Review

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Immunobiology of Anticancer Virotherapy With Newcastle Disease Virus in Cancer Patients

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ABSTRACT

Virotherapy with oncolytic viruses that preferentially infect and kill cancer cells is a novel and promising strategy for cancer treatment. Newcastle disease virus (NDV), which is pathogenic in birds, has beneficial clinical effects in cancer patients. NDV virotherapy is safe and elicits an antitumor response in patients affected by different types of cancers. The selective replication of NDV in tumor cells, the lack of genetic recombination, the lack of interaction with host cell DNA, and safety of NDV vaccination in cancer patients has resulted in NDV virotherapy to be accepted as a potentially attractive anticancer modality. However, more knowledge is needed to support the development of optimal NDV-based treatment modality for cancer. In this paper, the biological characteristics of NDV, the clinical effectiveness of NDV-based anticancer vaccination, immunobiology of NDV virotherapy in cancer patients, immune responses to NDV vaccines, and NDV-induced immunogenic cell death and apoptosis of cancer cells have been discussed in detail.

KEY WORDS: Virotherapy; Newcastle disease virus (NDV); Cancer treatment; Antitumor immune responses; Apoptosis.

ABBREVIATIONS: NDV: Newcastle Disease Virus; nm: Nanometer; NP: Nucleoprotein; P: Phosphoprotein; M: Matrix Protein; F: Fusion Protein; HN: Hemagglutinin-Neuraminidase; L: Large Protein; IFN: Interferon; RIG-1: Retinoic Acid-Inducible Gene 1; BCG: Bacillus Calmette-Guerin; DTH: Delayed Type Hypersensitivity; IL: Interleukin; PSA: Prostate-Specific Antigen; NK cell: Natural Killer cell, CTLA-4: Cytotoxic T-Lymphocyte Associated Antigen 4; PAMPs: Pathogen-Associated Molecular Patterns; PPRs: Pattern Recognition Receptors; dsRNA: Double Stranded RNA; PKR: Protein Kinase R; TLRs: Toll Like Receptors; NO: Nitric Oxide; TRAIL: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand; TNF: Tumor Necrosis Factor; IFNRA: Interferon Receptor Alpha; DC1: Type I Dendritic Cell; Th1: Helper 1 T-Cell; ELISPOT: Enzyme Linked ImmunoSpot.

INTRODUCTION

Conventional cancer therapy modalities, including surgery, chemotherapy, and radiotherapy, do not have sufficient clinical efficacy in the treatment of advanced cancers and introduction of more effective therapeutic approaches is essential for treating patients with advanced forms of cancer. Virotherapy with oncolytic viruses that preferentially infect and kill cancer cells is a promising therapeutic strategy for cancer treatment. Several viruses, including vaccinia virus, herpes simplex virus, measles, adenovirus, vesicular stomatitis virus, myxoma virus, reovirus, lentivirus, and Newcastle disease virus (NDV) have been identified as oncolytic viruses in preclinical and clinical studies.[1-3] Virotherapy approaches have the potential to be employed as monotherapy or be used in combination with conventional cancer therapy modalities to improve the overall chances of the patient's survival and increase the percentage of treated patients with long-term survival. Further investigation has shown that NDV may be a suitable oncolytic agent for virotherapy of cancers.

Anticancer properties of NDV have been intensively studied in the decades 1950s and 1960s.[4-6] Post-operative vaccination of mice with irradiated autologous tumor cells infected with NDV resulted in the disappearance of micrometastases from visceral organs, increased the survival of vaccinated mice, and helped cure the cancer in about 50% of the treated mice.[7] Favorable properties of NDV, including selective replication of NDV in tumor cells, lack of genetic recombination, lack of interaction with the host cell DNA, and safety of NDV vaccination in cancer patients, led to the clinical application of NDV virotherapy as an anticancer treatment of choice. In several clinical trials, NDV virotherapy has been medically implemented in patients with different types of cancer such as colorectal carcinoma, melanoma, renal cell carcinoma, breast cancer, ovarian cancer, glioblastoma multiform, head and neck squamous cell carcinoma, and prostate cancer. This virotherapy approach was considered as clinically safe and could help support antitumor effects in patients with advanced forms of cancer.[3]

BIOLOGICAL CHARACTERISTICS OF NDV

Biology of NDV

NDV, with a spherical shape, 150 nm diameter, and a lipid bilayer envelope, belongs to the genus *Avulavirus* in the family *Paramyxoviridae*. This virus has a single-stranded, negative-sense, nonsegmented RNA genome of approximately 15,186 nucleotides, which contains six genes, including nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein (L). These genes encode at least seven proteins. NDV harbors a single-stranded RNA-dependent RNA polymerase complex that consists of the L, P, and NP proteins.[8-10] The V protein, which is encoded by the P gene through an overlapping reading frame, functions as an IFN type 1 antagonist in avian hosts.[11,12] NDV is an RNA virus and it replicates in the cytoplasm of infected cells without a DNA stage, thus, the possibility for genetic recombination with host cell DNA is very rare.[13]

Pathogenic Classification of NDV

NDV is an animal pathogen which infects various avian species. Different strains of NDV causes a contagious viral disease in most domestic and wild avian species. NDV strains are classified into three pathotypes based on their virulence in birds, classified as velogenic, mesogenic, and lentogenic strains. Velogenic strains are one of the most commonly observed pathogenic NDV strains, responsible for causing a severe infection resulting in a high incidence of mortality in infected chickens. The common signs of ND include depression, fever, loss of appetite, abnormal thirst, severe dehydration, and emaciation. The mortality rate can reach up to 100% on account of this condition. Mesogenic strains are mid-virulent NDV strains, causing respiratory disease in chicks and young chickens and reducing their egg-laying ability. These strains may result in up to 25% mortality.

Lentogenic strains are non-virulent attenuated strains, causing mild symptoms in the respiratory tract of infected birds.[10,14] In humans, NDV alone can cause transient conjunctivitis and mild flu-like symptoms and poses no hazard to the human health. So far, several mesogenic and lentogenic strains of NDV have been successfully used in oncolytic virotherapy in mouse tumor models and cancer patients, such as *PV701* (strain *73-T*), *LaSota*, and *Ulster*.[3] NDV strains can also be categorised into lytic and nonlytic strains. Both oncolytic and non-oncolytic NDV strains have been used in the clinical treatment of patients affected with cancer.

Selective Replication of NDV in Human Cancer Cells

NDV replicates in most human cancer cells and destroys various types of cancer cells such as fibrosarcoma, osteosarcoma, cervical carcinoma, bladder carcinoma, neuroblastoma, pancreatic adenocarcinoma, pleural mesothelioma, and Wilm's tumor cell lines, both *in vitro* and *in vivo*.[15-17] It has been observed that the virus yield increases 10,000-fold within 24 hours in the tumor and chick embryo cells supernatant, but the titer values remains near zero in the normal fibroblast supernatant.[15] Human pancreatic tumor cell lines also show more than 700 times higher sensitivity than normal cells to the NDV killing *in vitro*.[16] Moreover, NDV is a biological agent with a potential to disrupt the resistance of cancer cells to therapy on account of its ability to replicate in non-proliferating tumor cells which are resistant to chemotherapy and radiotherapy.[18] NDV can also replicate in hypoxic cancer cells.[19]

Mechanisms Involved in the Selective Replication of NDV in Cancer Cells

The molecular mechanisms underlying the NDV-sensitivity of human cancer cells have been investigated in some studies. Selective replication of NDV in tumor cells is suggested to be associated with defects in the antiviral defense mechanisms in tumor cells. Decreased IFN expression and impaired induction of IFN-induced antiviral proteins in tumor cells have been shown to be correlated with efficient NDV replication.[20-22] But, there are some existing evidences that indicate that other mechanisms are also involved in the selective replication of NDV in human tumor cells. Some strains of NDV with intact IFN-antagonistic function, containing V protein, can replicate in normal human cells. In a multistage skin carcinogenesis model derived from nontumorigenic HaCaT cells, there was no significant difference in interferon signaling between virus-sensitive tumor cells and virus-resistant nontumorigenic parental cells. In this epithelial cancer cell line model, Rac1, a pleiotropic regulator of multiple cellular functions, was considered as an oncogenic protein that is essential for NDV replication in tumorigenic cells. Additionally, Rac1 expression was sufficient to render nontumorigenic cells susceptible to NDV replication and to oncolytic cytotoxicity.[23] In a nude mouse model of human fibrosarcoma, IFN-sensitive recombinant NDV was as effective as IFN-resistant virus in the elimination of tumor burden.[24] No correlation was observed

between defects in IFN pathways and NDV replication or NDV-induced cytotoxicity in 11 different human pancreatic adenocarcinoma cell lines. Pretreatment of cell lines with IFN resulted in diminished NDV replication and its cytotoxic effects in most cell lines.[25] Tumor selectivity of NDV has also been dependent on the expression of retinoic acid-inducible gene 1 (RIG-1), a cytosolic RNA sensor.[26] As a consequence, several mechanisms are associated with the selective replication of NDV in tumor cells such as defects in the activation of antiviral defense pathways especially type I interferon signaling pathways,[20-22] activation of Ras signaling and expression of Rac1 protein,[23] as well as defects in apoptotic pathways.[27]

ANTICANCER EFFECTIVENESS OF NDV-BASED VACCINATION IN CLINICAL TRIALS

To date, four NDV-based vaccination approaches have been implemented in clinical trials, including vaccination with free infectious NDV, vaccination with intact, irradiated, tumor cells infected in culture by NDV, vaccination with lysate from NDV-infected tumor cells, and vaccination with *ex vivo* generated dendritic cells pulsed with lysate from NDV-infected tumor cells.[3] In clinical trials of patients with solid cancers, administration of NDV particles resulted in some clinical responses. General virus induced-side effects were flu-like symptoms, tumor site-specific adverse events, and infusion reactions.[28-32] In a ten-year follow-up of stage II malignant melanoma patients treated postsurgically with NDV oncolysate (tumor cell lysate), post-operative vaccination with NDV oncolysate was able to improve the survival of stage II malignant melanoma patients.[33] In contrast, post-operative vaccinations with lysate from autologous melanoma cells infected with NDV *Ulster* strain in combination with administration of IL-2 did not show clinical efficacy in melanoma patients with resectable stage III disease.[34] In other clinical trials, vaccination with NDV-infected autologous tumor cells elicited clinical responses and increased the survival rate of patients particularly affected by colorectal cancer, renal cell carcinoma, breast cancer, ovarian cancer, glioblastoma multiform, and head and neck squamous cell carcinoma.[3] In general, vaccination with NDV-infected autologous tumor cell vaccines have showed greater therapeutic efficacy than vaccination with NDV particle vaccines and NDV-infected tumor cell lysate vaccines.[3]

In colorectal patients vaccinated post-operatively with autologous tumor cell vaccine and NDV vaccine, survival rates were more than that in patients treated with surgery plus radiotherapy or chemotherapy.[31] In addition, vaccination with NDV-infected autologous colorectal tumor cells was more effective than vaccination with tumor cells admixed with bacillus Calmette-Guerin (BCG) in eliciting antitumor responses in resected colorectal carcinoma patients. Also, NDV-infected tumor cell vaccines induced mild side effects while vaccination with BCG-admixed tumor cells led to the development of long-lasting ulcers and serious side effects.[35] Nevertheless, vaccination with NDV-infected autologous tumor cells did not improve the overall survival of stage IV rectal cancer patients following resection of liver metastasis when compared with non-vaccinated patients for a follow-up period of about 10 years.[36] Recent resources have shown that vaccination with dendritic cells pulsed with lysate from NDV-infected autologous tumor cells in cancer patients resulted in cancer regression.[37-38]

IMMUNOBIOLOGY OF NDV VIROTHERAPY IN CANCER PATIENTS

In a phase I/II trial in patients with recurrent glioblastoma multiform, anti-NDV hemagglutinin antibodies were detected following the administration of intravenous injections of NDV and viral particles which were recovered from the blood, saliva, urine samples, and one tumor biopsy.[39] However, neutralizing antibodies generated during NDV treatment may interfere with the antitumor effectiveness of NDV vaccines. In a clinical trial involving colorectal patients vaccinated post-operatively with autologous tumor cell vaccine and NDV vaccine, there was an association established between skin delayed type hypersensitivity (DTH) reaction and the prognosis of treated patients.[31] In other clinical trials involving patients of colorectal cancer with liver metastases, vaccination with NDV-infected, irradiated, autologous tumor cells following curative liver resection resulted in an increased sensitization against autologous tumor cells, as measured by DTH reactivity. Importantly, a strong correlation between increased skin DTH reaction against autologous tumor cells and recurrence-free interval was observed in the vaccinated patients.[40] Postsurgical vaccination of colorectal cancer patients with NDV-infected autologous tumor cell vaccine was also associated with increased skin DTH reactivity and a dense infiltration of predominantly helper T-cells in the vaccination site.[41]

In patients with glioblastoma multiform postsurgically vaccinated with NDV-infected autologous tumor cells, a significant increase in the skin antitumor DTH reactivity, improved survival, and increased numbers of tumor reactive memory T-cells in the peripheral blood and CD8^{+} tumor infiltrating T-cells were observed in the secondary tumors of vaccinated patients.[42] Significant increase in the antitumor skin DTH reactivity and the presence of tumor reactive T-cells in the peripheral blood, even 5 to 7 years after vaccination, were observed in a significant proportion of head and neck squamous cell carcinoma patients vaccinated postsurgically with NDV-infected autologous tumor cells.[43] Preconditioning of head and neck squamous cell carcinoma patients with IL-2 prior to vaccination was associated with an increase in the number of T-cells and augmented antitumor DTH reactivity.[44] In patients with advanced renal cell carcinoma with distant metastases, multiple vaccinations with NDV-infected autologous tumor cells after nephrectomy followed by administration of low doses of IL-2 and IFN-α resulted in a complete response in 12.5% and partial response in 15% of the vaccinated patients.[45] Genetic manipulation of NDV towards arming the virus with genes encoding cytokines or tumoricidal molecules is also being investigated to improve

the antitumor effects of NDV-based vaccines.[46-50]

In a patient with hormone-refractory metastatic prostate cancer who had failed to cope with standard cancer therapy, postsurgically intravenous administration of NDV in combination with vaccination with autologous monocyte-derived dendritic cells pulsed with NDV-infected tumor cell lysate and administration of IFN-γ, resulted in complete remission of prostate cancer, long-lasting dramatic decrease in prostate-specific antigen (PSA) levels, induction of antitumor memory T-cell response, and a reduction in bone metastases.[51] Similarly, long-term survival of another patient with invasive ductal breast cancer and primary liver metastases was observed upon the post-surgical application of radiofrequency for treating hyperthermia of the liver, intravenous administration of NDV, and vaccinations with autologous monocyte-derived DCs pulsed with lysate from NDV-infected breast cancer cells. Sustained tumor-specific memory T-cell response was observed upon the administration of dendritic cell vaccinations.[52]

Induction of immunogenic cell death as well as induction of apoptosis in cancer cells were involved in the NDV-mediated killing of cancer cells upon NDV vaccination in affected patients.

INDUCTION OF ANTITUMOR IMMUNE RESPONSES TO NDV VACCINATION AND IMMUNOGENIC CELL DEATH OF CANCER CELLS

Virus-induced stimulation of different immune cells can be responsible for strong antitumor responses of NDV in tumor-bearing hosts.[53] The prevention of metastatic spread by postsurgical vaccination with NDV has been paralleled with an establishment of specific systemic antitumor immunity.[54] Presentation of NDV-encoded antigens on the cell surface of infected cancer cells induces the stimulation of lymphocytes. Two of six NDV genes, HN and F, modify the tumor cell surface which leads to enhanced lymphocyte interactions. Other viral genes can also stimulate a number of host cell genes leading to the production of several cytokines and chemokines. Furthermore, double-stranded RNA produced in NDV-infected cells activates antiviral immune responses based on type I interferons such as IFN-α and IFN-β. Nonetheless, NDV selectively replicates in murine/human tumor cells as the V protein, which inhibits type I interferon responses in permissive NDV-infected avian cells, which does not interfere with the interferon response in mammalian cells.[11,12]

NDV has a capability to co-stimulate tumor-specific cytotoxic T-lymphocytes.[55,56] Tumor-specific cytotoxic T-cell response observed in mice immunized with NDV-infected tumor cells was mediated using IFN-α/β.[56] NDV infection of melanoma cell line completely restored the proliferative response of tumor tissue-derived CD4⁺ T-cell clone and inhibited the induction of T-cell anergy to melanoma by the induction of B7-1/B7-2-independent T-cell costimulatory activity in human melanoma cells.[57] It has been found that NDV-infected tumor cells enhance tumor-specific T-cell responses as a result of CD4⁺ and CD8⁺ T-cell cooperation.[58] NDV-infected tumor cell vaccine augmented tumor-specific cytotoxic CD8⁺ T-cell responses and CD4⁺ T helper activity in a mouse lymphoma model.[59] NDV induced long-term survival and tumor specific T-cell memory through induction of immunogenic cell death in an ortheotopic glioma model.[60]

NDV antigens expressed on antigen presenting cells or tumor cells can augment peptide-specific T-cell responses.[61] NDV-derived HN molecules facilitated adhesive interactions of lymphocytes with NDV-infected tumor cells.[62] Vaccination of late-stage metastasized colorectal carcinoma patients with NDV-infected tumor cells attached with NDV-specific single chain antibodies with specificity for the HN and CD28 induced tumor-specific T-cells in all vaccinated patients, and 28.6% of patients showed a partial response.[63] HN protein can activate natural killer (NK) cells. In a mouse tumor model, vaccination with a plasmid encoding the HN protein of NDV resulted in a significant increase in NK cell infiltration and a decrease in infiltration of myeloid-derived suppressor cells.[64] Combinational therapy with localized NDV and systemic anti-CTLA-4 blockade led to rejection of pre-established tumors and protection from tumor rechallenge in poorly immunogenic tumor models, melanoma (B16 cells) and colon cancer (MC38 cells). This combinational therapy resulted in distant tumor infiltration with CD4⁺ and CD8⁺ T cells and its therapeutic efficacy was dependent on the CD8⁺ T cells, NK cells, and type I interferon.[65]

Intratumoral injection of NDV in athymic mice resulted in complete regression of human fibrosarcoma and neuroblastoma xenografts,[66] indicating that other immune cells, other than T-cells, are involved in the NDV-induced antitumor immune responses. Pathogen-associated molecular patterns (PAMPs) of NDV can be recognized by pattern recognition receptors (PPRs) of innate immune cells, including cytoplasmic RIG-1, cytoplasmic dsRNA dependent protein kinase R (PKR), endosomal Toll-like receptors (TLRs), plasma membrane expressed NK cell receptor NKp46, leading to initiation of multiple signaling pathways, and subsequently, strong type I interferon response, release of proinflammatoy cytokines, and activation of other immune cells.[67]

NDV can activate macrophages. NDV induces nitric oxide (NO) synthesis in infected macrophages *via* activation of nuclear factor-kappa B.[68] NDV also stimulates tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated tumoricidal activity of human monocytes.[69] HN protein of NDV induces cell surface expression of TRAIL and secretion of IFN-α in human peripheral blood mononuclear cells.[70] NDV-activated murine macrophages upregulated antitumor molecules NO and TNF-α, and showed antitumor cytostasis and cytotoxicity *in vitro*. The antitumor cytotoxicity of NDV-activated macrophages was used against various tumor cell lines. Intravenous transfer of NDV-activated macrophages resulted in a significant

suppressive effect on pulmonary metastases in mammary carcinoma and lung carcinoma models.[71]

NDV can also activate dendritic cells. Viral RNA in the NDV oncolysate pulsed dendritic cells acts as a PAMP. Recognition of viral RNA in NDV-infected cells by endosomal TLRs, such as TLR-3, -7, and -8, and the cytoplasmic retinoic acid inducible gene1 (RIG-I) induces a strong type I interferon response.[72] IFN-α and IFN-β molecules secreted by NDV-infected cells interact with the cell surface type I interferon receptor (IFNRA) and initiate intracellular signaling pathways leading to the blockage of viral replication in the target cells.[73] *In vitro* stimulation of human monocyte-derived dendritic cells with NDV polarized dendritic cells towards type I dendritic cells (DC1) induce helper 1 T-cell (Th1) responses.[74] NDV oncolysate-pulsed dendritic cells potently stimulated autologous T-cells in breast cancer patients. They increased the expression level of costimulatory molecules in comparison to tumor lysate-pulsed dendritic cells and elicited greater IFN-γ ELISPOT responses. Supernatant from cocultures of NDV oncolysate-infected dendritic cells and bone marrow cancer reactive T-cells contained increased titers of IFN-α and IL-15.[75]

Recently, an NDV oncolysate-pulsed dendritic cell vaccine has been clinically administered to patients at the Immunological and Oncological Center (IOZK) in Cologne, Germany.[37,38] Before receiving the vaccination, patients were preconditioned by electrohyperthermia to activate the immune system and to enhance the virus tumor targeting and replication. It is possible that induction of NDV oncolysate-specific T-cells help recall T-cell responses upon dendritic cell vaccination and augment the generation of effective antitumor T-cell responses.[38]

NDV-MEDIATED INDUCTION OF APOPTOSIS IN CANCER CELLS

In addition to immunogenic cell death, induction of apoptosis appeared to be an important mechanism of NDV-mediated cancer cell killing. Intratumoral injection of recombinant NDV strains derived from the velogenic strain *Italien* induced syncytium formation and cell death as well as prolonged survival of the tumor-bearing mice.[76] Human tumor cell infection by NDV leads to upregulation of MHC and cell adhesion molecules, induction of interferons, chemokines and finally apoptosis.[77] Also, velogenic NDV AF2240 strain has been reported to induce apoptosis in a time-dependent manner on the mammary carcinoma cell line.[78]

In both the intrinsic and extrinsic pathways of apoptosis, caspases, cysteine aspartyl-specific proteases that cleave structural cytoplasmic and nuclear proteins, are activated, leading to the biochemical and morphological changes. Recombinant NDVs have mediated cytotoxicity against human tumor cell lines by inducing apoptosis through multiple caspase-dependent and IFN-independent pathways. NDV primarily triggered apoptosis by the activation of the intrinsic mitochondrial death pathway. Early activation of caspase-9 and effector caspase-3 was detected in NDV-infected tumor cells as early as 6-8 hours, indicating that intrinsic apoptotic pathways operate early in NDV-infected tumor cells. Activation of caspase-8 was detected in many of the tumor cell lines 48 hours after the NDV infection of cells but it was dispensable for inducing apoptosis. Cleavage of caspase-8, which is predominantly activated by the death receptor pathway, was a TRAIL-induced late event. Moreover, caspase-8 and caspase-9 inhibitors suppressed biochemical and morphological changes of the NDV-infected tumor cells. But, caspase-8 and caspase-9 inhibitors did not completely abrogate the signs of apoptosis in NDV-infected tumor cells. In addition, caspase inhibitors had no effects on virus replication.[79] Releasing multiple tumor antigens upon lysis of NDV-infected tumor cells is also responsible for inducing immune-mediated antitumor therapeutic response.

CONCLUSION

Various NDV strains selectively replicate in and kill human tumor cells. NDV-based vaccinations have helped increase the survival rate of cancer patients in several clinical studies. NDV vaccination in cancer patients can activate different immune cells with antitumor activity. Immunogenic cell death and induction of apoptosis are involved in the NDV-mediated killing of cancer cells. Interestingly, NDV virotherapy can be combined with other anticancer modalities, such as surgery, chemotherapy, and diverse immunotherapy approaches to induce stronger antitumor responses and eradicate residual tumor cells which persist following conventional therapy. Genetic manipulation of NDV to express genes encoding cytokines and other immunostimulatory molecules, and identifying NDV strains with potential antitumor effects are presently being investigated to improve the antitumor efficacy of NDV-based vaccines.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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