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IN VITRO AND *IN VIVO* CHEMOTACTIC EFFECT OF MIP-1α GENE TRANSFECTED TUMOR VACCINE ON IMMUNE EFFECTOR CELLS[1]

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Vaccination with chemokine gene-transfected tumor cells may be a new apporach to cancer treatment. Macrohage inflammatory protein-1α (MIP-1α) is a new type of chemokines which has chemotactic activity on effector cells. In the present study, the B16 melanoma cells were transfected with recombinant adenovirus harboring murine MIP-1α gene. The biological characteristics of the MIP-1α gene transfected B16 melanoma cells were investigated. The level of MIP-1α in the supernatant of gene-transfected melanoma cells was 368 ± 24 ng/ml/10^6/24hr. This supernatant showed strong chematactic activity for NK cells, CD4$^+$ T cells, CD8$^+$ T cells or the freshly isolated peritoneal macrophages. Though the *in vitro* growth were not altered, the tumorigenicity of the gene-transfected B16 melanoma cells decreased signicantly. The infiltration of inflammatory cells into the tumor mass formed by MIP-1α gene-transfected B16 cells were much more obvious than that by wild-type B16 cells or B16 cells transfected with the control gene. However, the survival time of the mice bearing B16 melanoma cells transfected with MIP-1 α gene was not prolonged and the NK, CTL activity remianed unchanged. We suggested that the *in vivo* phenomenon may be related to the high toxicity of the MIP-1 α as a strong non-specific inflam-matory mediator.

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Macrophage inflammatory protein-1 (MIP-1), a novel cytokine composed of alpha/beta subunits, is firstly characterized from the supernatants of macrophages stimulated with LPS. Strong chemotactic activity of MIP-1 for T lymphocytes, B lymphocytes, monocytes as well as neutrophils was showed by a number of *in vitro* and *in vivo* experiments.[1-3] Taub et al found that recombinant human rhMIP-1α and rhMIP-1β attracted only antiCD3 mAb- activated T cells and did not attract unstimulated lymphocytes.[4,5] Schall et al reported that MIP-1α and MIP-1β can attract distinct population of lymphocytes. They found that CD4$^+$ T cells were capable of migrating in response to rh MIP-1β, however, rhMIP-1α induced chemotaxis of CD8$^+$ T lymphocytes predominantly. The effects of MIP-1α on different types of lymphocyte were shown to be dependent on its concentration. MIP-1α at a concentration of 100 pg/ml attracts B cells and cytotoxic T cells, whereas at higher concentration (10 ng/ml) of MIP-1α, the migration of these cells to MIP-1α appears diminished, and the migration of CD4$^+$ T cells is enhanced.[6]

It has been reported recently that monocyte chemotactic protein-1(MCP-1) gene-transfected tumor cell vaccine may induce significant antitumor immunity *in vivo* through the increased inflammatory infiltration in the tumor site.[7] Up to now, there is no report about MIP-1α gene-transfected tumor vaccine. In the present study, the murine B16 melanoma cells

were transfected with recombinant adenovirus harboring MIP-1α gene, and their chemotaxis, tumorigenicity and immunogenicity were investigated.

MATERIALS AND METHODS

Animals and Cell Lines

Pathogen-free female C57BL/6 mice, 6-8 weeks old, were purchased from the Shanghai Center for Experimental Animal. B16 F10 cells, a highly malignant melanoma cell line, Yac-1 cells, NK sensitive plasmacytoma cell line, were cultured in the RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 4 mM glutamine, 1 mM sodium puruvate and 0.5% combined antibiotics.

Reagents

The replication-deficient, harboring the murine MIP-1α gene or the control LacZ gene were kindly provided by Dr. Hamada from the Japanese Foundation of Cancer Research. Recombinant murine MIP-1α and rat anti-murine MIP-1α monoclonal antibody were from R&D (USA). MiniMACS cell separation system was from Miltenyi Biotec GmbH (Sunnyvale, CA, USA). Boyden chamber was the product of Neuro Probe Inc (Cabin John, CA, USA). The 13 mm-diametered polycarbonate membrane filter (pore size 5 or 8 μm) not treated with PVP was from Poretics Corp. (Livermore, CA, USA).

Monoclonal Antibodies (mAbs)

The rat anti-mouse CD4 mAb, rat anti-mouse CD8 mAb and the HRP-labeled goat anti-rat IgG mAb, FITC labeled goat anti-rat IgG mAb were all purchased from Gibco. Mini-bead (<50nm) conjugated goat anti-rat IgG was from Miltenyi biotec GmbH (Sunnyvale, CA, USA).

MIP-1α Gene Transfection of B16 Melanoma Cells Mediated by Adenovirus

As described elsewhere,[8] B16.F10 cells were allowed to grow to confluent in 25cm^2 tissue culture vessels in the complete RPMI1640 medium. After wash with D-PBS solution, the culture was supplemented in small volume of serum free RPMI 1640 containing 20 MOI of recombinant adenovirus harboring murine MIP-1α or the control Lac Z gene. After 2 hours, the gene transfection medium was removed, and the complete RPMI 1640 medium was added. The gene-transfected B16 melanoma cells cultured for another 48 hrs were used in the following studies

Assay for MIP-1α

The MIP-1α gene-transfected B16 melanoma cells were seeded in a 6-well microplate (1×10^6 cells/well). After 24 hr's culture, the supernatants were collected. The concentration of MIP-1α in the supernatants of gene-transfected B16 melanoma cells was determined using an indirect ELISA.

Preparation of CD4$^+$, CD8$^+$ T Lymphocytes and NK Cells

Murine splenic mononuclear cells were isolated on Ficoll-Hypaque (density 1.088) gradients. After passing through Nylon wool column (Gibco), the nonadherent cells were collected and further purified using MiniMACS cell separation system according to the manufacturer's instruction. CD4$^+$ T lymphocytes and CD8$^+$ T lymphocytes were positively selected. The purity determined by FACS analysis was 89.5% and 95%, respectively. More than 85% of the negatively selected cells were NK cells (determined by FACS analysis).

Preparation of Peritoneal Macrophages

Peritoneal macrophages were collected from normal mice injected 2ml of 3% isothiocyanate broth medium (Sigma) ten days before. Peritoneal lavage was performed using 10ml of ice cold HBSS containing 10 U/ml heparin without calcium or magnesium. Macrophages in supplemented RPMI 1640 were plated in 100-mm diameter plastic Petri dishes, incubated for 3 h at 37°C in 5% CO_2 and then washed three times with 10 ml HBSS to remove nonadherent cells. The adherent cells containing high purified macrophages were harvested using a rubber policeman and resuspended in complete RPMI 1640 medium for further use.

Assay for Chemotaxis

The chemotaxis assay was performed in blind well chambers (Neuro Probe Inc., Cabin John, USA) as described previously.[9] Briefly, 200 μl of supernatants sample from gene-transfected B16 F10 melanoma cells were placed in the lower well of the blind well chamber. A polycarbonate membrane filter (pore size 5μm for T cells or NK cells, 8μm for macrophages) (Poretics Corp., Livermore, CA) was covered, and then 200 μl of cell suspension (2×10^6 cells) in the complete RPMI 1640 medium were added in the upper chamber. After 2 hrs of incubation at 37 ℃ in 5% CO_2, the medium in the upper chamber were removed, the upper layer of the filter was cleaned using a cotton swab carefully. The filters were taken out carefully, dehydrated and stained with 15% Wrights stain for 10 min and then mounted on glass slide for counting. The supernatants from wild-type B16 melanoma cells or B16-LacZ or the complete RPMI 1640 medium were used as the negative control. The chemotaxis index (CI) was calculated from the formula:

$$CI = \frac{\text{The number of migrated cells effected by supernatant or standard cytokine}}{\text{The number of migrated cells effected by the negative control}}$$

Inoculation of MIP-1α Gene-Transfected B16 Melanoma Cells and Observation of the Tumorigenicity

C57BL/6 mice of the experimental group were subcutaneously injected 1×10^5 B16 melanoma cells transfected with MIP-1α or Lac Z gene respectively. Mice inoculated with the same number of wild-type B16 cells were used as the control. Eight mice were used for each group, and their tumor growth properties and survival time were observed.

Assay for NK, CTL Cytotoxicity

Splenocytes were prepared from tumor-bearing mice 7 days after inoculation and used for assay of NK activity directly. The splenocytes were then stimulated with mitomyocin C inactivated B16 melanoma cells at a ratio of 20:1 in the complete RPMI 1640 medium supplemented with 10% FCS and 50 U/ml IL-2 for 7 days.The cells were harvested as CTL. The cytotoxicity of NK or CTL was determined by a standard 4-hrs ^{51}Cr release assay. Yac-1 cells were used as the NK sensitive target cells and the wild-type B16 melanoma cells were used as the target cells of CTL. The ratio of effector/target was 50:1.

Statistical Analyses

Data were analyzed with the Student's *t*-test. Significance was defined as $P<0.05$.

RESULTS

Chemotactic Activity of the Supernatants of MIP-1α Gene-Transfected B16 Melanoma Cells

1×10^6 MIP-1α gene-transfected B16 melanoma cells were seeded into the cell culture flask, and the supernatants were collected 24 hrs later for determination of MIP-1α by indirect ELISA method. MIP-1α were found to be 368±24ng/ml (n=6). Then the chemotactic activity of the supernatants on different kinds of immune effectors was assayed with the Bodyen chamber system. As shown in Figure 1, the supernatants of MIP-1α gene-transfected B16 melanoma cells showed stronger chemotactic activity on both NK cells, $CD4^+$ T cells, $CD8^+$ T cells and peritoneal macrophages.

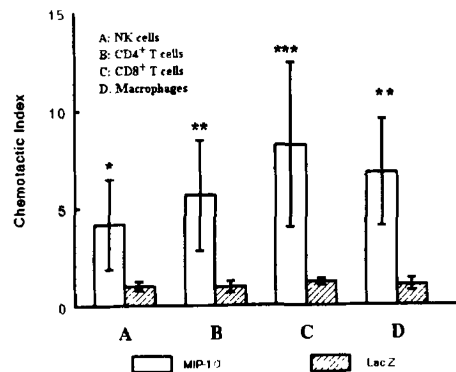

Fig. 1. Chemotactic activity on effector cells of the supernatants from MIP-1 α gene-transfected B16 melanoma cells.

Changes of Tumorigenicity of B16 Melanoma Cells Transfected with MIP-1α Gene and the Survival Time of Tumor-Bearing Mice

As shown in Figure 2, the growth of MIP-1α

gene-transfected B16 melanoma after subcutaneous inoculation was inhibited more significantly than that of wild-type B16 or the B16-LacZ cells. But the survival time of the mice bearing the MIP-1α gene transfectants was not prolonged, but even shortened compared with that of the controls (Figure 3). The mice bearing the MIP-1α gene-transfected B16 melanoma cell showed more obvious anoxia, malnutrition, disseminated skin petechia when compared with the mice bearing the wild-type B16 melanoma cell or the B16-LacZ.

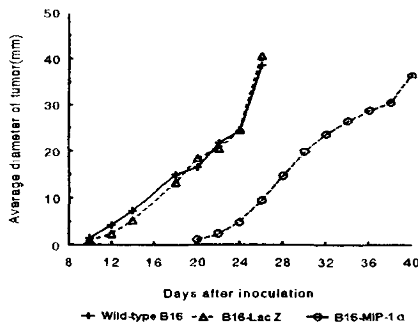

Fig. 2. The tumorigenicity of B16 melanoma cells transfected with MIP-1 α gene.

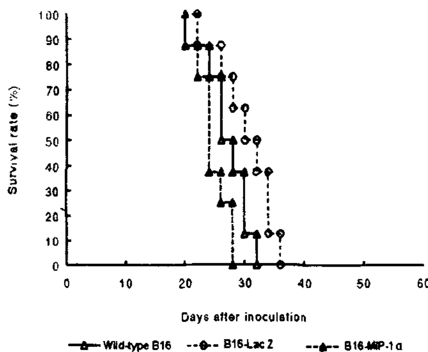

Fig. 3. The survival time of the mice bearing B16 mealnoma cells transfected with MIP-1 α gene.

NK, CTL Activity of Tumor-Bearing Mice

Then, the NK and CTL activities of splenocyte from the mice inoculated with MIP-1α gene-transfected B16 melanoma cells were determined. It was demonstrated that neither NK activity nor CTL activity of the mice bearing MIP-1α gene transfectants were increased (Table 1). These indicated that no antitumor immunity were induced by MIP-1α gene transfected B16 melanoma cells.

Table 1. The NK activity and CTL activity induced from the splenocytes of tumor-bearing mice

Groups	NK activity (%)	CTL activity (%)
wild-type B16	11.4 ± 2.8	8.4 ± 2.2
B16-Lac Z	9.3 ± 1.6	9.32 ± 3.4
B16-MIP-1 α	13.4 ± 1.8*	7.8 ± 0.6*

* P>0.05 as compared to control groups

Histological Analysis of the Tumor Tissue

7 days after subcutaneous inoculation with tumor cells, the histological analysis of tumor tissue were performed. More significant infiltration of inflammatory cells and obvious necrosis of tumor tissue were observed in the tumor mass formed by MIP-1α gene-transfected B16 melanoma cells. These results suggested that the slower growth property of MIP-1α gene-transfected B16 melanoma cells might be related to massive infiltration of inflammatory cells due to the chemotactic activity of MIP-1α secreted by the tumor cells in situ.

DISCUSSION

Cytokine gene therapy has been proposed as a new potential prospect in the active immunotherapy of cancer. Several studies have already shown that cytokine (such as IL-2, IL-4, IL-6, IL-7, IFN-γ, TNF-α) gene-transfected tumor cells could inhibited parental tumor cell growth and induce protective immunity in vivo following vaccination.[10,11] It has been well documented that the effects of systemic antitumor immune responses are closely correlated with the infiltration of effector cells, e.g. macrophages, T cells and NK cells, at the site of tumor.[12,13] Though the mechanisms of infiltration of immune effectors into the tumor tissue is still not fully understood, the chemokine and other chemoattractive factor produced in the tumor tissue may be partly responsible for the extravasation of the immune effectors. We supposed that chemokine gene-transfected tumor vaccine may be used to induce local as well as systemic antitumor immunity through induction migration of immune effector cells to the tumor site.

Huang et al. transfected MCP-1 gene into the murine colon carcinoma cells using a recombinant retrovirus vector. It was demonstrated that MCP-1 gene-transfected tumor cells showed decreased tumorigenicity and low metastatic potential when

inoculated into the mice subcutaneously. The tumor-bearing mice showed enhanced antitumor immunity and survived longer. Furthermore, increased infiltrations of lymphocytes and neutrophils were observed in the tumor site. These results suggested the potential role of the chemokine gene-transfected tumor vaccine in the induction of antitumor immunity *in vivo*.[7]

In the present study, we found that the supernatants of MIP-1α gene-transfected B16 melanoma cells have strong chemotactic activity on the $CD4^+$ T cell, $CD8^+$ T cells, NK cells and peritoneal macrophages. Though the *in vitro* growth of MIP-1α gene-transfected B16 melanoma cells was similar to that of the wild-type B16 melanoma cells, their growth *in vivo* after subcutaneous inoculation was inhibited significantly. Nevertheless, the survival time of the mice bearing MIP-1α gene-transfectants was not prolonged, and the NK and CTL of splenocytes from the tumor-bearing mice were not increased. It is known that MIP-1α may serve as a strong central inflammatory mediator through direct neuro-transmitter-like effect on the hypothalamus. Increased MIP-1α *in vivo* may give rise to much adverse effect to the host, such as fever, anorexia, adipsia and loss in body weight.[14] We also observed that the mice inoculated with the MIP-1α gene-transfected melanoma cells showed disseminated skin petechia, anorexia, malnutrition, which indicated the over-unregulated nonspecific inflammation reaction. Thus, we supposed that the shortened survival time of MIP-1α gene-transfected melanoma cell-bearing mice and the unchanged host antitumor immunity may be related to the adverse effect of MIP-1α. We concluded that the effectiveness of MIP-1α gene- transfected tumor vaccine in treatment of cancer is still required to be further confirmed. The choice of the ideal chemokine and control of its expression level *in vivo* should be considered for the further study of chemokine gene-transfected tumor vaccine.

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