REVIEW ARTICLE

AN OVERVIEW ON THE DEVELOPMENT OF NEWCASTLE DISEASE VIRUS AS AN ANTI-CANCER THERAPY

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Newcastle disease virus (NDV) is one of the most economically important avian virus which affects the poultry industry worldwide. Although NDV is being very actively studied in Malaysia, there are still no studies on its potential as an anticancer agent, a new approach to treating cancer known as virotherapy. Currently, a collaborative research is being undertaken between Universiti Putra Malaysia (UPM), Universiti Sains Malaysia (USM) and Majlis Kanser Nasional (MAKNA) in characterising various local NDV isolates as anticancer agent. This paper describes an overview of the research that have been carried out worldwide in the use of NDV for cancer treatment and also some of our findings in characterising local NDVs with oncolytic properties.

Key words *: Newcastle disease virus, anticancer agent, apoptosis*

Introduction

Cancer is one of the major killers in human in the world including Malaysia. It can affect any organ(s) of the body regardless of age, gender, race/ethnic background, diet, and the environment. The most predominant cancer affecting males in Malaysia are cancer of the lung, nasopharynx, mouth, stomach and liver, while amongst females, the most prevalent cancers, are cancer of the breast, cervix, lung and stomach (3). The conventional approach to the treatment of cancer is cytotoxic chemotherapy, either alone or in combination with surgery and radiotherapy. Another approach, known as immunotherapy is through the use of immunomodulatory factors such as cytokines and interferons. Viruses with inherent oncolytic activities have been used in the past as potential cancer therapeutics. Lately, the application of virus as virotherapy for cancer has been revived among the scientific community (40). This paper describes an overview on the application of NDV as an alternative approach to treat cancer in human.

Virotherapy in cancer medicine

It has been known for more than 70 years that some viruses such as adenovirus, herpes simplex virus (HSV), reovirus, rabies virus, poliovirus, measles virus, vesicular stomatitis virus, hepatitis A virus and NDV have the ability to destroy cancer cells. These viruses are either used without any genetic manipulation or undergo genetic engineering for increasing selectivity in animal models and human clinical trials (24, 32, 40, 50). NDV has been classified together with other viruses such as reovirus and parvovirus as viruses with inherent oncolytic effects, meanwhile, viruses such as HSV and adenovirus are examples of those that have been manipulated to enhance their cytolytic properties as anti-cancer agents (Table 1). These viruses were manipulated in such a way that they are attenuated in normal cells without altering their ability to lyse tumour cells. In some of the modifications, the engineered viruses were targeted to very specific cancer cells (24).

Molecular Biology of Newcastle Disease Virus (NDV)

Newcastle disease (ND) was first recorded in Jakarta, Indonesia (25) and Newcastle-upon-Tyne, England (16). ND has contributed to major losses to the poultry industry in Malaysia in terms of mortality and loss in egg and meat production (2). Although it is effectively controlled by vaccination and mass slaughtering, sporadic outbreaks are still threatening the industry (31). The disease is caused by NDV which has been classified into the order *Mononegavirales*, family *Paramyxoviridae*, sub-family *Paramyxovirinae* and genus *Rubulavirus* (21). The virus primarily infects poultry and can be categorized into three pathotypes; lentogenic strain which causes mild or inapparent respiratory disease, mesogenic strain which produces respiratory and nervous signs with moderate mortality and the viscerotropic or neurotropic velogenic strain which causes severe intestinal lesions or neurological disease resulting in high mortality.

The genome of NDV consists of non-segmented, single stranded RNA of 15.9 kb, which encodes for 6 viral proteins; phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), polymerase (L) and nucleoprotein (NP) (21).

Recently, the entire genome of several NDV strains has been completely sequenced (15). Subsequently, infectious cDNA clones of NDV were produced by using reverse genetic technology (41). The main feature that distinguishes the Paramyxoviruses from the other members of the same family lies in the presence of two surface projections, or spikes which extend from the envelope. The longest spike comprises the HN glycoprotein which is associated with hemagglutination (HA) and neuraminidase (NA) activities while the other spike contains the F glycoprotein which is involved in the fusion between the virus and infected host cells. These two proteins interact with each other and are involved in viral infectivity and virulence (51).

Oncolytic Newcastle Disease Virus

Among the first intentional use of NDV to treat cancer in humans was documented in the early 1950's where NDV and adenovirus were injected directly into uterine carcinoma which underwent partial necrosis and sloughing followed by regrowth (8). In another report, NDV was also shown to be oncolytic on Ehrlich ascites carcinoma (19). However, all of these trials were stopped because early oncolytic effects were lost with regrowth of the tumours when the patients produced virus

Table 1: *Genetically engineered and non-engineered viral agents used as treatment against cancers in clinical trials.*

Parental Strain	Agent	Tumour Target
Engineered		
Adenovirus	Onyx=015	Colorectal, ovarian and pancreatic in phase I, II, III clinical trials
HSV	CN706, CN787	Prostate in phase I clinical trials
Vaccinia virus	Wildtype	Melanoma in phase I clinical trials
Non-engineered		
Parvovirus	H-1	Variety of advanced tumour
Reovirus	Reolysin	SCCHN
NDV	73-T, PV701, Ulster, MTH/68H, La Sota	NDV use as live virus, VO and/or ATV against a variety of advanced tumours.

ATV = autologous live cell NDV modified tumour vaccine; SCCHN = squamous cell carcinoma; VO = viral oncolysate

neutralising antibodies. Similar results were also produced after the use of other oncolytic viruses such as mumps virus and influenza virus (1). Nevertheless, systemic administration of mumps virus for the therapy of human cancer in Japan caused partial tumour remission despite the production of virus-neutralising antibodies (6, 43). Similar results were also obtained with repeated systemic administration of NDV for the therapy of cancer in Hungary (14). It was then postulated that perhaps these oncolytic viruses lose their effects on the cancer cells because of humoral antiviral immunity of the host, whereas when an incompletely replicating or non-cytolytic virus establishes a persistent relationship with a tumour, antiviral immune components such as antibodies and activated T cells attack and eliminate tumour cells expressing viral antigen (50).

The oncolytic properties of NDV have been studied both in mouse models (Table 2) and in human clinical trials (Table 3). In both instances, favorable results from partial to complete regression of tumours were obtained for various types of tumours including those in the advanced stages that were not responsive to standard therapy. The ability of NDV to successfully infect and destroy cancer cells seemed to be dependent on many factors (50).

The use of NDV as non-viral oncolysate based treatment has been reported in Hungary (14). In that study, patients with advanced tumour received repeated administrations of high dose inhalation, ingestion, injection or enema of the attenuated NDV strain (MTH68/H) derived from Hertfordshire strain showed significant regressions of varying degrees. The use of high doses of live NDV has also been shown to be effective against non-responsive grade IV glioblastoma (11). However, not all NDV strains are able to induce direct oncolysis. For example, the most oncolytic NDV strain was Cassel's 73T whilst the NDV strain Ulster (which exhibits abortive replication in normal cells) induces host immunity towards tumour cells expressing the NDV antigen (50). It has been shown that the former NDV strain selectively replicates in tumour cells as compared to normal cells and new virions produced by infected tumour cells are non-infectious (49). They also indicated that the strain Ulster which infected various cancer cells gave least favorable trends in the induction of clinically evaluated antitumour responses. This finding lead to the postulation that x-ray irradiated viral infected tumour cells were more immunogenic than the viral oncolysates. However, it appeared that this effect was strain dependent. For example, the vaccine strains Roakin and B1 suppressed cellular DNA synthesis in Daudi Burkitt's lymphoma cells leading to cell death (53), but treatment on the same cells using strain 73T did not damage the cells (57).

In addition to irradiated infected xenograft tumour cells, autologous tumour cell vaccine (ATV)

Table 2:   NDV strains used as anti-cancer agents in animal model

Form and strain of NDV	Tumour target xenograft on athymic/nude mice	Remarks	Ref
Live virus-73T	Bladder carcinoma, Wilm's tumour, fibrosarcoma, cervical carcinoma neuroblastoma, ostcosarcoma	Complete regression after intra-tumoural inoculation	39
Live virus-73T	Neuroblastoma	Complete regression after intra-tumoural inoculation	27
Live virus-73T	Fibrosarcoma	Complete regression after intra-tumoural inoculation	28
Live virus-73T	Breast, prostate, epidermoid, neuroblastoma, large cell lung carcinoma, colon carcinoma,	Intra-tumoural, subcutaneous and intra-peritoneal administration	35
Live virus-73T, Italien ATV - Ulster	Metastases melanoma, renal carcinoma, colorectal, lymphoma	Regression after local but not systemic administration	48

have also been used by several researchers. In one study, Bohle *et al.* (7) demonstrated favorable results using ATV comprising a dose of 1 x 10^7 human colorectal tumour cells together with 32 hemagglutination unit (HAU) of non-irradiated NDV given intracutaneously to patients. Recently, a study in China indicated that patients who have received ATV and NDV vaccine strain La Sota IV have significant regression of advanced tumours of the digestive tract compared to the controlled group (26).

The oncolytic properties of NDV have also been studied in animal models by xenotransplanting tumour cells onto athymic and nude mice. It was found that the most oncolytic strain 73T completely destroyed human neuroblastoma or fibrosarcoma tumours xenotransplanted in athymic mice following intra-tumoural route of the virus (Table 2) (27, 28). Similarly, the oncolytic effects of 73T were also shown in other tumour cells such as bladder carcinoma, Wilm's tumour, osteosarcoma and cervical carcinoma (39). In addition, direct administration of 73T either through intra-tumoural and intra-peritoneal routes showed complete regression of various advanced tumours including neuroblastoma in nude mice model (Table 2) (35). It was found that only live virus showed better results than inactivated virus, and the oncolytic virus itself might provide additional benefits (10, 39). However,these results were strain dependent. A study by Schirrmacher *et al.* (48) on effects of NDV

strains to colon carcinoma showed that the non-lytic strain Ulster displayed stronger antitumour activity than the lytic 73T. On the other hand, intra-tumoural injection of NDV on human melanoma was found to be more effective when using lytic strain Italien compared to the non-lytic strain Ulster.

It can be concluded from the above studies that replication competency is necessary for maximal effects and multiple NDV doses are more effective than a single dose. It seems that depending on the NDV strains, intra- or peritumoural application is more effective than systemic application for various tumours in mice model. Compared to other viruses with inherent oncolytic properties, NDV therapy is safer, non-neurotropic with very minimal side effects. The only side effects that have been reported were low-grade fever, vomiting and fatigue (50). Nevertheless, the clinical usefulness of NDV need to be carefully evaluated since any cancer treatment depends on its antitumour potency and its therapeutic index between cancerous and normal cells. Additionally, it has been known that most of the therapies that are currently available for metastatic solid tumours are not effective in one or both of these areas.

Virotherapy modification in cancer medicine

The ability of NDV to selectively replicate in cancer cells is one of the most important features in the effectiveness of the virotherapy. Treatment with

Table 3: Application of NDV as virotherapy against advanced tumours in clinical trials

Form and strain of NDV	Clinical Phase	Tumour target	Ref
Live virus-PV701	I	Advanced solid tumours	35
Live virus-MTH68/H	.	Advanced tumours of the renal, colon and breast	14
VO- Cassel	II	Stage II malignant melanoma	9
VO- Ulster	II	Advanced tumours of the renal, colon and breast	46
ATV- Ulster	I	Colorectal carcinoma (Dukes stage B2, C, D)	7
ATV- Cassel	-	Renal carcinoma	23
ATV- La Sota IV	-	Advance tumours of digestive tract	26
ATV- PV701	III	Breast Carcinoma	45

ATV = autologous live cell NDV modified tumour vaccine; VO = Viral oncolysate

ATV in combination with low-dose of recombinant interleukin-2 (IL-2) and interferon-alpha 2 (IFN-2a) was able to improve relapse-free and overall survival of patients with locally advanced renal cancer cell (4). In another study, it was found that ATV transfected with major histocompatibility (MHC) genes was more effective in prevention of spread of malignant melanoma than regression of established micrometastases (36)

There are several approaches to enhance the cytolytic effects of replication-component viruses such as NDV namely by expression of cytotoxic proteins, drug-sensitivity genes and cytokine genes. These approaches are currently being developed and tested for oncolytic viruses such as adenoviruses, herpes simplex virus and vaccinia virus (40). Tumour selectivity can also be achieved by introducing an essential viral gene under the control of a tumour-specific promoter (20). So far, no studies have been published on the use of recombinant oncolytic NDV expressing foreign genes or tissue-specific promoter for genetic improvement of the viral oncolytic effects. However, with the recent development of reverse genetic technology for NDV (41) and the identification of non-essential regions in the NDV genome (30) the future is not far from the development of recombinant oncolytic NDV strain with improved oncolytic properties.

Oncolytic NDV-induced apoptosis

Apoptosis which is an energy-dependent process of cell suicide is also known as programmed cell death. It is a natural response of the cells when exposed to a variety of stimuli. Apoptotic cells have a characteristic morphology and show distinct biochemical processes that can be detected using transmission electron microscope and expression of apoptotic gene markers, respectively (29). A number of viruses have been shown to cause apoptosis in cells during infection (33). In general, the mechanisms associated with virus-induced apoptosis are associated with one or more of the host regulatory genes that function as an oncogene and/or tumour suppressor factor. Examples of such genes are CD95/FasR/APO-1, *bcl-2, c-myc, p53, Rb, p21WAF1* and *ICE/ced-3* (40, 52). The importance of these genes in NDV-induced apoptosis of cancer cells is, however, not known. It has been shown that strain MTH-68/H was found to be cytotoxic on rat phaeochromocytoma (PC12) cells (17) causing internucleosomal DNA fragmentation, the most characteristic feature of apoptosis. The role of the anti-apoptotic protein, *c-ras* in tumour cells has been implied since mutation(s) in the protein promoted reovirus replication leading to oncolysis (12, 13). However, such evidence has never been demonstrated for NDV induced oncolysis although

Table 4: Determination of optimum NDV titers that exhibit oncolytic effects on tumour cell lines. The IC_{50} values shown are from average values of at least 3 different experiments

NDV Strains	Virus IC_{50} titer on tumour cells			
	MCF-7	MDA-231	HT-29	HL-60
AF 2240	64	4	-	-
V4	128	96	4096	1024
S	Nil	Nil	Nil	Nil
F	2048	8	-	-
Ijuk	Nil	64	-	-
01/C	Nil	Nil	Nil	-

Note. IC_{50}= virus titer in HA unit that destroy 50% of the tumour cells following MTT cytotoxicity assay; Nil = IC_{50} value not achieved;- not tested

it is known that certain tumour cells such as fibrosarcoma and neuroblastoma which were susceptible to oncolysis by 73T NDV had certain forms of *ras* mutation (27, 28).

Immunology Perspective of Oncolytic NDV

NDV has pleiotropic immune stimulatory properties in addition to good cell-binding and selective proliferation in replicating cells. In addition, the virus has the ability to introduce T cell co-stimulatory activity and induce cytokines such as IFN-a, IFN-b and TNF-a that affect T cell recruitment and activation (46). Thus, some researchers considered virotherapy as a form of immunotherapy approach in treating cancer in humans. Immunotherapy is favored for prevention of tumour metastases among other postoperative treatment (37). However, the mechanism responsible for the immunotherapeutic effects of NDV is yet to be defined. Cellular cytotoxicity of peripheral blood mononuclear cells (PBMC) was enhanced significantly after co-incubation of NDV with effector cells (57). Through the study, natural killer (NK) cells were found to be the predominant mediator of lysis. Enhancement of cytotoxicity also correlated with the induction of IFN-a and TNF-a in PBMC by NDV.

A study by Schirrmacher *et al.* (47) to investigate the capacity of NDV to activate anti-tumour activity in murine macrophages revealed that macrophages were activated after infection with different strains of NDV. Various macrophage enzymes became upregulated and anti-tumour effector molecules such as nitric oxide and TNF-a were also found in the supernatant. The NDV activated macrophages displayed cytotoxic anti-tumour activity *in vitro* and were active against tumour cell lines such as mammary carcinoma, lung carcinoma and mastocytoma. Anti-tumour activity by NDV activated macrophages could also be transferred *in vivo*. These results demonstrated that NDV can strongly activate macrophages to perform anti-tumour activities *in vitro* and *in vivo*.

Besides induction of IFN-a and IFN-b, NDV also increases adhesive host tumour-cell interaction via its HN glycoprotein, thus enhances their binding affinity and/or avidity (44). Activation of NK cells might be the result from direct binding and activation through the HA gene product of the HN glycoprotein. Recently, it has been shown that the HA protein is more important for inducing IFN-alpha than the neuraminidase (NA) activity (55). In another study, the HN but not the F protein of NDV was shown to be a potent inducer of IFN-alpha production, and capable of upregulating the TNF related apoptosis inducing ligand (TRAIL) (56). The HN protein also activated human monocytes (Mphi) that kill various human cancer cell lines through the TRAIL-mediated tumouricidal activity (54). However, this tumouricidal activity was not associated with other apoptotic inducing related ligands such as CD95 and TNF-R2.

Oncloytic Malaysian isolates of NDV

Several different isolates of NDV have been isolated and characterised by researchers at UPM and the Veterinary Research Institute. In addition, the standard reference strains F and V4 have been modified and developed as commercial vaccines for poultry (21). The genome of the local velogenic NDV, strain AF2240 is about to be sequenced completely. In addition, several studies have been focused on strain AF2240 in developing novel approach to diagnose and control NDV. This includes the development of ELISA-PCR based diagnostic tool for NDV (22) and NP as universal carrier for subunit vaccine (37). Recently, several short peptides that inhibit NDV replication have been identified by using phage display technology (38). These findings will pave research on the development of new antiviral drugs for other paramyxoviruses including Nipah virus.

Even though advances have been made in characterising the biological and molecular characterisation of NDV, no studies have been carried out on the use of local NDV as the alternative approach to treat cancer in human in Malaysia. A collaborative project between UPM and USM funded by National Cancer Council (MAKNA) was launched in 2000 with the primary target on the development of local NDV vaccines with oncolytic properties. The oncolytic effects of six (AF2240, 01/C, Ijuk, S, F, V4) strains of NDV were screened on commercially available tumour cell lines, CEM-SS (T-lymphoblastic leukemic cells), MCF-7 and MDA-231 (breast cancer), HT29 (colorectal cancer) and HL60 (acute promelocytic leukemia). Based on the colorimetric microtiter (MTT) cytotoxicity assay, strains AF2240, F and V4 showed significant oncolytic effects on MDA-231 and MCF-7 cells whereas strain Ijuk showed significant killing of MDA-231 cells only (Table 4). Strain V4 also showed a significant killing effect on the CEM-SS, HT29 and HL60 tumour cells. Compared to V4 and

F, the strain AF2240 was far more superior in destroying breast cancer cells. In most cases, regardless of NDV strains and cancer cells, the oncolytic effects were demonstrated only on cancer cells but not on normal (3T3) cells. However, inactivation of NDV abrogates the oncolytic activity on cancer cells.

The mode of NDV strains AF2240, F and/or V4 in destroying the MCF-7 and MDA-231 cells is primarily by inducing apoptosis (18). Similar results were also obtained when CEM-SS and HL60 cells were treated with V4 strain. This was based on several analyses such as transmission electron microscopy, DNA fragmentation test, acridine orange/propidium iodide (AO/PI) staining and TUNEL (deoxynucleotidyl transferase mediated dUTP nick-end labeling) assay. The mechanisms of NDV-induced apoptosis are currently being investigated. A preliminary study indicated that neuraminidase treated MCF-7 cells remove its cell surface sialic acid did not lower the oncolytic effects of strain F. In addition, treatment of the latter with sialyllactose comprising lactose and sialic acid failed to prevent oncolysis of MCF-7. This finding indicated that surface expression of sialic acid on breast cancer cells was not essential for NDV induced oncolysis. However, studies have implicated that the high expression of sialic acid on the surface of neuroblastoma and fibrosarcoma is associated with *ras* mutation and susceptibility to NDV oncolysis (27, 28, 39). This suggestion was based on finding from reovirus induced oncolysis, where expression of *ras* and sialic acid was associated with virus replication and eventually lysis of the tumour cells (11, 12). Currently, the oncolytic effects of the local NDV is being tested on a variety of other cancer cells including brain, colorectal, lung and cervical. Studies are also underway in characterising the oncolytic effects of NDV strains AF2240 and V4 in animal models. Although, we have just started work on the use of NDV as an alternative means to treat cancer, the local NDV strains seem to have the potential to be developed as anti-cancer agents for the treatment of cancer in human.

Conclusion

The relationship of NDV with tumours may be extremely variable. Depending on the tumour cells, NDV may exhibit their oncolytic activities either directly or indirectly. In the former pathway, viruses such as 73T affect the physiology of the infected cells whilst infection through the latter pathway (by strain Ulster) initiates immunity of the host upon the virus and virus-infected cells. In addition, NDV has been tested in the form of live virus or in the form of autologous or allogenic tumour vaccines. In both cases, it initiated weak tumour antigens, breaking tolerance towards tumour and generate immune responses against tumour antigens. The upregulation of TRAIL in activated PMBC by HN protein indicated that TNF-induced apoptosis may be an important mechanism in oncolytic NDV-induced apoptosis. The relationship between the activation of oncogenes and/or loss of tumour suppressor genes that are commonly found in malignant human tumour and susceptibility to NDV oncolysis remains to be determined. Once the identification of such gene(s) and the sequence of immunological reactions that accompanies oncolysis or tumour rejection become known, it will be possible to construct genetically engineered NDV strains that are safer with improved oncolytic effects on cancer cells.

Acknowledgements

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