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ANTITUMOR EFFECT OF GRANULOCYTE-MACROPHAGE COLONY- STIMULATING FACTOR (GM-CSF)-GENE ENCODED VACCINIA MELANOMA ONCOLYSATE AND ITS IMMUNOLOGICAL MECHANISMS[1]

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Vaccinia melanoma oncolysate (VMO) prepared by infecting B16F10 melanoma cells with recombinant vaccinia virus encoding murine GM-CSF gene was tested for its therapeutic effect on the preestablished melanoma. C57BL/6 mice were inoculated s.c. with 1×10^5 B16F10 melanoma cells and received s.c. administration with VMO prepared with GM-CSF gene-encoded vaccinia virus(GM-CSFVMO), VMO prepared with thymidine kinase gene-deficient vaccinia virus(TKVMO), B16F10 melanoma oncolysate(BMO), or PBS 3 days after tumor inoculation. The same treatment was bolstered one week later. The results demonstrated that GM-CSFVMO treatment significantly inhibited the growth of subcutaneous tumor and prolonged the survival period of tumor-bearing mice. Further study elucidated that cytotoxicity of PBL and splenocytes towards B16F10 increased obviously after treatment with GM-CSFVMO, but NK activity remained unchanged. These results suggest that the tumor oncolysate vaccine prepared with GM-CSF gene-encoded vaccinia virus might exert potent therapeutic effect on the preestablished tumor through the efficient induction of specific antitumor immune response of the host.

Key words: Vaccinia virus, Gene therapy, Melanoma, Granulocyte-macrophage colony-stimulating factor, Oncolysate, Antitumor immunity

Cytokine gene therapy has been studied intensively for the treatment of cancer. Among various methods used for gene transfection, viral vectors have strong potentials as vehicles for the targeted delivery of cytokine gene into tumors in vivo. Candidate viruses include retrovirus, adenovirus, herpes simplex virus, adeno-associated virus and vaccinia virus.[1] Vaccinia virus is now widely used as a transitory expression vector within the cytoplasm of eukaryotic cells and high levels of protein can be synthesized by recombinant vaccinia virus.[2,3] Recent studies have shown that active specific immunotherapy with vaccinia virus-modified tumor vaccine could induce protective immunity in both animal tumor models and patients. Recombinant vaccinia viruses engineered to produce interleukin-4(IL-4), interferon (IFN), or interleukin-6 (IL-6) exhibited therapeutic effects on various murine tumor models.[3-5] Vaccinia oncolysates were found to inhibit tumor growth and prevent metastasis in animals with pre-established tumor. Oncolysate vaccine prepared from tumor cells infected with interleukin-2(IL-2)-secreting recombinant vaccinia virus was successfully used to treat murine colon adenocarcinoma and hepatic metastatic melanoma.[6-8]

Tumor cells transduced with cytokine gene showed decreased tumorigenecity and increased

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immunogenecity. Tumor vaccine prepared with tumor cells transfected with cytokine gene not only induced cellular immunity to protect against the same cells but also elicited protective immunity to the parental tumor cells. Granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting tumor cells were found to stimulate potent, specific and long lasting antitumor immunity.[9] In the present study, we have observed the antitumor effect of vaccinia melanoma oncolysate (VMO) prepared with GM-CSF expressing vaccinia virus in tumor-bearing mice with preestablished melanoma and investigated the immunological mechanisms.

MATERIALS AND METHODS

Animals and Cell Lines

Male or female C57BL/6 mice, 6-8 weeks of age, purchased from Joint Ventures SIPPR-BK Experimental Animal Co., Shanghai, China, were housed in a specific pathogen-free state for any experiment. B16F10, a subclone of B16 melanoma cell line from C57BL/6, VERO, a continuous cell line derived from African green monkey kidney, and YAC-1, a NK sensitive cell line, 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 the media were purchased from Gibco, USA, and FCS from Shanghai Institute of Biological Products, Shanghai, China.

Preparation of Vaccinia Melanoma Oncolysate (VMO)

Recombinant vaccinia virus containing murine GM-CSF gene was constructed and kindly provided by Dr. Bruce Acres in Transgene SA, France. The vaccinia viruses were propagated in VERO cells and their infectious titers were checked every two months by a standard plaque-forming unit (PFU) assay. Vaccinia melanoma oncolysates (VMO) using recombinant vaccinia virus were prepared by a method described elsewhere.[8]

Immunization of Mice with Melanoma Oncolysate

Four groups of mice with 5 mice in each group were immunized with melanoma oncolysate equivalent to 10^6 cells twice with an interval of one week. The mice were re-challenged s.c.with 10^5 B16F10 melanoma cells 7 days after the immunization and tumor occurrence observed.

Treatment of Tumor-Bearing Mice with VMO

C57BL/6 mice were inoculated s.c. with 1 x 10^5 B16F10 melanoma cells. 4 groups of mice with 15 mice in each group were used in each experiment. The mice in each group received an s.c. injection of any of the following preparations: PBS, 10^6 cell equivalents of B16F10 melanoma oncolysates without vaccinia virus infection (BMO), VMO(10^6 cell equivalents) prepared with thymidine kinase gene-deficient deficient vaccinia virus (TKVMO), or GM-CSFVMO (10^6 cell equivalents) prepared with GM-CSF gene-encoded vaccinia virus. Injections of the preparations in 0.2 ml PBS were performed 3 days after the inoculation of tumor cells and a booster of the same injections was given after another 7 days. The length and width of the tumor mass were measured with caliber every other day after tumor inoculation and the tumor volume were expressed as $1/2 \times \text{length} \times \text{width}^2$.

NK, CTL Cytotoxic Assay

Four-hour ^{51}Cr release assays were performed for cytotoxicity determination. 4 days after the 2nd treatment with melanoma oncolysate 5 mice in each group were sacrificed and peripheral blood lymphocyte (PBL) and lymphocytes in spleen were isolated utilizing Ficoll-Hypaque. The PBL and splenocytes were directly used as effector cells for NK activity assay. The splenocytes were stimulated in vitro with irradiated B16F10 cells for 6 days and used for CTL activity assay. The PBL were used for CTL assay directly. 2 x 10^6 B16F10 or YAC-1 cells in 0.5 ml RPMI-1640 with 20% FCS were labeled with 200 μCi $Na_2{}^{51}CrO4$ (Amersham, USA) for 2 h. The labeled cells were washed three times in serum-free medium. Ten thousand target cells were then mixed with the effector cells for 4 h at 37°C with E:T of 50:1. For the maximal or spontaneous ^{51}Cr release control, 0.1 ml of 0.1 N HCl or 0.1 ml medium were added to the target cells. The amount of released ^{51}Cr was determined by γ counting on a 1275 Minigamma Counter (LKB-Wallac, Finland), and cytotoxic activity was calculated as follows:

Cytotoxic activity (%) =

$$\frac{\text{Experimental cpm - spontaneous cpm}}{\text{Maximal cpm - spontaneous cpm}} \times 100$$

Statistics

Statistical analysis was performed using the Student's *t* test.

RESULTS

Induction of Protective Effect by Oncolysate Immunization

The data in Table 1 showed that the mice received PBS immunization developed palpable tumor nodule within 10 days after tumor inoculation. The mice immunized with BMO and TKVMO formed palpable tumor mass 2 weeks after tumor challenge. The immunization of the mice with GM-CSFVMO could obviously delay the development of tumors, with 2 of the total 5 mice being tumor-free. The mean survival periods for mice immunized with PBS, BMO, and TKVMO were 27.4 ± 5.1, 40.2 ± 9.4, 44.2 ± 10.1days , respectively. Two mice in GM-CSF-immunized group survived up to 90 days being tumor-free, and the mean time for the other three mice is 54.1 ± 12.0 days. The results indicated that oncolysate immunization could protect the mice against the subsequent challenge with wild-type tumor cells.

Inhibitory Effect of GM-CSFVMO on the Growth of Preestablished Melanoma

Then, we determined whether immunization with GM-CSFVMO has therapeutic effect on pre-established tumor. C57BL/6 mice were inoculated with 1 x 10^5 B16F10 melanoma cells on day 0 and treated with different melanoma oncolysates on day 3 and day 10. The results in Figure 1 demonstrated that GM-CSFVMO treatment inhibited the growth of subcutaneous melanoma significantly when compared with PBS, BMO, or TKVMO treatments($P<0.01$). BMO .or TKVMO therapy also showed inhibitory effect on murine melanoma growth when compared with PBS therapy ($P<0.05$). 10 mice in each group were remained to be observed for their survival period. The results in Figure 2 demonstrated that mice treated with GM-CSFVMO survived much longer than those treated with TKVMO, BMO or PBS($P<0.01$). 3 of 10 GM-CSFVMO-treated mice survived more than 90 days while all control animals continuously died within 45 days.

Table 1. Effect of GM-CSFVMO immunization on tumor occurrence.

Groups	Time of tumor appearance (n =5)	Tumor occurrence 14 days after tumor inoculation
PBS	10.4 ± 3.2	10/10
BMO	15.3 ± 2.7*	6/10
TKVMO	17.2 ± 4.8*	4/10
GM-CSFVMO	26.6 ± 8.5**#	1/10

* Compared with PBS group, P<0.05

** Compared with PBS, TKVMO and GM-CSFVMO groups, P<0.01

\# Two mice did not develop palpable tumors 40 days after tumor inoculation and were not included here.

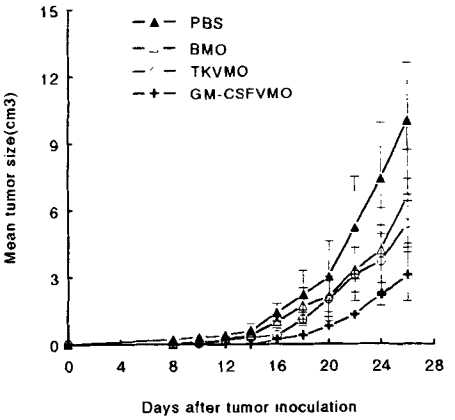

Fig 1.Growth curve of subcutaneous melanoma in mice treated with vaccinia melanoma oncolysate.

Induction of Specific Immunity against Melanoma

PBL or splenocytes from the sacrificed C57BL/6 mice were used in assays for cytotoxicity against YAC-1 cells or B16F10 cells at E:T of 50:1 by a 4-h ^{51}Cr release method. No increasing of NK activity was observed in mice after treatment with GM-CSFVMO when compared with the mice in other therapy groups, suggesting that the antitumor effect of GM-CSFVMO was not through the augmentation of NK cells (data

not shown). Activities of cytotoxic T lymphocytes in PBL and spleen were tested utilizing B16F10 tumor cells as targets. The data in Figure 3 demonstrated that PBL and splenocytes from GM-CSFVMO-treated mice showed an obvious increase of cytotoxic activity against B16F10 cells when compared with these lymphocytes from PBS, BMO, or TKVMO-treated mice ($P<0.01$). The cytotoxic activities of lymphocytes in TKVMO and BMO-treated mice against B16F10 cells were also found to be higher than these in PBS-treated mice($P<0.05$).

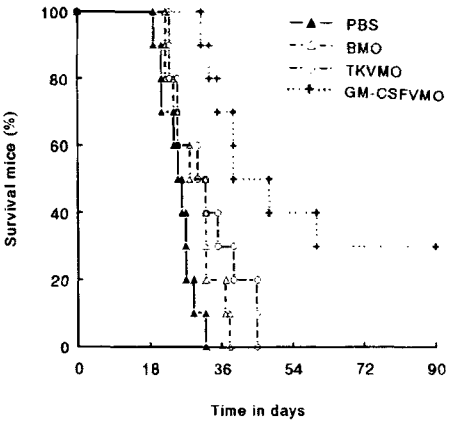

Fig. 2. Survival period of tumor-bearing mice treated with GM-CSFVMO, TKVMO, BMO, or PBS.

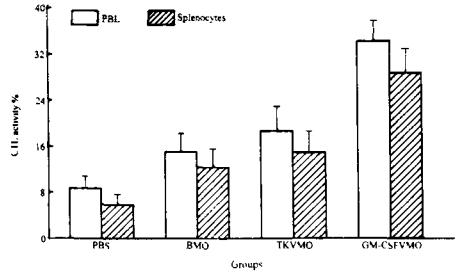

Fig. 3. Cytotoxicity of PBL and spleen lymphocyts from mice treated with GM-CSFVMO, TKVMO, BMO, or PBS. B16F10 cells were utilized as target cells with E:T of 50:1.

DISCUSSION

Vaccinia virus has many obvious advantages over other viral vectors. The recombinant vaccinia virus could infect a broad range of tumor cell types, and express foreign genes immediately after infection using its own enzyme systems. Vaccinia virus is restricted to local and transitory infection which favors its clinical application to deliver cytokines locally.[13,14] Direct injection of purified recombinant cytokines are successful in the treatment of cancer, but to achieve satisfactory therapeutic effects, high dose of cytokines must be administered, which often results in serious side effects. The utilization of recombinant viruses to express cytokines in vivo may address the limitation of toxicity by sustained delivery of the cytokines at the sites of viral infection. Recombinant vaccinia viruses were used to deliver cytokine genes such as IL-2, IL-4, IL-6 *in vivo*.[3-5,10] We have previously demonstrated that intratumoral injection of recombinant vaccinia virus could inhibit the growth of subcutaneous tumor and prolonged the survival period. The pulmonary metastasis of melanoma could also be inhibited by treatment of the tumor-bearing mice with vaccinia virus encoding GM-CSF gene.[11,12]

Tumor cells engineered to express certain cytokines such as ILs, IFN, TNF or GM-CSF have been demonstrated to induce potent and specific antitumor immunity. In a comparative study on the ability of various cytokines and adhesion molecules to induce host immune responses, C57BL/6 mice were immunized with irradiated gene-transduced B16 melanoma cells, followed 7 days later by a subcutaneous challenge with non-irradiated parent B16 cells. Among 22 molecules examined, GM-CSF was the most potent stimulator of systemic antitumor immunity.[9,13] Clinical trials with GM-CSF tumor vaccine have been carried out for the therapy of patients with renal cell carcinoma or melanoma. In this study we found that GM-CSFVMO administration could significantly inhibit the growth of subcutaneous tumor and prolong the survival period of tumor-bearing mice. Immunization with GM-CSFVMO could obviously delay the time of tumor formation when rechallenged by wild type B16F10 cells. Further study elucidated that cytotoxicity of PBL and splenocytes towards B16F10 increased obviously after treatment with GM-CSFVMO, but NK activity remained unchanged, suggesting that GM-CSFVMO might exert potent therapeutic effect through the efficient induction of specific antitumor immunity of the host.

Clinical trials have showed that treatment with melanoma oncolysate could improve survival of patients with stage II melanoma patients.[14] Experimental studies with melanoma oncolysate infected with IL-2 gene-encoded vaccinia virus

showed obvious induction of antitumor immunity of the host in tumor-bearing mice. So we may speculate that tumor oncolysate transfected with cytokine gene-encoded vaccinia virus might have therapeutic potentials for the immunotherapy of tumors in clinical trials.

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