

Study the Apoptotic Pathway Induced in Mammary Adenocarcinoma Tissue Treated by Local Iraqi Strain of Newcastle Disease Virus

Ahmed M. Al-Shamery*, Nahi Y. Yaseen*, Mohammed J. Alwan**

* Iraqi Center for cancer and medical genetics research, Almustansiriya University.

** College of veterinary medicine, Baghdad University.

Abstract :

In tumor pathogenesis and metastasis, defects in apoptosis are essential. They allow tumor cells to overcome nutrient deprivation, absence of growth-stimulating signals, and presence of growth-inhibitory signals, surrounding tissue barriers, hypoxia, oxidative stresses, and host immune responses.

Newcastle disease virus is interesting oncolytic agent with promising anti-tumor properties. One of the major anti-tumor properties is apoptotic induction.

This study aimed to find out the apoptotic pathway induced by Iraqi strain of NDV, *in vitro* and *in vivo* study were conducted to find out that Endoplasmic reticulum pathway is the dominant pathway for NDV in inducing apoptosis.

Introduction:

Apoptosis can be defined as a carefully regulated process, characterized by specific morphologic and biochemical features. It is initiated by both physiologic and pathologic stimuli, and its full expression requires a signaling cascade in which caspase activation plays a central role (1).

Apoptosis may be essential for the prevention of tumor formation, and its deregulation is widely believed to be involved in pathogenesis of many diseases, including cancer (2). In tumor pathogenesis and metastasis, defects in apoptosis are essential.

They allow tumor cells to overcome nutrient deprivation, absence of growth-stimulating signals, and presence of growth-inhibitory signals, surrounding tissue barriers, hypoxia, oxidative stresses, and host immune responses. Tumor cells survive beyond their physiologically intended

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Corresponding Address:

Ahmed M. Al-Shamery

Iraqi Center for cancer and medical genetics research,
Almustansiriya University.

Email: ahmed.majeed@iraqbiotech.com

lifespan, and accumulate genetic alterations that increase cell proliferation, angiogenesis, invasiveness, and interfere with differentiation.

Apoptotic defects are required to complement proto-oncogene activation, as many oncoproteins that increase cell proliferation also trigger apoptosis (3). There are three major caspase-dependent apoptotic pathways: 1. Death Receptor-mediated Apoptosis (The Extrinsic Pathway): Cell surface death receptors are members of the tumor necrosis factor receptor (TNFR) superfamily and transmit their apoptotic signals following binding of specific death ligands. The most extensively studied members of the TNFR superfamily are Fas and TNFR1 (4).

The upstream caspase in the death receptor-mediated pathway is caspase-8, which triggers apoptosis by activating downstream caspases, including caspase-3 (5). Complicating things is the observation that activated caspase-8 can trigger the intrinsic pathway by cleaving the BH3 protein, Bid, which translocates to mitochondria to induce cytochrome c release. Thus, extrinsic pathways have been further subclassified as "type I" or "type II" based on whether induction of apoptosis requires this so-

called mitochondrial amplification loop (as it does in type II cells) (6).

2. The Mitochondrial Pathway of Apoptosis (intrinsic pathway): The mitochondria-associated apoptotic pathway is initiated following damage to the mitochondria due to several anti cancer drugs (7). Damage to the mitochondria is mediated by proapoptotic members of the Bcl-2 family, including Bax, Bad, and Bid.

Under normal conditions, the antiapoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-XL, stabilize the mitochondria, inhibiting the efflux of cytochrome c, thereby preventing apoptosis induction. However, under apoptotic conditions, proapoptotic Bcl-2 family members destabilize the mitochondria, resulting in loss of the mitochondria membrane potential ($\Delta\Psi_m$) and release of cytochrome c (8). Cytochrome c then interacts with APAF-1, also released from damaged mitochondria, and procaspase-9, forming a complex known as the apoptosome. Procaspase-9 is cleaved into the active caspase-9, which activates caspase-3, thereby inducing apoptosis (9). 3. The endoplasmic reticulum (ER) pathway: This pathway is triggered by ER stress and involves activation of the upstream caspase, caspase-12. Caspase-12 subsequently activates downstream executioner caspases, including caspase-3 and -7, which induce apoptosis (10). Rao and co-workers (11) proposed that any cellular insult that causes prolonged ER stress may induce apoptosis through caspase-7-mediated caspase-12 activation. Caspase-12 is a recently described initiator caspase. Once activated, it induces the cleavage of caspase-3 in a cytochrome c-independent manner (12, 13).

Endoplasmic reticulum pathway can be linked to the mitochondrial pathway; the initial insult that caused the stress, results in efflux of calcium from the ER into the mitochondria, due to UPR signalling may also result in activation of additional, non-mitochondrial signalling pathways.

Increased calcium levels in the mitochondria stimulate release of pro-apoptotic molecules like Cytochrome c and Apaf-1 form the apoptosome to process procaspase 9, resulting in downstream caspase 3 cleavage in both the cytosol and ER lumen. Activated caspase 3 can induce loss of ($\Delta\Psi_m$), potentiating further cytochrome c release. One potential function of active caspase 3 in the ER might be processing of procaspase 12 bound to the membrane to amplify processing of caspases 9 and 3 in the cytosol (10).

Newcastle disease virus is interesting oncolytic agent with promising anti-tumor properties. One of the major anti-tumor properties is apoptotic induction. Fabian et al. (14) found vaccine strain MTH-68/H to cause internucleosomal DNA fragmentation on phaeochromocytoma cells which is the most characteristic feature of programmed cell death and 30 minute exposure sufficient. The apoptosis induction was dose-dependant manner (15).

Apoptotic process induced by MTH-68/H does not depend on p53. Apoptosis was accompanied by virus replication in

tumor cell lines tested and signs of endoplasmic reticulum stress were also detected in transformed cells (16). Elankumaran et al. (17), demonstrate that NDV triggers apoptosis by activating the mitochondrial/intrinsic pathway and that it acts independently of the death receptor/extrinsic pathway.

NDV is likely to act primarily through the mitochondrial death pathway. NDV infection results in the loss of mitochondrial membrane potential and the subsequent release of the mitochondrial protein cytochrome c, but the second mitochondrion-derived activator of caspase (Smac/DIABLO) is not released. This experiment aimed to investigate the ability of Iraqi strain of NDV to induce apoptosis both *in vitro* and *in vivo* and to give preliminary look about the main pathway of apoptosis that NDV induce.

Materials and Methods:

1. Ahmed-Mohammed-Nahi-2003 (AMN3) Cell Line:

This murine mammary adenocarcinoma cell line was derived from first *in vivo* passage for a spontaneous mammary adenocarcinoma of female BALB/c mice (18). Passages 75-78 of AMN3 cell line were used throughout this study and cells were maintained using RPMI-1640 with 5% FCS.

2. Ahmed Majeed-2003 (AM3) Transplantable mammary adenocarcinoma line:

This transplantable tumor line was established from Spontaneous murine mammary adenocarcinoma of aged female mouse that transplanted into immunosuppressed mice and successfully adapted for grown in immunocompetent mice for more than 38 passages *in vivo*. And used as animal tumor model in the development and testing of new anticancer agents in ICCMGR (18).

3. Experimental Animals:

Inbred female mice (8-10) weeks old, (20-25g) weight housed and maintained in ICCMGR animal house, with controlled conditions of temperature ($23 \pm 5^\circ\text{C}$). The animals were fed on special formula food pellets and given water ad libitum. Throughout the experiments, each five animals were housed in a plastic cage containing hardwood chip as bedding. The bedding was changed weekly to ensure a clean environment.

4. Virus Isolation and propagation:

Samples preparation and virus propagation:

Samples from suspected birds (liver, lung and trachea) (supplied by Poultry department /College of veterinary medicine / Baghdad University), were directly transferred to the lab in cooled box, cut and crushed. Measured amount of PBS with 10 fold antibiotic was added then centrifuged at 3000 rpm for 30 min.

The supernatant was injected (0.1ml) into 10days embryonated chicken eggs allantoic fluid. The egg was observed daily for mortality, immediately after the death of embryo, it was transferred to the refrigerator (4°C).

After 12-24hrs the allantoic fluid was collected by sterile syringe purified from debris by centrifugation (3000 rpm, 30 minute, 4C°). Then it dispensed into small tubes and stored at -20C°.

Virus Purification:

Virus was ultracentrifuged (50000xg, 60min, 4C°) by using (sorval ultracentrifuge, USA) the sediment was resuspended in PBS and purified over cushin Density gradient with 35% sucrose (BDH, England) was used with 97000 xg at 60min, 4C°. This was done twice. The virus was resuspended in PBS and stored at -196 C° (19).

Hemagglutination test:

Newcastle disease virus was quantified in which one hemagglutination unit (HAU) is defined as the smallest virus concentration leading to visible chicken erythrocyte agglutination.

5. Mitochondrial permeability transition apoptosis test:

The BioAssay™ Apoptosis Detection Kit (USbiological, USA) was fluorescent-based method for distinguishing between healthy and apoptotic cells by detecting the changes in the mitochondrial transmembrane potential. The kit utilizes a cationic dye that fluoresces differently in healthy cells and in apoptotic cells. In healthy cells, the dye accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, the dye cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and thus it remains in the cytoplasm in its monomer form, fluorescing green. The fluorescent signals can be easily detected by fluorescence microscopy using a band-pass filter (detects FITC and rhodamine).

Apoptosis induction *in vitro*:

Apoptosis induced in cells by infection with NDV and a control culture was incubated without induction. AMN3 cells were cultured in tissue culture slides (labtech – Denmark). After confluency (18-24 hrs) they were exposed for 2hrs to NDV at 256 HAU then washed and treated cells further incubated in 37C for another 24hrs. Control cells treated with Serum Free Media only. After the end of incubation 0.4 ml of the diluted MitoCapture solution was added to cultured cells and Incubated at 37C° for 15-20 min. Cells washed and 0.4 ml of the pre-warmed Incubation Buffer was added. Cells observed immediately under a fluorescence microscope using a band-pass filter (detects fluorescein and rhodamine).

MitoCapture that has aggregated in the mitochondria of healthy cells fluoresces red. In apoptotic cells, MitoCapture cannot accumulate in the mitochondria, it remains as monomers in the cytoplasm, and fluoresces green.

6. Transplantation of AM3 mammary adenocarcinoma Tumor Cells:

A method described by (18): Mice were anesthetized by intraperitoneal (I.P) injections of zylazine (40mg/kg) (laboratories Calier, Barcelona, Spain). The tumor mass region was well disinfected with 70% ethanol. Implantations of tumor tissue were carried out by aseptically aspirating

the subcutaneous tumors using needle gage 18.

The tissue fragments were placed immediately in sterile PBS and the tumor cells were allowed to settle down and the supernatant was discarded, and then the tumor fragments were resuspended in PBS at appropriate volume. Single cell suspension was made through mechanical disaggregation of the cells by vigorous pipetting.

Tumor suspension aspirated by syringe with needle gage 18 and inoculated with S/C injection of 10 x 10⁶ viable cells in 0.1ml cell suspension into shoulder region through puncture in thigh region.

7. Therapeutic Experiments on Mouse tumor model:

The above animal tumor model was divided to 5 animals per group starting at 10 days after tumor transplantation, day 1 was the day which measurements started which is 2 days before first injection: (this *in vivo* experiment repeated for 2 times)

Group One: Intratumoral injection (IT) with NDV (2 x 10⁷HAU) alone for a week, 2 days interval (4 IT injections).

Group Two: control injected with PBS only.

The experiments were ended 30 days after initiation of treatment, and the mice were sacrificed and tumor tissue samples of the treated and control groups were carefully dissected and fixed in 10% neutralized buffered formalin, paraffin embedded, and sectioned at 5µm thickness for histology and immunohistochemistry.

8. Histopathological samples processing:

The steps were followed according to (20) for tissue preparation, paraffin sections and carried out in Shandon automated histokinase system (Thermo, USA), they cut and marked and put in plastic box. Dehydration, embedding, sectioning and staining were done as described by Luna (20). The slides used for Immunohistochemistry were coated with gelatin, all slides kept in clean dry place until stained.

9. Apoptosis determination in tumor sections

For detection of apoptosis in tumor tissues, the following mAbs were used: Primary mAb: Rabbit anti-mouse caspase-12 (isotype IgG, concentration 0.02mg/ml) (USBiological, USA). Secondary antibody: Goat anti-rabbit IgG, H & L, X- adsorbed (Biotin), (concentration 2mg/ml) (USBiological, USA). Immunohistochemistry was performed according to USbiological recommended procedure were the slides rehydrated.

Antigen retrieval protocol for the tissue sections immunostained for caspase 12 was performed putting the slides in a closed jar containing antigen retrieval solution (10mM citrate buffer pH 6.0) then autoclaving at 121 °C for 2-5 minutes. The slides were allowed to cool in the antigen retrieval solution for 20-30 minutes at room temperature (20-25 °C). Then the slides were removed and placed in the washing buffer jar for 5 minutes.

After Peroxidase and Protein blocking treatment with primary antibody done by adding of 30-40 µl of diluted primary antibody onto each section. The slides were incubated in humid chamber for 2 hours at room

temperature (20-25 °C). Then the slides were washed and treated with biotinylated secondary antibody: where 30-40 μ l of diluted biotinylated secondary antibody was applied onto each section. The slides were incubated in humid chamber for 1 hour at room temperature (20-25 °C).

Then the slides were washed and Thirteen -40 μ l of diluted Avidin-HRP (1:500 in PBS) reagent was applied on each section. The slides were placed in the humid chamber and incubated at room temperature for 30 minutes. Then the slides were washed enough drops of freshly prepared DAB substrate mixture were applied to cover the tissue section. The slides were incubated in the humid chamber in dark for 20 minutes at room temperature (20-25 °C). Then the slides were rinsed gently with distilled water and Counterstained with Mayer's hematoxylin stain and dehydrated then mounted.

The quantitative scoring for assessment of caspase-12 staining was done by counting the number of positive and negative cells in several randomly selected fields in each

section. More than 1000 cells evaluated under 40X high power and the percentage of positive cells was graded.

3-3: Statistical Analysis

Repeated measures multivariate ANOVA test was used within the SPSS (2007) program to analysis of data to study the effect of group, time and concentration in the difference traits.

Results:

1. Mitochondrial Permeability Transition Apoptosis Test:

Newcastle disease virus infection induce apoptosis significantly when compared to control untreated cells, apoptotic green cells in infected mammary adenocarcinoma cells was dominant in the filed and live red cells in control untreated cells was dominant which can be seen in (figure-1a.b).

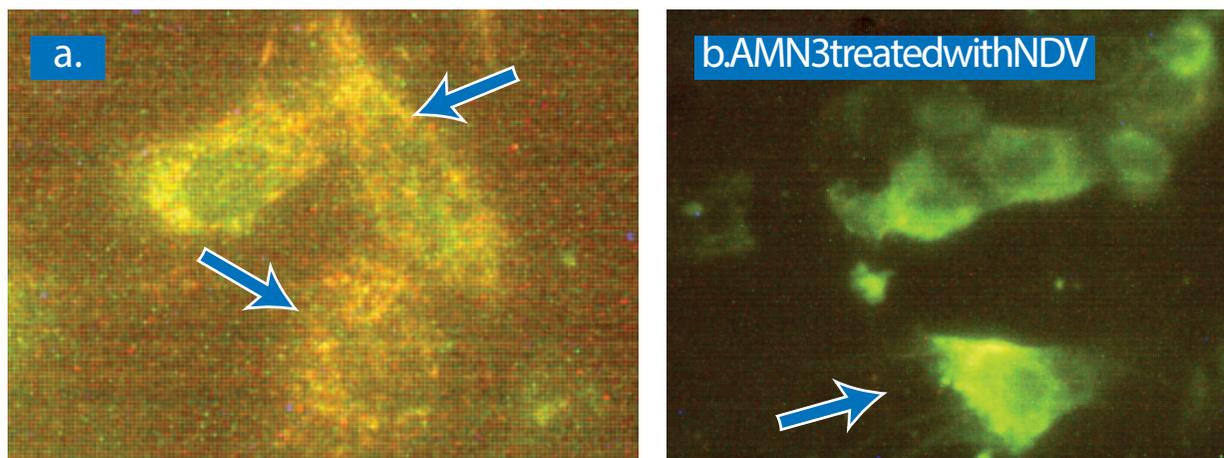


Figure-1a.b AMN3 cells were stained with MitoPT and viewed through a fluorescence microscope. Non-apoptotic cells exhibit red stained mitochondria (white arrow in b.). Apoptotic cells at varying stages of mitochondrial $\Delta\Psi$ appear green (yellow arrow) (in 1.a) after treatment with Newcastle disease virus for 24hrs.

2: Immunohistochemical Detection of Caspase-12 in Mammary Adenocarcinoma Tissues in Treated and Control Groups:

To assess apoptosis induced by treatment (endoplasmic reticulum pathway), caspase-12 protein expression was evaluated in tumor specimens by immunohistochemistry. As illustrated in table (1) and figure-2, the results revealed a significantly ($p < 0.05$) marked increase in the mean percentage of cells expressing caspase-12 in Newcastle disease virus treated group G1 compared with the untreated control group at day 7. At day 14 NDV treatment group

express caspase-12 significantly than the control group (G2). Immunohistological analysis at day 31 revealed that NDV treated group-G1 was more significant in inducing caspase-12 expression in compare to G2 control group. There was an increase in expression of caspase-12 after treatment ceased when measured at the end of the experiment. The results in general show that treatment with NDV induce apoptosis in tumor tissue, the results of frequency distribution of caspase-12 expression can be seen in figures-3 and 4. Cells expressed caspase-12 were brown.

Table (1): The mean percentage of caspase -12 expressions and the frequency distribution of expression scores in mammary adenocarcinoma tissues in treated and control mice.

| Type of treatment | Caspase-12 7 days mean | Caspase -12 14 days mean | Caspase -12 30 days mean |
|------------------------|------------------------|--------------------------|--------------------------|
| G1 $\mu\pm SD$ | 25 \pm 1.633A,a | 70 \pm 8.165 B,a | 75 \pm 12.247 B,a |
| G2 control $\mu\pm SD$ | 0 \pm 0.0 A,b | 10 \pm 4.082 A,e | 41.25 \pm 14.93 B,b |

Different small letter represents significant differences ($P\leq 0.05$) between means of the same column. Different capital letters represent significant differences ($P\leq 0.05$) between means of the same rows.

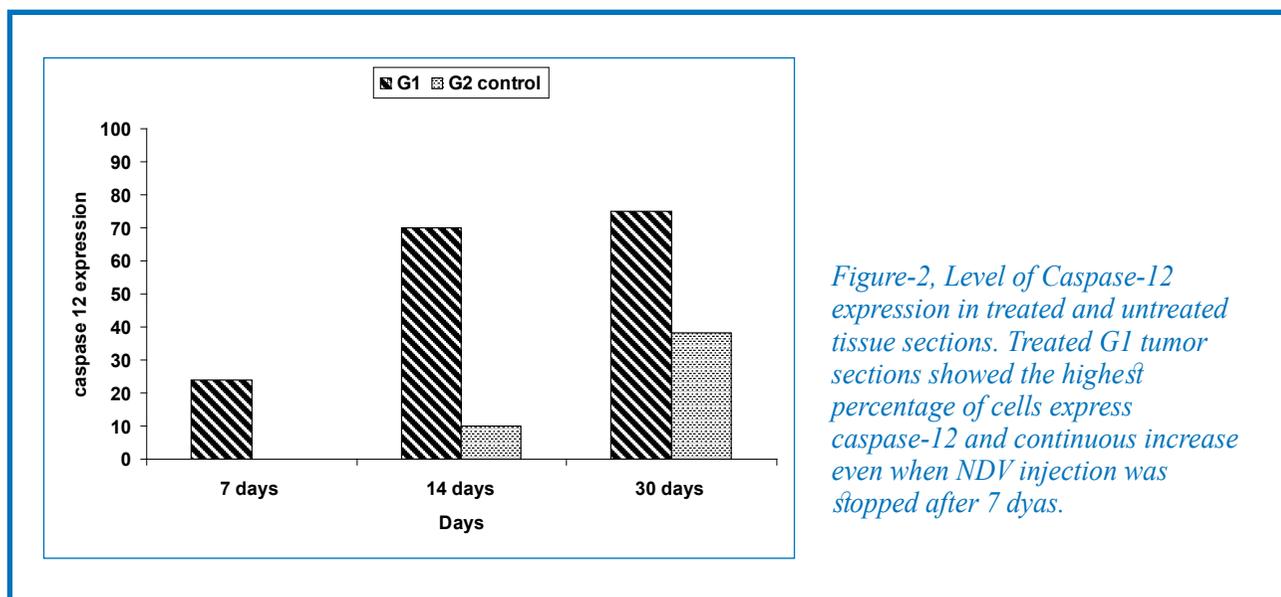


Figure-2, Level of Caspase-12 expression in treated and untreated tissue sections. Treated G1 tumor sections showed the highest percentage of cells express caspase-12 and continuous increase even when NDV injection was stopped after 7 days.

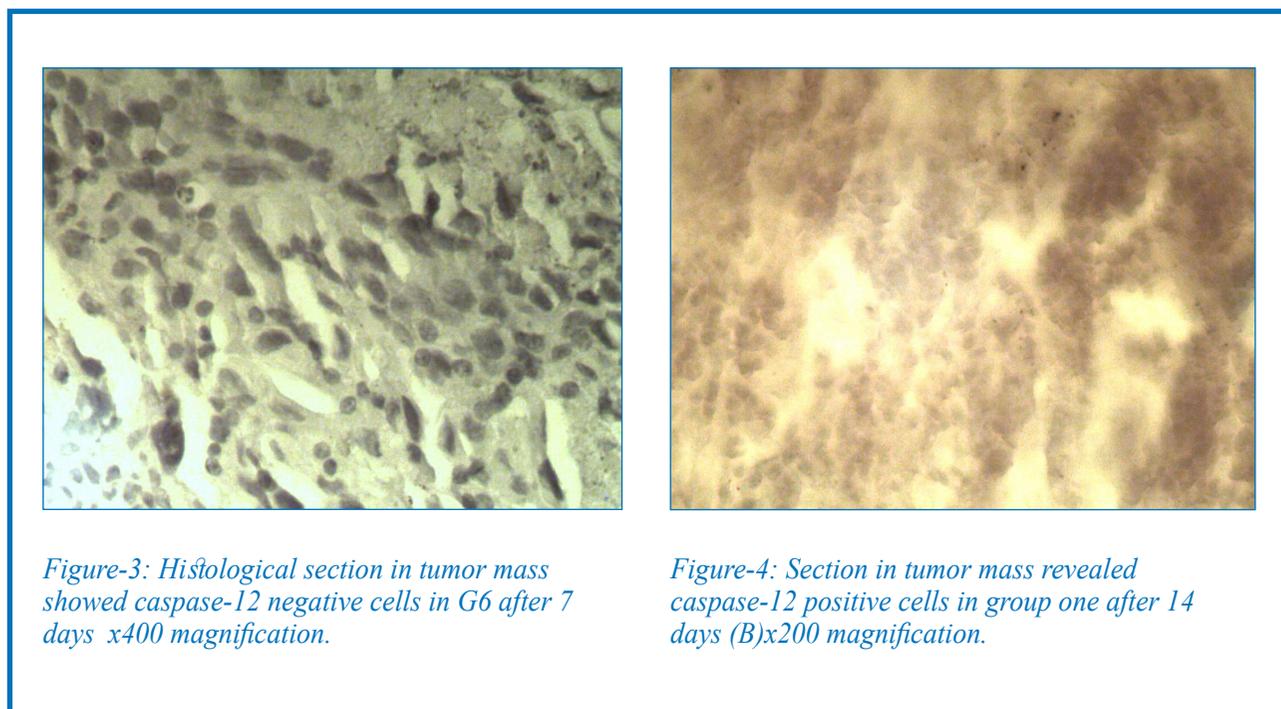


Figure-3: Histological section in tumor mass showed caspase-12 negative cells in G6 after 7 days x400 magnification.

Figure-4: Section in tumor mass revealed caspase-12 positive cells in group one after 14 days (B)x200 magnification.

Discussion:

The results revealed that NDV had powerful effect on inducing apoptosis in AMN3 mammary adenocarcinoma. Detection of the mitochondrial permeability transition event (PT) provides an early indication of the initiation of cellular apoptosis. Two studies (21, 22) described this process which typically defined as a collapse in the electrochemical gradient across the mitochondrial membrane, as measured by the change in the membrane potential ($\Delta\Psi$). Changes in the mitochondrial $\Delta\Psi$ lead to the insertion of proapoptotic proteins into the membrane and possible oligomerization of BID, BAK, BAX or BAD. This could create pores, which dissipate the transmembrane potential, thereby releasing cytochrome c into the cytoplasm (23, 24 and 25).

This event of mitochondrial pathway which tested in the present experiment has connection to the other apoptotic pathways: the death receptor-mediated pathway (extrinsic) and the endoplasmic reticulum (ER)-associated pathway. In regard to the death receptor-mediated pathway (extrinsic pathway), McConkey and Bondar (6) referred to activated caspase-8 (which activated due to surface receptor signals) can trigger the intrinsic pathway by cleaving the BH3 protein, Bid, which translocates to mitochondria to induce cytochrome c release. Masud with his team (10) linked Endoplasmic reticulum pathway to the mitochondrial pathway; the initial insult that caused the stress to the cell which induce ER apoptosis pathway, results in efflux of calcium from the ER into the mitochondria, Increased calcium levels in the mitochondria stimulate release of pro-apoptotic molecules like cytochrome c. Cytochrome c and Apaf-1 form the apoptosome to process procaspase 9, resulting in downstream caspase 3 cleavage in both the cytosol and ER lumen, activated caspase 3 can induce loss of ($\Delta\Psi_m$), potentiating further cytochrome c release.

One potential function of active caspase 3 in the ER might be processing of procaspase 12 bound to the membrane to amplify processing of caspases 9 and 3 in the cytosol (10).

The results showed that NDV alone induce apoptosis in AMN3 cells by induction the mitochondrial permeability transition event (PT) which is an early event of apoptosis led to a drop of ($\Delta\Psi_m$). Following a drop of ($\Delta\Psi_m$), cytochrome c can be released from mitochondria through the opened mitochondrial pores. Elankumaran et al. (17) investigated the localization of cytochrome c after NDV infection, and they found the level of cytochrome c in cytosol increased twofold after NDV infection to tumor cells. These results indicate that the intrinsic mitochondrial pathway is initiated after infection with NDV.

Fabian et al. (16) suggest that NDV-induced apoptosis involves inactivation of eIF2 α and activation of caspase-12 and caspase-3 but is independent of caspase-8 and caspase-9. In order to determine the apoptotic pathways involved in NDV-induced apoptosis, they observed Strong accumulation of cleaved caspase-3 after 10 h of incubation

in NDV-infected PC12 cells, but proteolytic activation of the initiator caspases caspase-8 and caspase-9 was not detected before the activation of effector caspase-3.

In contrast, activation and nuclear translocation of the endoplasmic reticulum enzyme caspase-12 correlated well with the accumulation of cleaved caspase-3.

Activation of caspase-12 suggests that NDV disturbs endoplasmic reticulum functions in virus-infected cells. In the present study to determine if ER stress is involved in NDV-induced cellular responses, the present study analyzed ER stress-related caspase-12 in tumor sections in NDV-treated groups, caspase-12 expression was high which confirm the previous results of Fabian et al. (16). With all explained results we suggest the following mechanism of apoptosis that induce cell death after infection of NDV: NDV disturbs endoplasmic reticulum functions in virus-infected cells results in efflux of calcium from the ER into the mitochondria, increased calcium levels in the mitochondria leading to drop of ($\Delta\Psi_m$) which opened mitochondrial pores to release cytochrome c. Caspase-12 which activated following to ER stress subsequently activates downstream executioner caspases, including caspase-3 and caspase-7 cleavage in both the cytosol and ER lumen, activated caspase 3 can induce loss of ($\Delta\Psi_m$), potentiating further cytochrome c release.

One potential function of active caspase 3 in the ER might be processing of procaspase 12 bound to the membrane to amplify processing of caspases 9 and 3 in the cytosol, Cytochrome c which was already released along with extra released due to amplified signal and Apaf-1 form the apoptosome to process procaspase 9, resulting in further amplified downstream caspase 3.

The downstream caspases (like caspase-3) induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins, and finally, destruction of "housekeeping" cellular functions. Caspases also affect cytoskeletal structure, cell cycle regulation, and signaling pathways, ultimately leading to the morphologic manifestations of apoptosis, such as DNA condensation and fragmentation, and membrane blebbing (26).

Fabian and co-workers (14) and Szeberenyi with his team (15) found NDV caused internucleosomal DNA fragmentation which is most characteristic feature of late event of programmed cell death. Yang and colleagues (27) reported induction of apoptosis by inactivated NDV, this report together with a Gaddy (28) result about M protein of VSV virus which induces apoptosis via the mitochondrial-associated pathway due to inhibition of host gene expression, can propose that one of NDV proteins can play role in apoptosis induction which needs more investigation.

Recent research by Yang and co-workers (29) on another oncolytic virus showed that oncolytic adenovirus induces mitochondrial apoptotic cell death. Newcastle disease virus treatment was effective modality in inducing apoptosis by increase expression of caspase-12, control group showed increased expression of caspase-12 at the end of the

experiment only due to the presence of necrotic area which cause hypoxic environment that induce stress apoptosis pathway of endoplasmic reticulum. While NDV – G1 induce high percentage of caspase-12 expression from the first 7days of treatment and kept increase indicating the

important role of NDV Iraqi strain in inducing apoptosis in cancer cell which is one of the major interesting feature that make NDV Iraqi strain is a good addition to antitumor arsenal.

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دراسة الموت المبرمج المحفز في انسجة سرطانة الغدد اللبئية المعالجة باستخدام العترة العراقية المحلية لفايروس النيوكاسل

احمد مجيد الشمري*، ناهي يوسف ياسين*، محمد جويد علوان**

* المركز العراقي لبحوث السرطان والوراثة الطبية / الجامعة المستنصرية
**كلية الطب البيطري / جامعة بغداد

الخلاصة:

في علاج الخلايا السرطانية سواءاً كانت الامراضية منها او الانتشار تعتمد على موت الخلايا المبرمج والذي يكون ضروري في عملية منع التغذية، الاشارات الخاصة بالنمو، بروز الاشارات المثبطة للنمو، احاطة الخلايا بحواجز، نقص اقبال الاوكسجين، الاكسدة الاجهادية والاستجابة المناعية للمضيف كلها عوامل تعمل على تثبط انتشار الخلايا السرطانية. ان فايروس النيوكاسل يعتبر من العوامل الواعدة كمضادات للسرطان لاحتوائه على خصائص مضادة للخلايا السرطانية، واحدة من اكبر هذه الخصائص هو الحث على الموت المبرمج للخلايا السرطانية. ان هدف هذه الدراسة هو معرفة آلية الموت المبرمج للخلايا في العترة المحلية لفايروس النيوكاسل داخل وخارج الزجاج، اذ تم دراسة الشبكة الاندوبلازمية الداخلية والتي تعتبر من العوامل الثابتة لفايروس النيوكاسل في الحث على الموت المبرمج.