Original Article

Evaluation of Apoptosis Induction by Newcastle Disease Virus LaSota Strain in Human Breast Carcinoma Cells

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ABSTRACT

The innovation of therapeutic modalities with better clinical efficacy is necessary for the treatment of patients with advanced cancers. Newcastle disease virus (NDV), an avian pathogenic virus, is one of the most promising oncolytic viruses that can replicate selectively in human cancer cells. In humans, NDV can cause transient conjunctivitis and mild flu-like symptoms. However, this virus poses no hazard to human health. The elucidation of the mechanisms of cancer cell killing by NDV is helpful for the clinical application of NDV in cancer patients. Regarding this, the present study was performed to evaluate apoptosis induction by NDV LaSota strain vaccine in human breast carcinoma cells. To this end, MCF-7 cells, a human breast adenocarcinoma cell line, were infected with NDV in vitro. Tumor cell cytotoxicity, apoptosis induction, and expression levels of apoptosis-related genes were examined in NDV-infected breast carcinoma cells. Tumor cell cytotoxicity was measured by 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazoliumbromide (MTT) assay. The induction of apoptosis was assessed by annexin V/propidium iodide staining. In addition, the expression levels of apoptosis-related genes were evaluated using real-time reverse transcription polymerase chain reaction (PCR) technique. The NDV showed cytotoxic effects on MCF-7 cells and induced apoptosis in the infected carcinoma cells. The gene expression levels of BAX, caspase-9, and caspase-3, but not BAK-1, were increased in NDV-infected cancer cells, compared to the gene expression levels in the non-infected cancer cells. These results suggest that the induction of the intrinsic pathway of apoptosis is one of the mechanisms that can contribute to cancer cell killing by NDV. Additional studies are required to investigate other probable mechanisms involved in NDV-mediated cancer cell killing.

Keywords: Cancer, Newcastle disease virus, Oncolytic virus, Apoptosis, Breast adenocarcinoma cell

Évaluation de l’Induction de l’Apoptose par la Souche LaSota du Virus de la Maladie de Newcastle dans les Cellules de Carcinome du Sein Humain

Résumen: L’innovation en matière des modalités thérapeutiques aboutissant à une meilleure efficacité clinique est nécessaire pour le traitement des patients atteints de cancers avancés. Le virus de la maladie de Newcastle (NDV), un virus pathogène aviaire, est l’un des virus oncolytiques les plus prometteurs capables de se réplicer sélectivement dans les cellules cancéreuses humaines. Chez les humains, le NDV peut provoquer une conjunctivite transitoire et des symptômes pseudo-grippaux légers. Cependant, ce virus ne présente aucun danger majeur pour la santé humaine. L’étude des mécanismes de destruction des cellules cancéreuses par le NDV pourrait mener à l’application clinique du NDV dans le traitement des patients cancéreux. À cet effet, cette étude a été réalisée pour évaluer l’apoptose induite par le vaccin contre la souche NDV LaSota dans des cellules
INTRODUCTION

Oncolytic viruses were first identified in the 19th century when tumor regressions were reported in cancer patients with natural viral infections (Kelly and Russell, 2007). In the past decades, cancer therapy with oncolytic tumor-selective replicating viruses has displayed several advantages over conventional cancer therapies, including surgery, chemotherapy, and radiotherapy. Virotherapy offers a new approach to the treatment of human cancers (Ries and Brandts, 2004; Ilkow et al., 2014; Russell and Peng, 2018). Various oncolytic viruses have been investigated for cancer treatment so far (Chiocca and Rabkin, 2014; Lang et al., 2018; Msaouel et al., 2018). Recently, a modified herpes simplex virus 1, termed talimogene laherparepvec, has been approved by the U.S. Food and Drug Administration for the treatment of recurrent melanoma after initial surgery (Kaufman et al., 2016). Newcastle disease virus (NDV) is one of the most promising oncolytic viruses. NDV, systematically named Avian Orthoavulavirus 1, is classified in the genus Orthoavulavirus, subfamily Avulavirinae, and family Paramyxoviridae of the order Mononegavirales. Although NDV causes serious infections in birds, it is nonpathogenic for humans. NDV can only induce transient conjunctivitis in humans, and there is no report regarding the human-to-human transmission of this virus (Alexander, 2000). The first reports on the anti-malignant effect of NDV date back to the 1950s (Moore et al., 1952; Prince and Ginsberg, 1957; Adams and Prince, 1959). In 1964, Wheelock and Dingle treated a patient with acute leukemia with the repeated injection of NDV (Wheelock and Dingle, 1964). In 1971, regression of metastatic colon cancer was reported in a Hungarian farmer following an outbreak of NDV (Csatary, 1971). Afterward, the NDV strains were studied as virotherapeutic agents for various human cancers in several preclinical and clinical trials (Farashi-Bonab and Khansari, 2017a). NDV attaches to the cell surface of malignant cells and enters into the cytoplasm through endocytosis (Cantin et al., 2007). The selective proliferation of NDV in tumor cells has been proposed to be associated with defects in the...
antiviral defense of tumor cells, especially due to the reduced capability of tumor cells in the induction of a strong type I interferon response (Fiola et al., 2006). The induction of antitumor immune responses to NDV antigens is accountable for tumor cell death (Farashi-Bonab and Khansari, 2017b). In addition, NDV has been recently found to induce apoptosis in infected cells (Ravindra et al., 2008a; Ravindra et al., 2008b). Viral proteins, such as hemagglutinin-neuraminidase (HN) glycoprotein, are involved in the induction of apoptosis in cells (Ghrici et al., 2013a). Apoptosis is a type of cell death induced by extrinsic (death receptor) or intrinsic (mitochondrial) pathways, in which the activation of mediators, such as caspases, cysteine aspartyl-specific proteases that cleave structural cytoplasmic and nuclear proteins, results in cell death (Elmore, 2007). Recently, NDV has been reported to induce apoptosis through the extrinsic pathway by activating death receptors. These receptors trigger the activation of initiator caspases, such as caspase-8, and executioner caspases, especially caspase 3 (Elankumaran et al., 2006; Kumar et al., 2012). NDV has also been reported to trigger the intrinsic pathway of apoptosis through activating p53 gene and strengthening apoptosis-inducing proteins which act to release cytochrome c from the mitochondrial matrix into the cytosol (Ozaki and Nakagawara, 2011). The elucidation of cell death mechanisms induced by NDV in human cancer cells can be helpful in improving the clinical application of NDV in cancer patients. With this background in mind, the present study was conducted to evaluate apoptosis induction by an attenuated NDV strain in human breast carcinoma cells.

**MATERIAL AND METHODS**

Newcastle disease virus strain and evaluation of its capability to infect cells. The NDV used in this study was an attenuated NDV LaSota strain. For the purpose of the study, the commercial NDV LaSota strain live-virus vaccine was purchased (Isovac LA SOTA, Italy). To confirm that the virus has the capability to infect cells, the NDV particles were injected into the allantoic cavity of embryonated chicken eggs aged 7-9 days. Each egg received $10^3 \text{EID}_{50}$ (i.e., 50% egg infectious dose) per 0.1 ml of inoculum. The eggs were incubated at 37 °C with 55% humidity for 4 days and then incubated at 4 °C overnight. In the next stage, the allantoic fluid was collected and cleared from debris by centrifugation at 3000×g for 10 min. The proliferation of NDV was determined by the hemagglutination assay (HA) using a 96-well V-shaped microtiter plate (Santry et al., 2018). The hemagglutination units were expressed per ml as HAU/ml.

Tumor cell infection with Newcastle disease virus. MCF-7 cells, a human breast adenocarcinoma cell line, were maintained in the Dulbecco’s Modified Eagle medium (DMEM; Gibco, UK), supplemented with 10% fetal bovine serum (Sigma-Aldrich, UK), 100 U/ml penicillin (Invitrogen, UK), and 100 µg/ml streptomycin (Invitrogen, UK) and incubated at 37 °C in a humidified atmosphere with 5% CO$_2$. When the cells were more than 80% confluent, they were harvested from tissue flasks using trypsin (Sigma-Aldrich, UK) and then seeded in cell culture plates. After the incubation of the cells for 24 h, the cultures were washed once with phosphate-buffered saline (PBS; Sigma-Aldrich, UK); subsequently, different doses of NDV (i.e., 2, 4, 8, and 16 HAU) were added to the cell culture wells. After 1 h, complete culture medium was added to the wells, and the cells were further incubated. The virus-infected tumor cells were examined at different time points for tumor cell cytotoxicity, apoptosis, and gene expression analysis.

Examination of cytotoxic effects of Newcastle disease virus on MCF-7 cells by MTT assay. The MCF-7 cells were plated onto 96-well cell culture plates at 5,000 cells/well. After 24 h, 10 µl of NDV at the doses of 2, 4, 8, and 16 HAU were added to the cells. The MCF-7 cells non-infected with NDV were considered as control. After further incubation for 72 h, 20 µl of 5 mg/ml MTT (Sigma-Aldrich, UK) was added to each well, and the cells were further incubated
at 37°C for 5 h to allow formazan crystals to form. The cells were then spun at 3,000 rpm for 5 min. After the removal of the supernatant, purple-colored formazan precipitates were dissolved in 100 µl dimethyl sulfoxide (Sigma-Aldrich, UK). After dissolution, the optical density (OD) of each well was measured using an automated microplate reader spectrophotometer (Awareness Technology Inc., USA) at a wavelength of 570 nm with a reference wavelength of 630 nm. The percentage of viable cells was calculated using the following formula:

\[
\% \text{Viability} = \frac{OD_{\text{Sample}}}{OD_{\text{Control}}} \times 100
\]

The experiment was performed in triplicate.

**Evaluation of apoptosis in Newcastle disease virus-infected tumor cells.** The MCF-7 cells were seeded in 7×10^5 cells/ml in each well of the 6-well cell culture plates. After incubation for 24 h, the cells were infected with NDV at the doses of 4 and 8 HAU. Apoptotic cells were measured by staining the MCF-7 cells with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) (Biolegend, CA, USA), followed by flow cytometric analysis according to the manufacturer’s protocol. The MCF-7 cells without NDV infection were considered as control cells. Briefly, after the incubation of the cells for 72 h, they were washed in PBS and resuspended in annexin V binding buffer (Biolegend, CA, USA). FITC-conjugated annexin V and PI were added to cell suspensions, and after 15 min of incubation in the dark, the cells were analyzed by the FACSCalibur flow cytometry instrument (Becton Dickinson, CA, USA). The experiments were carried out independently three times.

**Measurement of expression levels of apoptosis-related genes in Newcastle disease virus-infected MCF-7 cells.** The MCF-7 cells infected with NDV, as described above, were harvested 6, 12, 24, and 48 h after NDV infection. The expression levels of apoptosis-related genes were assessed by real-time reverse transcription polymerase chain reaction (RT-PCR) technique. To this end, total RNA was extracted from NDV-infected tumor cells, as well as tumor cells non-infected with NDV, using the Qiazole RNA extraction solution according to the manufacturer’s protocol (Qiagen, Germany). After the treatment of extracted RNA with DNase I (Qiagen, Germany), total RNA was reverse-transcribed to complementary DNA (cDNA) using the RevertAid™ First Strand cDNA synthesis Kit (Thermo Fisher Scientific, USA). Finally, real-time PCR was performed using the SYBR Green/Rox qPCR Master Mix Kit (Applied Biosystems, USA) and specific primers for 18S rRNA (housekeeping gene), BAK-1, BAX, caspase-3, and caspase-9 in a StepOnePlus real-time PCR system (Applied Biosystem, CA, USA). The PCR reaction volume was 20 µl. Primer sequences are shown in Table 1. The thermal program of real-time PCR lasted 15 min at 95 °C, followed by 45 cycles of denaturation step at 95 °C for 15 sec and annealing-extension for 1 min at 60 °C. Then, the melting curve analysis was performed to determine the specific amplification of target genes. The relative gene expression levels were calculated by analyzing the collected data with comparative CT (ΔΔCT) method. These experiments were repeated three times.

**Statistical analysis.** One-way analysis of variance with Tukey’s posthoc test was performed to determine statistical significance among groups using SPSS software, version 22. A p-value less than 0.05 was considered statistically significant.

| Table 1. Primer sequences used for gene expression analysis by real-time reverse transcription polymerase chain reaction |
|-------------------------------|-------------------------------|-------------------------------|
| **Primers** | **Sense primer (5'-3')** | **Antisense primer (5'-3')** |
| 18S rRNA | 5'-AGGAATTCCCAGTAAGTGCG-3' | 5'-GCCTCACTAAACCCTCCA-3' |
| BAK-1 | 5'-GGTCCTTGCCTACAATCTACC-3' | 5'-CCTGAGAGTCAACCTGCCA-3' |
| BAX | 5'-GGGGACGAACATTGGAAGTAA-3' | 5'-CATTTGAGTTGCCGCTACA-3' |
| Caspase-3 | 5'-TGTTTCATCCAGTGGCCTGTTG-3' | 5'-CATTCTGTTGCCACCTTTCG-3' |
| Caspase-9 | 5'-ACTCAGGAAGGCAAGACAGT-3' | 5'-ACCATCAACTTCCAGAAAC-3' |
RESULTS

Ability of Newcastle disease virus particles to infect cells. The allantoic fluid of the embryonated chicken eggs was collected 4 days after incubation. The HA assay was carried out immediately after the collection and clarification of allantoic fluids to confirm the propagation of NDV. The results revealed an increase in the virus titer (256 HAU/ml) after the inoculation of the virus into the allantoic cavity, compared to that before inoculation (16 HAU/ml). This result was indicative of the ability of NDV particles to infect cells and proliferate.

Cytotoxicity of Newcastle disease virus for breast carcinoma cells. The cytotoxic effect of NDV in human breast carcinoma cells was measured by the MTT assay after the infection of MCF-7 cells with NDV particles at different doses. The MTT assay is a colorimetric assay for assessing cell metabolic activity, reflecting the number of viable cells present. It is based on the ability of nicotinamide adenine dinucleotide phosphate-dependent cellular oxidoreductase enzymes to reduce MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazoliumbromide) to its insoluble formazan. The specific cell death after infection with NDV can be estimated by MTT assay. The MCF-7 cells infected with NDV at the doses of 8 and 16 HAU showed reduced viability, compared to the control cells cultured without virus infection. The infection of tumor cells with NDV at a dose of 8 HAU resulted in higher cytotoxicity (with a viability of 78.52%±6.65% of viable tumor cells) than infection with other doses of the virus. However, this increased cytotoxicity was not statistically significant (P>0.05). The viability rates of the tumor cells infected with NDV at the doses of 2, 4, and 16 HAU, compared to the control cells untreated with NDV, were obtained as 91.43%±4.31%, 83.74%±6.52%, and 85.12%±4.69%, respectively.

Apoptosis induction in Newcastle disease virus-infected breast carcinoma cells. Flow cytometric analysis illustrated early and late apoptosis in tumor cells after staining the cells with annexin V and PI. The MCF-7 cells infected with NDV showed increased apoptosis, compared to the control cells (Figure 1).

Figure 1. Flow cytometric analysis of apoptotic cells in MCF-7 cells infected with Newcastle disease virus (NDV); a) MCF-7 cells without NDV infection, b) MCF-7 cells infected with NDV at a dose of 4 HAU, and c) MCF-7 cells infected with NDV at a dose of 8 HAU (Cells in the early apoptotic stage were stained only with annexin V, and those in the late apoptotic stage were stained with both annexin V and PI).
The percentage of carcinoma cells in the early stage of apoptosis was higher in the cells infected with NDV at the doses of 4 and 8 HAU than that in the control cells that were not infected with NDV (P>0.05). A higher proportion of tumor cells infected with NDV at a dose of 8 HAU showed early apoptosis, as well as late apoptosis, compared to that in the tumor cells infected with NDV at a dose of 4 HAU (P>0.05). Furthermore, the proportion of tumor cells at the late stage of apoptosis was significantly higher in the cells infected with NDV at a dose of 8 HAU than that in the control non-infected cells (P<0.05; Figure 2).

Figure 2. Percentage of apoptotic cells in MCF-7 cells infected with Newcastle disease virus at the doses of 4 and 8 HAU (*: significant difference with the control group [P<0.05]).

Increased expression levels of apoptosis-related genes in Newcastle disease virus-infected breast carcinoma cells. The gene expression analysis of MCF-7 cells by real-time RT-PCR showed that the expression levels of BAX, caspase-3, and caspase-9 were increased in the NDV-infected MCF-7 tumor cells, compared to the MCF-7 cells cultured in the absence of NDV. These increased levels of expression were observed when NDV was added to tumor cells at a dose of 8 HAU and after the incubation of cells for 48 h. The expression of BAK1 was not noticeably affected in NDV-infected MCF-7 cells (Figure 3). The upregulation of BAX, the initiator caspase-9, and the executive caspase-3 indicated that NDV activated the intrinsic pathway of apoptosis in MCF-7 cells.

Figure 3. Expression levels of apoptosis-related genes in MCF-7 cells infected with Newcastle disease virus at a dose of 8 HAU.

In the intrinsic pathway of apoptosis, the opening of mitochondrial permeability transition pore (MPTP) and loss of mitochondrial membrane potential result in the formation of apoptosome, and consequently the recruitment and activation of the initial caspase-9, which triggers a cascade of events leading to apoptosis (Elmore, 2007). Elankumaran et al. (2006) demonstrated that recombinant NDV induces caspase-dependent apoptosis in human tumor cell lines. The activation of caspase-3 and caspase-9 was detected early after NDV infection. Additionally, caspase-8 was

DISCUSSION

Apoptotic evasion is one of the main causes of the development and survival of tumor cells (Gerl and Vaux, 2005; Zhivotovsky and Orrenius, 2006). Many tumor cells have defective apoptosis pathways (Wong, 2011). Nonetheless, tumor cells may retain an intact apoptosis execution system and die when receive an effective apoptotic signal. Many viruses impede apoptosis in order to replicate in infected cells. These viruses encode specific proteins that change the activity of programmed cell death regulators, such as p53 and pRb (Bouchet et al., 2006). The NDV has been reported to be able to induce apoptosis in infected cells through the activation of death receptor (extrinsic) and mitochondrial (intrinsic) pathways (Lam et al., 1995; Elankumaran et al., 2006). Recently, the HN protein of NDV has been found to induce apoptosis in a human cervical cancer cell line (i.e., HeLa cells) through the activation of SAPK/JNK pathway (intrinsic pathway) (Rajmani et al., 2015).
activated in the NDV-infected tumor cells; however, it was dispensable for apoptosis induction after NDV infection (Elankumaran et al., 2006). Fabian et al. (2007) reported that an attenuated NDV strain MTH-68/H caused apoptotic cell death in rat pheochromocytoma cells (PC12). This was associated with the activation of caspase-3 and caspase-12 in NDV-infected PC12 cells. However, caspase-8 and caspase-9 were not involved in NDV-induced apoptosis (Fabian et al., 2007). The upregulation of caspases-1, -9, -8, and -3, loss of mitochondrial transmembrane potential, and increased oxidative stress have been observed in chicken embryo fibroblast cells expressing the HN viral protein (Ravindra et al., 2008b). Ghrici et al. (2013b) observed that NDV strain AF2240 induces apoptosis in human breast carcinoma MCF-7 cells 1 h post-infection through the activation of mitochondrial transition pore opening and 2 h later through the activation of caspase-8. The NDV-induced apoptosis in human breast carcinoma cells was independent of virus replication and protein synthesis (Ghrici et al. 2013b).

In our study, the infection of MCF-7 cells with an attenuated NDV LaSota strain resulted in an increase in the expression of caspase-3 and caspase-9 after 48 h of infection. The increased expression of the initiator caspase-9 and executive caspase-3 indicated that NDV could activate the intrinsic pathway of apoptosis in MCF-7 cells. This result is in parallel with the results obtained by Elankumaran et al. (2006) and Ravindra et al. (2008b), showing the involvement of the upregulation and activation of caspase-3 and caspase-9 in NDV-induced apoptosis in infected cells.

The Bcl-2 family proteins have a major role in the induction or inhibition of the intrinsic pathway of apoptosis (Danial and Korsmeyer, 2004). These proteins are divided into two categories, namely pro-apoptotic and anti-apoptotic proteins. Pro-apoptotic proteins, such as Bax, Bak, Bid, Bad, Bim, Puma, and Noxa, induce apoptosis through releasing cytochrome c (Shimizu et al., 1999). On the other hand, the anti-apoptotic proteins, such as Bcl-2, Bcl-xL, and Mcl-1, practically act counter to the pro-apoptotic members and inhibit apoptosis (Shamas-Din et al., 2013). The Bak (Bcl-2 homologous antagonist/killer) protein is a pro-apoptotic protein that shares a functional homology with another pro-apoptotic member of Bcl-2 family, named Bax (Bcl-2-associated X protein). These proteins share an essential BCL-2 homology domain 3 (BH3) required for oligomerization-based killing activity (Chittenden et al., 1995). There is evidence regarding the increased Bax to Bcl-2 ratio, as well as caspase-3, -8, and -9 expression levels, in NDV-infected Vero cells, compared to that in mock-infected cells (Ravindra et al., 2009). Molouki et al. (2010) observed that the infection of human cervical cancer HeLa cell line with Malaysian velogenic NDV strain AF2240 led to a conformational change in Bax protein and its translocation from the cytoplasm to mitochondria, resulting in the releasing of cytochrome c into the cytoplasm and cell death. However, NDV did not affect the level of Bcl-2 protein in infected cells (Molouki et al., 2010). Further investigation showed that the presence of Bax and its subsequent interaction with viral proteins, such as matrix (M) protein, sped up the apoptosis.

However, NDV-induced apoptosis also occurred in the absence of Bax, suggesting that the apoptotic proteins upstream of mitochondria may be involved in NDV-induced apoptosis (Molouki and Yusoff, 2012). Because the M protein of NDV contains a BH3 domain that interacts with Bax, it may be possible that the M protein activates other pro-apoptotic members when the Bax protein is absent. Bak can independently release cytochrome c from mitochondria in the absence of Bax. Both Bax and Bak proteins facilitate the release of cytochrome c from mitochondria through MPTP pores. Although Bax is a cytosolic protein, Bak localizes to mitochondria (Degenhardt et al., 2002). In this study, we measured the gene expression levels of both BAX and BAK-1 in the MCF-7 cells infected with NDV LaSota strain. Similar to the results of the study performed by Ravindra et al. (2009), BAX, caspases-3,
and caspases-9 expression levels were increased in the NDV-infected carcinoma cells. Moreover, our results revealed that the gene expression level of BAK-1 was not altered noticeably in the NDV-infected carcinoma cells.

The results of the present study were indicative of the in vitro cytotoxic effect of NDV LaSota strain on MCF-7 tumor cells. We further evaluated the effect of NDV LaSota strain on apoptosis induction in MCF-7 tumor cells. The results showed that NDV increased the early and late apoptosis of MCF-7 human breast cells. The gene expression analysis of some apoptosis-related genes in the NDV-infected tumor cells revealed that the intrinsic pathway of apoptosis is involved in NDV-induced cancer cell apoptosis. As apoptosis is a safe way to eliminate cancer cells, and NDV replicates selectively in human cancer cells and induces apoptosis, it can be considered an excellent candidate for cancer therapy. However, further studies are required to investigate the other possible mechanisms of NDV in destroying cancer cells.

Ethics
We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest
The authors declare that they have no conflict of interest.

Authors’ Contribution
Study concept and design: Farashi Bonab, S., Keyvanfar, H.
Acquisition of data: Kalantari, A., Farashi Bonab, S.
Analysis and interpretation of data: Kalantari, A., Farashi Bonab, S.
Drafting of the manuscript: Farashi Bonab, S.
Critical revision of the manuscript for important intellectual content: Farashi Bonab, S., Keyvanfar, H.
Statistical analysis: Mortazavi, P.
Administrative, technical, and material support: Farashi Bonab, S.

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