

Combination of virotherapy with VSV and tumor vaccination significantly enhances the efficacy of antitumor therapy

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Summary. – Oncolytic virotherapy offers the potential to treat tumors both as a single agent and in combination with conventional therapies such as chemotherapy and immunological therapy. Here, we describe an effective treatment regimen which combines virotherapy with immunotherapy. IFN- α and co-stimulator IL-2 along with tumor cell lysate vaccination with intratumoral administration of oncolytic vesicular stomatitis virus (VSV) resulted in regression of established TC1 papilloma tumor model in C57BL/6 mice. The remarkable results especially in the group receiving tumor vaccination and virotherapy together (TC1-VSV) were obtained. Combination therapy synergistically enhanced CTL activity against tumor cells and reduced tumor size, although significant reduction in tumor size was observed in both groups receiving VSV or tumor vaccine alone. The presented data suggest that the effectiveness of virotherapy is enhanced when combined with immunotherapy by priming specific CD8 T cells against tumor antigens through tumor vaccination and boosting by exposure of antigens upon virus infection.

Keywords: virotherapy; VSV; tumor vaccine; immunotherapy; IFN- α ; IL-2

Introduction

Although recent advances in conventional cancer therapies such as surgery, chemotherapy and radiotherapy have resulted in increased patient survival, tumor resistance to conventional therapies has led to the design of new strategies (Saito *et al.*, 2009). In recent years, treatment of cancer using oncolytic viruses shows promising results (McCormick, 2005; Vähä-Koskela *et al.*, 2007). Oncolytic viruses are useful agents that infect malignant cells and damage them without remarkable effect on the normal tissues. Although, scientists found tumor regression following viral infection in 1893, most of the research in this area,

was done in the past 17 years. At this time, animal viruses such as vaccinia virus, adenoviruses, herpes simplex virus and reoviruses were tested for their potential oncolytic effect and numerous success in laboratory and clinical trials were reported (Hastie and Grdzlishvili, 2012). Selective cytotoxicity for tumor cells and safety of the host are the advantages of oncolytic virotherapy (McCormick, 2005; Saito *et al.*, 2009). Defects in antiviral mechanisms in tumor cells are the cause of preferential tropism of many viruses to cancer cells (Boisgerault *et al.*, 2010). Beside the direct killing of the tumor cells, virotherapy can overcome the immunosuppressive effects within the tumor and induce anti-tumor responses (Bourke *et al.*, 2011). Nonpathogenic nature of VSV for humans and the ability to effectively destroy permissive cells make the virus a suitable choice for cancer virotherapy. VSV is a negative sense RNA virus belonging to the *Rabdoviridae* family, that quickly replicates and shows an effective oncolytic potential in a variety of tumors in pre-clinical models (Hastie and Grdzlishvili, 2012; Hu and Lipshutz, 2012; Shinozaki *et al.*, 2005; Stan-

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Abbreviations: VSV = vesicular stomatitis virus, IFN- α = interferon alpha, IL = interleukin, CTL = cytotoxic T lymphocyte, CPE = cytopathic effect, MOI = multiplicity of infection, TCID₅₀ = tissue culture infectious dose 50%

ford *et al.*, 2010; Yamaki *et al.*, 2013). VSV is naturally considered as an oncolytic virus and needs no genetic manipulation (Saito *et al.*, 2009). VSV is very sensitive to interferon response induced by viral infections. Thus it can replicate selectively in tumors with defects in interferon responses (Hu *et al.*, 2011; Lun *et al.*, 2009; Saito *et al.*, 2009; Stanford *et al.*, 2010). Defects in p53 and ROS function are also reported to be the reason for virus selection of tumor cells. On the other hand, VSV matrix protein has intrinsic anti-tumor activity and induces apoptosis through interaction with p53 (Boisgerault *et al.*, 2010; Bourke *et al.*, 2011; Hastie and Grdzlishvili, 2012).

Combination of virotherapy and standard multiple therapies including surgery and chemotherapy creates an effective relation between oncolytic virotherapy and standard treatments which lead to an effective response against solid tumors (Bourke *et al.*, 2011).

Cancer immunotherapy and tumor vaccination that recruit tumor specific immune response are extensively used for tumor elimination (Drake *et al.*, 2013; Vähä-Koskela *et al.*, 2007). Activation of immune system against cancer is an important aim in immunology and oncology. In the past decades, immunotherapy has been known as a standard treatment for a variety of malignancies. But, immunotherapy against cancer is limited because of poor immunogenicity of tumor antigens (Hu *et al.*, 2007). Despite the immunological tolerance to tumor antigens, induction of effective immune responses against these antigens is desirable (Bridle *et al.*, 2010). Addition of immunostimulating factors to oncolytic therapy, including cytokines and chemokines such as IL-2 and IL-17 is a suitable method that contributes to the effectiveness of treatment (Stewart and Smyth, 2011). The use of cytokines such as IL-2 can lead to activation of a specific immune response against tumor cells through the activation of CTL response (Vähä-Koskela *et al.*, 2007).

Nowadays in biological therapy interferons along with chemotherapy are used to boost the immune responses. Among interferons, interferon alpha is mostly used (Lu *et al.*, 2009). IFN- α mediated cytotoxicity through stimulating anti-tumor immune effectors and indirect antitumor activity leads to the inhibition of tumor growth (Boisgerault *et al.*, 2010; Hu and Lipshutz, 2012).

Adjuvant therapy with IFN- α , has resulted in stimulation of CTL and NK cell functions in patients with high risk cancers in clinical trials (Oosterling *et al.*, 2006). IFN- α also shows other direct effects including negative regulation of oncogene expression and tumor suppressing induction that can help anti-growth activity of the cytokines (Belardelli and Gresser, 1996). Oncolytic viruses can release tumor antigens through cell lysis (Hu *et al.*, 2007; Stewart and Smyth, 2011). Virus mediated oncolysis alone induces a weak antitumor immune response (Bourke *et al.*, 2011; Bridle *et al.*, 2010). Combination of immunotherapy with

virotherapy has been considered to be a promising approach in improvement of treatment efficacy (Stewart and Smyth, 2011). Vaccination by tumor cells and boosting immune response against tumor antigens along with virotherapy increases treatment efficacy. The goal of this study is to investigate the effect of combination of virotherapy and immunotherapy.

Materials and Methods

Cells. Vero cell line was obtained from Pasteur institute of Iran and was grown in DMEM (Gibco, USA) supplemented with penicillin (100 unit/ml), streptomycin (100 mg/ml) and 10% FBS. Cells 48 h after the seeding were inoculated with VSV (gift from Dr Bell, Cancer Research Institute, Canada) (Breitbach *et al.*, 2011) of MOI 0.1 in serum free medium and maintained in an incubator at 37°C with 5% CO₂ until the CPE appears. Subsequently, cells were lysed by three cycles of freeze/thawing and centrifuged at 1,500 x g for 10 minutes. After titration, with Karber method, supernatant was stored at -70°C until further use. TC-1 cell line which expresses the HPV-16 E7 and E6 onco-proteins, was used to induce tumor growth in the mouse animal model (Song *et al.*, 1985).

Lymphocyte EL-4 cell line was used as target for CTL assay. Cell line was originally derived from lymphoma induced in C57BL/6 mice (Stremmel *et al.*, 1999). All cell lines were obtained from Pasteur cell bank of Iran.

Viral titration by TCID₅₀ (Karber method). Logarithmic serial dilutions of the virus (1 log) were prepared. One hundred microliters of each dilution were inoculated to 4 wells of a 96 wells microplate containing Vero cells. Microplates were controlled for CPE every day. Three days after inoculation, the number of wells with CPE in each dilution, were counted and Karber's formula was used to determine viral titer, which was 10⁸ TCID₅₀/ml (Wiedbrauk and Johnston, 1993).

Purification of expression plasmid CMV-MUFFIN- α 1 in large-scale for mice inoculation. Eukaryotic expression plasmid containing IFN- α (gift from Stefano Andracolo, Institute of Oncology, Italy) was used for mice inoculation (Fulci *et al.*, 2006; Lasfar *et al.*, 2006). The alkaline lysis method (Plasmid maxi prep kit, Sigma-Aldrich) was used for purification of plasmid DNA according to the manufacturer's instructions. One hundred micrograms of DNA were used for mice inoculations.

Animal study. Male C57BL/6 mice (6–8-week-old) were purchased from Pasteur Institute of Iran and were housed for one week before starting the experiment. All experiments were done according to the guidelines for the care and use of the laboratory Animal Ethical Commission of Tarbiat Modares University (IR.TMU.REC.1394.258). To develop tumors, TC1 cells were cultured in DMEM medium containing 5% FBS. In each flask around 5 million TC1 cells were grown and after two passages, the appropriate number of cells from the third passage (8×10⁵ cells) were injected to the left or right flank of each mouse. Mice were divided into 4

groups of 5 mice. When the size of tumors reached around 5–8 mm, two doses of IFN- α DNA (100 μ g/mouse), 0.05 μ g of IL-2 (Roche, Germany) and apoptotic tumor cells irradiated with UVB (wavelength of 312 nm for 20 min) (Bartholomae *et al.*, 2004; Kotera *et al.*, 2001) were injected subcutaneously to mice groups at 10 days intervals. Complete necrosis and apoptosis of irradiated cells was confirmed by Trypan Blue staining before injection and one million of cells in a volume of 200 μ l was prepared for injection. One week after immunization, virotherapy was done in two groups of VSV and TC1-VSV, in 3 doses at 4 days intervals. PBS was injected to control group. The TC-1 group only received apoptotic cells with IL-2 and IFN- α DNA. (Table. 1)

CTL assay. The lytic activity of spleen cells was assayed against TC1 cell line for evaluation of CTL response against tumor cells or against EL4 cell line inoculated with VSV (MOI of 10), as a highly sensitive target for the CTL assay in C57BL/6 mice (Hu *et al.*, 2011). Five days after the last virus injection, spleens were aseptically removed and homogenized in complete RPMI1600 medium. The viability of splenocytes was determined by Trypan Blue (0.4% w/v) exclusion, and the cells were used as effectors. The EL-4/TC1 target cells were distributed into a 96 well plate (2x10⁵ cells/well) in 50 μ l of RPMI1600 with 1% FBS. The effector cells were added to the target cells at ratios of 10:1, 50:1 and 100:1 for a standard lactate dehydrogenase (LDH) release assay. Wells containing 50 μ l of target cells plus 50 μ l of medium with 1% FBS served as the “low” control (lC), with less LDH release, while the “high” control (hC) contained 50 μ l of target cells plus 50 μ l of medium with 1% FBS and lysis solution (which was added at the end of the experiment). In the control well with the effector cells (EF), 50 μ l of the medium with 1% FBS and 50 μ l of spleen cells (the highest density was used) were added. All experiments were performed in triplicates. The plates were incubated for 6–8 h at 37°C with 90% humidity and 5% CO₂. Then, 100 μ l of a freshly prepared reaction mixture (LDH cytotoxicity detection kit plus, Roche, Germany) was added to each well and incubated for 30 min in the dark. Finally, by adding 50 μ l of the stop solution, the reaction was stopped, and optical density was measured at the wave length of 492 nm (A492). The percentage of cytotoxicity was calculated with the following formula:

$$\text{Percent specific release (\%)} = \frac{(\text{Effector} - \text{targetcellmix} - \text{Effectorcontrol}) - \text{low control}}{\text{Hight control} - \text{low control}} \times 100$$

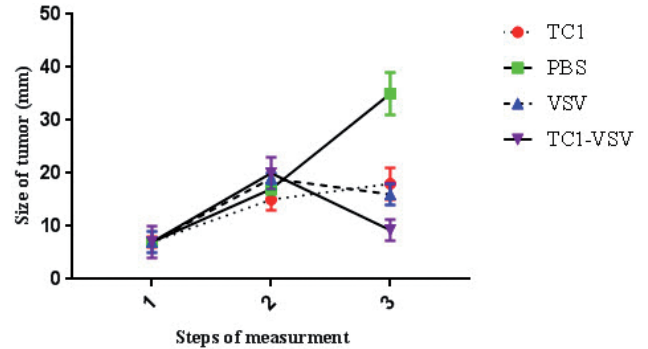


Fig. 1

Tumor size in different groups

Step1: Tumor cell lysate vaccination. Step 2: Start of virotherapy in all groups except TC-1 and PBS groups. Step 3: Mice sacrifice.

The median value for each triplicate was used for the calculations of the cytotoxicity.

Tumor size measurement. Digital Caliper was used to measure tumor size at different times of injections.

Statistical analysis. Data obtained from the size of tumor and CTL assay were analyzed by one-way ANOVA test (Tukey’s multiple comparison test) and SPSS software version 19. Values of p <0.05 were considered to be significant.

Results

Oncolytic VSV therapy decreased the tumor size

On the first day of injection, the tumor size of each group was 7±0.5 mm. At the end of treatment, before sacrifice, the size of tumors in control group (35 ± 2.6 mm) that had received PBS, showed statistically significant differences with other groups (p >0.05).

Although a significant reduction in tumor size was observed in both groups of VSV (16 ± 2.48 mm) and TC-1

Table 1. Groups of mice and injection schedule

| Names of group | Injection | Schedule of injection |
|--------------------|--|--|
| Control group: PBS | only PBS | |
| Group 1: PBS-VSV | only virotherapy | three doses with 4 days intervals |
| Group 2: TC1-VSV | IFN- α + IL-2 + tumor cell lysate vaccination + virotherapy | two doses of vaccination with 10 days intervals and three doses of virotherapy with 4 days intervals |
| Group 3: TC1 | IFN- α + IL-2 + tumor cell lysate vaccination | two doses of vaccination with 10 days intervals |

(18.0 ± 3.3 mm), an obvious reduction was recorded in TC-1-VSV group (9 ± 2.52 mm) compared to VSV or TC-1 groups. (Fig. 1). This result demonstrates that oncolytic VSV therapy along tumor vaccination has strongest therapeutic effect on tumors among the tested groups.

Combination of VSV and immunotherapy resulted in virus specific CTL activities

To evaluate the CTL activity against the virus, the cytotoxicity of mice spleen cells exposed to VSV inoculated EL4 target cells was determined.

After centrifugation of microplates containing effector and target cells and separation of supernatants, lactate dehydrogenase enzyme activity was evaluated. The best results were obtained in 50:1 ratio of E:T. Comparing to control group, immune response enhancement was detected in VSV group, as well as a significant rise in TC-1-VSV group compared to VSV injected group (Fig. 2).

Combination therapy resulted in tumor specific CTL activities

To evaluate CTL activity against tumor cells, the cytotoxicity of mice spleen cells exposed to TC-1 tumor cells was determined.

The cytotoxicity in all test groups was significantly higher than those of the control. TC-1 group showed higher CTL cytotoxicity compared to VSV group, but the difference was not significant (Fig. 3). Although the increased immune response in VSV group against TC-1 which was due to VSV virotherapy and following lysis of tumor cells, and the increased immune response in TC-1 group against TC-1 due to tumor cells injection were observed, the highest cytotoxic activity was recorded for TC-1-VSV group. This result showed that VSV effectively damages tumor cells, releases tumor antigens, and recalls antitumor T cell immunity induced by tumor vaccination.

Discussion

Oncolytic viruses are promising agents for tumor therapy. Beside the direct cytotoxicity of oncolytic viruses for tumor cells, the immunogenicity of the virus can partly revert the immune suppression and induces immune response against tumor antigens (Bourke *et al.*, 2011). As observed in VSV injected group in the present study that showed a reduction in tumor size and an enhanced CTL activity against tumor cells compared with the control group. VSV is a potent virus in stimulation of immune system, although it is reported that viral antigens elicit a strong immune response which may limit the immune activity against tumor cells. Induction

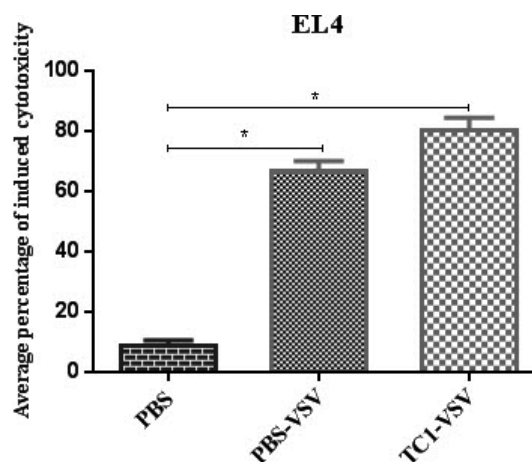


Fig. 2

Comparing the CTL cytotoxicity of spleen cells following stimulation with virus in different groups after virotherapy with VSV

The EL4 cells expressing viral proteins were exposed to the spleen cells of injected mice and the percent of LDH was measured. *Represents statistically significant difference of each group with control ($p < 0.05^*$).

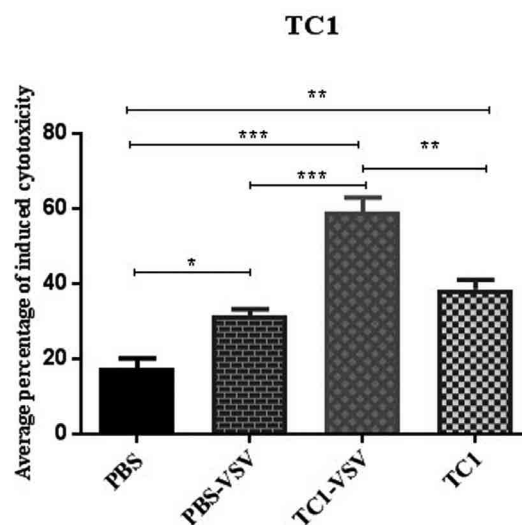


Fig. 3

Comparing the cytotoxicity induced by splenocytes against TC-1 in different groups of mice

TC-1 cells were exposed to the spleen cells of injected mice and the percent of LDH was measured. *Represents statistically significant difference of each group with control ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

of CTL response against VSV along with an elevated CTL activity against tumor cells, in VSV group confirmed that anti-tumor and antiviral response have contributed to the tumor size reduction and efficacy of the virotherapy.

There is no doubt that the specific immune response by viral infection leads to the creation of the anti-tumor ef-

fects, but usually direct viral oncolysis alone for treatment is not sufficient enough (Boisgerault *et al.*, 2010; Bridle *et al.*, 2010). Due to immunological tolerance to tumor antigens and poor presenting of the antigens in tumor environment, there are a variety of methods to expose tumor antigens to the immune system. These methods include complete cell transfer, application of lysates of cancer cells, tumor peptides and proteins loaded on DCs alone or in combination with a strong immunogen and RNA or DNA vaccines associated with an appropriate adjuvant (Liu and Kirn, 2008; Melcher *et al.*, 2011; Pouyanfarid *et al.*, 2012).

The increased efficiency of anti-tumor immunity in mouse models vaccinated with necrotic/apoptotic tumor cells, has been reported. The clinical effectiveness of autologous and allogeneic tumor cell vaccines has been repeatedly evaluated in phases I and II of clinical trials. This approach has been shown to be a very effective way, leading to tumor regression in mouse and human models of malignancies (Lasfar *et al.*, 2006).

Many cytokines have the ability to enhance the effect of tumor cell vaccination. Injection of irradiated tumor cells producing GM-CSF in several mouse models has been reported and in individuals with metastatic melanoma, resulted in the recovery of a large number of patients (Salgia *et al.*, 2003; Scheffer *et al.*, 2003). IFN- α was the first human cytokine, which is widely studied as a biological agent for the treatment of cancer (Goldstein and Laszlo, 1988). IFN- α has direct inhibitory effects on tumor cell growth (Choobin *et al.*, 2015), down-regulates oncogene expression and induces tumor suppressor genes, and also increases MHC class I expression and activities of T and dendritic cells (Santini *et al.*, 2009).

The use of other cytokines such as IL-2 was also reported to activate a specific immune response against tumor cells through activation of the CTL response (Lawler *et al.*, 2017). In 1984 the first evidence obtained by Pizza *et al.* (1984) showed that local administration of IL-2, inhibits the growth of human tumors and subsequent regression of tumors in tissues of bladder cancer. The amplifying effect of the cytokines IL-2 and IFN- α has been reported to result in a decrease in lung metastases in patients with advanced cancer (Rosenberg *et al.*, 1988).

In this study in order to enhance the immune response against injected tumor cells, IFN- α and IL-2 were used. The immune response was generated against the tumor antigens in group TC-1 that received TC-1 necrotic/apoptotic cells with co-stimulator IL-2 and adjuvant IFN- α and slowed tumor growth (18 mm) compared to control group. Use of IFN- α in an expression vector format led to a long-term presence of the cytokine in the tumor environment.

Combination virotherapy and anti-cancer agents such as radiation therapy, gene therapy and immunotherapy, have shown the amplifying level of the treatment efficiency

(Hemminki, 2014; Saito *et al.*, 2009; Vähä-Koskela *et al.*, 2007). Due to the role of oncolytic viruses in anti-tumor immune response stimulation and regarding this fact that CTL responses are essential for effective treatment of tumors with viruses (Melcher *et al.*, 2011), virotherapy and tumor vaccination are two treatment methods that are largely complementary to each other and combination of these methods can be exceptionally useful (Bridle *et al.*, 2010). Combination of VSV expressing melanoma tumor antigen and T cell specific adaptive transfer resulted in enhanced efficiency treatment in this model (Rommelfanger *et al.*, 2012). In the present study, combination of virotherapy with tumor cell lysate vaccination enhanced immune responses against the tumors comparing to each of these groups alone and resulted in a remarkable reduction in tumor size. Pre-immunization with tumor antigens along with stimulatory cytokines induces tumor specific CTL response which can be recalled with further exposure of the antigens upon virus destruction of tumor cells.

In general, combination of virotherapy with tumor vaccination could enhance the efficacy of treatment. Vaccination with tumor cell lysates along with co-stimulatory molecules induces immune response against the tumor, meanwhile tumor destruction by oncolytic virus enhances CTL activity against the tumor.

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