Newcastle disease virus-induced caspase-independent apoptosis pathway in BHK-21 cells

Chen-Wei Wang
Department of Veterinary Medicine, College of Veterinary Medicine, National Pingtung University of Science and Technology

Chia-Ying Lin
Da Dian Biotechnology Company Limited

Sheng-Chang Chung
Department of Veterinary Medicine, College of Veterinary Medicine, National Pingtung University of Science and Technology

Wen-Ling Shih
Department of Biological Science and Technology, National Pingtung University of Science and Technology

Tzu-Chieh Lin
International Degree Program in Animal Vaccine Technology, National Pingtung University of Science and Technology

Duangsuda Thongchan
Faculty of Agriculture and Technology, Rajamangala University of Technology Isan, Surin campus

hung-yi wu (✉ wuhy@g4e.npust.edu.tw)
National Pingtung University of Science and Technology Department of Veterinary Medicine

Research

Keywords: Newcastle disease virus, Apoptosis, Caspase-independent pathway

Posted Date: May 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-449586/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Newcastle disease virus-induced caspase-independent apoptosis pathway in BHK-21 cells

Chen-Wei Wang¹, Chia-Ying Lin², Sheng-Chang Chung¹, Wen-Ling Shih³, Tzu-Chieh Lin⁴, Duangsuda Thongchan⁵, Hung-Yi Wu¹*

1. Department of Veterinary Medicine, College of Veterinary Medicine, National Pingtung University of Science and Technology, No.1, Shuefu Rd., Neipu, Pingtung 91201, Taiwan.
2. Da Dian Biotechnology Company Limited, No. 21, Aly. 22, Ln. 77, Hengnan Rd., Hengchun Township, Pingtung County 946001, Taiwan
3. Department of Biological Science and Technology, National Pingtung University of Science and Technology, No.1, Shuefu Rd., Neipu, Pingtung 91201, Taiwan.
4. International Degree Program in Animal Vaccine Technology, National Pingtung University of Science and Technology, No.1, Shuefu Rd., Neipu, Pingtung 91201, Taiwan.
5. Faculty of Agriculture and Technology, Rajamangala University of Technology Isan, Surin campus, 145 Moo 15 Surin-Prasat Road, Nokmuang, Muang District, Surin Province, Thailand, 32000

*Correspondence author: Hung-Yi Wu (DVM, PhD); Department of Veterinary Medicine, College of Veterinary Medicine, National Pingtung University of Science and Technology, 1 Shuefu Road, Neipu, Pingtung 91201, Taiwan; wuhy@g4e.npust.edu.tw
Background: Newcastle disease virus (NDV) is an important virus for humans. It is highly lethal in fowl and is newly identified as an oncolytic virus for cancer treatment. In vivo, NDV induces spleen, thymus, bursa, and mesenteric gland cell apoptosis, thereby causing immunosuppression. In vitro, NDV can induce apoptosis by caspase-dependent pathways including the mitochondria-mediated pathway and death receptor-mediated pathway.

Methods: In this study, the major materials were baby hamster kidney (BHK-21) cells, NDV virus (Miyadera strain; $10^{3.8}$ TCID$_{50}$/μl), and pan-caspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD-fmk). All of the experiments used a viral infection MOI of 1. Apoptosis was confirmed using DNA fragmentation and TUNEL assay. Finally, the apoptosis-independent pathway was confirmed by western blot analysis and immunofluorescence.

Results: In this study, we differentiate between the caspase group and caspase inhibition group (added 100 μM pan-caspase inhibitor [z-VAD-fmk]) in BHK-21 cells treated with NDV at 0, 12, and 24 h. In
the DNA fragmentation and TUNEL assays, the apoptosis appears at 12 h and apoptosis increases over time, regardless of caspase or not. In protein level determination, the antiapoptotic protein Bcl-2 decreased over time, which is the opposite of how the proapoptotic proteins Bax, cytochrome C, Mst3, and AIF behaved. Further, using western blot and immunofluorescent staining, we checked the AIF and Endo G translocation from the mitochondria (cytoplasmic) to the nucleus. In addition to apoptosis, we found that NDV treatment of BHK-21 cells decreased actin, regardless of caspase. The actin always decreased in the NDV-treated BHK-21 cells at 12 and 24 h.

**Conclusions:** NDV-mediated BHK-21 cell apoptosis mechanisms involve complex pathway; when in the normal state, the caspase-dependent pathway is main apoptosis pathway; when the caspase is suppressed, the BHK-21 can switch on the caspase-independent pathway by AIF, Endo G, or Mst3 to allow apoptosis to continue.

**Keywords:** *Newcastle disease virus, Apoptosis, Caspase-independent pathway*
Background

Newcastle disease virus (NDV) is a single-stranded, negative-sense, non-segmented RNA genome of the genus Avulavirus in the family Paramyxoviridae [1]. It is virus with great significance; it has caused more outbreaks than any other virus and has a high lethality in fowl worldwide. However, the virus’ oncolytic efficacy is emerging as a novel cancer treatment and may give NDV a new research direction [2-4]. NDV was first used to treat cancer in the early 1950’s; NDV treatment induced tumor apoptosis or partial necrosis and sloughing in uterine carcinoma [5]. In 2013, Shobana et al. altered the viral fusion protein for exclusive cleavage by a prostate-specific antigen, making it the target of a tumor-specific signal for induction of apoptosis [6].

NDV in vivo can induce spleen, thymus, bursa, and mesenteric glands cell apoptosis for immunosuppression [7-9]. Most studies that have focused on apoptosis have determined whether or not it occurred, without further study of the in vivo mechanism. In this study, NDV has been discovered as a good oncolytic virus. NDV safely and effectively infects and kills
cancer cells. NDV is considered to be a good potential oncolytic agent in a clinical setting and is an experimental oncolytic agent [10]. Researchers have change NDV in vitro to target cancer cell specific markers and were confirmed in HT1080, KHOS, KB8-5011, HCV29T, IMR32, and G104 cell lines to cause cell apoptosis and lack of apoptosis in normal cells [5, 11, 12].

Apoptosis is a programmed multistep cell death that is intrinsic in all cells of the body; it is a necessary biological defense mechanism. Apoptosis has two pathways: a cysteine-aspartyl specific protease activation pathway (caspase-dependent) and an inactivated-caspase pathway (caspase-independent) [13]. Most apoptosis occurs because of multiple pathway interactions those that utilize a receptor, enzyme, regulatory protein, and signal transduction molecules. In the apoptosis research, the different cell induce cell death program are difference [14]. The viruses that induce apoptosis have two types. Direct pathways include the caspase-dependent pathway, mitochondria-mediated pathway, death receptor-mediated pathway, endoplasmic reticulum stress pathway, and
mitogen-activated protein kinases pathways. Indirect pathways utilize secretion of proinflammatory cytokines and chemokines and induction of the innate and acquired immune responses. The natural killer cells, lymphocytes, and macrophages are then stimulated to remove cells from the diseased area [15].

In 2006, Elankumaran et al. showed that NDV can use a caspase-dependent pathway for 16 different types of cell apoptosis, the major pathways being the mitochondria-mediated and death receptor-mediated pathway. Interestingly, when caspase is inhibited and cells are treated with NDV, apoptosis can still occur. It appears that NDV can induce apoptosis through a caspase-independent pathway [16]. After this, the other apoptosis pathways investigated were p53, Bax, and p3 MAPK during 2009 to 2011 [17-20]. However, no other signals from NDV induce apoptosis a caspase-independent pathway in the cell. Therefore, we wanted to examine the mechanism by which NDV induces cell apoptosis through a caspase-independent pathway and identify the proteins involved in it.
Methods

Cells and virus

The BHK-21 (baby hamster kidney) cell line was grown in Dulbecco’s modified Eagle medium with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and streptomycin at 37°C in a 5% CO₂ humidified incubator. NDV was isolated from a wild strain; after sequencing, it was determined to be Miyadera (gbjM 18456.1) and was then amplified. The final sample was quantified at a concentration of $10^{3.8}$TCID₅₀/μl, and the virus was stored at −80°C for further use. The samples were divided into two groups for this research; the normal NDV infection group (caspase group) and the caspase inhibition group. The caspase inhibition group was treated with 100μM pan-caspase inhibitor z-VAD-fmk (Z-Val-Ala-DL-Asp-fluoromethylketone) in the culture medium 1 h before virus treatment.

DNA fragmentation

The BHK-21 cells at a concentration of 2×10⁶ were infected with an NDV MOI of 1. The samples were separated as caspase and caspase
inhibition groups. After the 0, 12, and 24 h of incubation, the detached cells in the medium were collected. The pellet was washed with PBS twice, and lysed with lysis buffer (50 mM Tris–HCl, pH 7.5, 20 mM EDTA, and 1% Nonidet P-40). The supernatant was collected and incubated with RNaseA at a final concentration 5μg/μl for 2 h at 56°C. Proteinase K was added to a final concentration of 2.5 μg/μl, and the mixture was incubated at 37°C for 2 h. Furthermore, 3 M acetic acid (10 μl), 20 μl saturated sucrose, and cold 100% ethanol at a 2.5x volume was added, and the sample was stored at −80°C overnight. The sample pellet was collected and washed with cold 80% ethanol. The samples were dried at room temperature for 10 min, dissolved in deionization and distilled water, and diluted to a concentration of 5 μg per well. Finally, electrophoresis was performed using 2% agarose gel.

TUNEL assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling assay)

The BHK-21 cells were diluted at a concentration of 3 × 10^5 and infected with NDV MOI of 1. The sample was separated into the caspase and
caspase inhibitor groups. After the 0, 12, and 24 h of incubation at 37°C in an atmosphere of 5% CO₂, the TUNEL assay was carried out using a FragELTM DNAFragmentation Detection Kit (CALBIOCHEM, QIA33-1EA). The cover slip was sealed with 40% glycerol, and observation and counting were done under a microscope.

**Western blot analysis**

The western blot sample was applied to the two groups of caspase and caspase inhibition at 0, 12, and 24 h. In addition, the samples were differentiated between groups using total protein, cytoplasm protein, and nuclear protein groups at 0 and 24 h. The BHK-21 cells at a concentration of 2 × 10⁶ were infected with an NDV MOI of 1. The cytoplasm protein was extracted using buffer I (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM OTT, 0.05% NP-40, prepared at pH 7.9) and the other groups were extracted using RIPA lysis buffer. All samples were quantified using a Bio-Rad protein assay reagent with a protein of a uniform weight of 50 μg. All samples were separated by 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore, USA). The
membranes were blocked and incubated with antibody with NET buffer (Gelatin 2.5 g, NaCl 8.75 g, DETA-2Na 1.8 g, Tris 6.05 g, Tween 20 0.5 ml in 1 L water, pH 8.0). The primary antibody was incubated overnight at 4°C with α-tubulin (SC8035, Santa Cruz Bioechnology, CA), actin (MAB1501, Millipore, USA), AIF (SC1946, Santa Cruz Bioechnology, CA), Bax (SC493, Santa Cruz Bioechnology, CA), Bcl-2 (SC7382, Santa Cruz Bioechnology, CA), cleaved caspase-3 (SC22171, Santa Cruz Bioechnology, CA), cytochrome C (SC7159, Santa Cruz Bioechnology, CA), Endo G (SC26923, Santa Cruz Bioechnology, CA), GADPH (ab9483, Abcam®, UK), lamin B (SC6216, Santa Cruz Bioechnology, CA), Mst3 (SC21400, Santa Cruz Bioechnology, CA), and NDV (ab138719, Abcam®, UK). After washing, horseradish peroxidase (HRP)-conjugated secondary antibodies were added and the abundance of proteins was quantified via chemiluminescence assay using an ECL kit (WBULS0500, Millipore, USA). The image was acquired using an illumination imaging system (UVP, Upland, CA, USA) and the amount of proteins was estimated via densitometric analysis using the Bio-imaging camera system. The quantity of each protein was calculated
according to the GAPDH detected in each sample. The relative abundance of each protein was determined by dividing the arbitrary unit over that of their 0 h protein.

**Immunofluorescence**

The BHK-21 cells at a concentration of $3 \times 10^5$ and infected with an NDV MOI of 1. The cells were challenged with NDV at 0 and 24 h, the caspase and caspase inhibitor groups were incubated separately. The sample was added to Mitotracker (Invitrogen, M-7512) (50 μg/mL) and incubated at 37°C for 30 min, after which the medium was removed and the cover slip was washed with cold PBST. After that, the cells were fixed with 3.7% ice-cold paraformaldehyde for 10 min. Following, the slip washed three times with cold PBST. The sample was added to the primary antibodies (goat anti-AIF and goat anti-Endo G Santa Cruz, USA; chicken anti-ND Abcam®, UK) in PBST for at least 4 h at room temperature. After washing, the cells were incubated with FITC-conjugated donkey anti-goat IgG (Santa Cruz, USA) or goat anti-chicken IgY (Santa Cruz, USA) in PBST at room temperature for 1 h
in the dark. Then, the cells were added to DAPI 1 μg/μL in PBST at room temperature for 25 min in the dark. Finally, the cells were placed under the cover slip sealed with 40% glycerol, and visualization was carried out using a fluorescence microscope.

Results

Induction of apoptosis by NDV-mediated caspase and caspase-independent pathway in BHK-21 cells

The NDV-treated BHK-21 cells had been divided into two groups, one group was directly challenged by NDV, and the other group was treated with pan-caspase inhibitor z-VAD-fmk (final concentration 100 μM) to inhibit caspase-dependent pathway apoptosis before challenged with NDV. Cell apoptosis was induced by a caspase-dependent pathway with activation of caspase-3 by entering the nuclei; the caspase protein will cut the DNA according to nucleosome nucleotides length, causing DNA fragmentation. In Figure 1a, the caspase group indicated that the caspase family protein cut the DNA according to nucleosome nucleotides length that became the DNA markers in the 2% agarose gel. The caspase
inhibition group did not take the shape of DNA ladders after NDV infection, as the caspase group did (Figure 1b). The rate of cell apoptosis and cells fusion increased as time passed. The ratios of apoptosis of the caspase group from 0, 12, and 24 h were 6.50 ± 1.69%, 29.59 ± 3.52%, and 62.48 ± 3.69%, respectively. On the other hand, for the caspase inhibitor group, the apoptosis ratios from 0, 12, and 24 h were 5.94 ± 0.75%, 22.92 ± 1.66%, 32.57 ± 1.31%, respectively. The data are shown in Figure 3a–g. In addition, we observed that NDV infection increased cell fusion depending on the infection time. The ratios of cell fusion of caspase group at 0, 12, and 24 h were 0 ± 0%, 7.15 ± 1.32%, and 35.81 ± 3.53%, respectively, and in caspase inhibitor group, the ratios of cell fusion at 0, 12, and 24 h were 0 ± 0%, 4.85 ± 2.77%, and 17.55 ± 1.84%, respectively (Fig 2h).

NDV mediates BHK-21 cell apoptosis caspase-dependent and -independent pathway.

NDV induced apoptosis via caspase-dependent and caspase-independent pathways in BHK-21 cells. In the two groups, we used 0 h protein as a
starting point to confirm protein expression at 12 and 24 h. The results reveal that the proapoptotic proteins Bax, cytochrome C, Mst3, and AIF were increased over time and the antiapoptotic protein Bcl-2 decreased over time, regardless of the caspase-dependent or -independent pathway. Among them, Endo G was special; it was increased at 24 h in the caspase-dependent pathway and increased over time in the caspase-independent pathway; however, only the cleaved caspase-3 was different. It was inhibited by z-VAD-fmk in the caspase-independent pathway. In addition to apoptosis, we found that NDV-treated BHK-21 cells had less actin, regardless of having caspase or not. The actin always decreased in the NDV-treated BHK-21 cells at 12 and 24 h. This was because we used GAPDH to do protein internal control, not used actin. The western blot data were shown in Figure. 3.

In apoptosis caspase-independent pathway, Mst3, AIF, and Endo G are transferred from the mitochondria to the nucleus

In Figure 4a and 4b, the sample was separated it two groups; one was caspase apoptosis group that had 0 h total protein (0 h T), 0 h cytoplasmic
protein (0 h C), 0 h nucleus protein (0 h N), 24 h total protein (24 h T), 24 h cytoplasmic protein (24 h C), and 24 h nucleus protein (24 h N). The other group were caspase inhibited group that had same protein samples. The total protein internal control was actin, the cytoplasmic protein sample was α-tubulin, and the nucleus protein sample was lamin B. In Figure 4a, we confirm the transfer of Mst3, AIF, and Endo G from cytoplasm to the nucleus; Figure 4b shows that the caspase inhibition group was similar to the caspase group in terms of current protein expression. After that, we showed the image data in immunofluorescent staining in Figure 5a and 5b. In Figure 5a and 5b, the green color is target protein (FITC), red color is mitochondria (Mitoracker), and blue marks the nucleus (DAPI). The FITC three different target proteins were AIF, Endo G, and NDV. In Figure 5a and 5b, the 0 h, all samples show the protein of AIF and Endo G in cytoplasmic, and the color overlaps appear yellow. AIF and Endo G are transferred from the mitochondria to the nucleus 24 h after NDV infection. NDV replicated in cell cytoplasm and induced cell aggregation and fusion.
Discussion

NDV infect BHK-21 for 24 h caused growth inhibition in MMT assay. At 36 h, the MTT illustrated negative growth but this cannot explain how NDV can induce BHK-21 cell apoptosis, let alone through an apoptotic pathway. The findings must be confirmed by more specific experiments, like DNA fragmentation and TUNEL assay. When caspase-dependent pathway would be inhibited, NDV can induce BHK-21 apoptosis by caspase-independent pathway (Figure 3). In the tests, we used NDV added to BHK-21 cell before treatment with caspase inhibitor z-VAD-fmk to control caspase-dependent or independent pathway. NDV induced BHK-21 apoptosis via caspase-dependent and caspase-independent pathways (Figure 3). This result is the same as that obtained with THP-1, leukemia cell lines, HL cells, and HEG2 cell lines treated with NDV [15, 16].

Studies on the pathways of apoptosis induction in specific cells by NDV can cause different cells to undergo apoptosis in different pathways. Most NDV apoptosis papers showed that both the extrinsic and intrinsic pathways are activated in NDV-induced apoptosis. In 2011 the paper
confirmed that PC12 rat pheochromocytoma cell mitogen-activated protein kinase pathway was not involved in NDV-induced apoptosis, but in A549 human lung cancer, can induce apoptosis using the p38 and MAPK pathway, in addition, the endoplasmic reticulum apoptosis pathway no effect [17, 20]. When NDV induces cell apoptosis, Bax transfers from cytoplasm to mitochondria and cytochrome C transfers from mitochondria to cytoplasm. But Bcl-2 of protein expression is not any increase and decrease in NDV-induced apoptosis. If the caspase would be suppressed, the cell would be induced caspase-independent apoptosis pathway [16, 18, 21]. In summary, NDV-induced apoptosis has two mechanisms; one is NDV has different apoptosis pathways induced by different cells, and there is no absolute path followed. The other is NDV-induced apoptosis is a complex multiple pathway response. Our study showed that cytochrome C, AIF, Mst3, and Endo G were important because their proteins express increasingly form 0 to 24 h. Bax in caspase-dependent or independent pathway is increase protein expression, but the Bcl-2 is decrease.
The Bax protein configuration changes and aggregation can promote apoptosis; Bcl-2 can prevent that when the Bcl-2 protein expressed more than Bax and there is no apoptosis [22-25]. His study of NDV-induced HeLa cell apoptosis showed that Bax configuration changed before NDV infected the cell and that the Bax protein changed location and caused cytochrome C from the mitochondria to be released into the cytoplasm; the activity of Bcl-2 remained unchanged [18]. We observed the state of the Bax and the Bcl-2 was different. The Bax continued to increase with or without added inhibitors and Bcl-2 was exactly the opposite in our study. Expression of the other proteins Mst3, AIF, Endo G, and cytochrome C protein increased over time. Bax is an upstream regulatory protein in apoptosis that mainly affects the release of apoptotic proteins in the mitochondria and ultimately promotes the release of Endo G, AIF, and cytochrome C from the mitochondria, in which immunofluorescence staining results show that AIF and Endo G are two apoptotic proteins that are transferred to the nucleus because of NDV infection of BHK-21 cells.

During NDV infection, the cell surface often accumulates highly fused F
protein implants into infected cells to form a multinucleate state to facilitate virus transmission [26]. This phenomenon can be easily identified using a TUNEL assay and immunofluorescence, and the state of cell fusion has also been shown to effectively enhance NDV-induced cancer cell apoptosis in vitro or in vivo. How did the cell structure change affect our experiment? In the case of BHK-21 infected with NDV, the most special change was actin. Originally in 1977, NDV infection of BHK-21 was mentioned in a paper that actin will change with the infection. The paper uses immunofluorescence light staining to mark actin, and the results showed that actin has an increasing trend after adding virus [27]. However, the results of our experiment show that the actual performance of actin is declining, because the 1977 paper did not use western blotting and other more accurate protein detection methods to confirm; NDV can promote the infection of cells in vitro to cause cell fusion. When multiple cells are aggregated into one, the amount of actin should higher than that of a single cell. Actin is one of the cytoskeletal proteins in cells. Actin is involved in the various processes of virus infection, assembly, and release. Among the viruses of the
Paramyxoviridae family, actin is involved in the viral fusion protein (F protein). In the case of the matrix protein (M protein), when a large amount of F protein appears on the surface of the host cell, it will initiate the cell fusion between cells, and the M protein will transport the F protein and HN protein to the assembly process. The inner side of the cell membrane is fixed, and the fixation process also requires the participation of actin [27-30]. Actin will also be affected by caspase. When the cell breaks down, caspase-3, -6, and -9 can directly cleave poly (ADP-ribose) polymerase-1 (PARP), lamins, CAD, and actin, which may explain the lower actin content over time, coupled with cell fusion and various effects of virus assembly. This results in decreased actin protein content in BHK-21 cells after 24 h following challenge with NDV; the content is reduced to 0.60 times of 0 h.

Conclusions

In this study, we show the NDV-mediated BHK-21 cell apoptosis mechanisms involve complex pathway; when in the normal state, the caspase-dependent pathway is main apoptosis pathway; when the caspase is suppressed, the BHK-21 can switch on the caspase-independent
pathway by AIF, Endo G, or Mst3 to allow apoptosis to continue. And 
actin will also be affected by caspase, which may explain the lower actin 
content over time.

List of abbreviations

NDV Newcastle disease virus

BHK Baby hamster kidney

z-VAD-fmk Z-Val-Ala-DL- Asp-fluoromethylketone

caspase cysteine aspartyl specific proteases

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All relevant data are within the 
paper and its Supporting Information files.

Funding: The authors received no specific funding for this work.

Conflict of Interest: The authors declare no conflicts of interest.

Authors' contributions: Conceptualization: Wang CW, Wu HY; Data 
curation: Wang CW, Wu HY; Formal analysis: Wang CW, Wu HY, Lin
Acknowledgements: The authors would like to acknowledge the excellent technical assistance provided by Chiun Jye Yuan, Guan Ming Ke, Yen Li Huang, Wei Lin, Hung Li, Pei Ting Deng. We also thank the Nano Biology and Biosensing Lab, National Yang Ming Chiao Tung University.

Authors' information: Chen-Wei Wang¹, Chia-Ying Lin², Sheng-Chang Chung¹, Wen-Ling Shih³, Tzu-Chieh Lin⁴, Duangsuda Thongchan⁵, Hung-Yi Wu¹*

1. Department of Veterinary Medicine, College of Veterinary Medicine, National Pingtung University of Science and Technology, No.1, Shuefu Rd., Neipu, Pingtung 91201, Taiwan.
2. Da Dian Biotechnology Company Limited, No. 21, Aly. 22, Ln. 77, Hengnan Rd., Hengchun Township, Pingtung County 946001, Taiwan

3. Department of Biological Science and Technology, National Pingtung University of Science and Technology, No.1, Shuefu Rd., Neipu, Pingtung 91201, Taiwan.

4. International Degree Program in Animal Vaccine Technology, National Pingtung University of Science and Technology, No.1, Shuefu Rd., Neipu, Pingtung 91201, Taiwan.

5. Faculty of Agriculture and Technology, Rajamangala University of Technology Isan, Surin campus, 145 Moo 15 Surin-Prasat Road, Nokmuang, Muang District, Surin Province, Thailand, 32000

References

1. Mayo MA. A summary of taxonomic changes recently approved by ICTV. Arch Virol. 2002;147(8):1655-63. doi: 10.1007/s007050200039, PMID 12181683.

2. Southam CM, Hilleman MR, Werner JH. Pathogenicity and oncolytic capacity of RI virus strain RI-67 in man. Lab Clin Med.
3. Southam CM, Moore AE. Clinical studies of viruses as antineoplastic agents with particular reference to Egypt 101 virus. Cancer. 1952;5(5):1025-34. doi: 10.1002/1097-0142(195209)5:5<1025::aid-cncr2820050518>3.0.co;2-q, PMID 12988191.

4. Okuno Y, Asada T, Yamanishi K, Otsuka T, Takahashi M, Tanioka T, Aoyama H, Fukui O, Matsumoto K, Uemura F, Wada A. Studies on the use of mumps virus for treatment of human cancer. Biken J. 1978;21(2):37-49. PMID 749908.

5. Lam HY, Yeap SK, Pirozyan MR, Omar AR, Yusoff K, Suraini AA, Abd-Aziz Suraini, Alitheen NB. Safety and clinical usage of Newcastle disease virus in cancer therapy. J Biomed Biotechnol. 2011;2011:718710. doi: 10.1155/2011/718710.

6. Shobana R, Samal SK, Elankumaran S. Prostate-specific antigen-retargeted recombinant Newcastle disease virus for prostate cancer virotherapy. J Virol. 2013;87(7):3792-800. doi: 10.1128/JVI.02394-12, PMID 23345509.
7. Anis Z, Morita T, Azuma K, Ito H, Ito T, Shimada A. Histopathological alterations in immune organs of chickens and ducks after experimental infection with virulent 9a5b Newcastle disease virus. J Comp Pathol. 2013;149(1):82-93. doi: 10.1016/j.jcpa.2012.09.011, PMID 23369809.

8. Harrison L, Brown C, Afonso C, Zhang J, Susta L. Early occurrence of apoptosis in lymphoid tissues from chickens infected with strains of Newcastle disease virus of varying virulence. J Comp Pathol. 2011;145(4):327-35. doi: 10.1016/j.jcpa.2011.03.005, PMID 21511269.

9. Hu Z, Hu J, Hu S, Liu X, Wang X, Zhu J, Liu X. Strong innate immune response and cell death in chicken splenocytes infected with genotype VIIId Newcastle disease virus. Virol J. 2012;9:208. doi: 10.1186/1743-422X-9-208, PMID 22988907.

10. Csatary LK. Viruses in the treatment of cancer. Lancet. 1971;2(7728):825. doi: 10.1016/s0140-6736(71)92788-7, PMID 4106650.

11. Apostolidis L, Schirrmacher V, Fournier P. Host mediated anti-tumor
effect of oncolytic Newcastle disease virus after locoregional application. Int J Oncol. 2007;31(5):1009-19. PMID 17912426.

12. Sánchez-Felipe L, Villar E, Muñoz-Barroso I. Entry of Newcastle disease virus into the host cell: role of acidic pH and endocytosis. Biochim Biophys Acta. 2014;1838(1 Pt B):300-9. doi: 10.1016/j.bbamem.2013.08.008, PMID 23994097.

13. Donovan M, Cotter TG. Control of mitochondrial integrity by Bcl-2 family members and caspase-independent cell death. Biochim Biophys Acta. 2004;1644(2-3):133-47. doi: 10.1016/j.bbamcr.2003.08.011, PMID 14996498.

14. Horvitz HR. Genetic control of programmed cell death in the nematode Caenorhabditis elegans. Cancer Res. 1999;59(7);Suppl:1701s-6s. PMID 10197583.

15. Zamarin D, Palese P. Oncolytic Newcastle disease virus for cancer therapy: old challenges and new directions. Future Microbiol. 2012;7(3):347-67. doi: 10.2217/fmb.12.4, PMID 22393889.

16. Elankumaran S, Rockemann D, Samal SK. Newcastle disease virus exerts oncolysis by both intrinsic and extrinsic caspase-dependent
pathways of cell death. J Virol. 2006;80(15):7522-34. doi: 10.1128/JVI.00241-06, PMID 16840332.

17. Bian J, Wang K, Kong X, Liu H, Chen F, Hu M, Zhang X, Jiao X, Ge B, Wu Y, Meng S. Caspase- and p38-MAPK-dependent induction of apoptosis in A549 lung cancer cells by Newcastle disease virus. Arch Virol. 2011;156(8):1335-44. doi: 10.1007/s00705-011-0987-y, PMID 21625975.

18. Molouki A, Hsu YT, Jahanshiri F, Rosli R, Yusoff K. Newcastle disease virus infection promotes Bax redistribution to mitochondria and cell death in HeLa cells. Intervirology. 2010;53(2):87-94. doi: 10.1159/000264198, PMID 19955813.

19. Ravindra PV, Tiwari AK, Ratta B, Bais MV, Chaturvedi U, Palia SK, Sharma B, Chauhan RS. Time course of Newcastle disease virus-induced apoptotic pathways. Virus Res. 2009;144(1-2):350-4. doi: 10.1016/j.virusres.2009.05.012, PMID 19501124.

20. Szeberényi J, Fábián Z, Töröcsik B, Kiss K, Csatary LK. Newcastle disease virus-induced apoptosis in PC12 pheochromocytoma cells. Am J Ther. 2003;10(4):282-8. doi:
21. Aguilar HC, Henderson BA, Zamora JL, Johnston GP. Paramyxovirus glycoproteins and the membrane fusion process. Curr Clin Microbiol Rep. 2016;3(3):142-54. doi: 10.1007/s40588-016-0040-8, PMID 28138419.

22. Ravindra PV, Tiwari AK, Ratta B, Chaturvedi U, Palia SK, Chauhan RS. Newcastle disease virus-induced cytopathic effect in infected cells is caused by apoptosis. Virus Res. 2009;141(1):13-20. doi: 10.1016/j.virusres.2008.12.008, PMID 19152817.

23. Dlugosz PJ, Billen LP, Annis MG, Zhu W, Zhang Z, Lin J, Leber B, Andrews DW. Bcl-2 changes conformation to inhibit Bax oligomerization. EMBO J. 2006;25(11):2287-96. doi: 10.1038/sj.emboj.7601126, PMID 16642033.

24. Gross A, Jockel J, Wei MC, Korsmeyer SJ. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J. 1998;17(14):3878-85. doi: 10.1093/emboj/17.14.3878, PMID 9670005.

25. Nechushtan A, Smith CL, Hsu YT, Youle RJ. Conformation of the
Bax C-terminus regulates subcellular location and cell death. EMBO J. 1999;18(9):2330-41. doi: 10.1093/emboj/18.9.2330, PMID 10228148.

26. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol. 1997;139(5):1281-92. doi: 10.1083/jcb.139.5.1281, PMID 9382873.

27. Rutter G, Mannweiler K. Alterations of actin-containing structures in BHK21 cells infected with Newcastle disease virus and vesicular stomatitis virus. J Gen Virol. 1977;37(2):233-42. doi: 10.1099/0022-1317-37-2-233, PMID 200706.

28. Shaikh FY, Utley TJ, Craven RE, Rogers MC, Lapierre LA, Goldenring JR, Crowe JE, Jr. Respiratory syncytial virus assembles into structured filamentous virion particles independently of host cytoskeleton and related proteins. PLOS ONE. 2012;7(7):e40826. doi: 10.1371/journal.pone.0040826, PMID 22808269.

29. Taylor MP, Koyuncu OO, Enquist LW. Subversion of the actin cytoskeleton during viral infection. Nat Rev Microbiol. 2011;9(6):427-39. doi: 10.1038/nrmicro2574, PMID 21522191.
Wakimoto H, Shimodo M, Satoh Y, Kitagawa Y, Takeuchi K, Gotoh B, Itoh M. F-actin modulates measles virus cell-cell fusion and assembly by altering the interaction between the matrix protein and the cytoplasmic tail of hemagglutinin. J Virol. 2013;87(4):1974-84. doi: 10.1128/JVI.02371-12, PMID 23221571.
Figure 1. The 2% gel electrophoresis of genomic DNA from BHK-21 cell challenged with NDV. M is the 100 bp marker. Lanes 1, 2, and 3 represent the extraction time of DNA at 0, 12, and 24 h after NDV challenge. Figure 1a shows the caspase group and 1b shows the caspase inhibition group.
Figure 2a–f. Microscopic views of two groups of BHK-21 cells after NDV challenged for 0, 12, and 24 h; samples were treated with TdT-FragEL™ DNA Fragmentation Detection Kit, and cells with nuclear showing brownish color are undergoing apoptosis, while the cells with blue nuclear are living cells. 2a–2c are images of the caspase group and 2D–2F are images of the caspase inhibition group. Figure 2a–2f is magnified, scale bar = 200.0 μm (big) and scale bar = 50.0 μm (small).

2g and 2h. The trend of apoptosis rate in two groups of BHK-21 cells under different time periods of NDV challenge, the increasing tendency of apoptosis and cell fusion with or without addition of caspase inhibitor z-VAD-fmk is confirmed by TUNEL assay. Figure 2g x-axis represents time after NDV challenge while y-axis is percentage of cells undergoing apoptosis. Results are shown in the form of mean value ± SD for the three experiments. Figure 2h is the x-axis representing time after NDV
challenge while y-axis stands for percentage of cell undergoing fusion.

The results are shown in the form of mean value ± SD for the three experiments.
Figure 3 Influence of the expression of apoptosis-associated proteins at different times in BHK-21 cells treated with NDV and the caspase inhibitor z-VAD-fmk at the final concentration of 100 μM. The proteins detected were the proapoptotic proteins Bax, cytochrome C, Mst3, AIF, Endo G, and cleaved caspase-3, the antiapoptotic protein Bcl-2, internal control GADPH, and actin.

(3a) After BHK-21 treatment with NDV for 0, 12, and 24 h, the caspase-dependent apoptosis-associated proteins were expression.

(3b) After BHK-21 treatment with NDV for 0, 12, and 24 h, the caspase-independent apoptosis-associated proteins were expression.

(3c) The graph represents protein expression in the caspase-dependent group. The x-axis represents apoptosis-associated proteins and the y-axis
represents the relative fold expression, challenge time as 0, 12, and 24 h.

(3d) The graph represents protein expression in the caspase inhibition group. The x-axis represents apoptosis-associated proteins and the y-axis represents the relative fold expression, challenge time as 0, 12, and 24 h.
Figure 4 The cytoplasm and nucleus are shown separately to depict the changes in the positions of proteins. Lamin B is the internal control for the nuclear fraction, α-tubulin is the internal control for the cytoplasmic fraction, and actin is the internal control for the total cell. T: total protein; C: cytoplasmic protein; N: nucleus protein.

(4a) NDV challenged BHK-21 cells at 0 and 24 h (caspase group). Mst3, AIF, and Endo G were transferred from the cytoplasm to the nucleus in the cells.

(4b) NDV challenged BHK-21 cells at 0 and 24 h (caspase inhibition group). The Mst3, AIF, and Endo G proapoptotic proteins were similar to those in the caspase group, which help in performing apoptosis in BHK-21 cells.
Figure 5 The Endo G and AIF were transferred from the cytoplasm to the nucleus. The green color represents NDV, Endo G, and AIF (FITC). The red color represents the mitochondria (Mitoracker). The blue color represents the nucleus (DAPI). (5aA) The BHK-21 was treated NDV 0 and 24 h in apoptosis caspase-dependent study. (5bB) The BHK-21 was treated NDV 0 and 24 h in apoptosis...
caspase-independent study.
**Figure 1**

The 2% gel electrophoresis of genomic DNA from BHK-21 cell challenged with NDV. M is the 100 bp marker. Lanes 1, 2, and 3 represent the extraction time of DNA at 0, 12, and 24 h after NDV challenge. Figure 1a shows the caspase group and 1b shows the caspase inhibition group.
2a–f. Microscopic views of two groups of BHK-21 cells after NDV challenged for 0, 12, and 24 h; samples were treated with TdT-FragEL™ DNA Fragmentation Detection Kit, and cells with nuclear showing brownish color are undergoing apoptosis, while the cells with blue nuclear are living cells. 2a–2c are images of the caspase group and 2D–2F are images of the caspase inhibition group. Figure 2a–2f is magnified, scale bar = 200.0 μm (big) and scale bar = 50.0 μm (small). 2g and 2h. The trend of apoptosis
rate in two groups of BHK-21 cells under different time periods of NDV challenge, the increasing tendency of apoptosis and cell fusion with or without addition of caspase inhibitor z-VAD-fmk is confirmed by TUNEL assay. Figure 2g x-axis represents time after NDV challenge while y-axis is percentage of cells undergoing apoptosis. Results are shown in the form of mean value ± SD for the three experiments. Figure 2h is the x-axis representing time after NDV challenge while y-axis stands for percentage of cell undergoing fusion. The results are shown in the form of mean value ± SD for the three experiments.
Influence of the expression of apoptosis-associated proteins at different times in BHK-21 cells treated with NDV and the caspase inhibitor z-VAD-fmk at the final concentration of 100 μM. The proteins detected were the proapoptotic proteins Bax, cytochrome C, Mst3, AIF, Endo G, and cleaved caspase-3, the antiapoptotic protein Bcl-2, internal control GADPH, and actin. (3a) After BHK-21 treatment with NDV for 0, 12, and 24 h, the caspase-dependent apoptosis-associated proteins were expression. (3b) After BHK-21 treatment with NDV for 0, 12, and 24 h, the caspase-independent apoptosis-associated proteins were expression. (3c) The graph represents protein expression in the caspase-dependent group. The x-axis represents apoptosis-associated proteins and the y-axis represents the relative fold expression, challenge time as 0, 12, and 24 h. (3d) The graph represents protein expression in the caspase inhibition group. The x-axis represents apoptosis-associated proteins and the y-axis represents the relative fold expression, challenge time as 0, 12, and 24 h.
**Figure 4**

The cytoplasm and nucleus are shown separately to depict the changes in the positions of proteins. Lamin B is the internal control for the nuclear fraction, α-tubulin is the internal control for the cytoplasmic fraction, and actin is the internal control for the total cell. T: total protein; C: cytoplasmic protein; N: nucleus protein. (4a) NDV challenged BHK-21 cells at 0 and 24 h (caspase group). Mst3, AIF, and Endo G were transferred from the cytoplasm to the nucleus in the cells. (4b) NDV challenged BHK-21 cells at 0
and 24 h (caspase inhibition group). The Mst3, AIF, and Endo G proapoptotic proteins were similar to those in the caspase group, which help in performing apoptosis in BHK-21 cells.

**Figure 5**

The Endo G and AIF were transferred from the cytoplasm to the nucleus. The green color represents NDV, Endo G, and AIF (FITC). The red color represents the mitochondria (Mitoracker). The blue color represents
the nucleus (DAPI). (5aA) The BHK-21 was treated NDV 0 and 24 h in apoptosis caspase-dependent study. (5bB) The BHK-21 was treated NDV 0 and 24 h in apoptosis caspase-independent study.