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Original Article

Increased VSV oncolytic sensitivity in tumor cells by down-regulation of Mx1 gene expression

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Abstract: Viral oncolytic therapy, involving the use of viruses that specifically target and kill cancer cells, is a promising new strategy for cancer treatment. Previous studies have demonstrated that VSV selectively infects tumor cells due to defects of type I IFN pathway during their antiviral response. However, different cancer types display differential sensitivities to VSV oncolysis. In this study, we demonstrated that the resistance to VSV oncolysis was related to the up-regulation of Mx1 expression in the tumor cells during VSV infection. Furthermore, down-regulation of Mx1 gene expression by siRNA could increase the oncolytic sensitivity of the tumor cells to VSV, indicating that targeting Mx1 in tumor cells may reverse the resistance of the tumor cells to VSV oncolytic therapy.

Keywords: Oncolytic therapy, VSV, Mx1, siRNA

Introduction

Oncolytic virotherapy is an emerging platform for cancer therapy. Vesicular stomatitis virus (VSV), a small unmodified RNA virus is being developed as oncolytic agent for antitumor therapies [1]. VSV specifically targets and kills tumor cells due in part to defective innate antiviral host defense mechanisms in tumor cells. Because of its high sensitivity to type I interferon in host antiviral response, VSV is safe for normal tissues [2]. When VSV infects normal cells, interferon beta (IFN-β) is produced as an antiviral response by normal cells. IFN-β, then, upregulates the expression of IFN-stimulated antiviral genes (ISGs), such as Mx1 (myxovirus resistance 1), 2'5'-OAS (2',5'-oligoadenylate-synthetase) and PKR (double-stranded-RNA-dependent protein kinase) through autocrine/paracrine manner and induces IFN-α production in adjacent cells [2-4]. These proteins inhibit viruses' infection and replication and protect adjacent normal cells from infection. However, most tumor cells have defects in their type I interferon signaling network and as a result are sensitive to killing by VSV and other oncolytic viruses. VSV has shown therapeutic promise in a variety of preclinical tumor models, such as breast cancer, hepatocelluar carcinoma, leukemia, melanoma, ovarian cancer and prostate cancer. However, some primary human and murine tumor cells have been shown to exhibit significant resistance to VSV infection *in vitro* [5].

Myxovirus resistance 1 is a murine 78 kDa GTPase protein belonging to the super family of large GTPases, which accumulate in the cytoplasm in response to IFNα/β [6]. Mx1 protein is able to inhibit a broad spectrum of negative-stranded RNA viruses, including vesicular stomatitis virus, influenza virus, Thogoto virus, measles virus, and bunya virus *in vitro* [7]. Mx1 is exclusively induced in a dose-dependent manner by type I IFNs in 1-2 hours and reaches the maximum level within 36 hours. The counterpart of Mx1 is Myxovirus resistance protein A (MxA) in human, which has been identified as a marker correlated with resistance to oncolytic adenovirus [8].

Tumor cells resistance to oncolytic VSV could also be due to the activation of the type I interferon pathway and the down-stream proteins, such as Mx1, could mediate this process. In the current study, we confirmed that Mx1 expression was significantly induced in the resistant tumor cell line during oncolytic VSV infection, while the increase was relatively modest in the sensitive cell lines. Moreover, we demonstrated that siRNA mediated down-regulation of Mx1 gene expression in tumor cells could significantly improve the sensitivity of tumor cells to oncolytic VSV infection.

Materials and methods

Cell lines

RAW264.7 (murine leukemia virus-induced macrophage tumor cell line), Hepa1-6 (murine hepatocellular carcinoma), B16 (murine melanoma), and BHK-21 (hamster kidney fibroblasts) were all obtained from Cell Bank, Chinese Academy of Sciences (Shanghai, China) and were cultured in RPMI1640 or DMEM (Invitrogen, Carlsbad, CA) containing 50 mM 2-mercaptoethanol (2-ME), 1% penicillin, streptomycin, and 10% FBS (HyClone, Thermo Scientific, Tauranga, New Zealand) in a humidified atmosphere at 5% CO_2 and 37°C.

Virus amplification and quantification

The wild type VSV was amplified in BHK-21 cells. Briefly, the cells were infected with 100 μl-500 μl virus, and collected as 90% cytopathogenic effect (CPE) appeared. The freeze-thawed cells were then subjected to centrifugation at 12,000 rpm for 30 min. The virus-containing supernatants were then collected and stored at -80°C, and the virus titers were determined with standard plaque assay. The calculated virus titer used in the experiments was 9.5×10^6 pfu/mL.

$TCID_{50}$ assay

Cells were seeded in 96-well plates at a density of 5,000 cells per well. After 24 h, virus samples were serially diluted with basal DMEM and the tumor cells were infected with 100 μL of 10-fold serial dilutions of the virus samples. The cells were incubated at 37°C for 4 days. The cytopathic effect on cells in each well was observed using light microscopy. The TCID50 values were calculated by the Reed-Müench method.

MTT assay

RAW264.7, HEPA1-6 or B16 cells were seeded in 96 well plate at a density of 5,000 cells per well. Sets of 5-wells were used for each dose. 20 μl of MTT solution (5 mg/ml, Sigma Aldrich, St. Louis, MO) was added to each well at 24 hr and 48 hr. After cells were incubated at 37°C for another 4 h, the medium was removed and 150 μl DMSO was added to solubilize the formazan. Finally, the absorbance (OD) was measured at 490 nm using a multiwell plate reader (Bio-Rad Benchmark Plus, BioRad Laboratories, Gladesville, NSW, Australia).

RT-PCR assay

The total RNA of tumor cells were extracted with Trizol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized by using Superscript II® Reverse Transcription system (Invitrogen, Carlsbad, CA). Primer sequences of Mx1 and GAPDH were as follow: Mx1, 5'-GTATTGACCTCATCGACACACTG-3' (forward), 5'-CACTTGACTATCATGTAGCCCTT-3' (reverse); GAPDH, 5'-TCAACGGCACAGTCAAGG-3' (forward) and 5'-ACCAGTGGATGCAGGGAT-3' (reverse). Amplification conditions were as follows: a single cycle of 94°C for 5 min followed by 25 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s, and the final single cycle of 72°C extension for 7 min. PCR products were separated by electrophoresis in the 2% agarose gel.

siRNA synthesis and transfection

Transfection of tumour cells with siRNA was performed using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA). A siRNA Kit of Mx1 gene was purchased from Shanghai GenePharma Co, Ltd. siRNA duplexes used were as follows: control siRNA was non-targeting siRNA (UUCUCCGAACGUGUCACGUTT and ACGUGACACGUUCGGAGAATT) and were labeled with FAM. siRNA sequences targeting Mx1 (NM_010846.1) were designed by software, and 3 pairs of siRNA were synthesized by the Genepharma (Shanghai, China). Sequence of Mx1 siRNA_1 were CUAUUGGAAGAUCAAAUAATT and antisense, UUAUUUGAUCUUCCAAUAGTG; Mx1 siRNA_2 were GAAGUACGGUGCAGACAUATT and UAUGUCUGCACCGUACUUCTG, and Mx1 siRNA_3 were CAGGACCAGGUUUACAAGGAA, and UUCCUUGUAAACCUGGUCCUG, respectively. The transfection efficiency was determined by Laser scanning cytometry

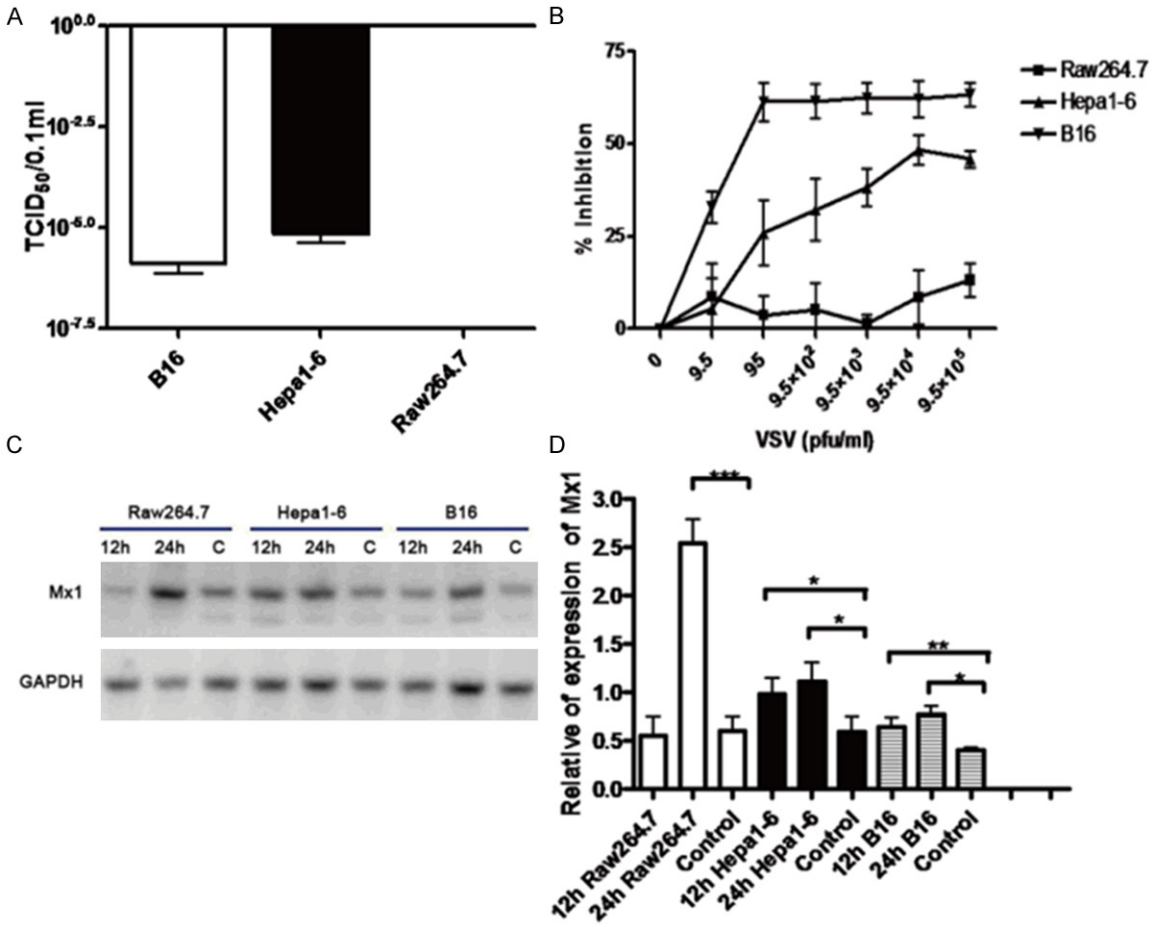

Figure 1. Inducible Mx1 expression in tumor cells was correlated with the sensitivity to VSV oncolysis. Tumor cell lines were treated with VSV for 48 h. A. The TCID50 was determined. B. The percent inhibition was calculated by MTT assay. C. The expressions of Mx1 were determined by RT-PCR before and after 1×10^4 pfu/mL VSV infection. GAPDH was used as the loading control. D. Quantification of Mx1 expressions normalized by GAPDH. The data were the representatives of three experiments.

(CompuCyte Corp, Cambridge, MA). The transfected nucleus was stained with DAPI.

Statistical analysis

Each experiment was repeated at least three times, and the data were expressed as the mean ± SD. Analysis was conducted with GraphPad Prism version 6.0 (GraphPad Software). Significance between two groups was analyzed using the unpaired two-tailed t test (*P<0.05).

Results

Inducible Mx1 expression in tumor cells was correlated with the sensitivity to VSV oncolysis

In order to identify the molecules critical for the sensitivity to VSV oncolysis, we performed assays to screen tumor cell lines for different sensitivities. The results showed that B16 cells had the highest viral load and the cell proliferation was significantly reduced with VSV infection (**Figure 1A**, **1B**). RAW264.7 cells showed the lowest viral load and the cell proliferation was not affected VSV infection, suggesting strong resistance to VSV oncolysis. Hepa1-6 was in the middle with the modest sensitivity to VSV. Therefore, the three cell lines exhibited differential sensitivity to VSV oncolysis. We then analyzed the expressions of Mx1 gene during VSV infection (**Figure 1C**, **1D**). The results showed that the Mx1 expressions were slightly increased in B16 and Hepa1-6 cells during VSV oncolysis. However, there was a five-fold increase of Mx1 expression in RAW264.7 cells 24 hours after VSV infection, suggesting the up-regulation of Mx1 may be involved in the resistance to VSV oncolysis.

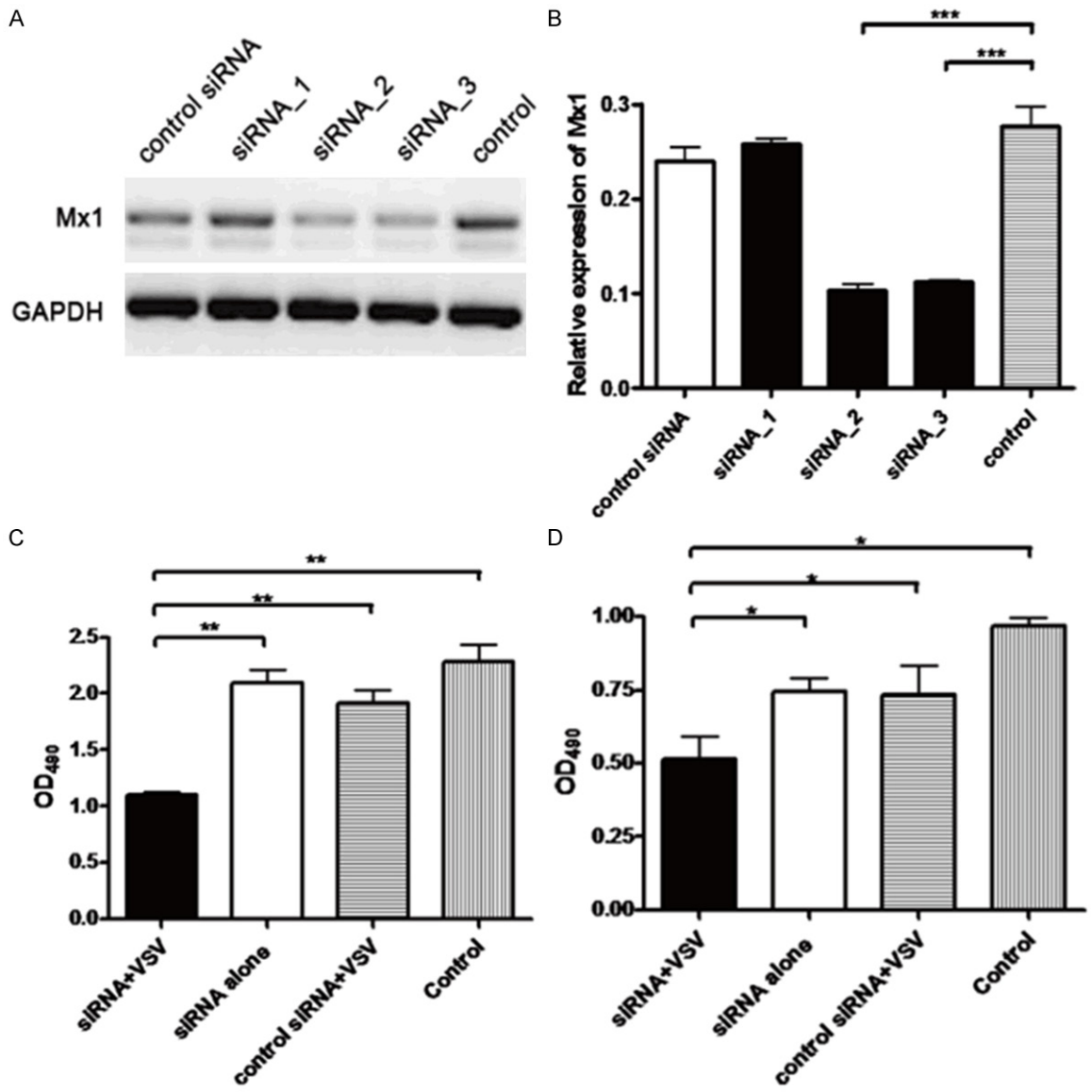

Figure 2. Down-regulation of Mx1 expression increased the oncolytic sensitivity of the tumor cells. A. Knockdown of Mx1 expression in RAW264.7 cells by siRNA determined by RT-PCR. B. Quantification of Mx1 expressions normalized by GAPDH. C. RAW264.7 cells with or without Mx1 knockdown were infected with 9.5×10^5 pfu/mL VSV and the cell viability was determined by MTT. D. B16 cells with or without Mx1 knockdown were infected with 9.5 pfu/mL VSV and the cell viability was determined by MTT. The data were the representatives of three experiments.

Down-regulation of Mx1 expression increased the oncolytic sensitivity of the tumor cells

In order to confirm the role of Mx1 in VSV oncolysis resistance, we transfected to RAW264.7 cells with siRNAs designed to specifically knock down Mx1. All 3 siRNAs have been successfully transfected into the target cells, and the transfection rate was about 70% as detected by the reporter fluorescence expression (data not shown). Two of the siRNAs could effectively knock down the Mx1 gene as detected by RT-PCR (**Figure 2A**, **2B**). SiRNA #3 was selected in the following experiments. The RAW264.7 cells transfected with siRNA #3 or control siRNA were challenged by VSV (**Figure 2C**) and the MTT assay was performed to analyze the oncolytic sensitivity of the tumor cells. The results showed that oncolytic sensitivity to VSV infection was increased about 35% by suppressing Mx1 expression in RAW264.7 cells. To test whether Mx1 down-regulation could also

increase the oncolytic sensitivity of the tumor cells which were already sensitive to VSV, the B16 cells were also transfected with siRNA #3 and infected with lower dose of VSV (**Figure 2D**). The result showed that there was also a 30% increase of the sensitivity to VSV oncolysis. Therefore, Mx1 was critically involved in the resistance of VSV oncolysis of the tumor cells. Down-regulation of Mx1 gene could be a feasible way to increase the tumor cell sensitivity to VSV oncolysis.

Discussion

VSV is known to infect a wide range of cell types. In normal cells, VSV replication is usually thwarted by innate immune response mechanisms. However, in tumor cells, VSV rapidly replicates to very high levels, and eventually lyse the tumor cells. This current study compared the oncolytic activity of VSV in several murine tumor cell lines, and investigated the relationship between the oncolytic resistance and the Mx1 expression in tumor cells. We found that the oncolytic efficiencies of VSV varied in different tumor cell types. This variation could be due to the different degrees of defects in antiviral innate immune responses in tumor cells, involving the IFN system or IFN-induced genes.

The Mx1 protein and its homologous protein in human, called Myxovirus-resistance protein A are induced specifically by type I IFN, which were known to exert an antiviral effect by targeting specific yet poorly defined steps of the viral replication cycle [9, 10]. It has been shown using the microarray analysis that MxA expression could be a marker correlating with resistance to oncolytic adenovirus [8]. Our data demonstrated that the basal level of Mx1 expression was not critical for the oncolytic resistance. However, the inducible Mx1 expression level was closely related with the oncolytic resistance of the tumor cells.

Many tumor cells exhibited significant resistance to VSV oncolysis [11-14], and high titers of VSV were needed for oncolytic therapy *in vivo*. However, some neurologic complications often appeared after high titers of VSV infection *in vivo* [15, 16]. Viral expansion *in vitro* is also a challenge for human oncolytic therapy. Therefore, increasing the tumor oncolytic sensitivity and lowering the therapeutic viral titers are necessary for future oncolytic treatment. Targeting Mx1 gene expression in tumor cells could provide an effective way of increasing the oncolytic sensitivity. Our results warrant further studies for combined Mx1 silencing and VSV oncolysis in animal tumor models.

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Disclosure of conflict of interest

None.

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