Synergistic Anti-Lung Cancer Effect and Mechanisms of NF-κb Signaling Pathway Inhibitor and Oncolytic Measles Virus

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Abstract

What is known and objectives: Lung cancer has become one of the leading causes of cancer-related morbidity and mortality. Oncolytic virotherapy is an emerging therapeutic modality that utilizes replication-competent viruses to destroy cancers. The aim of this study was to investigate the synergistic effect of NF-κb signaling pathway inhibitor and oncolytic measles virus vaccine against lung cancer and the involved mechanisms.

Methods: Using Western blots to detect expression level of p-IκBα, IκBα, PARP and BAX upon MV-Edm infection alone or used the NF-κb pathway inhibitor PS1145/autophagy related siRNA in A549 and H1299 lung cancer cells. Using flow cytometry to analysis the changes of apoptosis rate, and using MTT method to detect the cell survival rate.

Results and discussion: Inhibition of autophagy can inhibit the activation of NF-κb signaling pathway after the A549 and H1299 cells infected by MV-Edm. The expression level of p-IκBα increased in different degrees with the time of MV-Edm infection, while the expression level of IκBα decreased. NF-κb signaling pathways inhibitor PS1145 could promote apoptosis of human lung cancer cells, and enhance their oncolytic effect.

What is new and conclusion: The combination of NF-κb signaling pathway inhibitor PS1145 and oncolytic measles virus could promote the apoptosis of human lung cancer cells A549 and H1299 and enhance their oncolytic effect.

Keywords: Oncolytic measles virus; Autophagy; NF-κb pathway; Apoptosis

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Lung cancer is the first cause of death in all kinds of diseases, and non-small cell lung cancer is the highest incidence and mortality of cancer [1]. Although conventional treatment methods such as surgery, radiotherapy, chemotherapy, targeted therapy and so on continue to develop, but some drug-resistant or refractory end-stage lung cancer is still at a loss [2]. Therefore, we urgently need safe, effective and feasible treatment methods, such as gene therapy, biological therapy, and immunotherapy and so on.

Viruses with self-replication ability, tumor selective killing effect and safety, are called oncolytic virus [3,4]. In the past 10 years, there have been extensive and in-depth studies in clinical trials [5]. At present, at least six viruses have entered the I/II phase of clinical trials, which include Adenovirus in the treatment of head and neck tumors [6,7], Newcastle virus for recurrent glioma [8], measles virus treatment of multiple myeloma [9]. In addition, the clinical trials of Herpes, Reovirus and Vaccinia all show good safety and efficacy in human treatment [10,11].

MV-Edm was used to inoculate healthy uninfected people, have a solid safety record because there have been no reports of disease and death in the past 60 years [12]. Studies have found that measles virus vaccine has a selective killing effect on tumors, and has no damage or micro-damage to normal cells [13,14].

The elaboration of oncolytic mechanism is helpful to effectively improve the oncolytic effect of MV-Edm [15]. Our previous research found that MV-Edm could induce the production of autophagy in NSCLC to inhibit interferon or regulate the release of cytochrome C and inhibit cell apoptosis, which mediates non-caspase-independent cell death [16]. Autophagy plays a double-edged role in virus infection and replication [17,18]. On the one hand, it can activate innate immunity and adaptive immunity through direct degradation of viral components and proteins to resist some viruses [19]. On the other hand, some viruses can induce autophagy to resist cell death [20]. Previous studies have shown that cytoplasm can regulate the NF-κb pathway and then affect the expression of intracellular proteins [21,22]. It is suggested that whether the autophagy induced by MV-Edm can also regulate the NF-κb pathway to affect the oncolytic effect of virus?

In this study, we find that the autophagy induced by the oncolytic measles virus can regulate the activation of the NF-κb signaling pathway in vitro experiments. The inhibition of the NF-κb pathway by PS1145 can increase the apoptosis rate of the lung cancer cells strain A549 and H1299 induced by MV-Edm infection to enhance the oncolytic effects. These experiments provide a new strategy for
synergistic anti-lung cancer effect of the NF-κB signaling pathway inhibitors combined with MV-Edm. It also reveals a new oncolytic mechanism that oncolytic measles virus antagonizes the apoptosis of lung cancer cells through the NF-κB signal pathway regulated by autophagy, and provides a new idea for the further optimization of clinical transformation and application of oncolytic measles virus.

**Methods**

**Cell lines and cell culture**

Human non-small cell lung cancer cell line A549 (CCL-185) and Vero African green monkey kidney cells (CCL-81) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in DMEM supplemented with 0.1 mM nonessential amino acids, 5% fetal bovine serum, and 100 U/mL penicillin-streptomycin (all from Invitrogen, Carlsbad, CA). All cells were maintained in a humidified incubator with 5% CO2 at 37°C.

**Viruses**

Measles virus Edmonston vaccine lineage seed B (MV-Edm), kindly provided by S. Russell, Mayo Clinic, MN, USA, were propagated in Vero cells. The cells were infected with a Multiplicity of Infection (MOI) of 0.02 in 2 ml Opti-MEM (Invitrogen, 31985-062) at 37°C for 3 h. The medium was replaced with DMEM supplemented with 2% FCS, and the cells were incubated at 37°C for 1 day before being transferred to 32°C for another day. When 100% of the cell monolayer was fused into syncytia, the cells were harvested, and the viral particles were released by three cycles of freezing and thawing. The viral titers were determined by 50% end-point dilution assays (TCID50) on the Vero cells. The viral supernatant was centrifuged to remove cell debris and frozen at -80°C.

**Reagents and siRNAs**

The reagents used in this study are listed as follows. PS1145 (CFAD-P6624-5MG) were purchased from Sigma-Aldrich. All solvents were used directly without further purification. The siRNAs targeting ATG7 (Invitrogen, HSS116182), BECN1 (Invitrogen, HSS112731) and negative control (Invitrogen, 12935400) were all purchased by the Invitrogen Stealth RNAi collection. All reagents were formulated as recommended by their suppliers.

**Western blot analysis**

Cells were pelleted and lysed using RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany, 11873580001). The protein concentration was determined. The samples were migrated on SDS-PAGE and transferred onto PVDF membranes (Roche, 03010040001). After blocking with 5% nonfat milk, the membrane was incubated with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Signals were detected using an enhanced chemiluminescence reagent (Millipore, Darmstadt, Germany, WBKLS0500) and subjected to the Alpha Innotech Fluor Chem-FC2 imaging system (Alpha Innotech, San Leandro, CA). Antibodies were as follows: anti-GAPDH (Bioworld, Nanjing, China, 1:5000 diluted), anti-ATG7 (Cell Signaling Technology, #2631, 1:1000 diluted), anti-BECN1 (Cell Signaling Technology, #3738, 1:1000 diluted), anti-IxBa (Cell Signaling Technology, #4814, 1:1000 diluted), anti-p-IxBa (Santa cruz, sc-52943, 1:500 diluted) and anti-SQSTM1/p62 (Abcam, ab109012, 1:5000 diluted).

**Transfection**

100 nM of siRNA or 500 ng/ml expression plasmids coupled with Lipofectamine 2000 (Invitrogen; 11668-019) were used for transfection of A549 cells on a 6-or 12-well plate according to the manufacturer's instructions. For all experiments, MV-Edm in infection was performed 24 h after siRNA or plasmids transfection.

**Annexin V/PI analysis**

Aptotic cell death was detected by Annexin V/Propidium Iodide (PI) staining assay (Invitrogen, V13241) according to the manufacturer's protocols. Briefly, Cells were harvested by 0.25% trypsin, and washed once with PBS, then resuspended in 100 μl binding buffer followed by incubation with 2.5 μl Annexin V per test for 20 min. Then 1 μl PI per test was added and then cells were analyzed by a FACSCalibur (Becton, Dickinson and Company, USA). All data were analyzed using FlowJo software (Version 7.6.5, Tree Star Inc., and Ashland, Oregon).

**Cell viability/MTT assays**

Plate 1 × 10⁴ cells per well in 96-well plates and expose to serial concentrations of materials at 37 C for 24 h. Subsequently, 20 ml of MTT solution (5 mg/ml in PBS, pH 7.4) was added, and the cells were incubated for an additional 4 h. Then, the medium was replaced with 200 ml DMSO, and the absorbance was monitored using a Sunrise absorbance micro plate reader at the dual wavelengths of 570 and 650 nm. Cell viability was determined by comparison with untreated cells and calculated according to the following equation: Cell viability (%) = (Absorbance of the sample/Absorbance of the control) × 100%

**Statistical analysis**

All data are expressed as the mean ± Standard Error of the Mean (SEM). Student's t tests were used for statistical analyses. P-values less than 0.05 were considered to represent statistical significance.

**Results**

Oncolytic measles virus regulates the NF-κB signaling pathway by inducing autophagy

Previous studies have confirmed that MV-Edm can induce autophagy and inhibit cell apoptosis. Is the NF-κB signaling pathway involved in MV-Edm induced autophagy to inhibit apoptosis? In order to further explore the mechanism, the A549 and H1299 cell lines were infected with two siRNAs of the related genes ATG7 and BECN1 and the negative control siRNA without specific sinking function, then the SQSTM1 protein were detected after infection with MV-Edm. SQSTM1 protein is an important protein in the autophagy pathways. As an autophagy self-chaperone receptor, it can bind to cellular ubiquitinated proteins. At the same time, due to the interaction of autophagosome membrane proteins LC3, it selectively degrades the intracellular substrate. The results suggest that the two siRNAs of ATG7 and BECN1 can inhibit the autophagy of cells. A549 and H1299 cell lines infected with MV-Edm could induce autophagy compared with negative control group, and the IκBα protein level increased significantly after inhibiting autophagy-related genes (Figure 1), these results suggest that the autophagy induced by MV-Edm in infection is a major role in the regulation of NF-κB signal pathway in A549 and H122 cell lines infected with MV-Edm.

MV-Edm infects NSCLCs can activate the NF-κB signal pathway, and the application of IKK inhibitor PS1145 can inhibit the activation of the NF-κB signal pathway

In the resting state the NF-κB dimer bind to the inhibitor protein IκB and are stored in the cytoplasm in an inactive state. The IκB Kinase (IKK complex) trigger the induced stimulation, resulted in
The dissociation of NF-κB and IκB, and the IκB protein was then phosphorylated to form p-IκB. NF-protein dimers are then released into the nucleus and act on the primers of the corresponding genes, acting as major nuclear transcription factors that regulate inflammatory responses, oxidative stress and immunity. We infected A549 and H1299 cells with MV-Edm, then these cells were collected at different time points, and the expression of p-IκBα and IκBα were detected by Western blot to observe the activation of the NF-κB pathway. The result is shown in Figure 2. The quantity of p-IκBα protein in cells increased with the time of MV-Edm infection, and the quantity of IκBα protein decreased with the time of MV-Edm infection, which indicated that MV-Edm-infected A549 and H1299 cells increased the amount of p-IκBα protein significantly than the PS1145 untreated group. IκBα decreased significantly, suggesting that PS1145 could inhibit the activation of NF-κB signal pathway. The above results show that MV-Edm infected NSCLCs participated in the activation of the NF-κB signaling pathway. Using the inhibitor of IKK, PS1145 could inhibit the activation of NF-κB signal pathway.

Inhibition of NF-κB signal pathway can promote apoptosis induced by MV-Edm infection

As mentioned above, MV-Edm may regulate the NF-κB signal pathway by inducing autophagy, thus inhibiting cell apoptosis. So, can we induce apoptosis to promote oncolytic effects through inhibiting NF-κB signaling pathway? We further detected the apoptosis of the cells through inhibiting the NF-κB signal pathway by the IKK inhibitor PS1145. As shown in Figure 3, apoptosis of A549 and H1299 cells infected by MV-Edm was significantly increased after the PS1145 inhibits the NF-κB signaling pathway. These results suggest that inhibition of NF-κB signaling pathway can promote the induced cell apoptosis infected by MV-Edm.

Inhibition of NF-κB signaling pathway can enhance the oncolytic effects of MV-Edm

Since the inhibition of NF-κB signaling pathway can promote the apoptosis of MV-Edm-infected cells, NF-κB signaling pathway may also affect the survival of MV-Edm-infected cells. After inhibiting the NF-κB cell pathway by IKK inhibitor PS1145, we detected the oncolytic effects of MV-Edm-infected A549 and H1299 cells at different time point. The result is shown in Figure 4, the survival rate of cells was significantly reduced, and indicated that the combination of NF-κB signal pathway inhibitor and oncolytic measles virus could enhance the oncolytic effects.

Discussion

Despite the oncolytic measles virus MV-Edm has entered several clinical trials due to its proven safety and superior retention, the specific oncolytic mechanism of MV-Edm has not yet been clarified. Therefore, it is of great significance to understand its oncolytic mechanism for the continuous optimization of oncolytic viral treatment strategies. In this study, it was confirmed that the NF-κB signaling pathway was regulated by MV-Edm-mediated autophagy, and then the cell apoptosis was antagonized. Furthermore, the NF-κB signal pathway inhibitor PS1145 was combined with the oncolytic measles virus to promote the apoptosis of the human lung cancer A549 and H1299 cells, which enhanced the oncolytic effects. The new oncolytic mechanism of MV-Edm provided a new idea for further optimization of MV-Edm oncolytic measles virus therapy.

Apoptosis was considered to be the main mechanism of MV-Edm oncolytic effects [23]. In the study of human malignant glioma and breast cancer treated with MV-Edm, the specific changes of apoptosis were observed in infected tumor cells. However, the role of apoptosis in mediating MV-Edm oncolytic effects has not been thoroughly explored, and it is not clear whether there are other ways of oncolytic manner, and clarifying these mechanisms is of great significance for optimizing MV-Edm oncolytic strategy. In fact, in previous related studies, apoptosis caused by MV-Edm-infected tumor cells occurred in the late stage of infection. In MV-Edm infected tumor cells, the role of apoptosis in the oncolytic process has not yet been clarified.

The role of NF-κB signal pathway in cancer has not yet been fully elucidated. Activated NF-κB has been found in cancer research of...
lymphoma, liver, breast, colon and pancreatic cancer and so on [24]. NF-κB signaling pathways have also been found to be associated with tumor recurrence, drug resistance, and poor prognosis [25]. In addition, it has also been found that the upstream activators of the NF-κB signaling pathways or related transcription factors also have anti-tumor effects [26]. Therefore; it needs further study whether NF-κB inhibits or promotes the occurrence of tumors, or is related to the type of tumors, or is the potential interference factors of anti-tumor therapy. In this study, we found that MV-Edm infection can activate lung cancer cells A549 and H1299 NF-κB pathway and resisting the cell apoptosis, while the inhibition of NF-κB pathway can enhance the MV-Edm oncolytic effects. Recently, more and more studies have shown that autophagy and apoptosis pathways can affect each other because there are many intersections between them. Studies have shown that the apoptosis-related protein Bcl-xL or Bcl-2 can bind to the autophagy-related protein BECN1, thus antagonizing the autophagy of cells; Apoptosis-critical molecule Caspase-3 can inhibit autophagy by cleaving autophagy-related protein BECN1. Mitochondrial Bcl-xL can interact with the cleaved fragment of autophagy-related protein ATG5, and then control the release of cytochrome C into the cytoplasm to induce cell apoptosis [28,29]. These evidences provide a theoretical basis for the further study of the MV-Edm-infected tumor autophagy and apoptosis.

The autophagy-related protein BECN1 antagonizes apoptosis by inhibiting the degradation of Caspase-8 or the activation of apoptotic molecule Bid [27]. Mitochondrial Bcl-xL can interact with the cleaved fragment of autophagy-related protein ATG5, and then control the release of cytochrome C into the cytoplasm to induce cell apoptosis [28,29]. These evidences provide a theoretical basis for the further study of the MV-Edm-infected tumor autophagy and apoptosis.
Many previous studies have suggested that autophagy can alleviate the induction of apoptosis by external stimuli, thus protecting cells from or delaying death by decreasing senescent or damaged organelles, proteins, and so on [30,31]. Joubert's team found that chikungunya attenuated Caspase-dependent apoptosis by inducing apoptosis [32]. McLean's team found that flavivirus infection of epithelial cells induces autophagy, which protects the cells against apoptosis [33]. Recent studies by Richetta et al. [34] showed that the autophagy induced by MV-Edm infection cervical cancer cells Hela can resist apoptosis. Our previous studies have also suggested that MV-Edm controls the release of cytochrome C into the cytoplasm by inducing autophagy of damage mitochondria, thereby antagonizing apoptosis induced by key molecules such as Caspase [16]. Similarly, this study also confirmed that the activation of MV-Edm induced NF-κB signaling pathway was inhibited after autophagy inhibition, and the inhibition of NF-κB signaling pathway would significantly increase the cell apoptosis. These results may partly explain why the characteristics of apoptosis only occur in the relatively late stage of MV-Edm infection in previous studies. MV-induced autophagy can antagonize apoptosis, and there may be other possible mechanisms, which need further study. In order to improve the oncolytic effect of MV-Edm on different kinds of tumor cells and apply it to different kinds of tumor clinical treatments, it is still necessary to continue to study whether other tumor cell types have the same oncolytic mechanism.

What is New and Conclusion

In this study, we found for the first time that MV-Edm can antagonize cell apoptosis by regulating NF-κB signaling pathway via mediated autophagy by MV-Edm, and the combination of NF-κB signaling pathway inhibitor PS1145 and MV-Edm can exert a synergistic anti-tumor effect, for more efficient cancer therapy. The findings of this study are very important for the continuous development and optimization of MV-Edm-based oncolytic virus therapy, and provide a strong reference for the discussion of oncolytic mechanism related to other oncolytic viruses as well as the continuous optimization of oncolytic means.

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Conflict of Interests

The authors declare that they have no competing interests.

Author Contributions

Conception and design: Dong J, Xia M; Development of methodology: Xia M, Meng G; Acquisition of data (provided animals, provided facilities, etc.): Xia M, Meng G; Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Xia M, Meng G; Writing, review, and/or revision of the manuscript: Xia M, Dong J; Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Dong J, Xia M; Study supervision: Dong J.

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