Chinese Journal of Cancer Research 9(1):16-20,1997.

EFFECTS OF GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR GENE ENCODED VACCINIA VIRUS VECTOR ON MURINE PULMONARY METASTATIC MELANOMA

Ju Dianwen 鞠佃文 Cao Xuetao 曹雪涛 Wan Tao 万涛 Ma Shihua 马施华
Wang Baomei 王宝梅 Yu Yizhi 于益芝 Ye Tianxing 叶天星

Department of Immunology, Second Military Medical University, Shanghai 200433

A recombinant vaccinia virus expressing murine granulocyte-macrophage colony-stimulating factor (VVGM-CSF) was tested for its antitumor activity. Murine pulmonary metastasis was established by injecting 2×10^5 B16F10 melanoma cells into the tail vein of C57BL/6 mice. Three days after B16F10 inoculation, VVGM-CSF or VVTK, a thymidine kinase gene deficient control vaccinia virus, were injected intraperitoneally twice weekly for 2 weeks. Two weeks later, the mice were sacrificed and pulmonary metastasis foci counted. The results demonstrated that VVGM-CSF treatment significantly decreased the number of pulmonary metastasis and prolonged the survival time of tumor-bearing mice. Cytotoxic and phagocytic activities of the peritoneal macrophages were found to be markedly elevated in mice treated with VVGM-CSF. Nitric oxide released from the macrophages was also found to be increased. These data, together with our other results, strongly demonstrated that continuous secretion of GM-CSF and activation of macrophages might partially explain the therapeutic effects of VVGM-CSF on murine pulmonary metastasis.

Key words: Vaccinia virus, Gene therapy, Melanoma, Granulocyte-macrophage colony-stimulating factor.

Among various kinds of biological response modifiers (BRM) for the treatment of cancer, cytokines show very encouraging therapeutic effects. Injection of recombinant cytokines directly to human or experimental animals are able to markedly reduce or eradicate the tumor growth. But high dose of cytokines for tumor therapy often results in serious side effects. People have long been working to find much efficient way to deliver cytokines continuously and effectively. Cytokine gene therapy for cancer has been studied intensively. Different kinds of vectors have been used for cytokine gene transfer *in vivo*. Relatively low efficiency of gene transfection of liposome and gene gun limited their therapeutic potentials *in vivo*.[1] Replication-deficient viral vectors have obvious advantages as vehicles for the targeted delivery of cytokine expression to tumors *in vivo*. Candidate viruses include retrovirus, vaccinia virus, adenovius and adeno-associated virus et al.[2] Vaccinia virus (VV) was successfully used for the prevention of smallpox. It is now widely used as a transient expression vector within the cytoplasm of eukaryotic cells. Vaccinia virus infects a wide range of tumor cell types and expresses foreign gene within hours and recombinant vaccinia virus vector produces large quantities of foreign gene products with in the cytoplasm of host cells. Immunizations with recombinant vaccinia virus have been shown in murine tumor models to prevent and to actively treat murine tumors expressing human tumor antigen p97 and

*Accepted January 7,1997
This work was supported by grants from the National Natural Science Foundation of China (No. 39570668 and 39421009).

carcinoembryonic antigen (CEA).[3,4] The proven safety of vaccinia virus, which is restricted to local and transitory infection, favors clinical application of recombinant vaccinia to deliver immunomodulatory cytokines locally. In the present study we have observed the effect of a vaccinia virus expressing murine GM-CSF (VVGM-CSF) on pulmonary metastatic melanoma.

MATERIALS AND METHODS

Agents

Recombinant murine granulocyte-macrophage colony-stimulating factor(mGM-CSF) was purchased from Genzyme. MTT was bought from Fluka. LPS, neutral red, sulfanilamide, naphthylethylene diamine dihydrochloride were from Sigma. Culture medium was from Gibco.

Mice and Cell Lines

Male C57BL/6 mice, 6 weeks of age, were purchased form Joint Ventures SIPPR-BK Experimental Animal Co., Shanghai, China. The animals were kept for at least one week in a specific pathogen-free state prior to any experiments.

VERO, a continuous cell line derived from African green monkey kidney, and B16F10, a melanoma cell line clone from C57BL/6 mice and leukamia cell line L1210 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).

Generation of Recombinant Vaccinia Virus

Recombinant vaccinia viruses harboring murine GM-CSF gene (VVGM-CSF) were constructed in Transgene SA, France,[5] and kindly supplied by Dr. Bruce Acres. The murine GM-CSF cDNA was kindly provided by Dr. Widmer From Immunex Research and Development Co., USA. The cDNA was cloned into the thymidine kinase (TK) gene of the Copenhagen strain of vaccinia virus through homologous recombination. Expression of the murine GM-CSF gene is driven by 7.5 kDa vaccinia virus promoter. TK gene deficient vaccinia virus without insert (VVTK) was used as control. Both recombinant and control viruses were propagated in VERO cells and the infectious titer of the stock virus were checked semimonthly by a standard plaque-forming units (PFU)/ml.[6]

Treatment of Pulmonary Metastatic Mice with Recombinant Vaccinia Virus Expressing GM-CSF

B16F10 melanoma cells taken from continuous culture were washed twice and resuspended in PBS for injection into mice.[7] C57BL/6 mice given 2×10^5 B16F10 cells intravenously into the tail vein will develop obvious melanoma metastasis on the lung. Three groups of mice with 11 mice in each group were used in each experiment. Three days after tumor inoculation, each group received an intraperitoneal injection of control medium, VVTK or VVGM-CSF 10^6 PFU in 0.1 ml. The therapy was performed twice weekly for two weeks.

Macrophage Activity Assay

Peritoneal macrophages were washed from vaccinia virus treated mice 15 days after tumor inoculation.[8] The cells were washed twice and adherent macrophages were obtained by plating the cells into 24- and 96-well plate. Phagocytosis was determined employing the method of neutral red with minor modifications. For macrophage cytotoxicity assay, macrophages were incubated with L1210 cells at E/T=10 in a total volume of 0.1 ml/well in 96-well culture plate. After 20 h of coincubation the live remained L1210 cells were transferred to another culture plate and MTT was added to each well, the plates were cultured for 4 h followed by the addition of 0.1 ml 10% SDS in 0.01 N HCl. The formazan crystals were dissolved in SDS and absorbance read at 540 nm. Percentage cytotoxicity was calculated as follows:

$$\text{Percent cytotoxicity} = (1-A_{L1210\ remained}/A_{L1210\ control})\times100\%$$

Measurement of Nitric Oxide

Nitrite contents in macrophage supernatants were measured by a microplate assay method with Griess reagent.[9] Briefly, macrophages in 96-well plates were incubated with 5 μg/ml LPS for 24 or 48 h

in humidified atmosphere at 37 ℃. Fifty μl aliquots were collected from conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylene diamine dihydrochloride in 2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 550 nm was determined in BIO-RAD model 2550 microplate reader. Sodium nitrite was used as standard.

Statistical Analysis

The results were expressed as $\bar{x}\pm s$. Statistical analysis was performed using the Student's *t* test.

RESULTS

Effects of VVGM-CSF on B16F10 Pulmonary Metastatic Melanoma

C57BL/6 mice inoculated with 2×10^5 B16F10 tumor cells intravenously into the tail vein developed obvious pulmonary metastasis 15 days later. The results in Table 1 showed that VVGM-CSF treatment greatly decreased the number of metastasis foci on the lungs. VVGM-CSF-treated mice developed much less metastasis than those in RPMI-1640 and VVTK groups. Control virus VVTK showed no protection effects on metastasis formation.

Table 1. Effects of VVGM-CSF therapy on the number of murine pulmonary metastasis on the lung in tumor bearing mice

Group	Numbers of pulmonary metastasis
RPMI-1640	79.2± 8.9
VVTK	76.8± 9.1
VVGM-CSF	22.4± 5.2*

C57BL6 mice were inoculated with 2×10^5 B16F10 melanoma cells into the tail vein and received vaccinia injection intraperitoneally. Mice were sacrificed and metastasis calculated 15 days after the inoculation of B16F10 cells. (n=6 mice, $\bar{x}\pm s$). *$P<0.01$ compared with RPMI-1640 and VVTK groups.

Effect of VVGM-CSF on the Survival Period of Tumor Bearing Mice

Five mice in each group were used to be observed for their survival period. The results, as showed in Figure 1, showed that mice treated with VVGM-CSF was able to survive much longer than those injected with VVTK and RPMI-1640. Two of the VVGM-CSF treated mice lived more than 90 days and the mean survival period for another 3 mice was 46.7 days, while control animals continuously died within 38 days with mean survival period of 32.2 days.

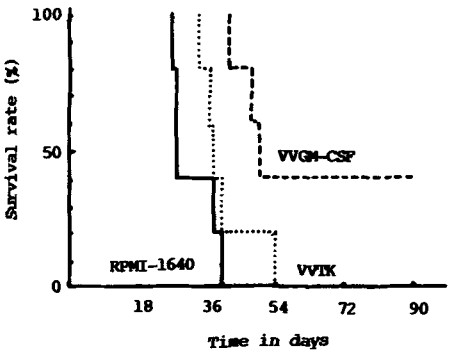

Fig. 1. Survival time of tumor-bearing mice treated with VVGM-CSF, VVTK and RPMI-1640. C57BL/6 mice were inoculated with B16F10 melanoma cells intravenously and received vaccinia virus injection intraperitoneally.

Increased Phagocytosis, Cytotoxicity of Macrophages from VVGM-CSF Treated Mice

In Table 2 the results showed that peritoneal macrophages were activated after repeated injection of VVGM-CSF intraperitoneally. The macrophages obtained from VVGM-CSF treated mice phagocytized more red dye when incubated with 1% neutral red. The macrophages derived from VVGM-CSF treated mice also showed much higher cytotoxicity against L1210 tumor cells compared with those from VVTK or solvent treated mice.

Nitric Oxide Releasing of Macrophages from VVGM-CSF Treated Mice

Nitric oxide (NO) was found to have a wide range of biologic effects, prominent among these effects is the cytotoxicity of NO to tumor cells. NO contents from LPS stimulated macrophages were measured using Griess agent. The results demonstrated

that the NO production in macrophages derived from VVGM-CSF treated mice was much higher than those from VVTK and medium treated mice ($P<0.01$) (Figure 2).

Table 2. Cytotoxic and phagocytic activity of peritoneal macrophages from mice treated with VVGM-CSF intraperitoneally

Groups	Cytotoxicity (%)	Phagocytosis (Absorbance)
RPMI-1640	12.5± 2.3	1.96± 0.25
VVTK	15.6± 3.1	2.32± 0.39
VVGM-CSF	38.5± 3.2*	4.25± 0.59*

Mice were sacrificed and peritoneal macrophage activity measured 15 days after the inoculation of B16F10 cells. *$P<0.01$ compared with RPMI-1640 and VVTK groups (n=6 mice, $\bar{x}\pm s$).

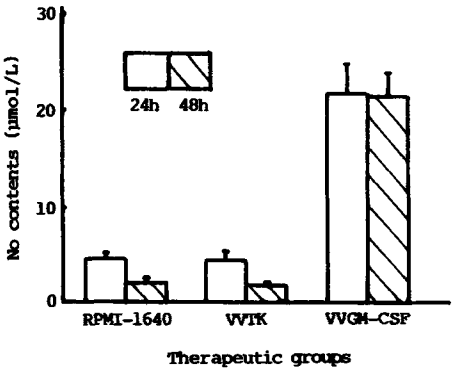

Fig. 2. Nitrite production by macrophages from mice inoculated with B16F10 tumor cells intravenously and received vaccinia virus vector intraperitoneally. Nitrite concentration in the samples was determined by reacting with an equal volume of Griess reagent and measuring the absorbance at 550 nm in a BIO-RAD plate reader.

DISCUSSION

In the past few years, various recombinant vaccinia viruses to express cytokine genes *in vivo* have been constructed and their perapeutic effects evaluated. Very satisfactory therapeutic effects were achieved with vaccinia vectors harboring certain cytokine genes such as interleukin 2(IL-2), interferon-γ(IFN-γ), interleukin-4(IL-4) and interleukin-6(IL-6).[10-14] Oncolysate vaccine 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] Elkins et al. successfully delivered IL-4 to mice inoculated with tumor cells by a recombinant vaccinia virus. They report that the vaccinia prevented tumor development in mice. Vaccinia virus encoding cytokine genes secreted high levels of cytokines immediately after its infection of tumor cells and both cellular and humoral antitumor immunity were induced. Tumor cells transfected with recombinant vaccinia virus not only induced cellular immunity to protect against the same cells but also produced protective immunity to the parental tumor cells that was used for cytokine gene transfection.

In this report, a vaccinia virus expressing the gene for murine GM-CSF was constructed and tested for its antitumor activity. Mice were inoculated with B16F10 murine melanoma cells intravenously, and 3 days later VVGM-CSF or VVTK, a thymidine kinase gene deficient vaccinia virus, were injected intraperitoneally to treat the tumor bearing mice. Our results demonstrated that VVGM-CSF treatment significantly reduced pulmonary metastasis formation and delayed the survival time of tumor-bearing mice. We have reported studies that intratumoral injection of GM-CSF or IL-2 gene encoded recombinant vaccinia virus could elicit potent antitumor response in murine melanoma model, and oncolysates prepared with GM-CSF gene encoded vaccinia virus infected melanoma cells significantly decreased the number of murine pulmonary metastasis and prolonged the survival period of the tumor-bearing mice.[13,14] GM-CSF is one of the cytokines involved in antitumor process. Vaccination with irradiated tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor can stimulate potent, specific and long lasting antitumor immunity. GM-CSF activated macrophages are important antitumor effector cells. In this report, cytotoxic and phagocytic activities of peritoneal macrophages were found to be greatly increased in mice treated with VVGM-CSF. Nitric oxide released from macrophages was also found to be increased. NO was thought to be key mediator in macrophage-mediated cytotoxicity. The role of NO in antitumor immunology has been studied intensively. Considering these data, combined with our previous

results we may speculate that activation of macrophages might partially explain the antitumor effects of VVGM-CSF when injected intratumorly.

REFERENCES

1. Whitman ED, Tsung K, Paxson J, et al. *In vitro* and *in vivo* kinetics of recombinant vaccinia virus cancer-gene therapy. Surgery 1994; 116:183.
2. Ram Z, Culver KM, Walbridge S, et al. *In situ* retroviral-mediated gene transfer for the treatment of brain tumors in rats. Cancer Res 1993; 53:83.
3. Estin CD, Stevenson US, Plowman GD, et al. Recombinant vaccinia-virus vaccine against human melanoma antigen p97 for use in immunotherapy. Proc Natl Acad Sci USA 1988; 85:1052.
4. Kantor J, Irvine K, Abrams S, et al. Antitumor activity and immune responses induced by a recombinant carcinoembryonic antigen-vaccinia-virus vaccine. J Natl Cancer Inst 1992; 84:1084.
5. Acres B, Dott K, Stefani L, Kieny MP. Directed cytokine expression in tumor cells *in vivo* using recombinant vaccinia virus. Therapeutic Immunol 1994; 1:17.
6. Sivanandham M, Scoggin SD, Tanaka N, et al. Therapeutic effect of a vaccinia colon oncolysate prepare with interleukin-2-gene encoded vaccinia virus studied in a syngeneic CC-36 murine colon hepatic metastasis model. Cancer Immunol Immunother 1994; 38:259.
7. Cao X, Yu Y, Xu Z, et al. Therapeutic effects of IL-4 gene transferred tumor vaccine on metastatic tumor *in vivo*. Chin J Microbiol Immunol 1994; 15:53.
8. Ju DW, Zheng QY, Wang HB, et al. Inhibitory effects of triazolodiazepine on mouse splenocytes and peritoneal macrophages *in vitro*. Acta Phamacol Sin 1994; 15:65.
9. Van de Loosdrecht AA, Nennie E, Ossenkoppele GJ, et al. Cell mediated cytotoxicity against U937 cells by human monocytes and macrophages in a modified colometric MTT assay. J Immunol Methods 1991; 141:15.
10. Bash JA. Recombinant vaccinia virus interleukin-2-infected tumor cell vaccines in immunotherapy of murine colon adenocarcinoma. J Immunother 1993; 1:269.
11. Arroyo PJ Bash JA, Wallack MK. Active specific immunotherapy with vaccinia colon oncolysate enhances the immunomodulatory and antitumor effects of interleukin-2 and α interferon in a murine hepatic metastasis model. Cancer Immunol Immunother 1990; 31:305.
12. Elkins KL, Ennist DL, Winegar RT, et al. *In vivo* delivery of interleukin. -4 by a recombinant vaccinia virus prevents tumor development in mice. Hum Gene Ther 1994; 5:809.
13. Ju DW, Cao X, Acres B. Active specific immunotherapy of pulmonary metastasis with vaccinia melanoma oncolysate prepared with granulocyte/ macrophage colony-stimulating factor-gene encoded vaccinia virus. J Cancer Res Cin Oncol 1996; 122:716.
14. Ju DW, Cao X, Acres B. Intratumoral injection of GM-CSF-gene encoded recombinant vaccinia virus elicits potent antitumor response in a murine melanoma model. Cancer Gene Ther 1997; (In press).