UNIVERSITI PUTRA MALAYSIA

CYTOLYTIC EFFECT OF NEWCASTLE DISEASE VIRUS STRAIN V4 (UPM) ON LEUKEMIC CELL LINES CEM-SS AND HL 60

MADIHAH ZAWAWI.

IB 2007 10

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By

MADIHAH ZAWAWI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Masters Science

January 2007

Dedicated to

my daughter, ***Syaima'***
&
my husband, ***Ahmad Shauki***

with love

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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December 2006

Chairman : Professor Abdul Manaf Ali, PhD

Institute : Bioscience

Newcastle disease virus (NDV) was classified into the order *Mononegalavirales,* family *Paramyxoviridae*, sub-family *Paramyxovirinae* and genus *Avulavirus*. The genome consists of a single stranded, non-segmented, enveloped negative sense RNA which consists of about 15 kb, encoding six viral proteins which are the phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), polymerase (L) and nucleoprotein (NP). NDV causes a highly contagious, generalized virus disease of domestic poultry and wild birds but only mild conjunctivitis and laryngytis in humans. Inoculation of live NDV strain V4(UPM), a local heat resistant variant of the Queensland vaccine strain, V4HR, showed visible cytolytic effects on CEM-SS and HL-60 leukemic cells. Therefore, three approaches were taken to study the effect of V4 (UPM) against the two leukemic cell lines which are via morphological observation, cytopathic effect and biochemical study. The morphological changes observed via inverted light microscopy include cell

shrinkage and blebbing of the cell membrane as well as membrane-bound apoptotic bodies. Results obtained from microtetrazolium cytotoxicity assay showed a titre of 110.6 and 150.9 HAU/ml of the virus reducing the cell population to 50% viability for HL 60 and CEM-SS, respectively. The virus affects cell proliferation in a way that it reduces viability abruptly at 24 hours postinoculation in HL 60 cell population while in CEM-SS cell population proliferation was inhibited almost immediately after inoculation. Morphological observation using the differential uptake of acridine orange and propidium iodide dyes showed the cells were undergoing apoptosis. The early apoptotic cells which which had intact membranes but have started to fragment their DNA, still had green cytoplasm and nuclei but condensation of the chromatin were visible as bright green patches at the brim of the nucleus membrane. Invagination of plasma membrane or blebbing appearance on the cell surface was also apparent. Late apoptosis showing bright red cells surrounded with apoptotic bodies were also observed in cell populations inoculated with the virus. The DNA of infected CEM-SS and HL 60 cells produced a DNA laddering profile on agarose-gel electrophoresis, a biochemical marker which is frequently regarded as the biochemical hallmark of apoptosis. Electron microscopy also confirmed the morphological structures indicating apoptosis was involved in the death of cells treated with the virus. In conclusion, based on the findings of these experiments, the mechanism by which live NDV strain V4(UPM) can induce cytolysis in CEM-SS and HL-60 cells is via apoptosis. Thus, it may be possible to further develop V4(UPM), a local oncolytic NDV vaccine strain, for the future choice of treatment in cancer patients.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN APOPTOTIK OLEH STRAIN V4(UPM) VIRUS NEWCASTLE DISEASE KE ATAS SEL-SEL LEUKEMIA CEM-SS AND HL 60

Oleh

MADIHAH ZAWAWI

Disember 2006

Pengerusi : Profesor Abdul Manaf Ali, PhD

Institut : Biosains

Virus Newcastle disease atau singkatannya, NDV, telah diklasifikasikan didalam order *Mononegalavirales,* famili *Paramyxoviridae*, sub-famili *Paramyxovirinae* and genus *Avulavirus* adalah sangat mudah berjangkit dan menyebebkan kematian serta kerugian dalam industri penternakan ayam dan burung di serata dunia. Genom NDV terdiri dari rantaian RNA yang bersense negative, tidak bersegmen serta berselaput. Genom ini mempunyai lebih kurang 16000 base yang mengkodkan 6 protein iaitu phosphoprotein (P), protein matrix (M), protein fusion (F), protein hemagglutinin-neuraminidase (HN), polymerase (L) dan nukleoprotein (NP). Strain V4(UPM) pada asalnya adalah strain vaksin Queensland V4HR yang telah diadaptasi untuk kesesuaian ternakan di Malaysia. Tiga pendekatan diusulkan untuk mengkaji kesan strain V4(UPM) iaitu melalui perubahan morfologi, kesan sitopatik dan kesan biokimia. Perubahan morfologi yang didatakan adalah pengecutan sel serta terbentuknya benjulan-benjulan di atas permukaan membran sel. Assai sitotoksik menggunakan garam MTT memberikan bacaan $TCID_{50}$ 110.6

HAU/ml bagi sel-sel HL 60 dan 150.9 HAU/ml bagi sel-sel CEM-SS. Virus ini mengakibatkan pengurangan peratusan bilangan sel-sel hidup dalam populasi sel HL 60 secara mendadak 24 jam selepas inokulasi. Manakala peratusan bilangan sel-sel hidup dalam populasi sel CEM-SS menurun hampir serta merta selepas inokulasi. Pengamatan yang lebih mendalam dari segi morfologi menggunakan prinsip penerapan membrane yang berbeza dua pewarna fluoresen akridin jingga dan propidium iodide menunjukkan sel-sel yang diinfeksi melalui proses apoptosis. Pengenal pastian sel-sel CEM-SS dan HL 60 terinfeksi yang telah diwarnai mengalami apoptosis pada peringkat awalan ialah pewarnaan sitoplasma dan nucleus dengan warna hijau serta hijau terang di pergigian membran nukleus seperti bulan sabit serta terhasil benjulan pada permukaan membran sel. Apoptosis peringkat akhiran pula, menunjukkan sitoplasma sel berwarna merah terang serta dikelilingi badan-badan apoptosis yang kecil. Apabila DNA sel-sel CEM-SS dan HL 60 yang telah di inokulasikan dengan virus diekstrak dan dijalankan elektroforesi agar, profil seperti tetangga DNA yang terbelah secara spesifik terhasil, iaitu salah satu daripada ciri penentu apoptosis secara biokimia. Pengamatan melalui mikroskop elektron juga mengesahkan lagi pemerhatian bahawa sel-sel tersebut mengalami apoptosis. Laporan saintifik terdahulu telahpun mendatakan bahawa beberapa strain NDV yang lain telah mampu mengecutkan sel barah manusia yang telah diinfeksikan ke atas tikus (xenograf) serta menyembuhkan pesakit barah kronik. Maka kesimpulannya, melalui kajian-kajian yang telah dijalankan ini, NDV strain vaksin tempatan V4(UPM) mampu mencetus pemusnahan sel leukemia CEM-SS dan HL 60 secara apoptosis dan berpotensi untuk dimajukan sebagai agen anti-barah pilihan pada masa yang akan datang.

ACKNOWLEDGEMENTS

بسم الله الرحمن الرحيم

Above all الحمدالله رب العالمين , I am thankful to Allah the Almighty, for all His blessings granted upon me. Without His will, I would not have been able to even start this in the first place.

I would like to express my deepest gratitude to Professor Dr. Abdul Manaf Ali for his priceless trust and guidance. To Professor Dr. Aini Ideris and Datin Professor Dr. Khatijah Mohd. Yusoff, my utmost thanks for their support as my committee members throughout my study years. May Allah Bless them all.

My appreciation and thanks goes to:

Majlis Kanser Nasional (MAKNA) for the financial support of the project.

Associate Professor Abdul Rahman Omar and his staffs at Biologic lab, Dr. Jaffri Malin and his staffs at USM Kubang Kerian Kelantan, Associate Professor Dr. Fauziah and her staffs at Unit Microscopy IBS, Associate Professor Dr. Raha Abdul Rahim, Dr Salman Hussein and Dr. Muhajir as well as all the staffs at IBS administration office for their advices, contributions and kind assistance during my study .

Senior members of ATCL; Dr. Shuhaimi, Dr. Anthony Ho, Dr. Lim Yang Mooi, Dr. Majid Eshagi, Dr. Siti Norlasiah for their advices, contribution and moral

support both at work and play. Dr. Noorjehan and Dr. Radziah whom have been my mentors and friends and to countless others whom I haven't mentioned here that have contributed in one way or another. I thank you all.

My colleagues and friends Tan Boon Kiat, Kee Cheng Ling, Kak Izan, Kak Asmah, Rohaya Ibrahim, Aida, Ainul, Ana, Mashitoh, Hasrol, Faridah, Rohaya Wahab, Zarina, Shakira, Ummi Kalthum, Safdi and Rozita. Knowing them have made the duration of my study more meaningful and durable. May they be safe and happy, wherever they may be.

My in laws, for being very understanding and supportive. May this be an inspiration to all my sisters, brothers and cousins especially Lutfiah, Fakhriah, Farhana and Tarmizi to realize all their dreams. Remember that nothing is impossible with Allah's will.

And lastly, but definitely not least, to my family; My loving parents, Faridah Ahmad & Zawawi Ahmad. My sister, kak Majdah (you're the best sis ever!!) and abang Amim and my lovely nieces, Inshirah & Asiah. Thank you for being the wind beneath my wings. My brother, Fauwaz who has listened to me patiently and stood by me. My husband, Ahmad Shauki Hj Ibrahim, little Kashif and little Syaima' for their huge sacrifices, support and love.

To all of them, I am forever gratefull and give *my* unconditional love.

I certify that an Examination Committee has met on 15th December 2006 to conduct the final examination of Madihah binti Zawawi on her Master of Science thesis entitled "Cytopathic Effect of Newcastle Disease Virus Strain V4(UPM) on Leukemic Cell Lines CEM-SS and HI-60" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Sabariah Abdul Rahman, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Siti Suri Arshad, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Rozita Rosli, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Mohd. Nizam Hj. Isa, PhD
Professor
Faculty of Medicine
Universiti Teknologi MARA
(External Examiner)

HASANAH MOHD. GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 3 August 2007

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

Abdul Manaf Ali, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Aini Ideris, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

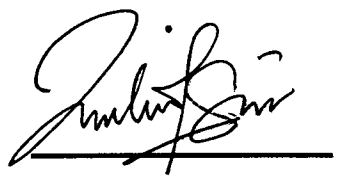
Khatijah Mohd Yusoff, PhD
Professor/Dean
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 9 August 2007

DECLARATION

I hereby 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 or concurrently submitted for any other degree at UPM or other institutions.



MADIHAH ZAWAWI

Date: 12 December 2006

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBRIEVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
AO	acridine orange
APMV	avian pneumonia m virus
CAM	complementary and alternative medicine
Ca^{2+}	calcium ion
CD_{50}	cytotoxic dose resulting in 50% reduction of cell population
CGM	complete growth medium
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CTL	cytotoxic T lymphocyte
CO_2	carbon doxide
ddH_2O	double distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ED	effective dose
ECACC	European Collection of Cell Cultures
EDTA	ethylenediaminetetraacetate
F	fusion protein
FBS	fetal bovine serum
HA	haemagglutination
HCl	hydrochloric acid

HN	hemagglutinin-neuraminidase
IL	interleukin
INF	interferon
IU	inhibition unit
KCl	potassium chloride
KH_2PO_4	potassium hydrophosphate
L	large protein
M	matrix protein
Mg^{2+}	magnesium ion
MAKNA	Majlis Kanser Nasional
MHC	major histocompatibility complex
MTT	3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium Bromide
N	nuclear protein
NaCl	sodium chloride
$NaHPO_4$	sodium phosphate
NDV	Newcastle disease virus
NIPC	natural interferon producing cells
NTE	NaCl-Tris HCl-EDTA buffer
NSF	N-ethyl maleimide-sensitive fusion protein
OD	optical density
P	phospho protein
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PI	propidium iodide

PS	phosphotidylserine
RBC	red blood cell
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	scanning electromicroscopy
SNARE	SNAP receptor
SNAP	Soluble NSF-attachment proteins
TE	Tris-EDTA buffer
TBE	Tris-base-EDTA buffer
TCID	tissue culture inhibition dose
TGF	tumor growth factor
TNF	tumor necrosis factor
UPW	ultrapure water
UV	ultraviolet
VAMP	vesicle associated membrane protein
VEGF	vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Newcastle disease virus (NDV) belongs to the *Avulavirus* genus, *Paramyxovirinae* sub-family *Paramyxoviridae* family and *Mononegalavirales* order (Mayo 2002a,b; Meyyappan, 2003; Wali, 2003). NDV was formerly known as APMV-1 and is a veterinary virus which infects chicken among other avian hosts, causing economic losses (http://www.epix.hazard.net/topics/animal/newcastl.htm). Although NDV causes a potentially fatal, respiratory disease (Newcastle disease) in birds, it causes only minor illness in humans. Several reviews have documented the mild to moderate side effects in humans exposed to NDV vaccines which are mild flu-like symptoms, conjunctivitis, and laryngitis (Alexander and Allen, 1974; Csatary et al., 1993; Kirn and McCormick, 1996; Nelson 1999; Sinkovics and Horvath, 2000; Omar *et al.*, 2002). Replication of NDV appears to be substantially better in human cancer cells than it does in most normal human cells (Reichard *et al.*, 1992). In numereous clinical trials, NDV-based immunotherapy therapy has been reported to be of benefit to the patients (Mobus et al., 1993; Csatary et al., 1993; 1999; Zorn *et al.*, 1997; Ockert *et al.*, 1996; Ahlert *et al.*,1997; Pecora *et al.*, 2002) .

Historically, Wheelock and Dingle were the first to report positive results using NDV in the treatment of an acute leukemic patient in 1964 (http://www.nci.nih.gov/cancerinfo/ pdq/cam/NDV). Since then many other NDV

strains were used in clinical trials to treat various human cancers. Among the NDV strains used for these clinical trials were Cassel's 73-T, MTH-68, Ulster, Hickman and PV701 (Sinkovics and Horvath 1993; 2000; Omar *et al.*, 2002). Administration of NDV in these clinical trails were in the form of oncolysates (73T) which were prepared from virus-infected cancer cells comprising of cell membrane fragments and viral and cancer cell proteins, whole cell vaccines (Ulster) which was prepared using autologous tumour cells infected with virus and infection of the patient with the virus itself (73T, MTH-68, Hickman and PV701).

The V4 strain was isolated and identified by G.Simmons in 1966 in Queensland, Australia. Being avirulent and able to induce immunogenic reactions, a thermostable and heat resistant vaccine strain known as V4HR was developed as a vaccine strain which could be administered orally by mixing onto chicken feed or other routes such as intranasal spray for commercial and village chickens (Spradbrow and Samuel, 1991). V4(UPM) is a heat tolerant variant of this vaccine strain and used on local chickens since 1985 (Aini *et al.*, 1986) .

Problem Statement and Hypothesis

NDV researchers have realized that it is crucial to determine the mode of action exerted by the virus as with any potential anticancer agent. Based on the history of the ability of a variety of international NDV strains which excelled as an oncolytic agent, a preliminary research was undertaken using V4(UPM) strain as a potential oncolytic agent in order to prove that it is able to induce

apoptotic cell death. An *in vitro* study of the effects of V4(UPM) or any other local NDV strains on acute leukemic cell lines have never yet been done. This has led to the design of the experimental approach implemented in this study. Both cellular and molecular changes in treated cell populations will be examined and compared with untreated populations in a time course manner. Hence, either necrotic or apoptotic cell death could be suggested.

Objectives

The main objective of the project is to establish whether the local NDV vaccine strain V4(UPM) is a suitable candidate to be developed as an anticancer agent by inducing apoptotic cell death. This preliminary study mainly focused on the evaluation of the *in vitro* effects of the virus strain on commercially established leukemic cell lines CEM-SS and HL 60. Experiments designed in this study were mainly to determine the mode of cell death *in vitro* via morphological, ultrastructural and biochemical studies.

Therefore, this study was undertaken to:

1. Assess the *in vitro* effects of the virus strain on commercially established leukemic cell lines CEM-SS and HL 60;
2. Determine the mode of cell death induced by NDV strain V4 (UPM) in leukemic cell lines.

Study Outline

Three steps of the study were used to achieve the objectives as stated above. The first step was the screening and preparation of the virus of choice and cell

lines to be used. A screening process of various types of cancereous cell lines was carried out to determine which cell line was to be used for this study, while several types of NDV strians were screened for the study. Cytotoxic assays using MTT was the chosen method for all screening purposes.

It was then determined that two leukemic cell lines, HL 60, an acute promyelocytic leukemia cell line, and CEM-SS, a T lymphoblastic leukemia cell line would be used in the study. The virus strain of choice was also determined to be NDV strain V4 (UPM), a heat stable vaccine strain. In the subsequent stage, determination of the suitable dose which is the ability of the virus to inhibit 50% of tissue culture proliferation ($TCID_{50}$), for the treatment of the cancereous cell lines was carried out. The determined dose was used throughout the rest of the study.

Finally the morphology, cytopathic effects and biochemical analysis of the treated cell were determined. Morphological studies included observations through inverted light microscopy, fluorescence microscopy and scanning electromicroscopy (SEM). Cytopathic effects of the virus against the selected cell lines was done through proliferative assays and scoring of viable, apoptotic and necrotic cells. In the biochemical analysis the DNA content of treated and untreated cells were compared.

CHAPTER II

LITERATURE REVIEW

Newcastle Disease Virus

In a review by Spradbrow, Newcastle disease (ND) was the name given by Doyle to a highly contagious viral infection of poultry in an outbreak on a farm near Newcastle upon Tyne in 1926. At about the same time, Kranveld observed similar symptoms in Jakarta, Indonesia (Spradbrow, 1987; Seal *et al.*, 2000). The virus was classified into the order *Mononegalavirales*, family *Paramyxoviridae*, sub-family *Paramyxovirinae*. Initially, NDV was considered as the prototype for the genus *Paramyxovirus* but was placed within the genus *Rubulavirus* in 1993 (Rima *et al.,* 1995; deLeeuw and Peeters, 1999; Seal *et al.,* 2000; Yusoff and Tan, 2001). It is now placed within the genus *Avulavirus* by the International Committee on the Taxanomy of Viruses (Mayo, 2002a,b; Meyyappan, 2003; Wali, 2003). The virus is a membrane-enveloped virus of roughly spherical spiky structure with a helical nucleocapsid surrounding the viral genome. The genome consists of a single stranded, non-segmented, negative sense RNA. The size of the genome consists of 15186 bases, encoding six viral proteins which are phosphoprotein (P, 53 kDa), matrix protein (M, 40 kDa), fusion protein (F, 67 kDa), hemagglutinin-neuraminidase protein (HN, 74 kDa), large protein (L, 200 kDa) and nucleocapsid protein (NP, 55 kDa) (Phillips *et al.*, 1998; Yusoff and Tan, 2001; Schirrmacher, 2005). Three proteins make up the nucleocapsid which mainly consists of the NP followed by L and P. The envelope is a lipid bilayer derived from the host cell plasma membrane with protrusions of the HN and F proteins giving a characteristic