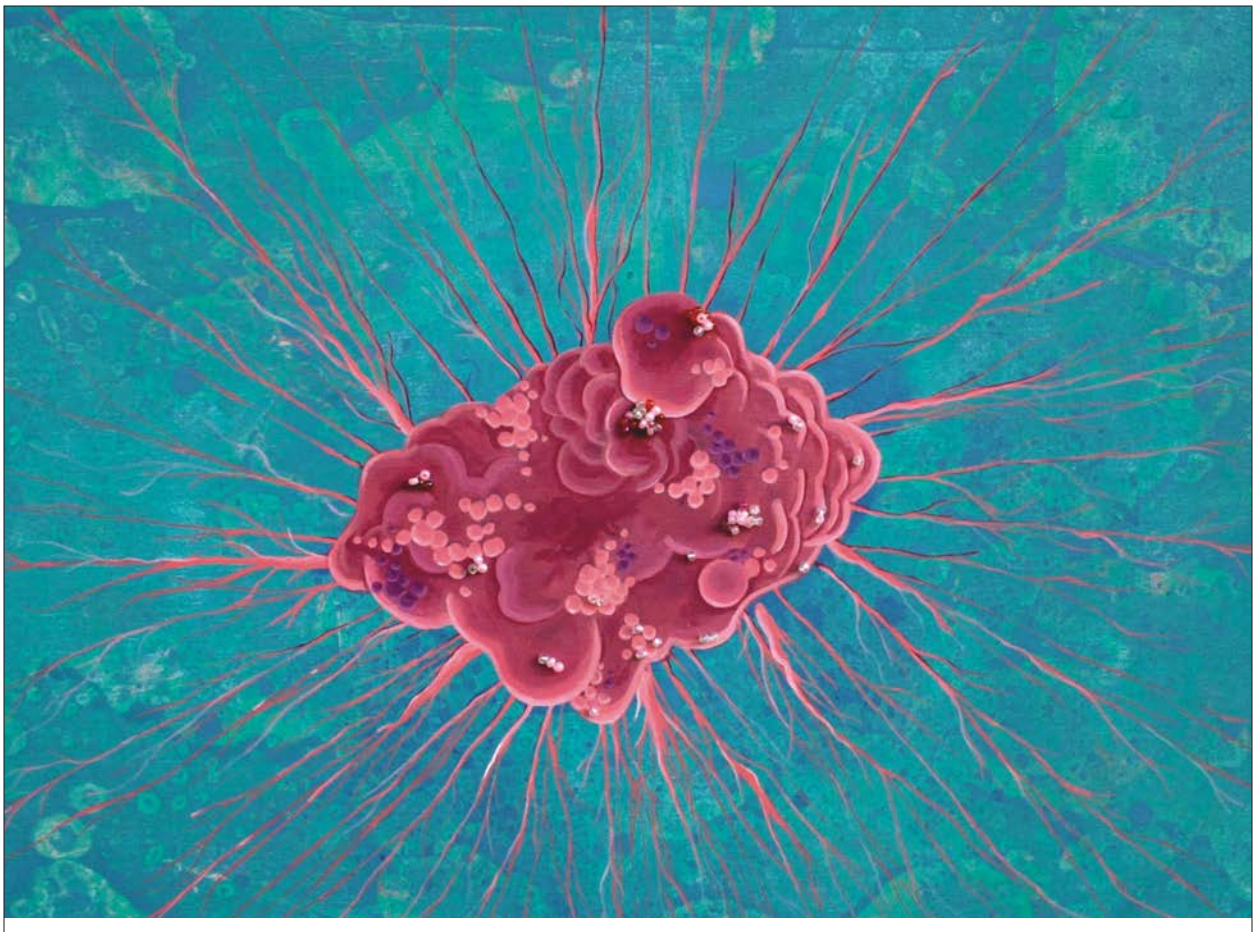

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Research Article
Antiproliferative and Apoptotic Effect of Newcastle Disease Virus (NDV) Strain AF2240 in Human Promyelocytic Leukemia Cells (HL60)

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Abstract

Background: Newcastle Disease Virus (NDV) is a negative-sense single stranded RNA virus that causes a Newcastle Disease (ND), a contagious disease of domestic poultry and wild birds characterized by gastro-intestinal, respiratory and nervous signs. Despite the negative effects of NDV to avian species, this virus was reported to possess significant oncolytic activity against mammalian cancerous cells. **Methodology:** In this study, the antiproliferative and apoptotic effect of NDV strain AF2240 on human promyelocytic leukaemia HL60 cell line were assessed using MTT proliferation assay, microscopic observation, DNA fragmentation, annexin V-FITC assay, caspase-3/7, 8, 9 assays and caspase-3/7, 8, 9 inhibition assays. **Results:** The proliferation of HL60 cells was inhibited when treated with cytotoxic titers (CD_{25}, CD_{50} and CD_{75}) of NDV AF2240 for a period of 72 h. Result from microscopic observation showed NDV AF2240 caused inhibition of cell growth and the treated cells exhibited morphological features of apoptosis and a ladder-like pattern of DNA, which is a hallmark of apoptosis. The proportion of cells in early and late apoptosis was quantified by using annexin V-FITC staining and analysed with flow cytometer. The percentage of cells in early apoptosis after treatment with NDV AF2240 at CD_{50} titer for 24 and 48 h were 16.27±0.25 and 25.93±1.2%, respectively. Late-apoptotic cells were increased from 3.15±0.07 and 6.85±1.05%, respectively. The mechanism of apoptosis through activation of caspases induced by NDV AF2240 was also analysed. The results suggested that apoptosis in NDV-infected tumor cells is dependent on caspase as both iniatiator caspases; caspase 8 and 9 were activated. Activation of caspase-3/7 were also detected in the cells treated with NDV AF2240. Furthermore, apoptosis by NDV AF2240 was effectively inhibited by ZVAD-FMK indicate that NDV AF2240-induced apoptosis is entirely dependent on caspase activation. **Conclusion:** To conclude, NDV AF2240 was found to have antiproliferative and apoptotic effects on HL60 cells. It has been shown from this study that NDV AF2240 infection resulted in the activation of both intrinsic and extrinsic apoptotic pathways.

Key words: Newcastle disease virus, AF2240, HL60, antiproliferation, apoptosis, caspase, intrinsic and extrinsic apoptotic pathway

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oncolytic viruses that replicate selectively in tumor cells but not in normal cells are used as agents to fight cancer known as oncolytic virotherapy[1]. Numerous oncolytic viruses such as Newcastle Disease Virus (NDV), reovirus, lentivirus herpes simplex virus, enterovirus, sindbis virus, semliki forest virus, seneca valley virus, adenovirus, vaccinia virus, myxoma and raccoonpox virus could display an antitumor activity in different animal models[2-4].

The NDV was first reported of having oncolytic activity in the mid-1950s[5]. In the mid-1960s, lysates from NDV-infected tumour explants have been administered to cancer patients in attempts to augment the anti-tumour immune response[6]. Using murine xenograft models, Phuangsab[7] demonstrated significant inhibition of tumour growth following intratumoural administration of NDV. The oncolytic activity of NDV has yielded encouraging results and inspiring further investigations into the potential use of NDV as an anti-cancer agent. Moreover, the perception that NDV possesses several unique properties has prompted much interest in this virus as a potential anticancer agent. The NDV has good cell-binding properties since it binds specifically to tumour cells, replicates selectively in tumour cell cytoplasm, independent of cell proliferation, relatively safe and can act as an adjuvant[8,9]. Due to this property, NDV has been exploited as a potential anti-cancer agent in humans[10,11].

The mechanisms governing cytotoxicity remain to be fully characterized although much preclinical work establishing that NDV could serve as a cancer therapeutic has been carried out. The NDV strains are also known to evoke cellular apoptosis[12,13]. In this study, the oncolytic and apoptotic effects of NDV AF2240 a heat stable virulent strain of Malaysian isolate were observed on HL60 human promyelocytic leukemia cells.

MATERIALS AND METHODS

NDV strain AF2240: The NDV strain AF2240 was propagated in allantoic fluid of 9-11 days-old embryonated chicken eggs at 37°C for 72 h. The allantoic fluid was harvested and the presence of virus was confirmed by the haemaglutination test[14]. The purification of virus was done as previously described by Chambers and Samson[15].

Cell lines: Human promyelocytic leukemia, HL60 was purchased from American Type Culture Collection (ATCC). The cells were maintained in Rosswell Park Memorial Institute, RPMI-1640 media (Sigma) containing 10% foetal bovine serum (PAA) and 1% penicillin/streptomycin (PAA) in a humidified 5% CO_2 incubator (Heracell 150, Thermo Electron Corp.) at 37°C. The cells were subcultured after they had achieved 80-90% confluency which can be observed under inverted microscope (Nicon Eclipse TS100). Cell viability was assessed using trypan blue (Sigma) exclusion test and was found to be greater than 99%.

MTT proliferation assay: The HL60 cells were seeded into wells of 96-well plates, treated at CD_{25}, CD_{50} and CD_{75} and incubated for 24, 48 and 72 h in humidified 5% CO_2 incubator at 37°C. The CD_{25}, CD_{50} and CD_{75} used in this study were previously determined by Bakar *et al.*[16]. After incubation time, the cell viability was assessed by MTT, (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole). Briefly, a volume of 20 µL MTT reagents was added to each well and further incubated for 3 h in a humidified 5% CO_2 incubator at 37°C. After the incubation time, the medium and MTT were removed and a volume of 200 µL of DMSO was added to each well. Absorbance at 570 nm of the mixture was detected using a microplate reader (Tecan 200).

Microscopic observation: The HL60 cells were seeded into wells of 12-well plates, treated with NDV at CD_{50} and incubated for 72 h in humidified 5% CO_2 incubator at 37°C. After incubation time, the morphological changes were observed using phase contrast microscope (Nikon Eclipse TE2000-U) and photos were taken.

Determination of DNA fragmentation: The HL60 cells (5×10^4 cells mL^{-1}) were treated with NDV AF2240 at CD_{50} in cultured flask and incubated for 24, 48 and 72 h in humidified 5% CO_2 incubator at 37°C. Both treated and untreated cells were extracted using a DNeasy Blood and Tissue Kit (Qiagen, USA). Electrophoresis of the DNA was done on 1.5% (w/v) agarose gel prepared in 1x TAE buffer (Tris base, acetic acid and EDTA). The voltage set for DNA separation was at 70 V for 2 h. Viewing and capture of laddering profile images was done using gel doc software.

Annexin V-FITC staining and flowcytometeer analysis: The HL60 cells were treated with NDV AF2240 at CD_{50} in the wells of 6-wells plate and incubated for 12, 24 and 48 h in

humidified 5% CO_2 incubator at 37°C. Treated and untreated cells were spun down 1000 rpm using rotor 12 151 (Sigma 2-16) for 10 min and washed twice with cold PBS, pH 7. A volume of 500 µL of 1x binding buffer (10 mM hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) was added to cell pellet and follow by cell counting using trypan blue dye. A volume of 100 µL of 1×10^6 cells mL^{-1} was transferred to a 5 mL culture tube. Then, a volume of 5 µL of annexin V-FITC and 5 µL of Propidium Iodide (PI) was added and incubated for 15 min at room temperature in the dark. A volume of 400 µL of 1x binding buffer was added into each tube and the cells were analyzed by flow cytometer (FACS Calibur) using CellQuest Software.

Analysis of caspase-3/7, 8 and 9 activities: Caspase-3/7, 8 and 9 activities were measured using Caspase-Glo® Assay Kit (Promega, USA). The kit provided a lumigenic caspase-3/7, 8 and 9 substrates, in a reagent optimized for caspase activity, luciferase activity and cell lysis. The HL60 cells were grown in white-wall, optical bottom 96-well plate and treated with NDV AF2240 at CD_{50} concentration for 0 and 12 h. After the incubation time, an equal volume of the reagent was added to the cells and further incubated for 1 h. The contents were mixed gently using a plate shaker at 300-500 rpm for 30 sec. Luminescene plate reader, Tecan (Infinite M200) was used to measure luminescene intensity. Blank values were subtracted from experimental values.

Inhibition of caspase-3/7, 8 and 9 assay: The HL60 cells were grown in white-wall, optical bottom 96-well plate. The general caspase inhibitor, 100 µM of ZVAD-FMK (Z-Val-Ala-Asp-FMK) was added 1 h prior to treatment with NDV AF2240. Cells were harvested after 0 and 12 h and analyzed for caspase inhibition activities. Lumigenic caspase-3/7, 8 and 9 substrates was added to the cells and further incubated for 1 h. The contents were mixed gently using a plate shaker at 300-500 rpm for 30 sec. Luminescene plate reader, Tecan (Infinite M200) was used to measure luminescene intensity. Blank values were subtracted from experimental values.

Statistical analysis: The data were presented as the mean±standard error of mean (SEM). Statistical analysis was performed with student's t-test. A $p<0.05$ was considered statistically significant.

RESULTS

Effects of NDV AF2240 on the proliferation of HL60 cells: The antiproliferative effects of NDV AF2240 on the culture of HL60 cells were examined by MTT assay. The HL60 cells were treated at CD_{25}, CD_{50} and CD_{75} incubated for 24, 48 and 72 h. From this assay, the Optical Density (OD) of untreated cells increased in a time dependent manner corresponded to the increment in the number of viable cells. However, treatments of cells with NDV AF2240 inhibited the cell proliferation. There was a decrease in the proliferation of HL60 treated with NDV AF2240 from 24-72 h (Fig. 1). The NDV AF2240 inhibited the proliferation of HL60 cells even at low titer of virus as treatment as low as CD_{25} concentration was found effective to cause growth inhibition in HL60 cells.

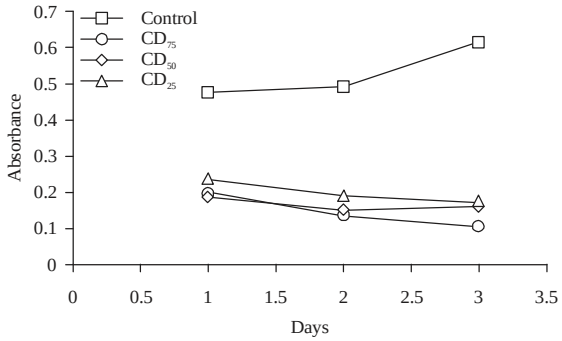

Fig. 1: Effects of NDV AF2240 on proliferation of HL60 cells. Data were expressed as Mean±SD of three experiments (n = 3). The HL60 cells were treated at CD_{25}, CD_{50} and CD_{75} and incubated for 24, 48 and 72 h

Microscopic observation: By using phase contrast light microscopy, the proliferation-inhibitory effect of NDV AF2240 in HL60 cells can be observed. When the cells were cultivated without NDV AF2240, they were proliferated normally with intact membrane appearance. However, with the addition of virus to the cells the inhibition of cell growth was observed with decrement in the number of viable cells and gradual change in cellular morphology. In comparison, the untreated control cells appeared budding on the surface of cell membrane and the present of apoptotic bodies in large numbers, indicating cell killing was by apoptosis (Fig. 2).

DNA fragmentation: To study the occurrence of apoptosis, the genomic DNA was isolated from the treated and untreated cells and studied using gel electrophoresis. Based on the result obtained, a single band appeared in the untreated cells (Fig. 3, lane 2). However, in treated NDV cells at 24, 48 and 72 h, a ladder-like pattern (typical character of DNA cleavage between nucleosomes) was visible which is a hallmark of apoptosis (Fig. 3).

Annexin-V FITC assay: In order to further confirm induction of apoptosis by NDV AF2240 in HL60 cells, annexin V-FITC/PI assay based on flow cytometry was used to examine the early apoptotic cells. Cells undergoing apoptosis will first express phosphatidylserine on the outer leaflet of the cell membrane, marked by annexin-V-FITC binding and followed by the membrane becoming compromised, marked by PI intercalation in the cellular DNA and RNA.

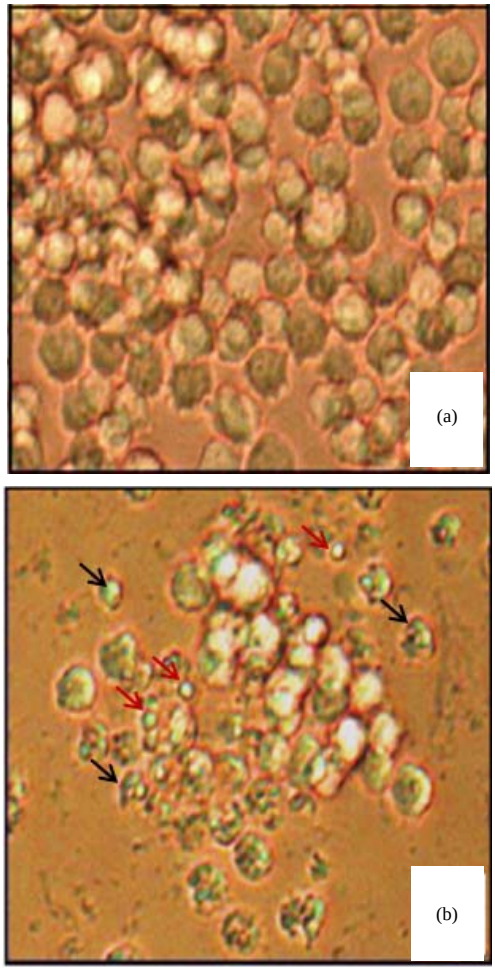

Fig. 2(a-b): Cytolytic effects of NDV AF2240 on leukemic cell lines viewed under inverted light microscope. (A) Untreated HL60 cells, (b) HL60 inoculated with AF2240 at CD_{50} (20x magnification). The addition of NDV AF2240 to the cultures of HL60 cells showed inhibition of cell growth with decrement in the number of viable cells. Cell shrinkages are shown in black arrow. Formation of apoptotic bodies are shown in red arrow

The HL60 cells treated with NDV AF2240 exhibited a significant increase proportionally to time from 12-48 h in the number of apoptotic cells (Fig. 4). In untreated cells, only 0.38±0.11% of early-apoptotic cells can be found, whereas 1.21±0.07% of late-apoptotic-cells was detected. As expected, more cells undergo early apoptosis after treatment with NDV AF2240 for 24 and 48 h where 16.27±0.25 and 25.93±1.2% were detected, respectively. Late-apoptotic cells were also increased following treatment for 24 and 48 h as 3.15±0.07 and 6.85±1.05% were detected, respectively.

Activation of imitator and effector caspases: The mechanism of apoptosis through activation of two initiator caspases, caspase-8 (extrinsic-mediated), caspase-9 (mitochondrial-mediated) and effector caspase, caspase-3 and 7 in NDV AF2240 treated cells were further investigated using lumigenic assay. Besides, the effect of general caspase inhibitor (ZVAD-FMK) was also examined in order to confirm the activation of related caspases.

It was demonstrated that, treatment of HL60 cells with NDV AF2240 for 12 h activated caspase-8, 9 and 3/7 at 109.6, 124.7 and 161.7%, respectively (Fig. 5). While pretreatment of HL60 cells with ZVAD-FMK indicated that cell death mediated by NDV AF2240 were effectively inhibited by ZVAD-FMK as 26.4, 39.8 and 48.1% inhibition of caspase 8, 9 and 3/7 were detected respectively after 12 h treatment compared to treatment NDV AF2240 alone (Fig. 5).

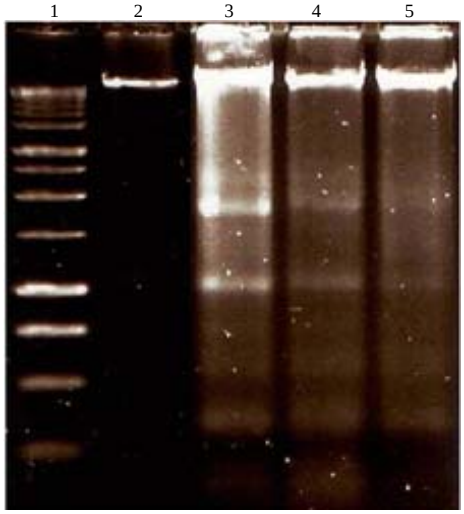

Fig. 3: DNA laddering profile of HL60 treated with NDV AF2240. Lane 1: 1 kb marker, 2: Untreated HL60, 3: Treatment for 24 h, 4: Treatment for 48 h and 5: Treatment for 72 h

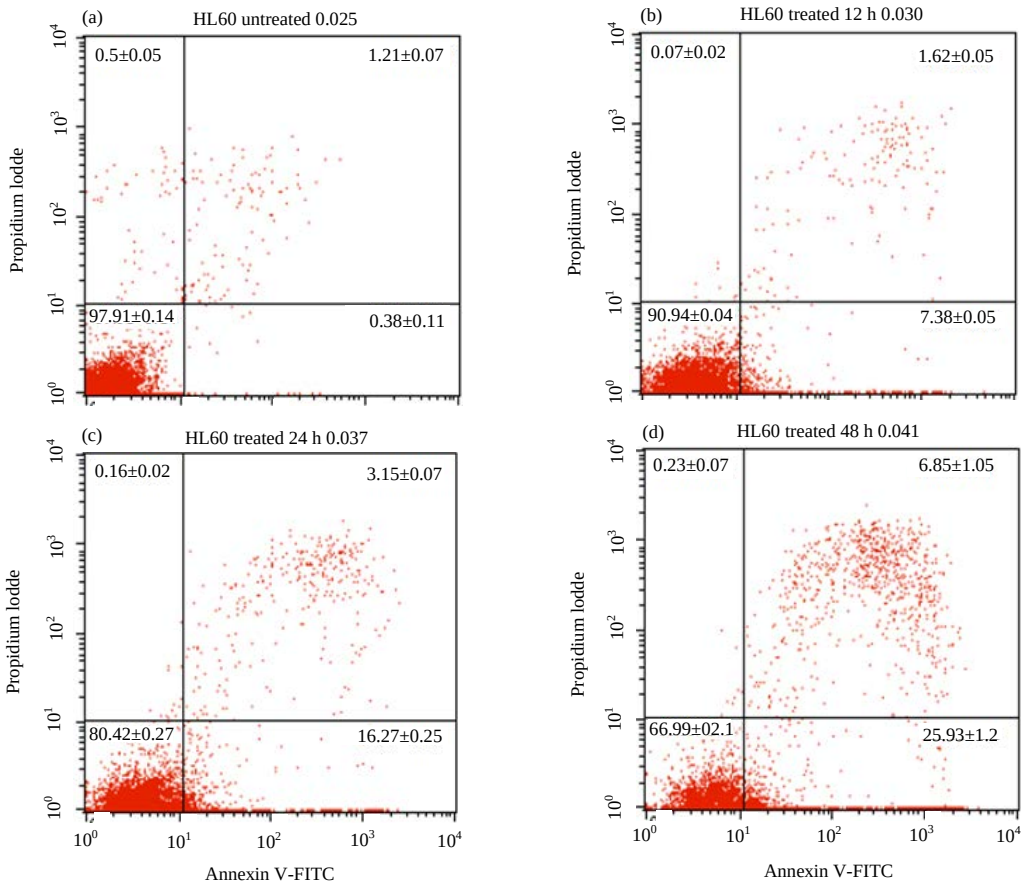

Fig. 4(a-d): Flow cytometry analysis of HL60 cells untreated and treated with NDV AF2240 at CD_{50} (B = 12 h, C = 24 h, B3 = 48 h) stained with annexin V-FITC/propidium iodide (PI). Viable cells are in the lower left quadrant, early apoptotic cells are in the lower right quadrant, late apoptotic cells are in the upper right quadrant and non-viable necrotic cells are in the upper left quadrant. Dot plots are a representative of 10,000 cells from a single replicate

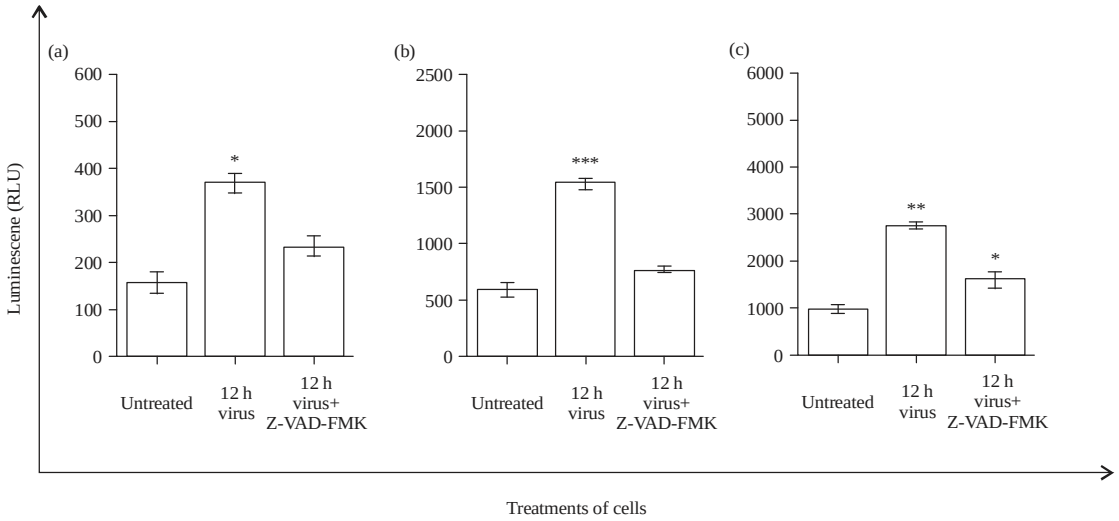

Fig. 5(a-c): Effects of NDV AF2240 on (a) Caspase 8, (b) Caspse 9 and (c) Caspase 3/7 in HL60 cells. Data were expressed by Mean±SEM of three experiments (n = 3). Student's t-test was used for statistical analysis of data and the significant differences from untreated control are indicated by *p<0.05, **p<0.01 and ***p<0.001

DISCUSSION

Newcastle Disease Virus (NDV) is considered to be a very promising oncolytic agent due to its selective property towards cancer cells[10,11]. The virus selectively replicates in tumor cells and induces death while sparing normal cells[17,18]. Several strains of NDV were reported to induce cytolysis and have apoptosis-inducing properties against various cancer cell lines[18-20].

The NDV strain AF2240 was found to induce tumor cytolysis in several cancer cell lines including HL60 cells[16,19]. The effects of different concentrations and exposure time of NDV AF2240 to the culture of HL60 cells were further examined in this study. Different virus titers at CD_{25}, CD_{50} and CD_{70} were found to inhibit the proliferation of HL60 cells. It was shown that the treatment as low as CD_{25} concentration was found to be effective to cause growth inhibition (Fig. 1). In addition, microscopic observations were also showed inhibition of cell growth with decrement in the number of viable cells. Typical features of cells in apoptosis such as cell shrinkage, nuclear fragmentation and the formation of apoptotic bodies were also observed (Fig. 2). This observation suggested that the treated HL60 cells with NDV AF2240 caused the cell to undergo apoptosis process.

The changes in apoptotic cells such as membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation[20] provide the basis for apoptosis detection. The DNA fragmentation and nuclear disorganization occurs in the final execution of the process, which has similarities between animal and plant cells[21,22]. In this study, two key apoptotic events were focused; DNA fragmentation and the alteration of the plasma membrane. The biochemical hallmark of apoptosis, the fragmentation of the genomic DNA was studied using agarose gel electrophoresis and the apoptotic changes on the cell membrane were examined by annexin V-FITC assay using flow cytometry.

It was demonstrated that apoptosis was the main mode of cell death in the treated HL60 cells with NDV AF2240. Fragmented DNA-laddering was found in all the treatment of cells with NDV AF2240 (Fig. 3) which represents the occurrence of random degradation of DNA. In addition, results from annexin V-FITC assay demonstrated that NDV AF2240 induced primarily apoptosis in treated cells at CD50 concentration. The number of early apoptotic cells was significantly increased in the time-dependent manner in HL60 cells treated with NDV AF2240. The combination of results from both of these assays strengthens the evident of apoptosis occurrence (Fig. 4). This result corroborated with the previous experiment using AO/PI staining which demonstrated induction of apoptosis in HL60 cells treated with NDV AF2240[16]. Besides, apoptosis was also reported as the main killing mechanism in the treated brain and breast cancer cell lines with the NDV AF2240[18,23].

Several other NDV strains used in pre-clinical and full clinical trials have been demonstrated to induce apoptosis in various tumor cells[24-26].

The activation of caspases can occur by two distinct pathways: An extrinsic death receptor-mediated pathway and an intrinsic mitochondrially-mediated pathway. In either of these pathways initiator caspases are activated by oligomerization following an apoptotic signal. Initiator caspases cleave and activate effectors caspases which then cleave diverse cellular proteins resulting in apoptosis[27]. The extrinsic pathway begins outside the cell through the activation of specific proapoptotic receptors on the cell surface. It is characterized by the binding of cell death ligands and cell death receptors and the subsequent activation of caspase-8 and 3, which then cleaves executioner caspase-3[27]. The intrinsic pathway as its name suggests, is initiated from within the cell. This pathway of cell death is mediated by Bcl-2 family proteins, which disrupt the mitochondria membrane potential and result in release of apoptogenic factors from the mitochondria into cytosol, such as cytochrome c, Smac/Diablo and AIF[28]. Caspase activations are observed in programmed cell death of both plants and animals and these activities could be inhibited with caspase inhibitors but not caspase-unrelated protease inhibitors[21].

It has been shown from this study that NDV AF2240 infection resulted in the activation of both death receptor and mitochondrion-associated pathways. Caspase-8 that responsible for extrinsic pathway and caspase-9 as initiator for intrinsic pathway were activated in the treated HL60 cells. Besides, the effectors caspase (caspase-3/7) was activated in the NDV AF2240 treated cells. The activation of caspase-3/7 may resulted from the cleavage of the initiator caspase, caspase-9, which goes on to activate caspase-3 and 7, triggering a cascade of caspase cleavage and activation, which are directly or indirectly responsible for the cleavage and degradation of several crucial cellular proteins and for the execution of cell death. In addition, results from caspase-3/7, 8 and 9 inhibition assay using general caspase inhibitor, ZVAD-FMK demonstrated that NDV AF2240-induced apoptosis is entirely dependent on caspase activation. The broad-specificity caspase inhibitor ZVAD-FMK was able to inhibit NDV AF2240-triggered cytolysis indicating that NDV-mediated cytotoxicity is caspase dependent.

The NDV has been demonstrated to induce apoptosis in various cancerous cells[13,29]. However, the pathways through which apoptosis is induced are still remains unclear. Some previous studies showed that NDV might activate apoptosis through the extrinsic death pathway. Cassel's 73-T NDV strain is reported to induce IFN-α and TNF-α in peripheral blood mononuclear cell and ulster strain is found to up regulate the expression of TRAIL receptors on cell surface of infected cells[30]. There are reports that NDV also augments the expression of induced nitric oxide synthase in infected cells. The MTH-68/H NDV strain induces cytotoxic anti-tumor effect through the induction of nitric oxide synthesis in rat peritoneal macrophages[31]. This suggested that NDV might induce apoptosis also through intrinsic death pathway.

It was also found that NDV trigger the activation of the intrinsic and extrinsic apoptotic pathway simultaneously[32,33]. Although, the extrinsic apoptotic pathway was preferred during the initial stages of infection, apoptosis induced by NDV was found to be predominantly mediated through the activation of intrinsic apoptotic pathway[33].

CONCLUSION

The NDV AF2240 was found to have antiproliferative effects on HL60 cells. The HL60 cells treated with NDV AF2240 at CD_{50} concentration, exhibited typically morphological features of apoptosis. A ladder-like pattern (typical character of DNA cleavage between nucleosomes) was visible in the NDV treated cells, which is a hallmark of apoptosis. The results from annexin V-FITC staining have shown that HL60 cells treated with NDV AF2240 at CD_{50} dose exhibited a higher percentage of apoptotic compared to necrotic cells. The mechanism of apoptosis through activation of caspase induced by NDV AF2240 was further determined. The NDV AF2240 infection activated caspase-8 (iniatiator caspase) in HL60 cells indicating that cells respond to NDV AF2240 with apoptosis may involved in death receptor signaling. However, caspase-9 was also found to be activated by a lumigenic substrate assay in HL60 cells after NDV AF2240 infection. Results from this study suggests that apoptosis in NDV AF2240-infected tumor cells may not only related with death receptor signaling but probably commences intrinsically, leading to mitochondrial membrane destabilization.

The selective nature of NDV makes it an ideal virotherapy agent. Its ability to replicate in tumour tissue allows for a massive amplification of the input dose at the tumour site and at the same time its lack of replication in normal tissues allows for efficient clearance and reduced toxicity. However, the specific mechanism by which NDV strains cause cell death selectively in cancer cells has not been completely elucidated. Further study need to carry out on the details mechanisms of action and more advanced clinical trials should be performed in the near future.

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