

# ANTI-METASTATIC EFFECT OF ONCOLYSATES FROM MURINE MELANOMA CELLS TRANSFECTED WITH RECOMBINANT VACCINIA VIRUS ENCODING HUMAN IL-2<sup>1</sup>

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Oncolysates, debris of tumor cells, have been proven to be effective in active immunotherapy of cancer. In this experiment, the oncolysates from murine melanoma cells B16-F10 transfected by recombinant vaccinia viruses encoding human IL-2(IL-2VBO) were used as vaccine. After treatment of tumor bearing mice with pulmonary metastases by intravenous injection of IL-2VBO or rVV-IL-2, higher level IL-2 activity was detected in the serum of IL-2VBO or rVV-IL-2 treated mice at 8h. Further experiment results demonstrated that IL-2VBO significantly reduced the number of pulmonary metastases and prolonged the survival time of tumor-bearing mice when compared with other preparations. Fresh peripheral blood lymphocytes from IL-2VBO treated mice showed potent cytotoxicity to B16-F10 cells and YAC-1 cells. But only cytotoxicity to B16-F10 cells is more marked than that in rVV-IL-2 group, indicating that the IL-2VBO could induce specific and non specific anti-tumor immunity. Because IL-2 expression was at the same level in the serum of IL-2VBO or rVV-IL-2 treated mice, the results suggested that the specific anti-tumor immunity induced by IL-2VBO might contribute to the enhanced therapeutic effect of IL-2VBO.

**Key words:** Vaccinia virus, IL-2, Oncolysate, Gene

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Recombinant vaccinia viruses(rVV), characterized with large accommodation and strong immunity, attract more attention in cancer gene therapy in recent years.<sup>1</sup> Cytokine gene, including IL-1, IL-2, IL-4, IL-5, IL-6, IL-12 or GM-CSF, encoded rVV have been constructed. This kind of cytokine gene-encoded rVV express synthesized protein and act as "adjuvant" themselves in stimulation of host immunity. Previous studies showed that direct injection of rVV-IL-2, rVV-IL4, rVV-IL6, rVV-GM-CSF had therapeutic effects on established tumor models.<sup>2-6</sup> But this kind of gene therapy couldn't stimulate the specific cellular immune responses, which was the most important for tumor rejection.

Cytokine gene-modified tumor cells were used as vaccine to induce specific cellular immunity. It was observed that cytokine-modified tumor cells acquired an enhanced immunogenicity and could induce high level of CTL activity. The experimental data and clinical trials showed that the vaccination of tumor-bearing host with this kind of tumor vaccine resulted in the rejection of tumor cells by host.<sup>7</sup>

Oncolysates, debris of tumor cells, have been used as vaccine to treat patients with cancer, Wallack et al. proved that the oncolysates infected with wild type vaccinia virus (wVV) could stimulate host

immunity to autologous tumor cells. In order to combine the advantages of cytokine gene-modified tumor cell vaccine with wVV-infected oncolysates, B16-F10 cell oncolysates transfected with IL-2 gene encoded recombinant vaccinia virus(IL-2VBO) were used in this experiment to induce anti-tumor immunity and the therapeutic effects of IL-2VBO were observed in murine melanoma model with pulmonary metastasis .

## MATERIALS AND METHODS

### Cell Lines

B16-F10 murine melanoma cells, VERO African green monkey kidney cells and YAC-1 cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100  $\mu$  g/ml streptomycin, 20mM HEPES at 37 °C, 5% CO<sub>2</sub> . IL-2 dependent CTLL-2 cells were maintained in RPMI1640 medium with above supplements and 20U/ml rIL-2.

### Mice

Male C57BL/6 mice, 6-8 weeks old, purchased from Joint Ventures Sippr BK Experimental Animal Co. Shanghai, China, were used in all experiments.

### Preparation of Recombinant Vaccinia Virus

rVV-IL-2 vector, kindly provided by Dr. Bruce Acres in Transgene SA(France), was constructed by homologous recombination. Human IL-2 cDNA was inserted into the non-necessary region (TK sequence), under the control of 7.5K promoter in Copenhagen strain of vaccinia virus. rVV-TK<sup>-</sup> was a control vaccinia virus vector which was TK gene deficient. Both viruses were expanded in VERO cells for 72h, and the virus infected cells were harvested by centrifuging at 800 $\times$ g for 10min, then the supernatant was collected and centrifuged at 20000  $\times$  g, the pepllet which contained the cell lysates and recombinant virus were harvested and tested by plaque-forming assay.

### Preparation of VBO

B16-F10 cells were cultured in RPMI1640

complete medium containing 10% FBS with rVV-IL-2 or rVV-TK<sup>-</sup> at the ratio of cell: virus =10:1 for 72 h. Then the transfected B16-F10 cells were harvested , adjusted to the concentration of 1 $\times$ 10<sup>6</sup>/ml and sonically disrupted at 300W for 3 min with Ultrasonication. The Sonication was repeated if any intact cell could be detected by trypan blue staining. The suspension was centrifuged at 800 rpm for 10 min, and the supernatant was harvested and centrifuged at 20000 rpm for 2 h, the pellets were collected and adjusted to the 1/10 of original volume. The resultant suspension was divided into aliquots 10<sup>6</sup> cell equivalents in 0.1ml RPMI1640 medium, stored at 4 °C for use.

### Treatment of Tumor-Bearing Mice

Tumor-bearing modle with pulmonary metastases was established by intravenous injection of 1 $\times$ 10<sup>5</sup> B16-F10 cells. 3 days later, the tumor-bearing mice were divided into four groups with 8 mice in each group, and then treated with intravenous injection of 0.1ml IL-2VBO, 0.1ml TK<sup>-</sup>VBO , 1 $\times$ 10<sup>7</sup>PFU rVV-IL-2 or normal saline(NS). The treatment was performed twice a week for 2 weeks. The tumor-bearing mice were sacrificed and the numbers of pulmonary metastases were counted 14 days after inoculation. Another serial groups with the same treatment as described above were maintained for the observation of survival time.

### Assay for IL-2 Production

Eight, 24, 48h after the first intravenous injection, the peripheral blood of mice were separated and the serum were collected. The IL-2 activity in the serum were tested by MTT bioassy. Briefly, 10<sup>4</sup> CTLL-2 cells were cultured with 0.1ml sample with different dilution for 24 h. A known concentration IL-2 (Genzyme) was used as positive control and the amount of IL-2 in supernatant was quantified by MTT method.

### Assay for Cytotoxicity

Fresh PBL were isolated from the mice by Ficoll-Hypaque centrifugation after 2 weeks of treatment . 4 h <sup>51</sup>Cr release assay were used to measure of cytotoxicity of NK and CTL. Briefly: B16-F10 (target cells for CTL assay) or YAC-1 (target cells for

NK assay) in 1ml RPMI 1640 medium with 20% FBS were labeled with 200 $\mu$ Ci Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (Amersham, Arlington Height, USA) for 4 h. The labeled cells were washed three times in RPMI1640 medium without FBS. 1  $\times$  10<sup>4</sup> target cells were mixed with effector cells for 4 h at 37 °C at the ratio indicated. 0.1ml 10% SDS were added to the labeled target cells for the maximal <sup>51</sup>Cr release control and 0.1ml RPMI1640 medium was added to the labeled target cells for spontaneous <sup>51</sup>Cr release control.  $\gamma$ count was determined on a 1275 MINGGAMA Counter (Wallac). Percentage of lysis was calculated as follows:

$$\text{specific lysis\%} = \frac{\text{cpm (experimental release)} - \text{cpm (spontaneous)}}{\text{cpm (maximal release)} - \text{cpm (spontaneous)}} \times 100$$

### Statistics

Statistics analysis was performed using unpaired student's *t* test. Significance was defined as *p*<0.05.

## RESULTS

### IL-2 Production in The Serum of Mice Treated with IL-2VBO

As shown in Figure 1, the IL-2 activity in the serum of mice treated by intravenous injection of IL-2VBO were detected as 24.3 $\pm$ 3.5U/ml at 8h, which was significantly higher than that from mice treated with TKVBO or NS, but it was at the same level when compared with that of mice treated with rVV-IL-2. The IL-2 expression of the mice treated with IL-2VBO or rVV-IL-2 was observed to decrease rapidly 24h after injection. There were no marked difference among the IL-2 expression from the mice treated with IL-VBO, rVV-IL-2, TKVBO or NS 48h after injection.

### Pulmonary Metastases of Tumor-Bearing Mice Treated with IL-2VBO

Two weeks after treatment with IL-2VBO, the mice were sacrificed and the number of pulmonary metastases was counted. The results shown in Figure 2 indicated that the number of pulmonary metastases reduced significantly by the intravenous injection of

IL-2VBO. Both of IL-2VBO and rVV-IL-2 inhibited the pulmonary metastases in the tumor-bearing model, however, the number of metastases in the mice treated with IL-2VBO was significantly less than that of mice treated with rVV-IL-2.

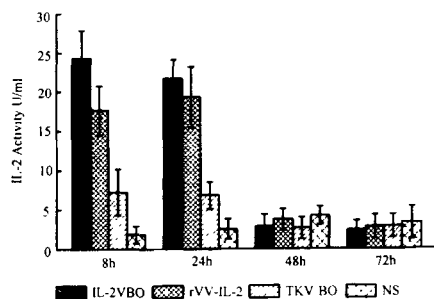


Fig. 1. IL-2 Expression in the serum of mice treated with IL-2VBO.

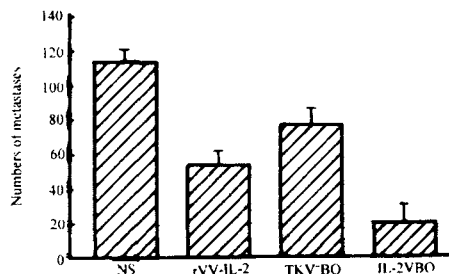


Fig. 2. Inhibition of the pulmonary metastases in tumor-bearing mice by the treatment with IL-2VBO.

### Survival Time of Tumor-Bearing Mice with Pulmonary Metastases Treated with IL-2VBO

Another serial groups of mice were treated with IL-2VBO, TKVBO, rVV-IL-2 or NS, the survival time of these mice were observed. As shown in Figure 3, the survival time of tumor-bearing mice treated with IL-2VBO prolonged obviously, 37.5% of mice survived more than 90 days, and the tumor-bearing mice in control groups died within 40 days. The injection of rVV-IL-2 alone was not effective in the extension of survival time of mice with pulmonary metastases.

### Cytotoxicity Of Fresh PBL From Tumor-Bearing Mice With Pulmonary Metastases Treated With IL-2VBO

In order to elucidate the mechanism of anti-

metastatic effect of IL-2VBO, the fresh peripheral blood lymphocytes were isolated and their cytotoxicities against B16-F10 cells and YAC-1 cells were determined. The data shown in Figure 4 indicated that the fresh PBL from IL-2VBO treated mice showed potent cytotoxicity to B16-F10 cells and YAC-1 cells. But only cytotoxicity to B16-F10 cells is more marked than that in rVV-IL-2 group. The results suggested that the IL-2VBO induced specific and non specific anti-tumor immunity, and the specific anti-tumor immunity might contribute to the enhanced therapeutic effect of IL-2VBO.

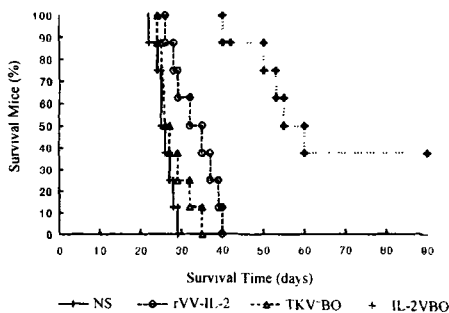


Fig. 3. Extension of survival time of tumor-bearing mice by treatment with IL-2VBO.

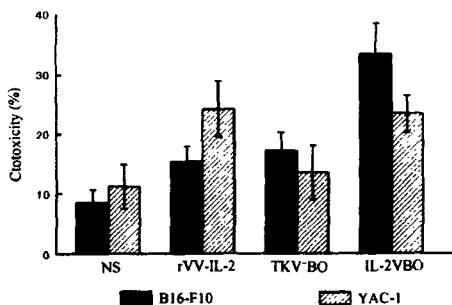


Fig. 4. Cytotoxicity to the B16-F10 cells and YAC-1 cells of peripheral blood lymphocytes from mice treated by IL-2VBO.

## DISCUSSION

Cancer active immunotherapy which is purposed to stimulate specific immunity depends on recognition of tumor antigen and activation of CTL. Previous studies have shown that the insertion of some kind of cytokine genes such as IL-2, IL-4, IL-6, IL-12, GM-

CSF genes into tumor cells decreased the tumorigenicity and increased the immunogenicity of these cells. This kind of cytokine gene-modified tumor vaccine has been proven to be able to induce specific anti-tumor immunity in animal model.<sup>7</sup>

Although the retroviral vector could integrate foreign gene into the chromosome of host cells and express synthetic protein stably, the whole cell vaccine which always transfected by retroviral vector had some limitations in preparation and storage. For example, it was difficult to prepare high titer of retroviral vector, several months would be taken to prepare the tumor vaccine. On the other hand the tumor cells must be inactivated by irradiation or some other agent, but still remain alive to secrete cytokine *in vivo*, so it was difficult in manipulation. With the development of molecular biological technology, various recombinant virus vectors were constructed in order to overcome these shortages. rVV is one of the candidate vectors which is maneuverable at the same situation. First, rVV can be easily prepared to  $10^9$ - $10^{11}$  PFU/ml and is highly efficient in gene transfection. Secondly, rVV is stable even at room temperature. Based on these characters, in this experiment, rVV was applied as a vehicle for IL-2 gene transfer. Higher level of IL-2 activity was observed during 24h in the serum of mice after intravenous injection of IL-2VBO and rVV-IL-2. It was thought to ascribe to the rVV-IL-2 and rVV-IL-2 present in IL-2VBO which could infect the host cells and express IL-2 *in vivo*. Consequently, The specific or non specific effector cells such as NK and CTL were activated and expanded to exert antitumor effect.

Oncolysates were considered containing some component of tumor associated or specific antigen, and have potential utility in the stimulation of specific cellular immune responses of host to autologous tumor.<sup>8</sup> Our previous study had proven that the MHC-I and ICAM-1 on the surface of B16-F10 cells were increased after the transfection of rVV-IL-2,<sup>9</sup> indicating that this kind of engineered tumor cell would stimulate stronger anti-tumor immune responses, and the "adjuvant-like" effect of vaccinia virus and IL-2 expression by rVV-IL-2 would be beneficial to the induction of the responses. In fact, IL-2VBO, being a mixture of gene modified cell lysates and rVV-IL-2, significantly inhibit the pulmonary metastases of murine melanoma in this experiment. The fresh PBL from IL-2VBO treated mice showed enhanced cytotoxicity to the B16-F10

cells and YAC-1 cells in comparison with that of NS or TKVBO treated mice, but only the cytotoxicity to B16-F10 cells was more marked than that of rVV-IL-2 treated mice. Because the IL-2 expression was at the same level in the serum of IL-2VBO and rVV-IL-2 treated mice, it suggested that the specific cytotoxicity induced by IL-2VBO was thought to be more important in the inhibition of pulmonary metastasis.

Recently, the rVVs encoding tumor specific or tumor associated antigen gene such as Muc-1, p97 or CEA were constructed and approved to do clinical trials in active immunotherapy of cancer. Because of the adjuvant effect induced by rVV vector, the antitumor response was induced more potently.<sup>10,11</sup> In view of the large accommodation of the rVV, multiple foreign genes such as cytokine gene plus tumor antigen gene or more than one kind of tumor antigen gene could be inserted into the rVV DNA and expressed these gene simultaneously. If so, use of rVV would be a more convenient and efficient approach in mediating the active immunotherapy of cancer.

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