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INDUCTION OF IMMUNE RESPONSE BY IL-6 GENE-MODIFIED LEUKEMIA CELLS*

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Human IL-6 gene was transduced into FBL-3 murine erythroleukemia cells *in vitro* by calcium phosphate co-participation. After selection in the presence of G418, limiting dilution and biological activity assay, G418 resistant clone that secreted the highest level of IL-6 (225.6 U/ml) was selected out of 24 IL-6-secreting clones. The FBL-3 cells secreting the highest level of IL-6 (FBL-3-IL-6) showed decreased growth potential and clonogenicity *in vitro*. Inhibition of cell growth and clone formation was found to be closely related to the level of IL-6 secretion. FBL-3-IL-6 cells grew more slowly than wild-type FBL-3 leukemia cells and FBL-3 cells secreting lower level of IL-6 (21.3 U/ml) when inoculated s.c. into C57BL/6 mice. The mice inoculated with FBL-3-IL-6 cells showed prolonged survival period than those inoculated with control leukemia cells. Increased cytotoxic activities of splenic NK and CTL were found in mice inoculated with FBL-3-IL-6 cells. The secretions of IL-2, TNF and GM-CSF from murine splenocytes were also found to be greatly elevated after the inoculation of FBL-3-IL-6 leukemia cells. These data suggested that transduction of IL-6 gene into FBL-3 cells magnificently decreased the tumorigenicity and increased the immunogenicity of the leukemia cells, could induce specific and nonspecific antitumor immune responses. IL-6 gene-modified leukemia cells might be of great interests to be used as vaccine for the treatment of leukemia.

Key words: IL-6, immunotherapy, gene therapy, leukemia, tumorigenicity, immunogenicity.

Intensive studies have been attributed to the immunotherapy of cancer with tumor vaccine transduced with cytokine genes or co-stimulating factor genes in the recent ten years. Tumor vaccine engineered to secrete various cytokines has been proved to be of particular interests for the treatment of certain kinds of tumors. In these studies cytokine genes were introduced into tumor cells to increase the immunogenicity of the tumor cells, and vaccination with the these cytokine gene-modified tumor vaccine could induce systemic immunity against a subsequent challenge with parent tumor cells or against the parental tumor cells in the host with preestablished tumor.[1,2]

IL-6, produced by a number of cell types and involved in the responses of immune response, hematopoiesis and inflammation, is one of the most important cytokines with opposite effects. First, IL-6 could act as a strong growth factor of certain cell types. IL-6 could elicit a wide variety of biological effects in hematopoietic system, stimulating the proliferation and differentiation of stem cells in bone marrow and spleen, resulting in the elevation of platelet and neutrophil counts in peripheral blood. It could elicit promoting effects on the growth of T cell and B cell lymphomas, Epstein-Barr virus transformed B cells,

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some plasmacytomas. Secondly, growth inhibition of carcinoma cell lines and leukemia cells was also found to be attributed to IL-6. IL-6 was reported to be involved in the differentiation of myloid leukemia cells. The growth of murine myeloid leukemia cells (M1) was completely arrested and was induced to differentiate morphologically into macrophage-like cells by IL-6. When IL-6 was combined with other cytokines like IL-1, IFN, GM-CSF or TNF, obvious differentiating potentials could be achieved in several leukemia models.[3-6]

To date no data have been reported concerning the immunotherapy of leukemia with leukemia cell vaccine transduced with IL-6 gene. The present investigation was aimed to find whether IL-6 gene-modified leukemia cell vaccine could have the potentials for the treatment of leukemia. We transduced IL-6 gene into murine erythroleukemia cell FBL-3, and then observed the changes of their tumorigenicity and immunogenicity.

MATERIALS AND METHODS

Cell Lines

FBL-3, a Friend virus-induced erythroleukemia cell line derived from C57BL/6 mouse was kindly provided by Prof. Wei Chen in Cleveland Clinic Foundation, Cleveland, Ohio, USA. FBL-3 cells, transduced with IL-6 gene, control neomycin (Neo) gene or without transduction, 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). YAC-1 cells, obtained from the Cell Bank, Chinese Academy of Science, were maintained in RPMI-1640 medium supplemented with above agents. IL-2-dependent CTLL-2 cells, IL-6-dependent B9 cells, and L929 cells were routinely maintained in our Department.

Reagents

G418, Con A, PHA and actinomycin D were products from Sigma USA. $Na_2{}^{51}CrO_4$ was purchased from Amersham, USA. MTT was from Fluka, France. Recombinant human IL-2 and IL-6 were provided by Genentech, USA. Recombinant murine GM-CSF was from Genzyme, USA. pSP64-T26K carrying human IL-6 cDNA was kindly provided by Prof. Fiers W, Belgium. Eukaryotic expression vector BMGNeo was provided by Dr. Karasuyama, Basel Institute for Immunology, Switzerland. All culture media were from Gibco-BRL, USA, and FCS provided by Shanghai Institute of Biological Products, Shanghai, China.

Mice

Male or female C57BL/6 mice, 6-8 weeks of age, purchased from Joint Ventures Sippr-BK Experimental Animal Co., Shanghai, China, were housed for at least one week in a specific pathogen-free state before their use in any experiment.

Transduction of IL-6 Gene into FBL-3 Cells

Plasmid BMGNeo, BMGNeo-IL-6 containing human IL-6 cDNA were transfected into FBL-3 cells by calcium phosphate co-participation method. The transfected leukemia cells were cultured in RPMI-1640 medium containing 600 μg/ml of G418 for 5 days and maintained in medium containing 200 μg/ml of G418 for another 15 days. G418-resistant cell clones were then selected by limiting dilution. 5×10^5 /ml G418-resistant cells were cultured for 24h and supernatants collected for IL-6 assay. The leukemia clone secreting highest level of IL-6 was assigned as FBL-3-IL-6. The control FBL-3 cells were transfected with BMGNeo gene and named FBL-3-Neo.

IL-6 Bioassay

IL-6 activity was determined utilizing IL-6-dependent B9 cells. Briefly, 2×10^4 cells in a volume of 0.05 ml were plated in the wells of a flat-bottomed 96-well plate. 0.05 ml of serial dilutions of the supernatants or recombinant IL-6 were added and cultivated in 5% CO_2 at 37°C for 36h. The proliferation of B9 cells was measured by MTT method. The unit of IL-6 was defined as standard recombinant human IL-6.

Growth and Clonogenicity Determination *in vitro*

Wild-type FBL-3 cells, FBL-3 cells transduced with Neo or IL-6 gene at 1×10^5 cells/ml were suspended in RPMI-1640 medium with 10% FCS and plated into wells of a flat-bottomed 96-well plate for the *in vitro* growth assay. The cells were incubated in 5% CO_2 at 37°C for 48 h and the cell proliferation was

measured by MTT method.

Semisolid culture method was used for clonogenicity assay. The cell mixture contains 1×10^5 cells/ml FBL-3 cells with or without gene modification, 25% double RPMI-1640, 45% RPMI-1640, and 30% house serum. The final cocktail was composed of cell mixture 5%, 0.3% agar and RPMI-1640 with 10% FCS. Cells were plated into 24-well plates and cultured in 5% CO_2 at 37°C. The colonies were counted under phase microscopy 1 week later.

Tumor Inoculation

C57BL/6 mice were injected s.c. with wild-type FBL-3 cells, FBL-3-Neo cells, or FBL-3-IL-6 cells. For tumorigenicity assay, 1.6×10^4, 8×10^4, 4×10^5, 2×10^6, and 1×10^7 cells were injected s.c.. For survival period observation and immunogenicity assay, 2×10^6 cells were injected s.c. into C57BL/6 mice.

Effector Cell Preparation

Two weeks after the C57BL/6 mice were inoculated with wild-type FBL-3 cells, FBL-3-Neo cells, or FBL-3-IL-6 cells, 8 mice in each groups were killed and splenocytes were isolated from the sacrificed mice. The fresh prepared splenocytes were washed three times in Hanks solution and the cells were used as NK cells. For CTL preparation, the cells were co-cultured with mitomycin C-inactivated FBL-3 cells at a ratio of 20:1 for 6 days and then used as CTL. YAC-1 cells and wild type FBL-3 cells were used as targets for the evaluation of NK and CTL cytotoxic activities. The cytotoxicity assay at effector : target (E:T) ratio of 50:1 was carried out by using the standard ^{51}Cr-release method[7]

Cytotoxic Assay

Four-hour ^{51}Cr release assays were performed for cytotoxic activity assay. Briefly, 2×10^6 wild-type FBL-3 cells or YAC-1 cells, in 0.5 ml RPMI-1640 with 20% FCS were labeled with 200 μCi $Na^{51}CrO_4$ for 2 h, The labeled cells were washed three times in serum-free medium. 10^4 target cells were then mixed with effector cells for 4 h at 37°C at the ratio indicated. For the maximal ^{51}Cr release control, 0.1 ml of 0.1 N HCl was added to the target cells, and for the spontaneous ^{51}Cr control, 0.1 ml medium was added to the labeled cells. The amount of ^{51}Cr released was determined by γ counting on a 1275 Minigamma Counter (LKB-Wallac, Finland), and percentage of specific lysis was calculated as follows:

$$\%\ \text{of specific lysis} = \frac{\text{Experimental cpm - spontaneous cpm}}{\text{Maximal cpm - spontaneous cpm}} \times 100$$

Cytokine induction from splenocytes

Splenocytes freshly prepared from the sacrificed mice were co-cultured at 5×10^6 cells/ml in complete RPMI-1640 medium containing 10 μg/ml of Con A. 24 h later the culture supernatants were collected for IL-2 assay, 48 h later the supernatants were gathered for the activity analysis of GM-CSF. The splenocytes were cultured at 5×10^6 cells/ml with 10 μg/ml PHA for 48 h at 37°C in 5% CO_2, and the supernatants were collected for TNF assay.

IL-2 Bioassay

IL-2 contents were assayed employing IL-2-depedent CTLL-2 cells. Briefly, 2×10^4 CTLL-2 cells in a volume of 0.05 ml were co-cultured with an equal volume of serial dilutions of the supernatants or recombinant IL-2 were plated into 96-well plate. The cells were then cultured in 5% CO_2 at 37°C for 36 h. The proliferation of cells was measured by MTT method. The activity of IL-2 was defined as standard recombinant human IL-2.

GM-CSF Assay

The GM-CSF level was determined using murine bone marrow cell proliferation method. In brief, murine bone marrow cells prepared from mice were plated in 96-well plate and dilutions of supernatants or standard recombinant murine GM-CSF were added, and the cultures incubated in 5% CO_2 at 37°C for 36h. Proliferation of bone marrow cells was measured by MTT method. The amounts of GM-CSF were defined as standard recombinant murine GM-CSF.

Statistics

Statistical analysis was performed using the

Student's *t* test.

RESULTS

Establishment of IL-6-Secreting FBL-3 cell clones

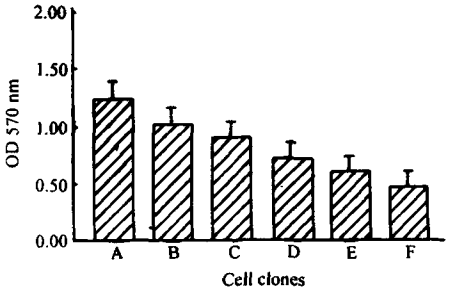
Human IL-6 gene was introduced into FBL-3 cells by calcium phosphate co-precipitation. We got 24 IL-6-secreting clones after gene transfection, G418 resistant selection, and IL-6 activity assay. The 24 clones were found to produce 26.6 U/ml, 23.2 U/ml, 64.6 U/ml, 36.4 U/ml, 41.3 U/ml, 102.8 U/ml, 225.6 U/ml, 184.2 U/ml, 73.2 U/ml, 120.4 U/ml, 33.6 U/ml, 26.7 U/ml, 24.9 U/ml, 86.8 U/ml, 113.6 U/ml, 43.2 U/ml, 83.6 U/ml, 35.8 U/ml, 21.3 U/ml, 49.6 U/ml, 130.4 U/ml, 94.5 U/ml, 43.6 U/ml, and 32.8 U/ml of IL-6 respectively into the culture supernatant. The clone with highest IL-6 secretion (225.6 U/ml) were selected and named FBL-3-IL-6 in the following experiments.

In vitro Growth Characteristics of IL-6-Secreting FBL-3 Cells

We selected the FBL-3 clones secreting highest level of 225.6 U/ml and lowest level of 21.3 U/ml, also another three clones that secrete 86.8 U/ml, 120.4 U/ml and 184.2 U/ml of IL-6, to evaluate their in vitro growth characteristics. The results in Figure 1 illustrated that growth inhibition of the positive clones were observed and the growth inhibition was found to be in a IL-6 level-dependent manner. The more IL-6 the FBL-3 leukemia cell clone secreted, the more inhibition was achieved with the *in vitro* growth of the clone. Magnificent growth inhibition was achieved with FBL-3 cells secreting 120.4, 184.2 and 225.6 U/ml IL-6 ($P<0.01$).

In vitro Clonogenicity of FBL-3 Cells Transfected with IL-6 Gene

FBL-3 clones secreting highest level of 225.6 U/ml and lowest level of 21.3 U/ml were selected and their clonogenicity in semisolid agar mixture was evaluated. As demonstrated in Figure 2, obvious clonogenicity inhibition of the positive clones were observed and the growth inhibition was more pronounced when the clones secreted higher level of IL-6 ($P<0.01$).



A: wild-type FBL-3 B: FBL-3-IL-6 21.3 μ/ml
C: FBL-3-IL-6 86.8 μ/ml D: FBL-3-IL-6 120.4 μ/ml
E: FBL-3-IL-6 184.2 μ/ml F: FBL-3-IL-6 225.6 μ/ml

Fig. 1. Growth inhibition of FBL-3 cells genetically modified to express human IL-6.

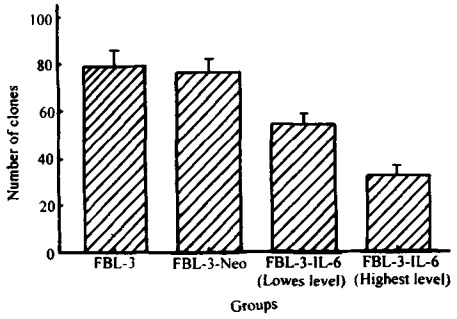


Fig. 2. Inhibition of clonogenicity by FBL-3 cells engineered to secrete human IL-6.

Tumorigenicity of IL-6 Gene-Modified Leukemia Cells

In order to determine whether the local IL-6 secretion from the genetically modified leukemia cells could affect their tumorigenicity *in vivo* after subcutaneously injection of FBL-3-IL-6 cells into syngeneic C57BL/6 mice. 1.6×10^4, 8×10^4, 4×10^5, 2×10^6, and 1×10^7 of wild-type FBL-3 cells, FBL-3-Neo cells, FBL-3 cells secreting highest and lowest levels of IL-6 were inoculated and the appearance of the palpable tumor was monitored. 90 days after the tumor inoculation of 2×10^6 and 1×10^7 leukemia cells led to the formation of tumor in all tested mice, and inoculation of 1.6×10^4 FBL-3 cells resulted in no tumor growth in all the mice (Table 1). After 8×10^4 of different leukemia cells were inoculated, no mice

inoculated with FBL-3-IL-6 cells formed tumor, but most mice inoculated with wild-type FBL-3 cells, FBL-3-Neo cells and low IL-6-secreting FBL-3 cells formed obvious tumor. When 4×10^4 leukemia cells were inoculated, mice inoculated with FBL-3 cells secreting high or low IL-6 formed less tumors and all mice inoculated with wild-type FBL-3 cells, Neo gene transfected FBL-3 cells formed palpable tumors.

Table 1. Percentage of tumor occurrence after C57BL/6 mice were inoculated with FBL-3 cells with or without IL-6 gene transduction

Number of leukemia cells inoculated	Mice with detectable tumor (%)			
	Wild-type FBL-3	FBL-3-Neo	FBL-3-IL-6 (lowest)	FBL-3-IL-6 (highest)
1.6×10^4	0	0	0	0
8×10^4	75	87.5	75	0
4×10^5	100	100	87.5	75
2×10^6	100	100	100	100
1×10^7	100	100	100	100

In vivo Growth and Survival Observation

The results in Figure 3 showed that all the mice inoculated with wild-type FBL-3 cells, FBL-3-Neo cells and FBL-3 cells secreting lowest levels of IL-6 developed tumors 7 days after tumor cell inoculation. The mice inoculated with FBL-3 cells secreting highest level of IL-6 showed obvious inhibitory effects on tumor growth when compared with the mice inoculated with wild-type FBL-3 cells, FBL-3-Neo cells and FBL-3 cells secreting lowest levels of IL-6 ($P<0.01$). Eight mice in each group were observed for their survival period. The results, as showed in Figure 4, demonstrated that mice inoculated with FBL-3 cells secreting highest levels of IL-6 were able to survive much longer than those injected with wild-type FBL-3 cells, FBL-3-Neo cells and FBL-3 cells secreting lowest levels of IL-6 ($P<0.01$).

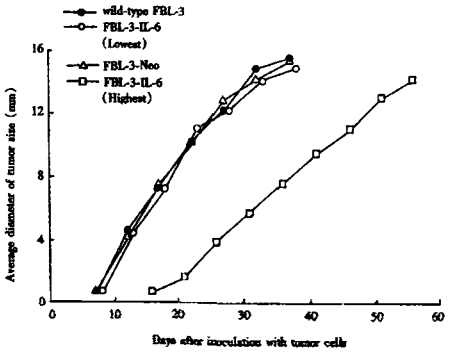


Fig. 3. Effect of IL-6 gene modification on the growth of FBL-3 erythroleukemia cells *in vivo*.

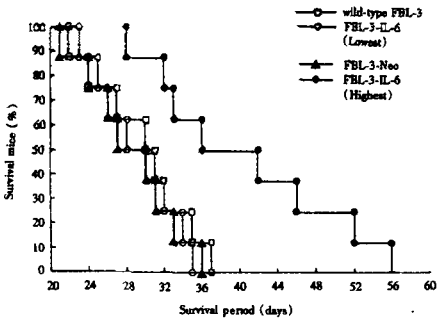


Fig. 4. Survival rate of mice inoculated with leukemia cells transduced with IL-6 gene.

Cytotoxic Activity

The results in Figure 5 (A, B) demonstrated that the cytotoxicity of NK and CTL were all showed to be greatly elevated after the mice were inoculated with FBL-3-IL-6 cells compared with normal mice or mice inoculated with wild-type FBL-3 cells ($P<0.01$).

Cytokine Release

Splenocytes prepared from sacrificed mice were co-cultured with Con A to induce IL-2 and GM-CSF, and were cultured with PHA for TNF induction. As illustrated in Table 2, IL-2, GM-CSF and TNF production were found to be markedly increased after the mice were inoculated with FBL-3-IL-6 cells when compared with normal mice or mice inoculated with wild-type FBL-3 cells ($P<0.01$).

Table 2. Cytokine release from splenocytes of leukemia-bearing mice inoculated with FBL-3 cells engineered to secrete IL-6

Groups	IL-2 (U/ml)	TNF (U/ml)	GM-CSF (ng/ml)
RPMI-1640	36.2± 4.6	24.4± 5.3	15.2± 2.6
Wild-type FBL-3	34.9± 5.0	23.3± 4.7	9.3± 2.8
FBL-3-IL-6	56.2± 4.9	28.1± 4.5	24.7± 4.3

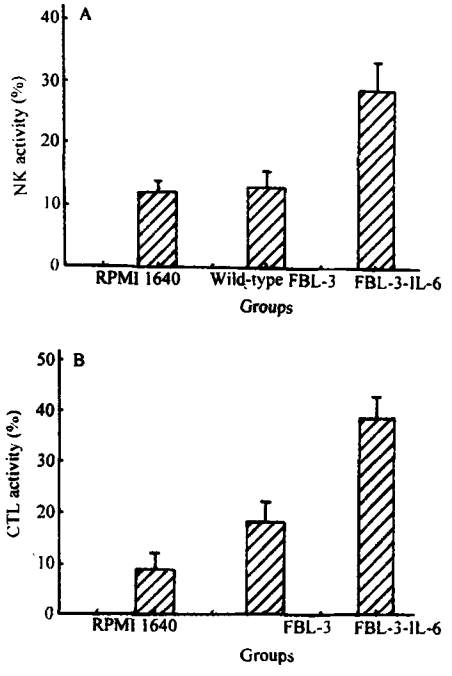


Fig. 5 (A, B). The cytotoxicity of NK (A) and CTL (B) from splenocytes derived from leukemia-bearing mice.

DISCUSSION

With the rapid development of cytokine gene therapy as an approach for the treatment of cancer, various experiments have been carried out to observe the biological characteristics and antitumor effect of IL-6 gene-transfected tumor cells. IL-6 gene-transfected lung carcinoma cells were found to show *in vitro* growth inhibition, which was directly correlated with the levels of IL-6 secretion. Fibrosarcoma cells transduced with IL-6 gene exhibited reduced tumorigenicity, increased immunogenicity and decreased metastatic potential. Tumor cells transduced with IL-6 gene generally showed reduced tumorigenicity and enhanced immunity of the host, and could employed as useful tumor vaccine for the treatment of cancer.[8-13]

In this report we first introduced the IL-6 gene into FBL-3 erythroleukemia cells and observed their characteristics *in vitro* and *in vivo*.The FBL-3 cells secreting highest level of IL-6 (FBL-3-IL-6) showed decreased growth potential and clonogenicity *in vitro*. Inhibition of cell growth and clonogenicity was found to be closely related to the level of IL-6 secretion. FBL-3-IL-6 cells grew more slowly than wild type FBL-3 leukemia cells when inoculated subcutaneously into C57BL/6 mice. The mice inoculated with FBL-3-IL-6 cells showed prolonged survival period than those inoculated with wild-type FBL-3 cells. These data suggested that transduction of IL-6 gene into FBL-3 cells magnificently decreased the tumorigenicity of the leukemia cells.

Direct killing of tumor cells have been found with different kinds of cytokines such as TNF and IL-6. IL-6 was found to inhibit the growth of human breast carcinoma and leukemia/lymphoma cell lines. *In vivo* administration of IL-6 also augmented the cure rate of C57BL/6 mice inoculated with syngeneic FBL-3 cells by the efficient induction of FBL-3 specific CTL. In this experiment we demonstrated that NK and CTL cytotoxic activity and cytokine production from splenocytes increased significantly in mice inoculated

with FBL-3 secreting highest level of IL-6. These data suggested that the locally produced IL-6 could not only kill leukemia cells directly, but also increase cellular immunity of the host, act as a co-stimulator of T and B lymphocytes, enhance cytotoxic effects of NK and CTL cells, thus increasing the specific and non-specific antitumor effects of the host.

FBL-3-IL-6 cells with increased immunogenicity and decreased tumorigenicity were inoculated into mice and the leukemia growth was obviously arrested, but the mice continuously died within 2 months. Primary results with inactivated FBL-3-IL-6 vaccine showed some inhibitory effects on leukemia-bearing mice, but the therapeutic effects were not satisfactory. This may be caused by the fact that local immunosuppression of the leukemia-bearing mice affected the therapeutic efficacy of the host. We speculated that combination of FBL-3-IL-6, low dose IL-2 and low dose cyclophosphamide (CY) might have potent therapeutic effects on leukemia-bearing mice. So in our lab mice inoculated with FBL-3 erythroleukemia cells were treated with inactivated IL-6 gene-modified leukemia vaccine in combination with low dose IL-2 and low dose CY. The results demonstrated that mice received combined therapy of FBL-3-IL-6, low dose IL-2 and low dose CY developed tumors most slowly and survived much longer when compared with mice in control groups. Pathological analysis found that the muscle, liver, spleen and bone marrow in mice treated with hanks solution, low dose CY, low dose IL-2 or wild-type FBL-3 vaccine were all seriously infiltrated with live proliferating FBL-3 cells, but after combined therapy with low dose IL-2, low dose CY and FBL-3-IL-6 vaccine, no obvious tumor infiltration was found (manuscript in preparation).

In conclusin, This study demonstrated that IL-6 gene introduction into FBL-3 leukemia cells significantly decreased the tumorigenicity and increased the immunogenicity of the tumor cells, and obviously induced specific and nonspecific antitumor immune responses of the host. IL-6 gene-modified leukemia cells might be efficient tumor vaccine for the treatment of tumor.

REFERENCES

1. Anderson WF. Gene therapy for cancer. Hum Gene Ther 1994; 5:1.
2. Colombo MP, Forni G. Cytokine gene transfer into tumor inhibition and tumor therapy: where are we now ? Immunol Today 1994; 15: 48.
3. Muller JJ, McIntosh Jk, Jablons DM, et al. Antitumor activity of recombinant interleukin-6 in mice. J Exp Med 1990; 171: 629.
4. Gallahger G, Stimson WH, Findlay J, et al. Interleukin 6 enhances the induction of human lymphokine activated killer cells. Cancer Immunol Immunother 1990; 31:49.
5. Givon T, Slavin S, Haran-Ghera N, et al. Antitumor effects of human recombinant interleukin 6 on acute myloid leukemia in mice and in cell cultures. Blood 1992; 79:2392.
6. Nebber J, Rosenberg SA. Phase I studies of the pharmacokinetic, toxicities and biological effects of interleukin-6 in patients with refractory advanced malignancies. J Immunother 1993; 13:74.
7. 曹雪涛, 章卫平, 郑玲莉, 等. IL-2 基因转移的瘤苗与 IL-1、低剂量环磷酰胺合用后的肿瘤治疗作用及其免疫学机理. 中国免疫学杂志 1994; 10(5): 289.
8. Sun WH, Kreisle RA, Phillips AW, et al. *In vivo* and in vitro characteristics of interleukin-6-transfected B16 melanoma cells. Cancer Res 1992; 52:5412.
9. Porgador A, Tzehoval E, Katz A, et al. Interleukin-6 gene transfection into Lewis lung carcinoma tumor cells suppresses the malignant phenotype and confers immunotherapeutics competence against parental metastatic cells. Cancer Res, 1992;52:3679-3686
10. Ohe Y, Podack ER, Olsen KJ, et al. Interleukin-6 cDNA transfected Lewis lung carcinoma cells show unaltered net tumor growth rate but cause weight loss and shorten survival in syngenic mice. Br J Cancer 1993;67:939-944
11. Mullen CA, Coale MM, Levy AT, et al. Fibrosarcoma cells transduced with IL-6 gene exhibit reduced tumorigenicity, increased immunogenicity and decreased metastatic potential. Cancer Res 1992; 52: 6020.
12. Dougherty GJ, Thacker JD, Lavey RS, et al. Inhibitory effect of locally produced and exogenous interleukin-6 on tumor growth *in vivo*. Cancer Immunol Immunother 1994; 38:339.
13. Cao X, Zhang W, Gu S, et al. Induction of antitumor immunity and treatment of preestablished tumor by interleukin-6-gene-transfected melanoma cells combined with low-dose interleukin-2. J Cancer Res Clin Oncol 1996; 121:721.