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EFFECTS OF NEWCASTLE DISEASE VIRUS STRAINS AF 2240 AND V4-UPM ON CYTOLYSIS AND APOPTOSIS OF LEUKEMIA CELL LINES

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EFFECTS OF NEWCASTLE DISEASE VIRUS STRAINS AF 2240 AND V4-UPM ON CYTOLYSIS AND APOPTOSIS OF LEUKEMIA CELL LINES

By

AIED MOHAMMED AL-ABSI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

September 2008
Specially dedicated to

My beloved parents
My loving wife
My daughters
My brothers
My sisters
My country
Newcastle disease virus (NDV) is a member of the *Paramyxoviridae* that has caused severe economic losses in the poultry industry worldwide. Several strains of NDV were reported to induce cytolysis to cancerous cell lines. In this study, two NDV isolates namely, AF 2240 and V4-UPM were evaluated for their anti-leukemic properties against four leukemic cell lines - HL60 (Promyelocytic leukemia), WEHI 3B (Mouse myelomonocytic leukemia), CEMSS (Human T-lymphoblastic leukemia) and K562 (Erythromyeloblastic leukemia). The cytolytic effects of NDV strains AF 2240 and V4-UPM towards WEHI 3B, HL60, CEMSS and K562 cell lines were determined using microtetrazolium (MTT) assay. The cytolytic dose - fifty percent (CD50) for WEHI 3B, HL60 and CEMSS treated with AF 2240 strain were 2, 25, 16 HAU, respectively, while the CD50 for WEHI 3B, HL60 and CEMSS treated with V4-UPM were 8, 110 and 64 HAU, respectively.

Comparatively, both NDV strains showed very low cytolytic activity against K562 and non-leukemic cell lines namely, 3T3 (mouse fibroblasts), mouse lymphocytes
and human peripheral lymphocytes. Further studies were done to observe the morphological changes in the WEHI 3B treated cells using light, transmission and scanning electron microscopes. The apoptosis and necrosis were examined under fluorescence microscope, where the cells were stained with acridine orange (AO) and propidium iodide (PI). The treated cells with NDV strains AF 2240 and V4-UPM showed apoptotic features such as cell shrinkage, cell blebbing, and formation of apoptotic bodies compared to the control cells that did not express any features for apoptosis and necrosis. The early apoptosis was also observed under fluorescence microscope, where the cells were stained with Annexin V and PI.

The virus effect on cell proliferation was determined by MTT assay and BrdU techniques. Furthermore, at molecular level, both NDV strains caused internucleosomal DNA cleavage producing a multiple of 180-200 bp fragments, that were visible as a ladder on the agarose gel. Early apoptosis was also observed using Annexin V flow cytometry method. The percentage of apoptosis for WEHI 3B cells treated with NDV strains AF 2240 and V4-UPM had increased with time. Cell cycle and apoptosis were also determined using flow cytometry PI method. Both NDV strains were not able to arrest WEHI 3B at specific cell cycle phases using flow cytometry PI method.

In this study anti-leukemic activity of both NDV strains was evaluated on BALB/c mice induced leukemia with WEHI 3B cells. A day later they were treated with NDV strains AF 2240 and V4-UPM and arabinocytocine, a commercial drug, as positive control. The mice groups treated with arabinocytocine, NDV strains AF 2240 and V4-UPM showed significant killings (p<0.05) of leukemic cells compared to the
mice group without any treatment. The total white blood cell and percentage of blasts cell in the blood, bone marrow and spleen smears were significantly low (p<0.05) in mice treated with the arabinocytocine, NDV strains AF 2240 and V4-UPM compared to the mice group without treatment, that showed high number of leukemia. Spleen and liver weights were significantly low (p<0.05) in the mice groups treated with arabinocytocine, NDV strains AF 2240 and V4-UPM compared to the mice group without treatment, that showed significant (p<0.05) splenomegaly and hepatomegaly.

Histopathological studies carried out had confirmed haematological results. From the results obtained, the mice groups treated with both NDV stains AF 2240 and V4-UPM showed similar results as arabinocytocine, which is a commercial drug for leukemia. Immunoperoxidase staining, haemagglutination test and real time PCR were carried out to detect NDV in mice organs after treatment with NDV. The results showed no NDV particles were detected in the organs. This study showed that NDV strains AF 2240 and V4-UPM had caused cytolytic effects against WEHI 3B leukemia cell line in vitro and in vivo.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah.

KESAN VIRUS PENYAKIT NEWCASTLE STRAIN AF 2240 DAN V4-UPM TERHADAP SITOTOSIK DAN APOPTOSIS PADA SEL LEUKEMIA
Oleh
AIED MOHAMMED AL-ABSI

September 2008

Pengerusi: Profesor Abdul Manaf Ali, PhD
Fakulti: Bioteknologi dan Sains Biomolekul

Virus penyakit Newcastle (NDV) merupakan ahli Paramyxoviridae yang menyebabkan banyak kes kemelesetan ekonomi di industri penternakan di seluruh dunia. Beberapa strain NDV telah dilaporkan dapat mencetuskan sitolisis terhadap sel barah. Dalam pengajian ini, dua strain NDV yang diasingkan adalah AF2240 dan V4-UPM telah diuji kesan anti-leukemic terhadap empat jenis sel leukemia yang dinamakan HL60 (Promyelocytic leukaemia), WEHI 3B (myelomonocytic leukaemia tikus), CEMSS (T-lymphoblastic leukaemia manusia) dan K562 (Erythromyeloblastic leukaemia). Kesal sitotosik oleh NDV strain AF2240 dan V4-UPM terhadap sel WEHI 3B, HL60, CEMSS dan K562 telah ditentukan melalui analisa methyl thiazolyl tetrazolium (MTT). Kesal sitotosik lima puluh peratus (CD$_{50}$) untuk WEHI 3B, HL60 dan CEMSS yang dirawati dengan AF2240 adalah 2, 16, 35 HA unit masing-masing, manakala CD$_{50}$ untuk sel WEHI 3B, HL60 dan CEMSS yang dirawati dengan V4-UPM adalah 8, 32 dan 64 HA unit masing-masing.
Secara perbandingan, kedua-dua strain NDV menunjukkan kesan sitotoksik yang sangat rendah terhadap K562 dan sel bukan leukemia yang dinamakan, 3T3 (fibroblasts tikus), limfosit tikus dan limfosit pinggiran manusia. Kajian selanjutnya telah dijalankan untuk memperhatikan perubahan mofologi dalam sel WEHI 3B yang dirawati dengan menggunakan cahaya, pancaran dan imbasan elektron mikroskop. Sifat morfologi, apoptosis dan nekrosis juga diperiksa dengan mikroskop pendarfluor di mana sel diwarnai dengan akridin oren (AO) dan propidium iodida (PI). Sel yang dirawati dengan NDV strain AF 2240 dan V4-UPM menunjukkan sifat apoptotik seperti kecutan sel, blebbing sel, pembentukan jasad apoptotik dan sel nekrotik berbanding dengan sel pengawal negatif tidak menunjukkan sebarang sifat apoptosis dan nekrosis. Apoptosis awal juga diperhatikan melalui mikroskop pendarfluor di mana sel telah diwarnakan dengan Annexin V dan PI.

Kesan virus terhadap pembelahan sel telah dikenal pasti dengan teknik MTT dan BrdU. Di samping itu, dari segi molekul, kedua-dua strain virus ini dapat menyebabkan pemotongan DNA internukleus untuk menghasilkan pelbagai serpihan 180-200 bp yang dapat dilihat sebagai tangga pada gel agarosa. Awal apoptosis juga diperhatikan melalui Annexin V kaedah aliran sitometri. Peratus apoptosis untuk sel WEHI 3B yang dirawati dengan NDV strain AF2240 dan V4-UPM telah meningkat dari semasa ke semasa. Kitar sel dan apoptosis juga ditentukan melalui kaedah aliran sitometri PI. Kedua-dua strain NDV tidak dapat menahankan WEHI 3B pada fasa kitar sel yang tertentu dengan menggunakan kaedah aliran sitometri PI.
Dalam kajian ini aktiviti anti-leukemik pada kedua-dua strain NDV telah dikaji dengan tikus BALB/c yang telah dicetuskan leukemik dengan sel WEHI 3B. Pada hari keesokan mereka telah dirawati dengan NDV strains AF2240 dan V4-UPM dan arabinositosin sebagai ubat kormesial yang dijadikan pengawal positif. Kumpulan tikus yang dirawati dengan arabinositosin, NDV strain AF 2240 dan V4-UPM, masing-masing menunjukkan kesan pembunuhan yang ketara (p<0.05) terhadap sel leukemic berbanding dengan tikus untuk kumpulan tanpa sebarang rawatan. Jumlah sel darah putih dan peratus sel blast di sapuan darah, sum-sum tulang dan limpa adalah rendah (p<0.05) di tikus yang dirawati dengan NDV strain AF 2240 dan V4-UPM, masing-masing, berbanding dengan kumpulan tikus tanpa rawatan yang menunjukkan nombor leukimia yang tinggi. Keberatan limpha dan hati adalah rendah secara ketara (p<0.05) di kumpulan tikus yang dirawati dengan arabinocytocine, NDV strain AF 2240 dan V4-UPM, masing-masing, berbanding dengan kumpulan tikus tanpa sebarang rawatan yang menunjukkan spleenomegaly dan hepatomegaly yang ketara (p<0.05).

Kajian histopatologikal juga dijalankan dan mengesahkan keputusan hematologikal. Daripada keputusan yang diperolehi, kumpulan tikus yang dirawati dengan kedua-dua NDV strain AF 2240 dan V4-UPM menunjukkan keputusan yang seiras dengan arabinocytocine yang merupakan ubat kormesial untuk leukemia. Pewarna immunoperoxidase, analisa hemaggulatin dan real time PCR telah dijalankan untuk mengesan NDV di organ tikus setelah dirawati dengan NDV. Keputusan telah menunjukkan tiada zarah NDV dikesan di organ tikus. Kajian ini menunjukkan NDV
strain AF 2240 dan V4-UPM menyebabkan kesan sitolitik terhadap sel leukemia WEHI 3B secara in vitro dan in vivo.
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In the name of Allah, the most gracious, the most merciful

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Last but not least, I would like to express my deepest gratitude to my beloved parents, wife, sisters and brothers for their endless encouragement, patience and sacrifices which had helped me throughout my student life.
I certify that a Examination Committee has met on 22 September 2008 to conduct the final examination of Aied Mohammed Al-Absi on his Doctor of Philosophy thesis entitled “Cytolytic Effects and Apoptosis Induction of Newcastle Disease Virus Strains AF 2240 and V4-UPM on Leukemia Cell Lines In vitro and In vivo” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Doctor of Philosophy. Members of the examination committee were as following:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the supervisory committee were as follows:

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Date: 19 December 2008
DECLARATION

I declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at UPM or at any other institutions.

________________________
AIED MOHAMMED AL-ABSI

Date: 26 February 2009
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<td>3.8</td>
<td>Percentage of cell viability of HL60 cells treated with NDV V4-UPM virus strain after 72 hrs.</td>
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<tr>
<td>3.9</td>
<td>Percentage of cell viability of 3T3 treated NDV AF 2240 and V4-UPM virus strains after 72 hrs.</td>
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<tr>
<td>3.10</td>
<td>Percentage of cell viability of mouse spleen lymphocyte cell treated with NDV AF 2240 and V4-UPM virus strains after 72 hrs.</td>
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<tr>
<td>3.11</td>
<td>Percentage of cell viability of peripheral blood cell treated with NDV AF 2240 and V4-UPM virus strains after 72 hrs.</td>
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<tr>
<td>3.12</td>
<td>Percentage of cell viability of HL 60, CEMSS, WEHI 3B, 3T3, K562, and human lymphocyte blood cells treated with Doxorubicin after 72 hrs.</td>
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</table>
3.13 Percentage of cell viability of HL 60, CEMSS, WEHI 3B, K562, human lymphocyte blood cell and 3T3 cells treated with arabinocytocine after 72 hrs.

3.14 MTT proliferation assay, effect of different concentrations (CD_{50} and CD_{75}) of NDV AF 2240 virus strain on the proliferation of WEHI 3B cell line at 24, 48 and 72 hrs post-inoculation.

3.15 The percentage of viable and non-viable WEHI 3B cells in the population after treatment with CD_{75} value of NDV AF2240 virus strain at various time intervals.

3.16: The percentage of viable and non-viable WEHI 3B cells in the population after treatment with CD_{50} value of NDV AF2240 virus strain at various time intervals.

3.17 MTT proliferation assay, effect of different concentrations (CD_{50} and CD_{75}) of NDV V4-UPM virus strain on the proliferation of WEHI 3B cell line at 24, 48 and 72 hrs post-inoculation.

3.18 The percentage of viable and non-viable WEHI 3B cells in the population after treatment with CD_{75} value of NDV V4-UPM virus strain at various time intervals.

3.19 The percentage of viable and non-viable WEHI 3B cells in the population after treatment with CD_{50} value of NDV V4-UPM virus strain at various time intervals.

3.20 BrdU Proliferation assay, effect of different concentrations (CD_{50} and CD_{75}) of NDV AF 2240 virus strain on the proliferation and viability of WEHI 3B cell line at 24, 48 and 72 hrs post-inoculation.

3.21 BrdU Proliferation assay, effect of different concentrations (CD_{50} and CD_{75}) of NDV V4-UPM virus strain on the proliferation and viability of WEHI 3B cell line at 24, 48 and 72 hrs post-inoculation.

4.1 Agarose-gel-electrophoretic patterns showing DNA fragmentation of WEHI 3B cells treated with NDV strain AF 2240 at CD_{50} (2 HAU).

4.2 Agarose-gel-electrophoretic patterns showing DNA fragmentation of WEHI 3B cells treated with NDV strain V4-UPM at CD_{50} (8 HAU).
DNA fluorescence histograms of WEHI 3B cells treated with NDV strain AF 2240 at CD50 value (2 HAU) after staining with propidium iodide.

Density plots showing Annexin-V FITC staining of WEHI 3B cells treated with NDV strain AF 2240 at CD50 value (2 HAU)

Density plots showing Annexin-V FITC staining of WEHI 3B cells treated with NDV strain V4-UPM at CD50 value (8 HAU)

Experimental design of in vivo study

Amplification curve of SYBR Green I real time PCR detecting NDV strains AF 2240 and V4-UPM in the spleen, heart, liver and kidney of BALB/c mice.

Melting curve of SYBR Green I real time PCR detecting NDV strains AF 2240 and V4-UPM in spleen, heart, liver and kidney of BALB/c mice.

Percentage of necrotic, apoptotic and viable of WEHI 3B cells in the population after treated with CD50 of NDV AF2240 virus strain at different times.

Percentage of necrotic, apoptotic and viable of WEHI 3B cells in the population after treated with CD50 of NDV V4-UPM virus strain at different times.

Cell cycle analysis of WEHI 3B cell population treated with NDV strain AF 2240 after staining with propidium iodide.

Cell cycle analysis of WEHI 3B cell population treated with NDV strain V4-UPM after staining with propidium iodide.

Effects of NDV strain AF 2240 on apoptosis of WEHI 3B cells analysed by Annexin-V FITC staining flow cytometry.

Effects of NDV strain V4-UPM on apoptosis of WEHI 3B cells analysed by Annexin-V FITC staining flow cytometry.

The effects of Ara-C, NDV strains AF 2240 and V4-UPM on survival time of BALB/c mice inoculated with WEHI-3B leukemia cells.
B.2 The effects of Ara-C, NDV strains AF 2240 and V4-UPM on body weight of BALB/c mice inoculated with WEHI-3B leukemia cells

B.3 The effects of Ara-C, NDV strains AF 2240 and V4-UPM on the total leukocyte count in peripheral blood of BALB/c mice inoculated with WEHI-3B leukemia cells.

B.4 The effects of Ara-C, NDV strains AF 2240 and V4-UPM on the percentage of Blasts cells in peripheral blood of BALB/c mice inoculated with WEHI-3B leukemia cells.

B.5 The effects of Ara-C, NDV strains AF 2240 and V4-UPM on the percentage of blast cells in bone marrow of BALB/c mice inoculated with WEHI-3B leukemia cells for 30 days.

B.6 The effects of Ara-C, NDV strains AF 2240 and V4-UPM on the percentage of blast cells in spleen of BALB/c mice inoculated with WEHI-3B leukemia cells for 30 days.

B.7 The effects of Ara-C, NDV strains AF 2240 and V4-UPM on the spleen weight in the BALB/c mice inoculated with WEHI-3B leukemia cells for 30 days.

B.8 The effects of Ara-C, NDV strains AF 2240 and V4-UPM on the spleen length in the BALB/c mice inoculated with WEHI-3B leukemia cells for 30 days.

B.9 The effects of Ara-C, NDV strains AF 2240 and V4-UPM on the liver weight in the BALB/c mice inoculated with WEHI-3B leukemia cells for 30 days.