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Induction of Apoptosis by Newcastle Disease Virus Strains AF220 and V4-UPM in Human Promyelocytic Leukemia (HL60) and Human T-Lymphoblastic Leukemia (CEM-SS) Cells

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***Abstract*—**Newcastle Disease Virus (NDV), an avian paramyxovirus, is a highly contagious, generalised virus disease of domestic poultry and wild birds characterized by gastro-intestinal, respiratory and nervous signs. In this study, it was shown that NDV strain AF2240 and V4-UPM are cytolytic to Human Promyelocytic Leukemia, HL60 and Human T-lymphoblastic Leukemia, CEM-SS cells. Results from MTT cytolytic assay showed that CD_{50} for NDV AF2240 against HL60 was 130 HAU and NDV V4-UPM against HL60 and CEM-SS were 110.6 and 150.9 HAU respectively. Besides, both strains were found to inhibit the proliferation of cells in a dose dependent manner. The mode of cell death either by apoptosis or necrosis was further analyzed using acridine orange and propidium iodide (AO/PI) staining. Our results showed that both NDV strains induced primarily apoptosis in treated cells at CD_{50} concentration. In conclusion, both NDV strains caused cytolytic effects primarily via apoptosis in leukemia cells.

***Keywords*—**Apoptosis, Cytolytic, Leukaemia, Newcastle Disease Virus

I. Introduction

ONCOLYTIC viruses are viruses that infect and replicate in cancer cells, destroying these harmful cells and leaving normal cells largely unaffected [1]. Newcastle disease virus (NDV) is one such virus with an inherent oncolytic property [2]. NDV or avian paramyxovirus 1 belongs to the genus Avulavirus in the family Paramyxoviridae [3, 4]. NDV can cause Newcastle disease in a wide variety of birds most notably in chickens, transmitted by ingestion or inhalation and may result in substantial loss to the poultry industry. NDV can be classified into three pathotypes based on the severity of the disease, lentogenic, mesogenic and viscerotropic or neurotropic velogenic [3, 5].

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The selective nature of NDV makes it an ideal virotherapy agent. It is demonstrated that NDV selectively replicates in tumour cells and induces death while sparing normal cells. Due to this property, NDV has been exploited as a potential anti-cancer agent in humans [6-8].

In Malaysia, there are two NDV isolates, AF2240 and V4-UPM, which mostly studied so far. V4-UPM is a virulent and heat-resistant NDV strain, also known as Queensland strain [9, 10]. AF2240, a viscerotropic-velogenic NDV strain was first isolated from a local field outbreak in the 1960s [11]. Its diameter ranging from 150 to 600nm contains 17nm nucleocapsids and exposes spikes of 10 to 12 nm from its envelope [11]. AF2240 differs from the other NDV isolates by having a different HN protein length [14]. Both of V4-UPM and AF2240 strains show significant higher thermostabilities in HA, NA and infectivity [12, 13].

The oncolytic effects of NDV strains AF2240 and V4-UPM have been studied on MCF-7 and MDA-231 breast cancer cell lines [14, 15]. Besides, NDV AF2240 also found to have cytolytic effects on CEM-SS and brain tumor cell lines [16, 17]. However, no studies have yet been made on NDV AF2240 against HL60 and NDV V4-UPM against HL60 and CEM-SS.

II. Materials and Methods

A. Propagation of NDV strain AF2240 and V4-UPM

NDV strain AF2240 and V4-UPM were 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 [18]. The purification of virus was done as previously described by [19, 20].

B. Cells and cell cultures

Human Promyelocytic Leukemia, HL60 and Human T-lymphoblastic leukemia cells, CEM-SS were 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 37^0C. 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%.

C. MTT assay

Cell viability was assessed by MTT, (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole). Briefly, after the treatment of cells, a volume of 20 µl MTT reagent was added to each well and further incubated for 3 hours in a humidified 5% CO_2 incubator at 37^0C. 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). Concentration of virus that can inhibit 50% of cell viability (CD_{50}) was determined from a graph of percentage cell viability versus virus titer.

D. Acridine orange/propidium iodide (AO/PI) staining

The cells were seeded into wells of 12-well plates, treated with NDV at CD_{50} and incubated for 24, 48 and 72 hours in humidified 5% CO_2 incubator at 37^0C. The cells were harvested and centrifuged at 1000 rpm using rotor 12 151 (Sigma 2-16) for 10 minutes. Then, the supernatant was discarded and fluorochrome dye, Acridine Orange, AO (10 µg/ml) and Propidium Iodide, PI (10 µg/ml) were added in the ratio of 1:1 into the pellet. The mixture was resuspended well and then a volume of 20 µl of the suspension was dropped onto the slide and covered with cover slip. The cells were observed within 30 minutes using fluorescence inverted microscope (Nikon Eclipse TE2000-U) and photos were taken. The percentages of viable, apoptotic and necrosis cells were determined for more than 200 cells.

E. Statistical analysis

The data were presented as the mean±standard error of mean (S.E.M.) except for MTT cytotoxic assay where the data were expressed as mean±standard deviation (S.D.). Statistical analysis was performed with Student's t-test for data from MTT cytotoxicity and AO/PI assay. A p value of <0.05 was considered statistically significant.

III. Results

A. Effects of NDV AF2240 and V4-UPM on the viability of HL60 and CEM-SS cells

The cytotoxicity of NDV AF2240 on HL60 cells was investigated using MTT assay. After treatment with NDV AF2240 for 72 hours, the cell viability decreased from 100% to 17.43±2.337% in HL60 cells. There was a significant decrease of viable cells at virus titer as low as 80 till 320 HAU in HL60 cells (Fig. 1). The results indicated that NDV AF2240 induced growth inhibition acts in a dose-dependent manner on HL60 cells.

The response of HL60 and CEM-SS cells towards increasing V4-UPM titer in HA unit/ml in terms of percentage of viability is represented in Fig. 2. Cytotoxic dose that reduced cell population to 50% viability estimated from the curve were 110.6 and 150.9 HAU for HL60 and CEM-SS respectively. A relatively lower CD_{50} value in HL60 compared to CEM-SS indicated that HL60 was more sensitive to the virus.

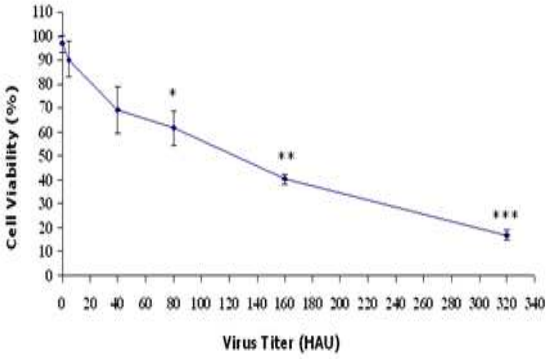

Fig. 1 The Percentage of Cell Viability of HL60 cells after treated with different concentration of NDV AF2240. Data were expressed as mean ± S.D. of three experiments (n = 3). Significant differences from untreated control are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

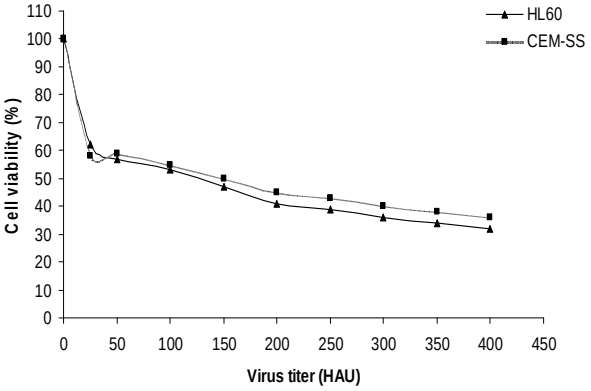

Fig. 2 The percentage of cell viability for CEM-SS and HL 60 after treatment with V4-UPM for 72 hours. Note: Data shown were deduced from means of triplicate absorbance reading determined using the MTT assay. The CD_{50} value for V4-UPM on each cell line was derived from this cytotoxic curve.

D. AO/PI staining

Fluorescent photomicrograph of untreated and treated HL60 cells and CEM-SS cells showed prominent apoptotic features in the treated cells, thus demonstrating the effect of the NDV on both cell lines. At 12 hours post treatment the earliest observable features of apoptosis had been documented here which are the condensation of the chromatin as well as budding of the membrane and formation of apoptotic bodies. Green fluorescent stain of membrane bound apoptotic bodies indicated intact membrane function. Necrotic cells and cells undergoing late apoptosis, which were stained red, seem to occur more after 24 hours post treatment (Fig. 3).

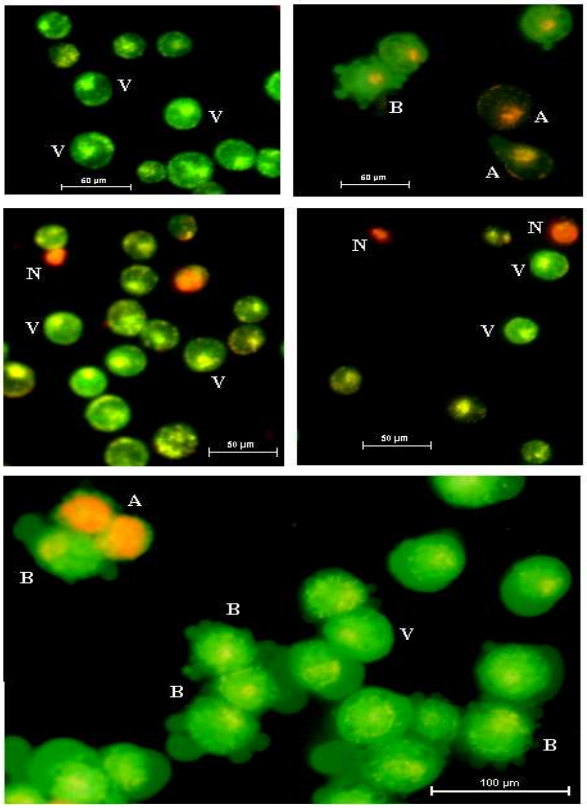

Fig. 3 AO/PI stained micrographic images of the WEHI-3B cells treated with NDV AF2240. (V) Viable cells with intact and translucent chromatin observed as a green fluorescence cells. (B) Blebbing cytoplasma (A) An apoptotic nucleus with chromatin fragmentations fluorescin orange-green (N) Necrotic cells

NDV AF2240 induced primarily apoptosis in HL60 cells at CD_{50} concentration. HL60 cells treated with NDV AF2240 exhibited a higher percentage of apoptotic compared to necrotic cells. As shown in Fig. 4C, the percentage of viable, apoptotic and necrotic cells in NDV AF2240 treated HL60 cells at CD_{50} treatment for 24 h were 77.27±3.33, 17.50±3.32 and 5.23±0.37% respectively and 48 h period were 58.03±0.33, 28.81±0.66 and 13.16±0.75% respectively. At 72 h treatment, the percentage of viable, apoptotic and necrotic cells were 56.38±1.96, 36.12±4.32 and 7.50±2.36%, respectively. The percentage of apoptotic and necrotic cells were significantly different ($p < 0.05$) from negative control at all time points.

NDV V4-UPM induced primarily apoptosis in both cells at CD_{50} concentration. HL60 and CEM-SS cells treated with NDV V4-UPM exhibited a higher percentage of apoptotic compared to necrotic cells. The percentage of viable, apoptotic and necrotic cells in NDV V4-UPM treated HL60 cells at CD_{50} treatment for 24 h were 61, 36 and 2% respectively and 48 h period were 60, 39 and 1% respectively (Fig. 4D). At 72 h treatment, the percentage of viable, apoptotic and necrotic cells were 59, 40 and 1%, respectively. Meanwhile in NDV V4-UPM treated CEM-SS cells, the percentage of viable, apoptotic and necrotic cells after 24 h treatment were 59.5, 38.5 and 2% respectively, for 48 h period were 57, 40 and 3% respectively and for 72 h treatment were 60.5, 38 and 1.5% respectively (Fig. 4E).

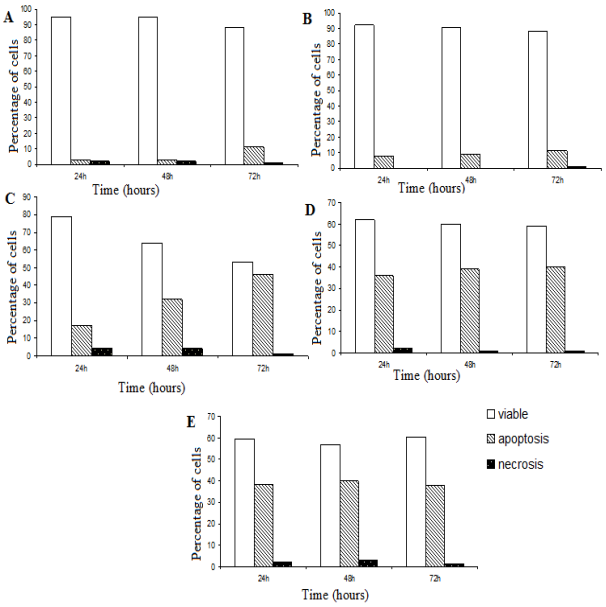

Fig. 4 The percentage of viable, apoptosis and necrosis of (A) Untreated HL60 (B) Untreated CEM-SS (C) NDV AF 2240 treated HL60 (D) NDV V4-UPM treated HL60 (E) NDV V4-UPM treated CEM-SS

IV. Discussion

In this study, the ability of NDV strain AF2240 and V4-UPM to induce tumor cytolysis were assessed towards leukemia cells. From our study, both NDV strains showed cytotoxic effects in HL60 and CEM-SS cells as obtained in MTT assay. Previous studies found that NDV AF2240 also have cytolytic effects against CEM-SS (Human T-lymphobalstic), MCF-7 and MDA-231 breast cancer cell lines and brain tumor cell lines [14, 16, 17]. However, it did not affect normal human lymphocyte and 3T3 (Mouse fibroblast) cells [17]. NDV V4-UPM was found to have cytolytic effects against MCF-7 and MDA-231 [14, 15].Several different strain of NDV also showed cytolytic effects against human tumor cells. NDV strain F and Ijuk were found to have cytolytic effects against MCF-7 and MDA-231 [14, 15]. NDV strain 73-T has been shown to kill the following types of human cancer cells in vitro: fibrosarcoma, osteosarcoma, neuroblastoma, bladder carcinoma, cervical carcinoma, melanoma, Wilms tumor, and myeloid leukemia [21, 22]. Lytic strain Roakin has been reported to kill human B-cell lymphoma cells and T cells transformed in vitro from a Hodgkin lymphoma patient four to five times faster than it kills normal, resting human white blood cells [23]. Another lytic strain, NDV Italien (or Italian) has been shown to kill human squamous cell lung carcinoma, melanoma, breast carcinoma, and larynx carcinoma, but not cervical carcinoma [24].

NDV AF2240 and V4-UPM has a potential to be an effective anticancer agent due to its cytotoxicity to cancer cells with less effects to normal cells. This criteria plays an important role in any type of anticancer agents. However, this assay is a basic screening test in demonstrating a pioneer intervention of NDV AF2240 and V4-UPM as antileukemia agent. The loss of viability in NDV AF2240 treated cells can be considered as one of the primary criteria for apoptosis. However, these cells may also undergo necrosis as a result of cell lysis or stop active growth and division. Thus, further experiments were carried out to identify the mode(s) of cell death induced by NDV AF2240 and V4-UPM treatments.

NDV has been demonstrated to induce apoptosis in various cancerous cells [25, 26]. Apoptosis was originally defined by a sequence of morphologic features [27]. Morphological changes are still considered the "gold standard" for apoptosis although recent progress in apoptosis research already at the biochemical and molecular levels. During apoptosis, nucleus of the cell undergoes dramatic morphological changes including chromatin condensation, peripheral margination, nuclear shrinkage, and subsequent fragmentation [27]. Wyllie et al., (1980) characterised morphological changes during apoptosis into two stages. The first stage was characterized by condensation and fragmentation of the nuclear chromatin and the second stage involve fragmentation of both cytoplasm and nucleus, and the cells separated into small membrane bound vesicles known as apoptotic bodies [28]. Budding and formation of apoptotic bodies can be observed visually by simple phase contrast light microscopy.

The mode of cell death either by apoptosis or necrosis was further analyzed using acridine orange (AO) and propidium iodide (PI) staining. This assay has been developed for the simultaneous visualization of both viable and nonviable cells [29]. The uptake of acridine orange dye was seen in viable and apoptotic cells while necrotic cells were stained by propidium iodide. The morphological changes and different fluorescence of fluorochrome in cells were used to distinguish viable, apoptotic and necrotic cells. Our results showed that NDV AF2240 and V4-UPM induced primarily apoptosis in treated cells at CD_{50} concentration. Acridine orange intercalates into the DNA, giving it a green appearance. This dye also binds to RNA but because it cannot intercalate, the RNA stains red-orange. Thus, a viable cell will have a bright green nucleus and a red cytoplasm. Propidium iodide, on the other hand, is taken up only by nonviable cells or cells with damaged plasma membrane. This dye also intercalates into DNA, making it to appear orange in colour, but binds weakly only to RNA, which appear as slightly red. Thus, a dead cell will have a bright orange nucleus (the propidium iodide overwhelms the acridine) and its cytoplasm, if it has any contents remaining, will appear as dark red [30, 31].

In conclusion, our study suggests that NDV AF2240 and NDV V4-UPM caused cytolytic effects primarily via apoptosis in leukemia cells**.** However, the specific mechanism by which NDV strains cause cell death selectively in cancer cells has not been completely elucidated. Further research 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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