigated the feasibility of direct G-CSF gene transfer *in situ* mediated by liposome and the antitumor effect of *in vivo* G-CSF gene therapy.

In our experiment, direct intratumoral injection of rhG-CSF could inhibit the tumor growth and extend the survival time of C-26 bearing mice, and intratumoral injection of liposome encapsulated G-CSF gene could inhibit the tumor growth and prolong the survival more significantly than the mice administrated of rhG-CSF. Then we investigated the therapeutic effects of G-CSF gene therapy on C-26-bearing mice receiving high-dose chemotherapy (5-Fu 150 mg/mice i.p.). More effective results could be observed in C-26-bearing mice receiving high dose chemotherapy after G-CSF gene therapy, and tumor regression could be observed in about 30% C-26-bearing mice. The results suggested that liposome-mediated G-CSF gene therapy has potent therapeutic effect on cancer.

By the analysis of the antitumor mechanisms, we found that anti-G_{418} (600 μg/ml) clones could be selected from the tumor cells freshly separated from the treated C-26 tumor mass, and secretion of G-CSF in the supernatant could be detected. Northern blot analysis also confirmed that G-CSF gene could been transfered into tumor cells *in situ*. After G-CSF gene transfection, high expression of MHC class I (H-2k^d) molecules and much more ICAM-1 on the tumor cells could be observed. For MHC I antigen and ICAM-1 are important molecules participating in tumor antigen presentation and recognition. It has been confirmed that the CD8$^+$ cytotoxic T lymphocyte is restricted to MHC class I in recognition and killing the tumor cells, and the ability of cytotoxic T lymphocyte recognizing tumor cells was enhanced by the up-regulation of MHC class I molecule. ICAM-1, a ligand of lymphocyte function-associated antigen 1 (LFA-1), is confirmed to play an important role in the antigen presentation and mediation of the effector cytotoxicity to target cells. When the tumor antigen combined with the MHC molecules, the complex is recognized by the T cell receptor, and then the interaction between ICAM-1 and LFA-1 makes the tumor cell and T lymphocyte combination tightly to facilitate the activation of T lymphocytes. Therefore, the increasement of ICAM-1 expression on the tumor cell after G-CSF gene transfection could promote the adhesion of tumor cells to T lymphocytes. It has bee proved that G-CSF can promote ICAM-1 expression *in vitro* and *in vivo*, and our results is in keeping with the reports.[9,10] In this study, high expression of MHC class I and ICAM-1 molecules after *in vivo* G-CSF gene therapy may be the crucial mechanism of antitumor effect of G-CSF gene therapy.

Alternatively, we also observed that the cytotoxicity of tumor-infiltrating lymphocytes, macrophage, neutrophils to C-26 adenocarcinoma cells could be enhanced after G-CSF gene therapy *in situ*, and the secretion of IL-1, TNF, NO by macrophages or neutrophils were also significantly enhanced (data not shown). The results demonstrated that G-CSF gene therapy could activate the immune functions by different mechanisms, in direct or indirect ways. The results show that liposome can effectively transfer G-CSF gene into tumor cells *in situ*, and then increase the immunogenicity of the tumor cells and activate the local and systemic immune response effectively.

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