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Newcastle Disease Virus Infection Promotes Bax Redistribution to Mitochondria and Cell Death in HeLa Cells

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Key Words
Newcastle disease virus · AF2240 · Apoptosis · Bax · Bcl-2 · HeLa

Abstract
Background/Aims: Newcastle disease virus (NDV) is an avian paramyxovirus that has gained a lot of interest in cancer viro-therapeutic applications because of its ability to selectively induce apoptosis in human cancer cells. However, the underlying mechanisms by which NDV induces apoptosis in human cancer cells are still not entirely understood. ***Methods:*** In this study we examined the effect of a Malaysian velogenic strain of NDV, known as AF2240, on some elements of the intrinsic pathway of apoptosis. ***Results:*** We show that NDV infection leads to conformational change of Bax protein. This is associated with the translocation of Bax from the cytoplasm to mitochondria and the release of cytochrome c into the cytoplasm. Interestingly, the level of Bcl-2 protein was not affected by NDV treatment. ***Conclusion:*** We have shown that Bax conformational change and subcellular distribution is involved in the intrinsic pathway of apoptosis induced by NDV.

Introduction

Newcastle disease virus (NDV) belongs to the genus *Avulavirus* from the family of *Paramyxoviridae* [1] which causes fatality in most species of birds, resulting in great economic impact on the poultry industry [2]. NDV has a non-segmented single-stranded negative-sense RNA genome of nearly 15 kb [3–5]. Upon endocytosis into the host cell, NDV genome is subjected to two separate mechanisms of transcription and replication; transcription into mRNAs to produce viral proteins [6, 7] and synthesis of a full-length positive-sense RNA which acts as a template for replication of more negative-sense RNAs [8]. Together, these two mechanisms function as a machinery to produce more viruses. Many strains of NDV have been isolated but this virus is mainly categorized by its pathotype and clinical effects rather than its RNA structure. Basically, lentogenic NDV strains cause clinically mild respiratory disease, mesogenic strains result in some respiratory and neurological symptoms with moderate mortality, and velogenic strains cause severe intestinal lesions and neurological effects with high mortality rates [9]. Strain AF2240 which is mostly used as the challenge virus in vaccine trials in Malaysia, is a viscerotropic velogenic strain of NDV that was isolated during an outbreak in the country in the 1960s [10]. This virus has a different hemagglutinin-neuraminidase protein compared to other strains [11].

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Although NDV is not a human pathogen, its effect on human cancers has been widely reported [12–18]. In general, NDV appears to selectively kill cancer cells while sparing normal human cells [19]. It is believed that NDV kills avian cells and human cancer cells by apoptosis [20, 21].

Apoptosis is a form of cell death which is initiated via either intrinsic or extrinsic pathway [22]. Mitochondria are involved in both apoptotic signaling pathways [23]. The Bcl-2 family of proteins plays an important role in regulation of mitochondrial checkpoints. These proteins display both pro- and anti-apoptotic functions [24, 25]. Bcl-2 displays primarily anti-apoptotic characteristics [22, 26] and it is a membrane protein that has been localized to nuclear outer membrane, endoplasmic reticulum and mitochondrial outer membrane [24, 27, 28]. Bax, on the other hand, is a pro-apoptotic member of this family [24]. It resides mainly in the cytoplasm of healthy living cells and it translocates to mitochondria upon the induction of apoptosis [29–31]. The conformational change of Bax, followed by the exposure of both of NH2- and COOH-terminal segments, leads to its translocation to mitochondria [32]. It has been proposed that Bax forms oligomers on mitochondrial surface and Bcl-2 could block this oligomerization process [33]. Thus, it has been proposed that the ratio of Bax/Bcl-2 serves as a rheostat that regulates cellular survival vs. death [24, 34].

Previous observations show that, during NDV-induced apoptosis, both extrinsic and intrinsic pathways are activated and that the mitochondria membrane potential drops [35]. Furthermore, another study suggests that this event is independent of the role of p53 [36]. These observations prompted us to investigate the role of mitochondria and Bcl-2 family of proteins in NDV-induced apoptosis. We mainly focused on the Bax protein to see if infection with the AF2240 strain of NDV leads to its conformational change and subcellular distribution. We also investigated the level of Bcl-2 protein to see if there is a disturbance in Bax/Bcl-2 rheostat and mitochondrial dysfunction as in the release of cytochrome c following NDV infection.

Materials and Methods

Virus and Cells

The Malaysian velogenic NDV strain AF2240 was grown in 9-day-old embryonated chicken eggs and purified as previously described [37]. The virus titer was measured by hemagglutination activity (HA) assay. Human cervical cancer cell line, HeLa, was purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U of penicillin/ml and 100 μg of streptomycin/ml in 5% CO_2 at 37°. The infection of cells was performed according to a standard protocol with 30 HA units of NDV per 10^6 cells [38]. All viral works were carried out inside a culture hood at the Department of Microbiology of Universiti Putra Malaysia.

Detection of Apoptosis by DNA Laddering and Acridine Orange/Propidium Iodide Staining of Apoptotic Nuclei

DNA fragmentation was assessed using the Suicide Track™ DNA Ladder Isolation Kit (Calbiochem, USA). Briefly, cells were cultured to 80% confluency, and were then infected with NDV virus and harvested at different time points. Cellular DNA was extracted using the rapid extraction protocol as specified by the manufacturer and then ran on a 2% agarose gel stained with ethidium bromide. Acridine orange/propidium iodide (AO/PI) staining was performed as previously described [39]. At different time points, infected cells were harvested, pelleted down, and resuspended in a solution of AO/PI (1 μg/ml of each stain). Stained cells were immediately visualized by fluorescence microscopy to observe apoptotic nuclei.

Subcellular Fractionation

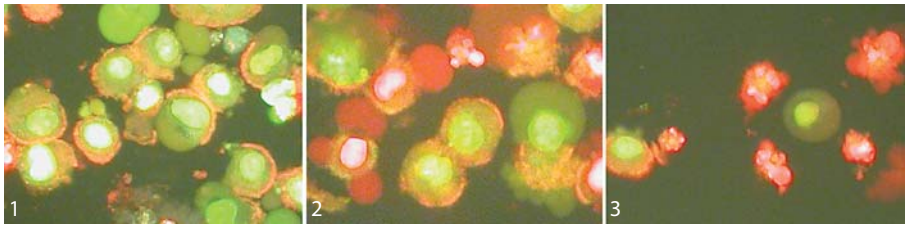
Subcellular fractionation was performed as previously described [40], but with minor modifications. Infected cells were collected at different time points and resuspended in mitochondrial buffer (70 mM Tris-HCl, 0.25 M sucrose and 1 mM EDTA, pH 7.4). An equal volume of ice-cold digitonin lysis buffer (2 mg/ml, 19.8 mM EDTA, 0.25 M D-mannitol and 19.8 mM MOPS, pH 7.4) was added for 90 s. Samples were then centrifuged twice at 300 *g* for 5 min to pellet the nuclei. The supernatant was further centrifuged at 17,000 *g* for 20 min to separate mitochondria from the cytosol.

Immunofluorescence and Confocal Microscopy

HeLa cells were plated on poly-D-lysine-coated glass coverslips. The cells were infected 24 h later with the NDV strain AF2240. At different time points post-infection, cells were fixed with 3.7% ice-cold paraformaldehyde for 10 min, washed twice with ice-cold PBS, and then permeabilized with 0.25% Triton X-100 in PBS for another 10 min. The cells were subsequently blocked with 3% BSA in PBST for 30 min–1 h and then incubated with primary antibodies (anti-Bax clone 6A7 and anti-Bcl-2 clone 100, Invitrogen, USA; anti-cytochrome c clone 7H8, Biovision, USA) in PBST and 1% BSA for at least 4–12 h at 4°. After washing, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse (Invitrogen, USA) or FITC-conjugated goat anti-mouse (Santa Cruz, USA) in 1% BSA in PBST at room temperature for 1 h in dark. Coverslips were washed and finally were treated with ProLong® gold antifade reagent (Invitrogen, USA) and put on glass slides. Visualization was carried out using the Leica DM RA2 fluorescence microscope or Olympus FV1000 confocal microscope.

Detection of Bax Conformational Change by Immunoprecipitation

HeLa cells (1×10^7) were infected with NDV and analyzed at different time points. Infected dead floating cells were collected separately from infected adherent cells. All adherent cells, both infected and uninfected, were harvested by scraping. The collect-



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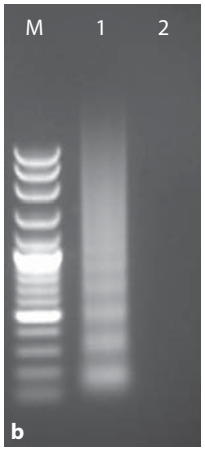

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Fig. 1. Detection of apoptosis in NDV-infected HeLa cells. **a** Uninfected and NDV-infected HeLa cells were stained with AO/PI. Panels 1–3 are HeLa cells before infection, 6 h post-infection and 18 h post-infection respectively. Living cells were stained green while apoptotic cells were stained red (for colors see online version). **b** NDV infection induced DNA laddering in infected HeLa cells. Lane M: VC 100 bp plus DNA marker (Vivantis, Malaysia); lane 1: DNA extracted from HeLa cells 18 h post-infection; lane 2: DNA extracted from uninfected HeLa cells.

ed cells were washed twice in ice-cold PBS before being lysed in 200 μl CHAPS buffer (1% CHAPS, 10 mM Hepes pH 7.4, 150 mM NaCl, and protease inhibitor cocktail) [41, 42]. The cell lysate was spun at 10,000 *g* (Sorvall, Legend Micro 17 R) for 15 min to pellet the nuclei and then incubated with antibody-coated protein G beads overnight at 4° on a rotator. The beads were then washed three times with the solubilization buffer containing 0.5% CHAPS buffer. The beads were then mixed with 20–50 μl SDS-PAGE sample loading buffer and boiled at 95° for 10 min. The supernatant was subjected to SDS-PAGE and Western blotting with rabbit polyclonal anti-Bax N20 antibody.

SDS-PAGE and Western Blotting

For Bax, Bcl-2, and β-actin studies, a 12.5% SDS-polyacrylamide gel and for cytochrome c studies, a 15% gel was used. Gels were electrotransferred onto Immobilon-P membrane (Millipore Corp., USA) and after transferring, membranes were blocked with a casein buffer (Pierce, USA). The membranes were incubated with the primary antibodies at dilutions suggested by the manufacturers from 4 h to overnight at 4°. The blots were then incubated with secondary antibodies (AP-conjugated goat anti-mouse, Bethyl, USA; or AP-conjugated goat anti-rabbit, Abcam, USA) for 1 h at room temperature. NBT/BCIP alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$, containing 3 mg/ml NBT and 1.5 mg/ml BCIP) was used to develop the blotted membranes (Fermentas, USA). Membranes were also stripped and re-incubated with anti-β-actin antibody (Sigma, USA) to confirm equal protein loading. Furthermore, protein bands were scanned and a densitometric analysis was performed with Bio-Rad's Quantity One software version 4.2.2.

Results

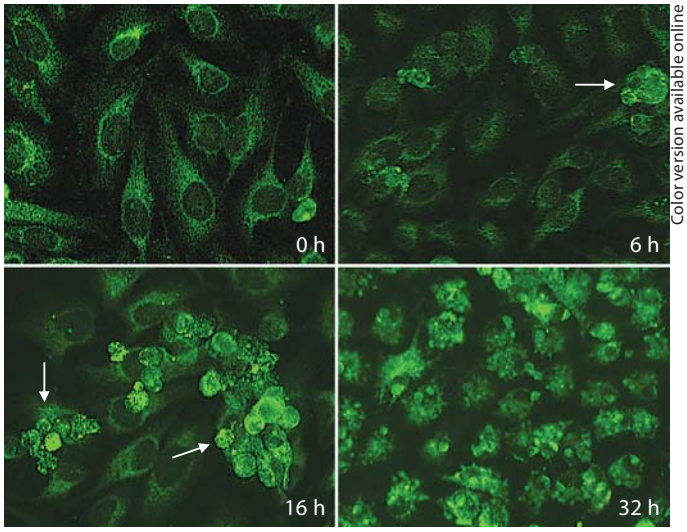
NDV Infection Leads to Cell Morphological Change and DNA Fragmentation

HeLa cells were infected with NDV and at different time points, the cells were subjected to AO/PI labeling as described [39] (fig. 1a). Living cells only took up the acridine orange stain and could be seen as green under fluorescence microscope. Apoptotic cells which underwent morphological change and membrane blebbing were stained by propidium iodide and the condensed and fragmented nuclei could be seen as red. In our study, we found that morphological change of HeLa cells started to occur at 6 h post-infection.

In order to confirm the AO/PI staining of apoptotic HeLa cells, a DNA fragmentation assay was performed (fig. 1b). Total cellular DNAs from infected cells were isolated and then the fragmented DNA was separated from the heavy weight genomic DNA and analyzed by agarose gel electrophoresis. At 18 h post-infection, endonuclease-mediated nucleosome excision was clearly observed as a DNA ladder (multimers of approximately 180–200 base pairs) which is characteristic of apoptosis [43]. No fragmented DNA was observed before infection by NDV.

NDV Infection Leads to the Release of Cytochrome c

HeLa cells infected with NDV were subjected to immunofluorescence labeling with an anti-cytochrome c 7H8 antibody at various time periods post-infection. The labeled cells were visualized by fluorescence microscope. As shown in figure 2a, cytochrome c was localized primarily to mitochondria before infection. But 6 h post-infection, it became evident that in a number of cells, cytochrome c was released from mitochondria into the cytoplasm. A number of these cells were rounded up. At 16 h post-infection, more cells showed morphological changes and many started to detach and of the attached cells, more have shown a diffused cytoplasmic labeling of cytochrome c. At 32 h post-infection, almost all cells showed a diffused cytoplasmic cytochrome c labeling pattern.



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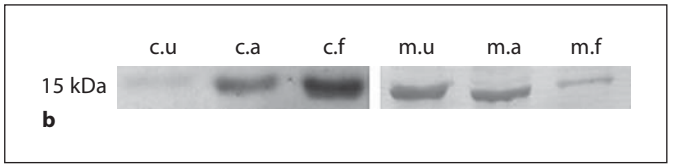

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Fig. 2. NDV infection leads to the release of cytochrome c in HeLa cells. **a** Fluorescence images of HeLa cells labeled with an anti-cytochrome c 7H8 antibody at various times (0–32 h) after NDV infection. At 0 h post-infection, almost all cells had cytochrome c residing in mitochondria. At 6 h post-infection, a number of cells showed diffused labeling of cytochrome c and rounding up (white arrows). At 16 h post-infection, more cells showed morphological changes (white arrows) and many started to detach. At 32 h post-infection, almost all cells showed a cytoplasmic cytochrome c labeling pattern. **b** Detection of cytochrome c localization by subcellular fractionation. HeLa cells were subjected to NDV infection for 20 h and then subjected to subcellular fractionation. Infected adherent cells were collected separately from infected floating cells. The protein samples were analyzed by Western blotting analysis with an anti-cytochrome c 7H8 antibody (c = cytosolic fraction, m = heavy membrane fraction, u = uninfected cells, a = infected adherent cells, f = infected floating cells). Cytochrome c was primarily localized to mitochondria in uninfected cells. NDV infection leads to the release to cytochrome c in both infected adherent and floating cells.

To corroborate the immunofluorescence labeling results, subcellular fractionation was performed followed by Western blotting with an anti-cytochrome c antibody. HeLa cells were infected with NDV. Floating and adherent cells were collected separately at 20 h post-infection. As demonstrated in figure 2b, the cytosolic fraction of uninfected cells had little or no cytochrome c. On the other hand, in infected cells, especially the floating dead cells, a significant quantity of cytosolic cytochrome c was detected. Taken together, these results indicate the release of cytochrome c into the cytoplasm is an intrinsic event during the induction of apoptosis by NDV.

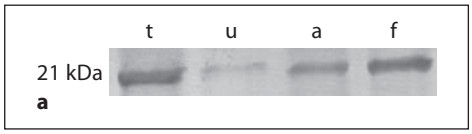
Bax Conformational Change during NDV-Induced Apoptosis

During apoptosis, a change in Bax conformation could be detected by a conformation-sensitive antibody 6A7 [29, 32]. In order to investigate the role of Bax in NDV-induced apoptosis, an immunoprecipitation analysis was carried out (fig. 3a). Cell lysates from uninfected and NDV-infected cells were subjected to immunoprecipitation with anti-Bax 6A7 antibody [42]. The immunoprecipitate samples were analyzed by Western blotting with an anti-Bax antibody. The results showed that little Bax binding to the 6A7 antibody was observed in uninfected HeLa cells. However, at 18 h post-infection, especially in lysates from infected floating cells, a significant binding of Bax to the 6A7 antibody was observed. This suggests that Bax underwent a conformational change in HeLa cells during infection with the virus.

NDV-infected cells were also subjected to immunofluorescence labeling with the anti-Bax 6A7 antibody (fig. 3b). Very weak labeling was observed in untreated cells (fig. 3b; black arrow). However, as early as 6 h post-infection, 6A7 labeling became apparent in infected HeLa cells (fig. 3b; white arrow). This labeling appeared to occur prior to membrane blebbing. The intensity of labeling and the number of cells being labeled by this antibody were increased over time (fig. 3b; arrowheads). These results indicate that Bax undergoes a conformation change following apoptosis induction by NDV infection.

Translocation of Bax from the Cytoplasm to Mitochondria

Subcellular fractionation was carried out to study the localization of Bax following NDV infection. The protein fractions were subjected to Western blotting analysis with the anti-Bax 2D2 antibody. As shown in figure 4, Bax was primarily localized to the cytosolic fraction before infection. However, after infection with NDV, the Bax level was decreased in the cytosolic fraction of infected adherent and this decrease was more evident in infected floating cells. In contrast, very little Bax was detected before infection in the heavy membrane fraction, which contains mitochondria. Bax level was increased in the heavy membrane fraction of infected cells, especially that obtained from the infected floating cells.



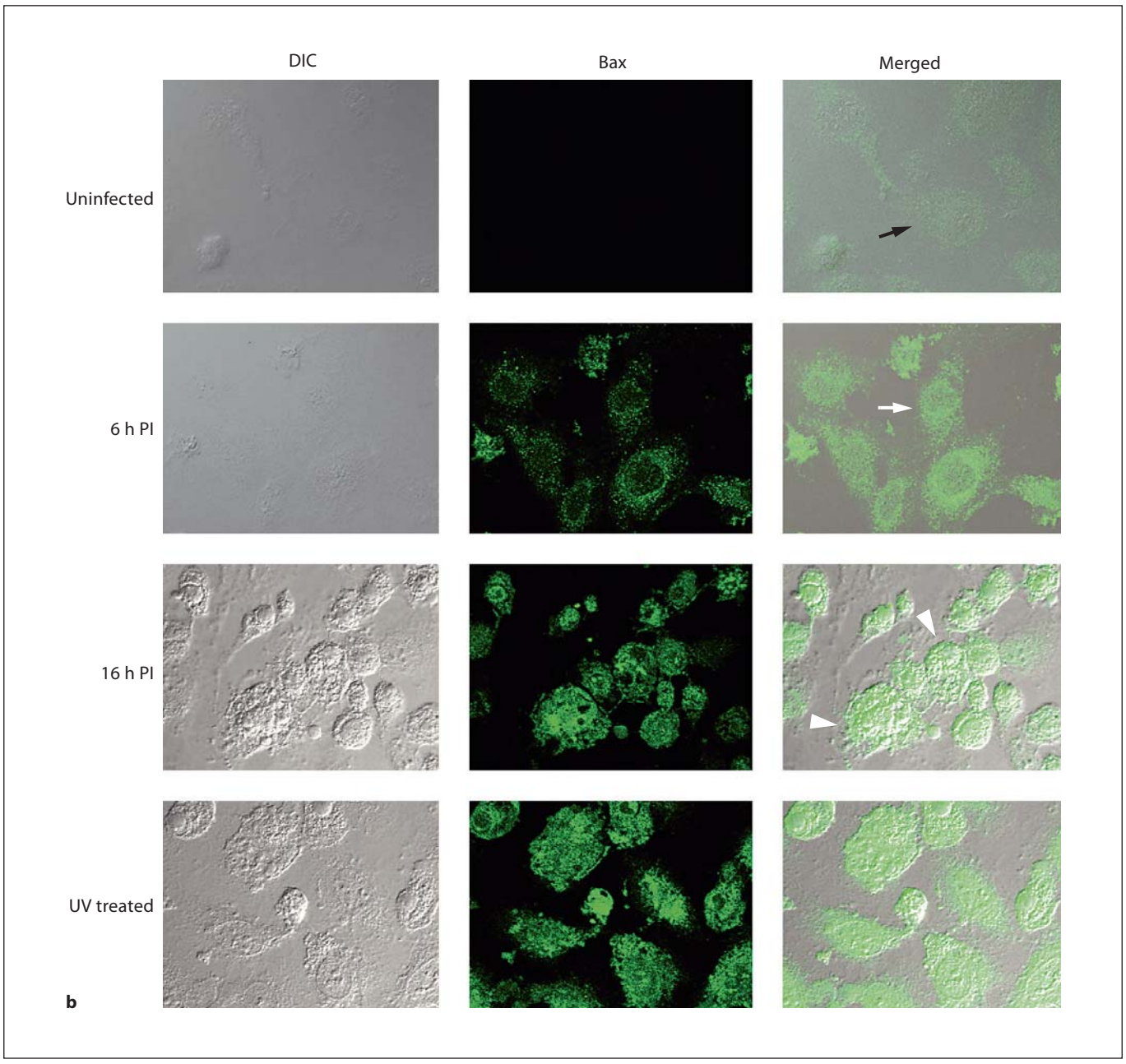


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Fig. 3. Detection of Bax conformational change in HeLa cells infected with NDV. **a** Uninfected and NDV-infected HeLa cells were solubilized in Chaps and subjected to immunoprecipitation with the conformation-sensitive 6A7 antibody. The immunoprecipitated samples were subjected to Western blotting analysis with rabbit anti-Bax clone N20. t = Total HeLa lysate, u = IP sample from uninfected cells, a = IP sample from infected adherent cells, f = IP sample from infected floating cells. A significant change in Bax conformation was detected in infected floating cells. **b** Confocal microscopy image of infected HeLa cells labeled with anti-Bax 6A7 antibody. Very week labeling was observed in uninfected cells (black arrow). At 6 h post-infection, 6A7 labeling became apparent (white arrow). At 16 h post-infection, a number of cells were labeled by the 6A7 antibody (arrowheads). UV-treated cells were used as control. NDV infection leads to activation and conformational change of Bax protein.

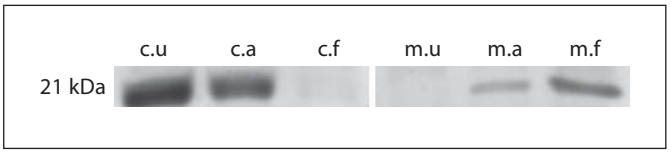


Fig. 4. Subcellular localization of Bax in NDV-infected HeLa cells. Uninfected and NDV-infected HeLa cells were subjected to subcellular fractionation. The protein samples were then analyzed by Western blotting analysis with the anti-Bax 2D2 antibody (c = cytosolic fraction, m = heavy membrane fraction, u = uninfected cells, a = infected adherent cells, f = infected floating cells). Bax was primarily localized to the cytosolic fraction before the infection but it translocated to the heavy membrane fraction after infection with the NDV strain AF2240, especially in infected floating cells.

NDV Infection Did Not Alter Bax and Bcl-2 Protein Expression Levels in HeLa Cells

An immunofluorescence labeling study was performed with the anti-Bcl-2 clone 100 antibody to assess whether Bcl-2 level was altered following NDV infection. We found that the intensity of Bcl-2 did not change between uninfected and infected cells (up to 18 h post-infection; data not shown). In addition, we have carried out a subcellular fractionation analysis of uninfected and infected cells. As shown in figure 5a, the heavy membrane fraction from uninfected and infected HeLa cells contained equivalent levels of Bcl-2. No Bcl-2 was detected in the cytosolic fractions in agreement with previous studies.

Furthermore, cell lysates from uninfected and NDV-infected HeLa cells were subjected to Western blotting analyses with anti-β-actin, Bax 2D2, and Bcl-2 clone 100 antibodies. The labeled Bax and Bcl-2 bands were subjected to densitometric scans and normalized to β-actin. The results showed that following NDV infection, there was no significant change in the levels of Bax and Bcl-2 proteins, and that Bax to Bcl-2 ratio remained relatively constant (fig. 5b).

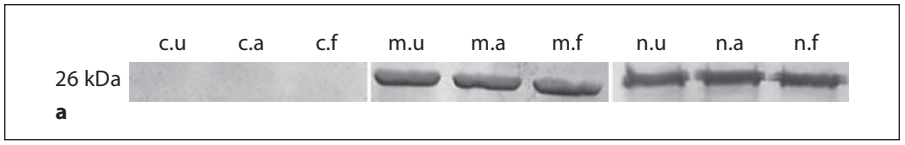
Discussion

Apoptosis is a form of cell death that could be initiated via either intrinsic or extrinsic signaling pathways [22]. Several viruses have been shown to inhibit apoptosis by the strategies that they have evolved in order to secure their propagation in the infected cells [44]. On the other hand, many viruses are known to induce apoptosis in cells via the extrinsic, intrinsic, or both signaling pathways [44]. NDV is believed to promote cellular apoptosis via both pathways [35]. In this report, we show that NDV infection leads to apoptosis as determined by the release of cytochrome c, DNA laddering, and nuclear fragmentation; release of cytochrome c into cytoplasm was monitored during NDV-induced apoptosis in HeLa cells by immunofluorescence and subcellular fractionation experiments. In addition, we have shown that Bax redistribution from the cytoplasm to mitochondria is involved in the signaling mechanism leading to cell death. Moreover, we show that NDV infection did not alter the Bax/Bcl-2 ratio at the protein level.

During apoptosis, Bax protein N-terminal and C-terminal regions are exposed [29, 32]. This is associated with Bax translocation from the cytoplasm to mitochondria [30–32]. Mitochondrial-bound Bax undergoes oligomerization on mitochondrial outer membranes, facilitating the release of apoptogenic factors such as cytochrome c [45, 46]. In our study, by immunoprecipitation and immunofluorescence methods, we found that NDV infection leads to the exposure of the Bax N-terminal epitope, the redistribution of Bax to mitochondria, and the release of cytochrome c. It is conceivable that NDV infection initiates a signaling pathway leading to conformational change of Bax protein resulting in its redistribution to mitochondria. This mitochondrial bound Bax likely leads to mitochondrial permeabilization resulting in cytochrome c release.

Bcl-2 has been shown to inhibit Bax oligomerization process [46]. It has been proposed that the ratio of Bax/Bcl-2 serves as a rheostat that dictates cell survival vs. death [24, 34]. By real-time PCR it has been recently reported that in NDV-infected Vero cells, Bax/Bcl-2 ratio is increased [47]. In our study, by Western blotting analysis of cell lysates, we did not detect significant changes in the total levels of these two proteins before and after infection with the NDV strain AF2240, indicating that the ratio of Bax/Bcl-2 at the protein level remained constant. This suggests that the endogenous Bcl-2 was not sufficient to inhibit the pro-apoptotic function of Bax in HeLa cells upon infection and also the alteration in Bax/Bcl-2 rheostat is not involved in NDV-mediated cell death.

Tumor suppressor p53 has been proposed as a regulator of Bax and Bcl-2 proteins during apoptosis [48]. However, it has been previously shown that NDV kills cancer cells independently of p53 [36], suggesting that p53 is not in NDV-mediated Bax redistribution. On the other hand, BH3 domain-only proteins of the Bcl-2 family such as



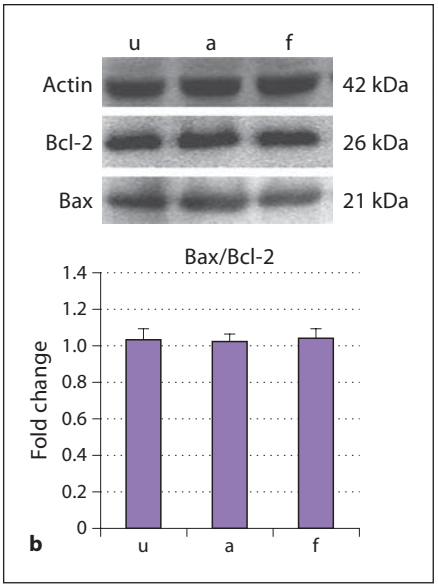


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Fig. 5. No significant change in the protein levels of Bax and Bcl-2 after infection with NDV. **a** Subcellular localization of Bcl-2 in NDV-infected HeLa cells. Uninfected and NDV-infected HeLa cells were subjected to subcellular fractionation. Protein samples were then analyzed by Western blotting with anti-Bcl-2 100 antibody (c = cytosolic fraction, m = heavy membrane fraction, n = nuclear fraction, u = uninfected cells, a = infected adherent cells, f = infected floating cells). No significant change in the level of Bcl-2 was observed in HeLa cells' heavy membrane and nuclear fractions before and after infection with the NDV strain AF2240. **b** Cells were lysed and analyzed by Western blotting for levels of Bax, Bcl-2, and β-actin proteins. Bar graph represents a densitometric scan of the Western blots of the ratio of Bax/Bcl-2 as normalized to β-actin (mean ± SEM, n = 4).

Bad, Bid and Bim are believed to activate and displace Bax [49–52]. It is possible that NDV infection activates these BH3-only proteins, leading to Bax conformational change and subcellular redistribution.

Recent studies show that viruses have mixed effects on Bax protein as some induce apoptosis by activation of Bax [53, 54] and some block apoptosis by inhibition of conformational activation of Bax [55, 56]. Our study shows a conformational change and redistribution of Bax after NDV infection. In the future, it would be interesting to determine the signaling mechanism by which NDV mediates this process.

Acknowledgements

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