Enhancement of the Proapoptotic Properties of Newcastle Disease Virus Promotes Tumor Remission in Syngeneic Murine Cancer Models

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Abstract

Newcastle disease virus (NDV) is considered a promising agent for cancer therapy due to its oncolytic properties. These include preferential replication in transformed cells, induction of innate and adaptive immune responses within tumors, and cytopathic effects in infected tumor cells due to the activation of apoptosis. To enhance the latter and thus possibly enhance the overall oncolytic activity of NDV, we generated a recombinant NDV encoding the human TNF receptor Fas (rNDV-B1/Fas). rNDV-B1/Fas replicates to similar titers as its wild-type (rNDV-B1) counterpart; however, overexpression of Fas in infected cells leads to higher levels of cytotoxicity correlated with faster and increased apoptosis responses, in which both the intrinsic and extrinsic pathways are activated earlier. Furthermore, in vivo studies in syngeneic murine melanoma models show an enhancement of the oncolytic properties of rNDV-B1/Fas, with major improvements in survival and tumor remission. Altogether, our data suggest that upregulation of the proapoptotic function of NDV is a viable approach to enhance its antitumor properties and adds to the currently known, rationally based strategies to design optimized therapeutic viral vectors for the treatment of cancer. Mol Cancer Ther; 14(5): 1247–58. ©2015 AACR.

Introduction

Newcastle disease virus (NDV) is a negative sense single-stranded RNA virus classified as an avian paramyxovirus in the Avulavirus genus of the Family Paramyxoviridae (1). In the absence of vaccination, NDV outbreaks in poultry can cause devastating economic losses. However, NDV infections in humans are infrequent, and only cause mild conjunctivitis. The antitumor potential of NDV was first described during the 1960s (2). Since then, its natural oncolytic capabilities have been demonstrated in different mammalian cancer cell lines, animal tumor models, and clinical trials (3–5). NDV selectively replicates in cancer cells inducing cell death and stimulating innate and adaptive immune responses against tumor cells. Together with the lack of preexisting immunity in the general population, NDV is a suitable candidate to be used as an oncolytic therapeutic agent (6). As with many oncolytic viruses, the establishment of reverse genetics systems for NDV facilitated the development of new genetically modified recombinant NDV viruses with improved antitumor properties (7, 8). The principal strategy followed by most research groups including ours has been focused on the enhancement of NDV immunostimulatory properties through the generation of recombinant NDV viruses that express cytokines (IL2, IFNγ, TNFα), tumor-associated antigens, or tumor-specific antibodies (9–13). In our quest to design an optimized therapeutic NDV vector, here we focused our efforts on enhancing the cancer killing potential of NDV by increasing its potential for apoptosis activation.

Apoptosis is a highly regulated form of death that cells undergo after activation by different, but specific, external or internal stimulation (14). There are two main apoptosis pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (15). Each pathway is executed in a caspase-dependent manner, and both pathways communicate by cross signaling in such a way that molecules from one pathway can influence the other. Apoptosis resistance is a major hallmark of most, if not all, types of cancers due to defects in the apoptotic pathways (16, 17).

Indeed apoptosis resistance has recently been identified as a marker for resistance to chemotherapy and poor prognosis in cancer patients (18, 19). Nevertheless, the oncolytic activity of NDV has been shown to correlate with activation of the intrinsic or mitochondrial apoptosis pathway even in cancer cells resistant to apoptosis induction (20–23).

In this study, we propose a novel approach to enhance the oncolytic properties of NDV. By generating a recombinant NDV...
that encodes the human tumor necrosis factor receptor Fas, we hypothesized that we would enhance the apoptotic and antitumor properties of NDV (24). Fas is one of the most important and better characterized death receptors due to its role in homeostasis, elimination of pathogen-infected cells, and activation of the immune response (25–29). The transduction of the Fas-dependent death signal initiates from binding of its ligand FasL that results in receptor-mediated apoptosis signaling complex formation and caspase 8 activation (30–33). Fas-mediated cytotoxicity is not only restricted to the activation of the extrinsic pathway, but is also required for CTL-mediated perforin–granzyme cytotoxicity (34). Defects in the Fas-FasL system have been documented in many tumor types as a major feature of malignant progression, tumor immune evasion, and resistance to cytostatic drug treatment (35, 36).

In our current study, we evaluated the antitumor potential of a newly generated rNDV-B1/Fas virus. We postulated that by over-expressing Fas in NDV-infected cells we will introduce a strong extrinsic proapoptotic stimulus that will synergize for apoptosis induction with the intrinsic pathways activated by NDV infection, translating into an enhancement of its antitumor phenotype in vivo.

Materials and Methods

Cell lines, antibodies, and other reagents
Vero (African green monkey kidney epithelial cells; ATCC Cat# CCL-81, 2014), B16-F10 (mouse skin melanoma cells; ATCC Cat# CRL-6475, 2013), HeLa cells (human cervical adenocarcinoma epithelial cells; ATCC Cat# CCL-2, 2012), NIH/3T3 (murine embryonic fibroblast; ATCC Cat# CRL-1658, 2014), HuH-7 (human hepatocarcinoma; gentlyly provided by Dr. Matthew Evans research group (Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, 2014), and A549 cells (human lung carcinoma; ATCC Cat# CCL-185, 2014) were maintained in DMEM medium supplemented with 10% FBS, l-Glutamine (1% Glutamax-100X; Invitrogen), penicillin and streptomycin (DMEM 10% FBS P/S). CT26 cells (ATCC Cat# 2638, 2013) were maintained in RPMI-NaHCO3 5% medium supplemented with 10% FBS, penicillin and streptomycin. C-33A cells (human cervical carcinoma epithelial cells; ATCC Cat# HTB-31, 2012) were maintained in EMEM medium supplemented with 10% FBS and 5% of penicillin and streptomycin (Gibco by Life Technologies; Thermo Scientific). A master cell-bank was created for each cell line after purchase, and early-passage cells were thawed in every experimental step. Once in culture, cells were maintained not longer than 2 months to guarantee genotypic stability and were monitored by microscopy. As a specific feature of the HuH-7 cells, ability to support HCV replication was demonstrated by Matthew Evans group (37).

Cell lysates for SDS-Page were obtained using the ProteoJET Mammalian Cell Lysis reagent purchased from Fermentas (Thermo Scientific). ECL Western blotting system and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from GE Healthcare. ECL Western Blotting Substrate (Thermo Scientific) was used for detection of HRP in immunoblots.

Polyclonal antibodies to human/Fas (C18C12), anti-caspase 3, anti-caspase 9, and anti-PARP, as well as monoclonal antibody to caspase 8, were purchased from Cell Signaling. Monoclonal anti–β-actin (AC-15) was purchased from Abcam. Rabbit polyclonal serum to NDV was previously described (38). Monoclonal antibody to human APO-1/Fas was for Bender MedSystems.

Generation of recombinant NDV
Plasmid pNDV-B1, encoding the full-length antigenic cDNA of Hitchner B1 lentogenic strain of NDV, has been previously described (8). The open reading frame (ORF) of human Fas receptor was amplified by PCR using specific primers including the required regulatory signals for its functional integration into the NDV genome and then cloned into the XbaI site between the P and M genes of pNDV-B1. Recombinant rNDV-B1/Fas was rescued using previously described techniques (8). Insert fidelity was evaluated by amplification of the Fas sequence by reverse transcription PCR and virus genomic RNA followed by sequencing. Viral stocks of rNDV-B1/Fas and rNDV-B1 were propagated in 9-day embryonated chicken eggs and purified from the allantoic fluid in a potassium tartrate gradient.

Fluorescence microscopy
For indirect immunofluorescence staining, cells seeded in 24-well standard plates or glass-bottomed 12-well plates were infected for 1 hour at a multiplicity of infection (MOI) of 1 PFU/cell, after which the inoculum was removed and replaced with 1 mL of DMEM with 10% FBS P/S. At 20 hours after infection, cells were fixed with 2.5% paraformaldehyde for 15 minutes and blocked in PBS with 1% BSA for 1 hour. Primary antibodies were incubated with the samples for 1 hour at room temperature. Secondary antibodies (goat anti-mouse Alexa Fluor 568 or 633 or goat anti-rabbit Alexa Fluor 488; purchased from Invitrogen) were used at a 1:1,000 dilution for 45 minutes before imaging using an Olympus IX41 microscope or a Zeiss LSM 510 Meta confocal microscope.

Growth curves and titers
HeLa, Vero, A549, HuH-7, C-33A, and B16-F10 cells monolayers in 6-well plates were infected with the virus suspension at an MOI of 0.01 PFU/cell in OPTIMEM-I. After 1 hour, the infection media were removed and the cells were incubated with 3 mL of DMEM with 0.3% BSA and 1 μg of TPCK-treated trypsin/mL to allow the production of fusion-competent viruses. Supernatants were collected at 24, 36, 48, and 72 hours (72 hours in A549, HuH-7, C-33A, and B16-F10) after infection and titrated by immunofluorescence assay on Vero cells seeded on 96-well plates by using a polyclonal anti-NDV serum.

MTT cytotoxicity assay
HeLa, A549, HuH-7, C-33A, and B16-F10 cells were cultured at a confluence of 50% in 24-well dishes and infected at an MOI of 1 PFU/cell. Infection media were removed 1 hour after infection, and cells were incubated 24, 36, and 48 hours in 1 mL of supplemented DMEM (or EMEM for C-33A). Since every time point, the media were aspirated and cells were subsequently incubated 1 hour 15 minutes with 300 μL of 2.5 mg/mL MTT solution at 37°C and under restricted light conditions. Subsequently, the MTT solution was aspirated, and cells were incubated with 500 μL of...
Isopropanol for 10 minutes in a shaker. The absorbance of each sample was recorded at 570 nm using a BioTek plate reader.

**Annexin-V/propidium iodide flow cytometric analysis**

Cells were plated in 35-mm dishes and infected at an MOI of 1 PFU/cell. Infection media were removed 1 hour after infection and cells were incubated 24 and 48 hours in supplemented DMEM. Apoptosis induction was determined using the Immuno-Step DY-634 Annexin-V Apoptosis Detection Kit (ImmunoStep), according to the manufacturer’s instructions. Cell acquisition was made on a FACSCalibur flow cytometer (Becton Dickinson). Data analysis was performed using CellQuest, from BD Biosciences, and FlowJo software (Tree Star).

**Caspase activity assay**

HeLa cells were plated into 96-well plates and infected at an MOI of 1 PFU/cell. One hour after infection, the media were replaced by conventional DMEM or DMEM supplemented with caspase-specific inhibitor at different final concentrations. For specific caspase activity inhibition, the reagents InSolution Caspase 8 or InSolution Caspase 9 (EMD Millipore; Ref 218840 and 218841, respectively) were used. At 24 hours after infection, caspase 8, 9, and 3 activity was quantified by Caspase-Glo 8, 9, or 3/7 assay systems (Promega; G8210, G8211, and G8090, respectively) following the manufacturer’s instructions.

**Interferon response assay**

B16-F10 and NIH/3T3 cell monolayers in 6-well plates were infected with the virus suspension at an MOI of 1 PFU/cell in OPTI-MEM-I. After 1 hour, the infection media were removed and the cells were incubated with 3 mL of DMEM. Total RNAs from cultured cells were isolated 8 hours after infection with a Qiagen RNeasy Mini kit (Qiagen). Mean n-fold expression levels of cDNA from three individual biologic samples, each measured in triplicate, were normalized to 18S rRNA levels and calibrated to mock-treated samples according to the 2^−DDCt method (39).

The primer sequences were as follows: for the murine gene IFNβ, the forward primer was 5′-CAGCTCCAAGAAGGACGACAC-3′ and the reverse primer was 5′-GGCAGCTGAACCTCTCTG CAT-3′. For murine IFNα, the forward primer was 5′-CTGACAGF GTCACTTCGACATGGAA-3′ and the reverse was 5′-GTGCACTG CCAAAGGGTTCT-3′. For murine gene OAS1, the forward primer was 5′-ATGGACAGCCAGTACGAGA-3′ and the reverse was 5′-TCAACACAGGATGTTGAGG-3′. For murine IRF7 gene, the forward primer was 5′-GTGACCGTTGCTATGCGGAG-3′ and the reverse primer was 5′-GACGGAAAAGTTGCTAGCAGGG-3′.

The 18S forward primer was 5′-GTAACCCGTGTAACCCCATF-3′, and the 18S reverse primer was 5′-CCATCCAATCCTAGTACGG-3′.

**Syngeneic melanoma tumor model**

C57/BL6J female mice 4 to 6 weeks of age used in all our in vivo studies were purchased from The Jackson Laboratory. A B16-F10 cell suspension (5 × 10^6 cells in 100 µL of PBS) was intradermally inoculated into the flank of the right posterior leg of each C57/BL6J mouse. After 10 days, the mice were treated by intratumoral injection of 50 µL of 5 × 10^6 PFU of the indicated recombinant NDV virus or PBS. The intratumoral injections were administered every 24 hours for a total of three treatment doses. Tumor volume was monitored every 48 hours or every 24 hours when the last volume estimation was approaching the experimental endpoint of 1,000 mm^3. Mice were humanely euthanized the day in which the volume exceeded the predefined endpoint. Tumor measurement was determined using a digital caliper, and total volume was calculated using the formula: Tumor volume (V) = L × W^2, where L, or tumor length, is the larger diameter, and W, or tumor width, is the smallest diameter.

**Immunohistochemistry and immunofluorescence staining of tumor samples**

A suspension of B16-F10 cells (5 × 10^5 cells in 100 µL of PBS) was intradermally inoculated into the flank of the right posterior leg of C57/BL6J mice. After 10 days, one intratumoral injection of PBS or recombinant NDV virus suspension (5 × 10^6 PFU in 50 µL of PBS) was administered. At 24 hours after inoculation, the tumors were removed and preserved by formalin fixation and paraffin embedding for immunohistochemistry (IHC) analysis. IHC staining for active caspase 3 was performed on 5-µm-thick tumor sections. The slides were incubated in H2O2 solution for 15 minutes, and antigen retrieval was performed by steam heating in 10 mmol/L citrate buffer (pH 6.0) for 45 minutes. After epitope recovery, the slides were then treated with 10% of normal goat serum for 60 minutes, followed by incubation with caspase 3 antibody (1:500 dilution; Cell Signaling; ref. 9664) incubation overnight at 4°C. The slides were washed and incubated with secondary biotinylated anti-Rabbit IgG antibody (H+L; Vector Laboratories, Inc.) at 1:500 dilution for 1 hour followed by incubation with avidin–biotin conjugate (1:25 dilution; ABC complex; Vector Laboratories, Inc.) incubation for 30 minutes. The samples were treated with the chromogen diaminobenzidine for antigen detection, and the final counterstaining was performed with hematoxylin.

**Analysis of myeloid cell populations present in infected tumors**

For the characterization of the immune cells within the tumor in response to the virus treatment, B16-F10 melanoma syngeneic model was carried out in C57/BL6J female mice as described before. The animals received a total of three intratumoral injections of PBS or recombinant NDV virus suspension (5 × 10^6 PFU in 50 µL of PBS), one every 24 hours, and the tumors were isolated 24 hours after the last injection.

For the immune cell isolation, the tumors were minced, digested with collagenase IV (Roche) for 1 hour at 37°C, and passed through 70-µm cell strainers to obtain single-cell suspensions. Cells were layered in a 40% and 90% Percoll gradient (GE Healthcare) and centrifuged at 1,260 × g for 40 minutes without brake. The interphase was collected and analyzed by flow cytometry.

Flow cytometry analysis: fluorochrome-conjugated antibodies against CD44 (clone IM7) were purchased from BD Pharmingen, against Ly-6C (AL-21) from BD Biosciences, against CD4 (RM4-5), CD8α (53-6.7), CD11b (M1/70), CD11c (N418), CD25 (PC61.5), CD62L (MEL-14), and Foxp3 (FJK-16s) from eBioscience, against CD3 (17A2), CD45 (30-F11), CD64 (X54-5/7.1), CD103 (2E7), I-A/I-E (M5/114.15.2), and Ly-6C (1A8) from BioLegend, against CCR2 (475301) from R&D Systems. Cells were incubated with specific antibodies in DPBS containing 0.5% BSA and 2 mmol/L EDTA for 20 minutes at 4°C. Intracellular staining for Foxp3 was performed using the transcription factor fixation/permeabilization concentrate and diluent from eBioscience. Samples were acquired on a BD LSRFortessa (Becton
Dickinson) using the FACSDiva Software and analyzed with FlowJo software (Tree Star).

Statistical analysis
Statistical significance between results from triplicate samples was determined by the two-tailed Student t test. The results are expressed as mean values ± SDs. The comparison of survival curves for the data obtained in the B16-F10 melanoma syngeneic model was performed using the long-rank (Mantel–Cox) test. The analysis of the myeloid cell populations presented within the treated tumors was performed using a one-way ANOVA (Dunn Multiple comparison test).

Ethics statement
All animal procedures performed in this study are in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines, and have been approved by the IACUC of Icahn School of Medicine at Mount Sinai.

Results
Detection of human fas receptor expression in rNDV-B1/Fas–infected cells
In our study, a new recombinant NDV virus, rNDV-B1/Fas, was generated and assessed for improved oncolytic potential. For this purpose, the ORF of the human Fas receptor was inserted into the backbone of the lentogenic NDV-B1 virus genome between the P and M genes (Fig. 1A) to ensure high level of protein expression during virus replication (1). Insert stability within the viral genome was assessed after six viral growth passages in eggs, and the fidelity of its sequence was confirmed by RT-PCR. Comparison of growth properties of rNDV-B1/Fas with those of the parental rNDV-B1 in the human cancer cell lines HeLa, A549, HuH-7, and C-33A, as well as in Vero cells showed no differences (Fig. 1B), with both viruses replicating to similar titers and kinetics. Specific expression of Fas receptor after infection with the newly generated virus was detected by immunofluorescence in HeLa cells (Fig. 1C), as well as in other human (A549, HBL, C-33A, Hep2, HuH, PC3) cancer–derived cell lines, and in the African Green monkey cell line Vero (data not shown). As expected, the Fas protein was broadly distributed on the cell surface, where areas of high-density receptor accumulation could be clearly distinguished (Fig. 1D, top).

The intracellular localization of the receptor during rNDV-B1/Fas infection was determined by confocal microscopy following immunofluorescent labeling (Fig. 1D, bottom). Inside the cell, Fas could be found colocalizing with the early endosome marker Rab5 in the cytoplasmic endosomal compartment, one of the hallmarks of Fas activation. These localization patterns suggest that overexpression of Fas receptor by the recombinant virus results in its activation in the absence of its ligand.

Enhanced cytopathic effect in rNDV-B1/Fas–infected cells correlates with an early activation of the apoptosis response
To determine whether overexpression of Fas receptor during rNDV-B1/Fas virus infection could enhance the inherent proapoptotic activity of NDV, we studied specific morphologic and biochemical features of this type of cell death. First, apoptosis–specific morphologic modifications in rNDV-B1 or rNDV-B1/Fas–infected cells were monitored by microscopy. At 24 hours after infection, the number of adherent cells was dramatically reduced during rNDV-B1/Fas virus infection as compared with those infected with rNDV-B1. The remaining attached cells presented typical apoptosis morphologic landmarks like cytoplasmic membrane blebbing and DNA fragmentation as observed by immunostaining (Fig. 2A). Cells that undergo apoptosis gradually lose metabolic functions leading to cell death. To quantify rNDV-B1/Fas and rNDV-B1 replication–associated cytotoxicity, we performed an MTT viability assay that measures the activity of mitochondrial reductase enzymes which are only active in living cells. rNDV-B1/Fas virus infection resulted in more than 50% reduction in HeLa cells viability at 24 hours after infection (Fig. 2B, right). Even at the latest time point of our study, 48 hours after infection, rNDV-B1 virus did not promote more than 50% reduction in cell viability. Both cytomorphologic and viability studies suggest that cells infected with rNDV-B1/Fas undergo an earlier and more potent apoptosis response. This enhanced cytotoxicity was also validated in other human (A549, HuH-7, C-33A) and murine (CT26) cancer–derived cell lines (Fig. 2B, left).

To further characterize the apoptosis response induced by rNDV-B1/Fas infection, we performed Annexin-V/propidium iodide (PI) stains in HeLa-infected cells. Since Annexin-V staining precedes PI staining during apoptosis, late stages of apoptosis are characterized by double Annexin-V/PI stain (Fig. 2C). As early as 24 hours after infection, rNDV-B1/Fas–infected cells showed higher number of cells progressing into the final stages of late apoptosis than cells infected with rNDV-B1.

Overexpression of Fas leads to activation of both intrinsic and extrinsic pathways in rNDV-B1/Fas–infected cells
We next studied the caspase activation pattern during infection with either rNDV-B1/Fas or rNDV-B1 (Fig. 3A). Protein extracts collected at different time points after infection were tested to detect active forms of the principal caspases involved in both extrinsic and intrinsic pathways. As compared with rNDV-B1–infected cells extracts, rNDV-B1/Fas–infected cells showed activation of caspases 8, 9, and 3 at 24 hours, which is in contrast with their late (48 hours) activation in rNDV-B1–infected cells. This was also accompanied by the presence of cleaved Poly (ADP ribose) polymerase PARP that is a marker for the final stages of programmed cell death.

We next wanted to investigate the contribution of the extrinsic (caspase 8–dependent) pathway into the new modified apoptotic response due to Fas overexpression. Adding specific caspase inhibitors to the postinfection media, we could observe that after 24 hours the inhibition of caspase 8 had the strongest effect restricting both caspase 9 and 3 activities in rNDV-B1/Fas–infected cells (Fig. 3B). However, caspase 9 inhibition led to a slight inhibition of caspase 8 and 3 activities. No effects were seen in rNDV-B1–infected cells, since at 24 hours after infection, there is still no detectable caspase 3, 8, and 9 activation. The apoptosis activation pattern seen in rNDV-B1/Fas–infected cells indicates that the upregulation of Fas receptor results in coactivation and cooperation in cell death of both the extrinsic (caspase 8–dependent) and intrinsic (caspase 9–dependent) pathways with an important and earlier contribution of the Fas-mediated caspase 8–dependent extrinsic pathway. This cooperative effect is likely responsible for the increase in the general apoptosis response observed during rNDV-B1/Fas infection as compared with rNDV-B1 infection.
rNDV-B1/Fas virus infection leads to interferon response activation and enhances apoptosis induction in murine B16-F10 melanoma cells

Previously to the evaluation of the potential therapeutic effect of the rNDV-B1/Fas virus in vivo, we wanted to know if the oncolytic properties displayed by the rNDV-B1/Fas virus in human cancer cell lines in vitro would be preserved also in cancer cells of murine origin.

Both, rNDV-B1 and rNDV-B1/Fas viruses were able to replicate at similar levels in B16-F10 showing similar titers and kinetics (Fig. 4B). By confocal microscopy, we could observe that the human Fas receptor was highly expressed and broadly distributed on the cell surface as well as in the cytoplasmic endosome compartment of the murine cell line (Fig. 4A), same way as we described before for the human cancer cell line HeLa (Fig. 1C and D). rNDV-B1/Fas virus exerted higher and earlier cytotoxicity in the murine cancer cell line (Fig. 4C), and this cytotoxicity was also correlated with an earlier activation of the apoptosis response, with the presence of active forms of caspase 3 detected 24 hours after infection (Fig. 4D).

Because the NDV therapeutic effect in vivo involves an active interferon response induction upon virus infection (5), we wanted to evaluate the potential stimulation of the rNDV-B1/Fas virus in the murine B16-F10 melanoma cells. To assess this question, B16-F10 cells with rNDV-B1 or rNDV-B1/Fas viruses at an MOI of 1 and total RNA were isolated 8 hours after infection. Immortalized NIH/3T3 murine fibroblasts were infected in similar conditions to be used as a positive control. The levels of INFβ
mRNA as well as those for the interferon stimulated genes IFT1, IRF7, and OAS1 were evaluated by qPCR (Fig. 4E). No difference was found between the viruses, and the B16-F10 cells showed similar levels of interferon induction as the immortalized fibroblasts, 8 hours upon infection.

Intratumoral treatment with rNDV-B1/Fas virus enhances survival, promotes complete tumor remission and protection against rechallenge in melanoma syngeneic murine model

To assess whether the improvement of the proapoptotic activity of rNDV-B1/Fas would enhance the inherent oncolytic properties of NDV in vivo, we tested its antitumor capacity in melanoma syngeneic tumor models. Murine melanoma B16-F10 cells were subcutaneously implanted in the leg flanks of C57Bl/6 mice. Tumors were allowed to develop until palpable, and a total of three intratumoral injections of each virus or PBS were administrated, as described in Materials and Methods. Treatment with rNDV-B1/Fas virus restricted tumor progression (Fig. 4F), leading to a significant improvement in survival compared with mice treated with rNDV-B1 or PBS. rNDV-B1 treatment delayed tumor growth, but only resulted in complete tumor remission in 12% of treated animals. However, complete tumor remission was observed in 83% of mice treated with rNDV-B1/Fas (Fig. 4F). These remaining tumor-free animals...
did not show tumor recurrence, loss of weight, or any other sign of sickness.

We also wanted to know if the enhancement of the apoptotic response could also influence the emergence of a long-time protection against cancer relapse. To assess this question, a new set of animals were used to induce a syngeneic melanoma model, and once they presented tumors, the animals were subjected to the same treatment conditions described before. The rNDV-B1/Fas virus treatment induced an overall enhancement of survival of 43%, and complete tumor revision was also reported within the experimental group (Fig. 4G). However, in this study, none of the animals treated with the wild-type virus underwent tumor remission. The survivors treated by the rNDV-B1/Fas virus that demonstrated complete recovery for up to 30 days were then rechallenged against melanoma by subcutaneous reinjection of B16-F10 melanoma cells in the flank of the opposite leg. Those animals, as a part of a long-term study, were under periodical observation, and no sign of tumor reversion or any other sign of sickness was reported for up to 6 months.

Early and enhanced apoptosis response during rNDV-B1/Fas virotherapy could be a key point for a more specific immune response against the tumor

We wanted to know if the earlier proapoptotic activity exerted by rNDV-B1/Fas virus in vitro could be also a main feature of the rNDV-B1/Fas–treated tumors in vivo. For that propose, murine melanoma B16-F10 cells were intradermally implanted on the flank of the leg in C57BL/6 mice. After 10 days, the generated tumors were intratumorally treated with a single dose of PBS or virus suspension. At 24 hours after inoculation, the mice were culled and the tumors were removed and processed for general histopathology analysis, virus immunodetection, and apoptosis markers determination (Fig 5A, B, C and Supplementary Fig. S1A and S1B). At this time point (24 hours after treatment), tumor histopathology analysis did not show any relevant differences in markers of advanced melanoma progression, such as high vascularization and necrosis, between treatment groups (PBS, rNDV-B1, or rNDV-B1/Fas–treated tumors), as monitored after hematoxylin and eosin staining (Fig. 5A). Only rNDV-B1– or rNDV-B1/Fas–infected tumors were positive for anti-NDV F protein detection (Fig. 5B), but there were no significant differences related to infection distribution or virus spread between these two groups. However, we could detect a notable presence of active caspase 3–positive cells in rNDV-B1/Fas–treated samples compared with both PBS and wild-type virus treatments (Fig. 5C). This indicates that the earlier and improved apoptosis response previously described in vitro for rNDV-B1/Fas (Figs. 2, 3, and 4) also occurs after intratumoral inoculation in vivo (Fig. 4A and B).

Last, we wanted to refine the characterization of the therapeutic effect exerted by the recombinant rNDV-B1/Fas in the early phase of the treatment by analyzing the innate immune cells populations resident within the tumors upon virus treatment. For that propose, we carried out a new syngeneic melanoma assay in C57BL/6 mice, following the same proceedings previously described. In this particular experiment, the animals received the complete treatment (3 doses of PBS or recombinant virus injected intratumorally) and were culled 24 hours after the administration of the last dose.

The tumors were removed and specifically processed for the isolation and analysis by Flow cytometry of the different myeloid cells lineages, as is mentioned in Materials and Methods. As expected, the response to both viruses demonstrated to be more immunogenic compared with the PBS treatment, with a decrease in dendritic cells and an increase in proinflammatory macrophages and neutrophils resident at that moment in the infected tumors (Fig. 5D). Further investigations would be needed to evaluate the contribution of the innate immune response in our
Figure 4.
nNDV-B1/Fas virus exerts higher oncolytic capacity and increases survival in a syngeneic melanoma murine model. A, immunofluorescence detection of human Fas receptor in rNDV-B1/Fas-B16-F10-infected cells. Confocal microscopy image of murine melanoma B16-F10 cells infected with rNDV-B1/Fas. Cells were infected at an MOI of 1 PFU/cell, fixed 20 hours after infection, and stained with monoclonal anti-human Fas antibody (red), polyclonal anti-NDV serum (green), and Hoechst for nuclear contrast. Scale bar, 50 μm. White arrow, endosome compartment localization of the recombinant human Fas receptor. B, multicycle growth curves. B16-F10 monolayers were infected at an MOI of 0.01 PFU/cell. At different time points after infection, viral titers in the supernatant were determined. Data points show mean values from three replicates with error bars representing SDs. C, cytotoxicity. B16-F10 cells were infected at an MOI of 1 PFU/cell, and viability was determined by MTT viability assay at different time points (24, 36, and 48 hours after infection; n = 3; **, P < 0.0005). D, time course of caspases activation and Western blot. B16-F10 cells were infected with rNDV-B1/Fas and rNDV-B1 at an MOI of 1 PFU/cell, and lysates were obtained at different time points after infection. Apoptosis activation was assessed by Western blot using a specific anti-caspase 3 antibody. Human Fas receptor expression was detected using an anti-human Fas monoclonal antibody. Viral replication (NP levels) was detected using an anti-NDV polyclonal serum. (Continued on the following page.)
Oncolytic Activity of rNDV-B1/Fas

model. An improved and more specific innate immune response due to a better immunological cell death of the tumor cells would be the major determinant in the long-term protection observed in the rNDV-B1/Fas–treated mice.

Discussion

During the last decade, new studies exploring NDV antitumor characteristics have emerged, and a new generation of recombinant NDVs has been engineered attempting to enhance its natural oncolytic capacity (4). In our current study, to design an improved therapeutic agent, we attempted to increase cell death induced in NDV-infected tumors. To do so, we generated a recombinant NDV encoding the human tumor necrosis factor receptor Fas (TNFRSF6, CD95). Neither Fas nor its ligand FasL had come out in the list of host elements involved in the NDV-stimulated apoptosis response. Our rationale in designing such vector is that overexpression of Fas receptor in infected cells would lead to the activation of the extrinsic apoptosis pathway which would both complement and increase the proapoptotic capacity of NDV otherwise mainly mediated by the activation of the intrinsic pathway (30–32). This may enhance the antitumor activity of NDV in several ways, first enhancing tumor cell death during direct infection, second, increasing immune-mediated cell death of infected cells, and third, promoting the release of proinflammatory mediators that promote immune activation of antitumor responses.

At this moment, we do not know which of these factors or combination of factors is the main driver in the increased therapeutic effect of rNDV-B1/Fas.

Our in vitro studies with rNDV-B1/Fas demonstrated high levels of the recombinant human Fas receptor expression in different human- and mouse-infected cancer cell lines. Fas receptor expressed by NDV exhibited widespread distribution throughout the cell membrane with areas of receptor aggregates, consistent with Fas receptor activation by overexpression. The recruitment and stabilization of preassembled receptors in signaling protein oligomerization structures (SPOTS) in the cytoplasmic membrane are necessary to initiate signal transduction. This is physiologically mediated through Fas/Fasl interaction (40). However, during rNDV-B1/Fas infection, the overexpressed receptor was able to form these proapoptotic SPOTS in the cytoplasmic membrane in absence of FasL stimulus. A similar phenomenon was previously described in other approaches in which the membrane in absence of FasL stimulus. A similar phenomenon was previously described in other approaches in which the membrane and enhanced apoptosis response might play a key role in the extrinsic pathway mediator of Fas receptor activation.

In our in vivo studies, we tested the oncolytic activity of rNDV-B1/Fas and rNDV-B1 against B16 melanoma, one of the more aggressive syngeneic murine tumor models. rNDV-B1/Fas virotherapy demonstrated an extraordinary efficacy, not only improving survival time but also inducing complete tumor remission and long-term protection against tumor relapse. A single dose of the virus suspension was enough to unleash a strong antiapoptosis response in tumor cells perceptible as early as 24 hours after virus administration. Supported by our in vitro observations, the in vivo results strongly suggest that an early and enhanced apoptosis response might play a key role in the rNDV-B1/Fas successful virotherapy. This enrichment in apoptotic cell death within the tumor in early stages during the treatment and the consequent modification of the tumor microenvironment could be boosting a better and more specific innate immune response against the tumor and therefore
provide the perfect scenario to the emergence of a long-term adaptive immune response.

An improvement in survival and complete tumor remission was also achieved by rNDV-B1/Fas treatment of colon carcinoma syngeneic murine tumor model (data not shown). Melanoma and colon carcinoma are some of the human cancers with the worst prognosis due to their metastatic capacity and resistance to chemo therapeutic drugs in which defects on the Fas/FasL system are known to be responsible for the tumor progression (35, 48, 49). The list of malignant tumors in which Fas/FasL deficits have been implicated also includes pancreatic, thyroid and lung carcinomas, breast and ovarian cancer, and blood cancers, among others (35, 50). Our results obtained in the murine model open the possibility of a new approach for the
treatment of such aggressive tumors, providing a local therapy that combines the specificity of viral infection of cancer cells and the enhancement of the proapoptotic capacity of NDV.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References

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Molecular Cancer Therapeutics

Enhancement of the Proapoptotic Properties of Newcastle Disease Virus Promotes Tumor Remission in Syngeneic Murine Cancer Models

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