Genetic Modification of Oncolytic Newcastle Disease Virus for Cancer Therapy

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ABSTRACT

Clinical development of a mesogenic strain of Newcastle disease virus (NDV) as an oncolytic agent for cancer therapy has been hampered by its select agent status due to its pathogenicity in avian species. Using reverse genetics, we have generated a lead candidate oncolytic NDV based on the mesogenic NDV-73T strain that is no longer classified as a select agent for clinical development. This recombinant NDV has a modification at the fusion protein (F) cleavage site to reduce the efficiency of F protein cleavage and an insertion of a 198-nucleotide sequence into the HN-L intergenic region, resulting in reduced viral gene expression and replication in avian cells but not in mammalian cells. In mammalian cells, except for viral polymerase (L) gene expression, viral gene expression is not negatively impacted or increased by the HN-L intergenic insertion. Furthermore, the virus can be engineered to express a foreign gene while still retaining the ability to grow to high titers in cell culture. The recombinant NDV selectively replicates in and kills tumor cells and is able to drive potent tumor growth inhibition following intratumoral or intravenous administration in a mouse tumor model. The candidate is well positioned for clinical development as an oncolytic virus.

IMPORTANCE

Avian paramyxovirus type 1, NDV, has been an attractive oncolytic agent for cancer virotherapy. However, this virus can cause epidemic disease in poultry, and concerns about the potential environmental and economic impact of an NDV outbreak have precluded its clinical development. Here we describe generation and characterization of a highly potent oncolytic NDV variant that is unlikely to cause Newcastle disease in its avian host, representing an essential step toward moving NDV forward as an oncolytic agent. Several attenuation mechanisms have been genetically engineered into the recombinant NDV that reduce chicken pathogenicity to a level that is acceptable worldwide without impacting viral production in cell culture. The selective tumor replication of this recombinant NDV, both *in vitro* and *in vivo*, along with efficient tumor cell killing makes it an attractive oncolytic virus candidate that may provide clinical benefit to patients.

Interest in oncolytic viruses (OV) for cancer virotherapy has been increasing due to a better understanding of viral biology, tumor immunology, and molecular genetics of viruses and to the success of OV clinical outcomes. Various oncolytic viruses from diverse virus families have been developed and have advanced into clinical evaluation (1, 2). Recently, a modified herpes simplex virus 1 (HSV-1) termed talimogene laherparepvec (Imlygic; Amgen) was approved by the U.S. Food and Drug Administration (FDA) for the treatment of recurrent melanoma after initial surgery (3).

Newcastle disease virus (NDV) is classified as *Avian paramyxovirus type 1* (APMV-1) in the genus *Avulavirus* of the *Paramyxoviridae* family that contains highly pathogenic strains in avian species. Infection of humans with NDV occurs infrequently in poultry workers and results in mild influenza-like symptoms. The seroprevalence of NDV in the general human population is very low. Despite agricultural concerns about NDV in poultry, the virus has been investigated as an oncolytic virus due to its inherent selective replication in tumor cells and associated cell death eliciting innate and adaptive antitumor immune responses (4). Prior to 2008, NDV had shown safety and some effectiveness in preclinical studies (5, 6) and in human trials to treat a wide variety of tumor types (4, 7–9). The mechanism of NDV cancer cell selectivity is not entirely understood. NDV binds to sialic acid on host cells and can infect a broad range of cell types but can replicate efficiently in and kill only tumor cells. Many tumor cells have defects in antiviral responses such as interferon (IFN) production or responses to IFN signaling (10), allowing virus to replicate and

spread. Indeed, N-ras oncogene (5) or Rac1 expression has been shown to be sufficient to render nontumorigenic cells susceptible to NDV replication and cytotoxicity (11). Apoptosis-resistant cancer cells are particularly sensitive to NDV-mediated cell death (12) because NDV can activate both extrinsic and intrinsic apoptotic pathways (13).

NDV is an enveloped virus containing a linear, nonsegmented, negative-sense RNA genome with eight gene cassettes in the order 3'-NP-P/V-W-M-F-HN-L-5'. NDV genome transcription and replication are mediated by the viral RNA polymerase complex consisting of the large polymerase protein (L), the nucleocapsid protein (NP), and the phosphoprotein (P). The NP gene at the 3' end is the most abundantly expressed, and the L gene at the 5' end is the least abundantly expressed. The P gene also encodes V and W through an RNA editing mechanism. The V protein is an IFN antagonist that contributes to viral virulence in the avian host (14, 15). The fusion (F) protein is an integral glycoprotein that is syn-

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thesized as an inactive precursor (F0), and proteolytic cleavage of F0 into two disulfide-linked polypeptides (F1 and F2) by host cellular proteases is essential for virus infectivity and pathogenesis. The binding of the integral hemagglutinin-neuraminidase protein (HN) to sialic acid on the cell surface triggers conformational changes in the cleaved F protein that drive the virus-cell membrane fusion required for viral entry (16). The matrix protein (M) is involved in the viral budding process (17).

Based on chicken pathogenicity, NDV is classified into three pathotypes: lentogenic (avirulent), mesogenic (intermediate), and velogenic (virulent). The F protein cleavage site (FPCS) is a major virulence determinant (18). Lentogenic viruses contain a monobasic amino acid within the FPCS such that the F0 protein can be cleaved into F1 and F2 subunits only by a trypsin-like protease that is present extracellularly in the respiratory and intestinal tract. In contrast, both mesogenic and velogenic viruses have multibasic amino acids at the FPCS that can be cleaved intracellularly by the ubiquitous furin-like protease (18, 19), resulting in systemic infections. Other factors such as the HN glycoprotein, viral RNA polymerase complex, interferon antagonist V protein, and intergenic sequences (IGS) (20) have been shown to also contribute to viral virulence in chickens (21).

The pathogenic properties of NDV strains correlate with their oncolytic properties. Velogenic and mesogenic strains can efficiently replicate in multiple human cancer cells, resulting in effective cell lysis, whereas lentogenic strains cannot (22). Because the mesogenic and velogenic NDV strains can cause significant economic losses to the poultry industry, they were classified as select agents in 2008 per federal regulation (23). To our knowledge, the use of the mesogenic NDV strains for virotherapy has not progressed since due to concerns associated with handling and working with select agents. We have generated several recombinant oncolytic viruses derived from the mesogenic NDV 73T strain (24) and identified a lead candidate that is no longer virulent in chickens, representing a step toward using NDV for virotherapy.

MATERIALS AND METHODS

Virus and cells. African green monkey kidney (Vero) cells, human fibrosarcoma (HT1080) cells, human cervical adenocarcinoma (HeLa) cells, normal human skin (CCD1122Sk) fibroblast cells, chicken embryo fibroblast (DF-1) cell lines, and human embryo kidney (293T) cells were obtained from the American Type Culture Collection (ATCC). Baby hamster kidney cells expressing T7 RNA polymerase (BSR-T7) were obtained from Karl-Klaus Conzelmann (Ludwig Maximilians University). The mesogenic NDV 73T viral RNA was a gift from Mark Peeples (Nationwide Children's Hospital, Columbus, OH). A USDA permit was obtained to work with the NDV 73T strain and recombinant 73T derivatives in the biosafety level 2⁺ (BSL2⁺) laboratories.

cDNA construction and recovery of infectious recombinant NDV strain 73T. The antigenomic cDNA of NDV 73T was assembled from six subgenomic cDNA fragments generated by reverse transcription-PCR (RT-PCR) of viral genomic RNA using the restriction enzyme sites shown in Fig. 1A. The antigenomic cDNA is under the control of the T7 RNA polymerase at the 5' end followed by a ribozyme cleavage site and T7 terminator at the 3' end as determined by the method described for respiratory syncytial virus (RSV) reverse genetics (25). The transgene cassette with gene start and gene end sequences together with human granulocyte-macrophage colony-stimulating factor (hGM-CSF), a luciferase gene, or a green fluorescent protein (GFP) gene was inserted into viral antigenomic cDNA between the P and M genes at nucleotide (nt) 3150 via a created AfeI site. To insert sequences at the HN and L intergenic sequence (IGS), different lengths (60, 144, or 198 nt) of random sequence synthesized by

DNA 2.0, 198 nt of the nucleocapsid protein (NP) gene sequence of the RSV A2 strain (25), or 318 nt of the nucleoprotein (N) gene of avian paramyxoviruses (APMV) was inserted at nt 8361 via a created AfeI site (Fig. 1A). The sequence information is available upon request or can be found at <http://www.google.co.in/patents/WO2009095167A1?cl=und>. For virus rescue, 0.4, 0.4, and 0.2 µg of NP, P, and L protein expression plasmids, respectively, under the control of the T7 RNA polymerase promoter and terminator were cotransfected with 1.6 µg of antigenomic cDNA into BSR-T7 cells. The recovered virus was confirmed by sequencing RT-PCR-amplified cDNA. Genetic stability of the FPCS was examined by *in vitro* passage in Vero and HT1080 cells at multiplicity of infection (MOI) of 0.01. The identified changes at the FPCS were introduced into 73T antigenomic cDNA, and recombinant viruses were recovered as described above. Viruses were grown in embryonated chicken eggs or HeLa S3 suspension cells and purified by 20% to 60% sucrose gradient centrifugation for *in vivo* studies.

Virus plaque morphology and growth kinetics. Viral growth kinetics in 9-to-10-day-old embryonated chicken eggs was examined by inoculating 1,000 PFU/egg of NDV and incubating at 37°C. Allantoic fluid was harvested from 3 eggs at 48 h, 72 h, and 96 h postinoculation. Vero or DF-1 cells on 6-well plates were infected with serially diluted virus and incubated under a 1% methylcellulose overlay at 37°C for 36 h or 6 days. Viral plaques were visualized by immunostaining with chicken anti-NDV polyclonal antibody followed by horseradish peroxidase (HRP)-conjugated anti-chicken antibody (Dako) and imaged by light microscope under ×10 magnification. The plaque size of each variant was obtained by calculating the mean area of 10 plaques and expressed as a value relative to that of the strain 73T wild-type (73T-wt) virus. Experiments to examine virus growth kinetics in tissue culture cells were conducted by infecting cells with virus at an MOI of 0.01 in duplicate, and aliquots of culture media were collected daily for 4 days. Viral titer was determined by plaque assay on Vero cells in the presence of trypsin. hGM-CSF and IFN-β production in infected cell culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) according to the manufacturer's instructions.

Chicken pathogenicity test. The pathogenicity of the 73T viruses in chickens was determined by the egg mean death time (MDT) test in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs using a series of 10-fold dilutions as described previously (26) at MedImmune (Mountain View, CA). The MDT was calculated as the mean time (h) for the minimum lethal dose of virus to kill all the inoculated embryos. The intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks was conducted at the USDA's National Veterinary Service Laboratory (NVSL; Ames, IA) using virus at a hemagglutination (HA) titer of 32.

Northern and Western blot analysis. Total intracellular RNA was isolated from virus-infected cells at an MOI of 3.0 at 20 h postinfection using an RNeasy minikit (Qiagen) and electrophoresed in a 1.5% agarose gel containing 0.5 M formaldehyde, transferred to a nitrocellulose membrane, and then hybridized with a biotin-labeled riboprobe specific to the NDV L, HN, or NP gene or to negative-sense viral genomic RNA. For Western blot analysis, virus-infected cells or F plasmid-transfected 293 cells was separated on 4% to 20% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with viral protein-specific antibodies followed by HRP-conjugated secondary antibodies. The antibodies used in the study were produced in rabbit (polyclonal antibody against anti-NDV F1 or HN recombinant protein or synthetic L protein peptide) or guinea pig antiserum to synthetic peptides (NP, M, and V) or P monoclonal antibody (from Takemasa Sakaguchi of Hiroshima University). The Northern and Western blots were imaged with ImageQuant LAS 4000 (GE Healthcare Sciences), and RNA or protein band intensity was quantitated using GelQuantNET software (BiochemLabSolution).

Cell killing assay to examine viral oncolytic activity. Cells plated in 96-well plates at 5×10^3 cells/well overnight were infected with virus at various MOIs. Cell viability on day 3 postinfection was determined by the use of a CellTiter Glo kit (Promega) per the manufacturer's instruction.

FIG 1 Generation of recombinant NDV 73T and its derivatives. (A) Schematic representation of NDV 73T antigenomic DNA. Viral gene cassettes, including the gene start (green triangle) and gene end (red rectangle), restriction enzyme sites used for cDNA assembly, insertions of the transgene transcriptional cassette, and nucleotides at the respective P-M and HN-L intergenic regions, are indicated. (B) The amino acid sequences of the FPCS of the NDV 73T-wt strain and its derivatives, the 73T-L, 73T-S, 73T-S-km, and 73T-R mutants, are indicated. The F2 and F1 cleavage site is indicated as a slash (“/”). (C) Plaque size and morphology of 73T-wt (wt), 73T-L (L), 73T-S (S), 73T-S-km (S-km), and 73T-R (R) in DF-1 and Vero cells in the absence of trypsin supplement. The number under each plaque image represents the plaque size relative to 73T-wt. (D) F protein cleavage in different cell types by Western blotting. F protein expression plasmids bearing mutations at the FPCS were transfected into 293T cells, and the cells were harvested at 24 h posttransfection. The DF-1 and HeLa cells infected with the corresponding 73T variants at an MOI of 5.0 were harvested at 24 h postinfection. The F protein was detected with F-specific antibody. The percentage of cleaved F1 (%F1) protein from the total F protein (F0 + F1) was calculated.

The relative percentage of live cells was determined by comparing the ATP level of each testing sample to the level of the mock-infected cells (set at 100% viability).

Evaluation of NDV oncolytic activity in an HT1080 xenograft tumor model. Mouse studies were conducted in an AAALAC-accredited facility under a specified protocol (ACF-13-001) approved by MedImmune’s Institutional Animal Care and Use Committee (IACUC). Athymic nude mice (Taconic) (5 to 10 weeks old) were implanted subcutaneously (s.c.) with 100 μ l of 5×10^6 HT1080 cells in 50% Matrigel into one flank. When tumors reached a volume of approximate 110 to 200 mm³, 2×10^7 PFU or 1×10^8 PFU virus in 50 or 100 μ l was administered by intratumoral (i.t.) or intravenous (i.v.) injection into the tail vein, respectively. The control animals were injected with 50 or 100 μ l phosphate-buffered saline (PBS). Tumor growth was measured using a digital caliper, and tumor volume was calculated as $0.5 \times (\text{height}) \times \text{width} \times \text{length}$ (in cubic millimeters).

Virus biodistribution in HT1080 xenograft mice after i.v. injection with 10^8 PFU of virus was determined by sacrificing 3 mice at various times postinjection followed by testing of homogenized tissues or blood samples for viral infectivity by plaque assay on Vero cells. hGM-CSF was quantitated by the use of a DuoSet ELISA kit (R&D); cytokines and chemokines in tumor tissues were quantitated by the use of a custom-made Luminex kit (Millipore). To image viral distribution in live mice, 1×10^8 PFU of 73T-R-198-Luc virus was i.v. injected. Mice were injected i.p. with luciferin (30 mg/kg of body weight) and anesthetized (2% isoflurane) prior to use of an IVIS imaging system (PerkinElmer). Immune cell

infiltration (neutrophil, NK, and macrophage cells) into the tumor on day 4 post-i.v. injection was examined by staining a single-tumor-cell suspension with anti-Gr-1, NKp46, and F4/80 (BD Biosciences), respectively, and the results were analyzed using FACSCanto II (BD Biosciences) and FACS Diva (BD Biosciences) software.

Data analysis. All statistical analyses were performed using GraphPad Prism 6.0 software. The unpaired *t* test was used to assess differences in tumor regression between groups. GraphPad Prism software was also used to calculate the quantity of virus resulting in a 50% reduction in cell viability (IC₅₀) in *in vitro* cell killing assays in normal and tumor cells.

RESULTS

Generation of recombinant NDVs with modified F protein cleavage site. The antigenomic cDNA of the NDV 73T strain was assembled from six reverse-transcribed and PCR-amplified cDNA fragments from viral genomic RNA. A transcriptional unit encoding granulocyte macrophage colony-stimulating factor (GM-CSF), luciferase, or GFP was inserted at the P and M junction of the 73T antigenomic cDNA (Fig. 1A). Unless specifically mentioned, all recombinant viruses used in this study correspond to human GM-CSF (hGM-CSF) transgenes. To reduce viral virulence in chickens, the FPCS of 73T-wt (amino acids 111 to 117) was modified to that of a lentogenic strain (L; Fig. 1B) or alternatively to that of the modified cytomegalovirus (CMV) glycoprotein

TABLE 1 Recombinant NDV nomenclature, viral titers, and chicken pathogenicity

Virus ^a	FPCS ^b sequence	HN-L intergenic extension (no. of nt)	Viral peak titer (log ₁₀ PFU/ml) ± SD		MDT ^c (h)	ICPI ^d
			Eggs	Vero		
73T-wt	¹¹¹ GRRQKR ¹¹⁶ /F	None	8.7 ± 0.42	6.7 ± 0.29	57	1.67
73T-L	¹¹¹ GGRQER ¹¹⁶ /L	None	8.8 ± 0.14	3.9 ± 0.28	>168	0.0
73T-S	¹¹¹ HNRTKS ¹¹⁶ /F	None	8.7 ± 0.07	5.5 ± 0.07	>168	0.0
73T-S-km	¹¹¹ HNKMKS ¹¹⁶ /F	None	8.6 ± 0.07	6.6 ± 0.07	>168	0.0
73T-R	¹¹¹ HNRTKR ¹¹⁶ /F	None	8.3 ± 0.06	6.8 ± 0.02	66	0.65
73T-R-60r	Same as 73T-R	60 (random)	8.1 ± 0.14	6.8 ± 0.21	64	0.78
73T-R-144r	Same as 73T-R	144 (random)	8.4 ± 0.28	6.9 ± 0.07	78	0.74
73T-R-198r	Same as 73T-R	198 (random)	7.8 ± 0.15	6.9 ± 0.04	>168	0.0
73T-R-198	Same as 73T-R	198 (viral)	7.7 ± 0.16	6.8 ± 0.01	>168	0.04–0.15
73T-R-318	Same as 73T-R	318 (viral)	8.0 ± 0.10	6.9 ± 0.10	>168	0.0–0.27

^a All viruses contained hGM-CSF at the P-M junction, grown in eggs at 37°C for 72 h or Vero cells without trypsin supplementation for 3 to 5 days at 37°C.

^b Fusion protein cleavage site amino acid sequence. Underlined amino acids differ from the wt sequence.

^c Mean death time (MDT) in eggs measured in hours. Velogenic strains, <60 h; mesogenic strains, 60 to 90 h; lentogenic strains, >90 h.

^d Pathogenicity of NDV in 1-day-old pathogen-free chicks measured by ICPI. ICPI scale, 0 to 2.0. A strain with an ICPI value of <0.7 or <0.4 (United Kingdom) is not a select agent.

tein B (S); the recovered viruses were designated 73T-L or 73T-S, respectively. Both viruses replicated well in embryonated chicken eggs, but 73T-L grew poorly in Vero cells, whereas 73T-S replicated to a level comparable to that of 73T-wt virus (Table 1).

Genetic stability of the modified FPCS was assessed by passaging 73T-S virus in Vero and HT1080 cells 10 times, and the F and HN genes were sequenced. Two types of mutations at the FPCS emerged at Vero passage 7 or HT1080 passage 9 (Fig. 1B): ¹¹¹HNRTKS-F¹¹⁷ (S) was changed to ¹¹¹HNKMKS-F¹¹⁷ (S-km), resulting in the loss of a potential N-linked glycosylation site (NRT) in the FPCS; another mutation was a serine-to-arginine substitution at residue 116 (¹¹¹HNRTKR-F¹¹⁷) (R). No other mutations were detected in the F and HN genes. 73T-S-km and 73T-R replicated as efficiently as 73T-wt in embryonated chicken eggs and Vero cells (Table 1) and had larger plaque sizes than 73T-L and 73T-S, both of which could not spread from cell to cell efficiently in avian and human cell lines. 73T-R had a plaque size larger than that of 73T-S virus but smaller than that of 73T-wt virus in both DF-1 and Vero cells (Fig. 1C).

The effect of FPCS mutation on F protein cleavage efficiency was examined using transiently expressed F proteins (Fig. 1D). 73T-wt F protein was processed most efficiently by host protease, as approximately 52% of the total F protein (F0 + F1) detected on Western blots was in the cleaved F1 form (the F2 polypeptide was not detected by the anti-F antibody). The F protein with the L or S FPCS sequences was not cleaved, while S-km and R variants had approximately 33% and 25% of the total F protein cleaved, respectively. The uncleaved F0 protein of the R or S variants exhibited slower electrophoretic mobility, likely because of a potential N-linked glycosylation site present at the FPCS. In virus-infected cells (Fig. 1D), 73T-wt F was most efficiently cleaved in chicken DF-1 (89% F1) and human HeLa (99% F1) cells. The F protein of 73T-R (45% to 46% F1) was more efficiently cleaved than those of 73T-L (1% F1), 73T-S (2% to 3% F1), and 73T-S-km (7% to 11% F1). Almost all F0 proteins were cleaved when exogenous trypsin was added to the culture of virus-infected cells (data not shown). We did not examine the impact of F protein cleavage efficiency on F protein surface expression since the F protein cleavage is mainly considered to be responsible for viral cell-to-cell spread.

FPCS modification and HN-L intergenic sequence extension reduce viral pathogenicity in chickens. The impact of the FPCS mutations on viral pathogenicity in chickens was determined using MDT and the ICPI test (Table 1). 73T-wt virus had a MDT of 57 h and an ICPI value of 1.67, whereas 73T-L, 73T-S, and 73T-S-km were avirulent in chickens, with MDT values greater than 168 h and an ICPI value of 0.0. However, the 73T-R virus had a MDT of 66 h and an ICPI of 0.65. This ICPI value is marginally lower than the select agent threshold of 0.7 defined by the USDA (https://www.aphis.usda.gov/animal_health/emergency_management/downloads/sop/sop_nd_e-c.pdf) but is above the level required by some countries outside the United States (e.g., the United Kingdom considers NDV strains with ICPI values of >0.4 to be select agents). While the 73T-L and 73T-S viruses met the international requirement for chicken virulence, they posed development issues as oncolytic agents due to poor growth of 73T-L in cell culture and instability of the FPCS of 73T-S. Although 73T-S-km and 73T-R replicated well in Vero cells, the virulence of 73T-R in chickens was not low enough for worldwide development. Thus, we decided to modify 73T-R to further reduce its avian pathogenicity.

Modifications of sequences in NDV intergenic regions have been shown to reduce viral transcription, replication, and pathogenicity (20). To further attenuate 73T-R, random sequences of 60 nt to 198 nt in length (R-60r, R-144r, and R-198r) or viral sequences derived from other paramyxoviruses of 198 nt (73T-R-198) or 318 nt (73T-R-318) were inserted into the HN-L intergenic junction of the 73T-R virus (Table 1). Sequence insertion of 60 nt (73T-R-60r) or 144 nt (73T-R-144r) did not reduce ICPI values, and the slight difference in ICPI values for 73T-R, 73T-R-60r, and 73T-R-144r likely reflects variability in the ICPI assay. 73T-R-198r, 73T-R-198, and 73T-R-318 had MDT values of >168 h and ICPI values ranging from 0.0 to 0.27. Their levels of replication in embryonated chicken eggs ranged from 7.7 to 8.0 log₁₀ PFU/ml, lower than the levels seen with 73T-R (8.3 log₁₀ PFU/ml) and 73T-wt (8.7 log₁₀ PFU/ml), but their peak titers in Vero cells were not reduced (Table 1). Thus, sequence insertions of 198 or 318 nt at the HN-L junction reduce NDV replication and virulence in chickens without affecting growth in

FIG 2 Virus growth kinetics in eggs and cells. Embryonated chicken eggs were infected with 100 PFU/egg of virus and incubated at 37°C for 2 to 4 days. DF-1 cells and HeLa cells in six-well plates were infected with the indicated virus in duplicate at an MOI of 0.01 PFU/cell, and the culture supernatants were collected daily for 4 days. Virus titers were determined by plaque assay in Vero cells.

mammalian cells, a desirable characteristic for oncolytic virus production in a cell culture system.

Growth kinetics of recombinant 73T derivatives in cell culture. As 73T-R-198r and 73T-R-198 exhibited similar levels of growth in cell culture and low avian virulence, we chose 73T-R-198 for further evaluation. 73T-S-Km and R-198 were compared with 73T-wt and 73T-R viruses for their growth in embryonated chicken eggs and in chicken DF-1 and human HeLa cells. In embryonated chicken eggs, 73T-wt, 73T-S-km, and 73T-R viruses reached comparable peak titers on day 3 or 4. However, R-198 had significantly reduced viral titers in eggs compared to the viruses without the intergenic insertion. 73T-wt had faster growth kinetics, and its titer declined from day 3 in both DF-1 and HeLa cells (Fig. 2). 73T-S-km had the slowest growth kinetics and the lowest titer in both cell lines. 73T-R-198 had slower growth kinetics, but its peak titer was similar to that of 73T-R in DF-1 cells and HeLa cells, and yet it achieved a slightly higher titer on day 3 in HeLa cells. Thus, the modifications introduced into 73T-R-198 did not affect viral yield, an important feature for viral manufacture.

Viral RNA and protein synthesis of NDV with a sequence insertion at the HN-L junction. Northern and Western blot analyses were performed to examine whether intergenic insertion would affect viral RNA transcription and protein expression in infected cells (Fig. 3). Viral genomic RNA levels and L, HN, and NP mRNA levels in 73T-R-198-infected DF-1 cells at an MOI of 3.0 were generally lower than those of 73T-R (Fig. 3A). In contrast, although 73T-R-198-infected HeLa cells had lower L mRNA levels, the genomic RNA and NP and HN mRNA levels were not reduced or were even higher than those of 73T-R. Viral proteins produced in 73T-R- and 73T-R-198-infected DF-1 and HeLa cells also had different patterns (Fig. 3B). The level of L protein in 73T-R-198-infected DF-1 cells was 0.2-fold of the level seen with 73T-R, and yet the amounts of the HN, NP, and V proteins were reduced only slightly compared to those seen with 73T-R (0.5-fold to 0.8-fold of the 73T-R levels). In contrast, despite a lower (<0.1-fold) level of L protein expressed by 73T-R-198, expression of the viral genes upstream of the L gene in HeLa cells was increased (1.1-fold to 2.0-fold of that of 73T-R). Similar results were obtained when DF-1 and HeLa cells were infected at a lower MOI (0.3) (Fig. 3C). The L protein level in 73T-R-198-infected DF-1 and HeLa cells was below the detection limit, but levels of other viral proteins were decreased (an average of ~0.4-fold) in DF-1 cells and increased (an average of ~1.5-fold) in HeLa cells. As men-

tioned earlier, all recombinant 73T viruses were engineered to express the hGM-CSF transgene. Similarly to the observed viral protein expression, 73T-R-198 produced more hGM-CSF than 73T-R in infected HeLa cells and human fibrosarcoma HT1080 cells (Fig. 4A).

73T-R-198 induces a lower interferon level in infected cells. A higher level of V protein production in 73T-R-198-infected HeLa cells (Fig. 3B) could result in reduced host innate immune responses due to its interferon agonist activities (27, 28). As shown in Fig. 4B, 73T-R-198 IFN- β production was about 2-fold lower than 73T-R IFN- β production in both the HeLa and HT1080 cell lines. Since both HeLa and HT1080 are cancer cell lines and the HT1080 cell line has been shown to have defects in responding to IFN (29), it was difficult to determine if the increased V protein level in 73T-R-198-infected cells was the only factor that accounted for the difference in interferon production levels in these cells. Nevertheless, the reduced IFN- β production in 73T-R-198-infected cells might lead to fewer paracrine antiviral responses, thereby compensating for low L protein expression and enabling efficient virus replication in human cells.

FIG 3 Analysis of viral RNA and protein synthesis *in vitro*. (A) DF-1 and HeLa cells were infected in duplicate with 73T-R or with 73T-R198 at an MOI of 3.0 PFU/cell for 20 h, and total intracellular RNA was extracted for Northern blot analysis with probes specific for the L, HN, and NP genes. gRNA, genomic RNA. (B and C) The infected DF-1 and HeLa cell lysates from experiments conducted on a different day were examined by Western blot analysis using viral protein-specific antibodies; infection was conducted at an MOI of 3.0 (B) or 0.3 (C). Viral protein levels in 73T-R-198-infected cells are expressed as the fold change relative to the protein levels of 73T-R-infected cells. The data are representative of the results of three independent experiments.

FIG 4 Quantitation of hGM-CSF and IFN- β in infected cells. HT1080 and HeLa cells were infected with 73T-R and 73T-R-198 at an MOI of 0.5 or 5.0 PFU/cell. Aliquots of cell culture supernatants were taken at 16 h or 32 h postinfection (hpi). The hGM-CSF (A) and IFN- β (B) levels were determined by ELISA. The data are representative of the results of two independent experiments.

Selective cancer cell killing and oncolytic activity of 73T variants. The oncolytic activity of 73T derivatives, i.e., 73T-S-km and 73T-R-198 viruses, was compared with that of the 73T-wt virus by *in vitro* cell killing assay using a pair of cancer and normal cell lines, HT1080 fibrosarcoma cells and normal human skin fibroblast CCD1122Sk cells (Fig. 5A). Cell viability and viral titers were determined at 72 h postinfection. In HT1080 cells, the quantity of the 73T-S-km virus resulting in a 50% reduction in cell viability (IC_{50}) was 0.006 PFU/ml, which was at least 6-fold greater than the IC_{50} values calculated for 73T-wt (IC_{50} = 0.001 PFU/ml) and 73T-R-198 (IC_{50} = 0.001 PFU/ml). 73T-R-198 was similar in this respect to 73T-wt, which possessed more potent oncolytic activity than 73T-S-km. In normal CCD1122Sk cells, all viruses were inefficient at cell killing, with IC_{50} values that were 100-

fold (73T-S-km) or about 500-fold (73T-wt and 73T-R-198) higher than the IC_{50} values calculated for the same virus-infected HT1080 cells. 73T-R virus was also evaluated for selective cell killing in these two cell lines, and its IC_{50} values in HT1080 and CCD1122Sk were similar to those of the 73T-wt virus. In addition, all 3 viruses grew in the HT1080 cancer cells to titers that were about 75-fold to 100-fold higher than those observed in noncancerous CCD1122Sk cells at an MOI of 0.01 (Fig. 5B). In addition, selective viral replication in cancer cells was also shown by infection of HT1080 and CCD1122Sk cells with 73T-S-km or 73T-R-198 (containing a GFP transgene inserted at the P-M junction instead of hGM-CSF) (Fig. 5C). Expression of the GFP transgene in 73T-S-km- and 73T-R-198-infected HT1080 cells was greater than in CCD1122Sk normal

FIG 5 Selective replication and cytotoxicity of 73T derivatives in tumor cells. (A) HT1080 cells (hashed lines) and CCD1125K cells (solid lines) were infected with the indicated viruses at MOIs ranging from 0.0001 to 10 PFU/cell, and cell viability was determined at 72 h postinfection. Viability is expressed as the percentage of viable cells in infected cells relative to uninfected cells. (B) Viral titers in supernatant of 73T-wt-, 73T-S-km-, and 73T-R-198-infected HT1080 and CCD1122Sk cells at an MOI of 0.01 PFU/cell at 72 h postinfection were determined by plaque assay. (C) HT1080 and CCD1122Sk cells were infected with 73T-S-km-GFP and 73T-R-198-GFP virus at an MOI of 0.01 PFU/cell and imaged with a fluorescence microscope at 48 h postinfection.

FIG 6 Tumor growth inhibition and selectivity in a HT1080 xenograft mouse tumor model. (A and B) Groups of seven athymic nude mice with subcutaneously implanted HT1080 tumors received a single dose of 2×10^7 PFU of 73T-wt, 73T-S-km, 73T-R-198, or PBS control intratumorally (A) or 1×10^8 PFU 73T-R-198 or PBS intravenously (B). The tumor volumes were measured every 3 to 4 days. The arrow indicates the time of virus administration. *, tumor size of virus-treated group relative to control group (PBS) ($P < 0.05$). (C) Groups of three athymic nude mice with subcutaneously implanted HT1080 tumors received 1×10^8 PFU 73T-R-198-Luc (R-198-luciferase) or PBS intravenously. Tumor volumes measured over the 25-day study period and imaging of luciferase expression in two mice on days 1, 4, and 15 after virus administration are shown.

cells, confirming better viral replication in cancer cells than in normal cells.

Selective viral replication in tumors leads to tumor growth suppression in a mouse xenograft model. The levels of oncolytic activity of recombinant NDVs (73T-S-km and 73T-R-198) were then evaluated *in vivo* in the HT1080 xenograft tumor model (Fig. 6A). 73T-R-198 was nearly as effective as 73T-wt in tumor growth inhibition whereas 73T-S-km was less efficacious when a single dose of 2×10^7 PFU was administered intratumorally. When a single dose of 1×10^8 PFU was administered intravenously (i.v.) (Fig. 6B), 73T-R-198 significantly slowed tumor growth during the 25-day study period. Viral distribution and replication in the mouse model were visualized by i.v. administration of 73T-R-198-luc, which had the luciferase gene inserted at the P-M junction. Bioluminescence was detected only in tumors and not in normal tissues of mice treated with 73T-R-198-luc as early as 1 day after virus administration, with the highest intensity detected on day 4 posttreatment (representative images from two animals are shown in Fig. 6C). The bioluminescence signal was still detectable on day 15 postdosing, while no signal was detected in other parts of the bodies. Tumor growth inhibition in 73T-R-198-luc-treated animals was similar to that seen in animals treated with 73T-R-198 (Fig. 6B and C).

The biodistribution of the 73T-R-198 virus in animals bearing

HT1080 tumors after i.v. dosing was also evaluated by viral titration (Fig. 7A). The infectious virus was detected only in the liver, lung, spleen, and serum at 2 h and on day 1 postdosing and had cleared from all normal tissues tested by day 4; no virus was detected in ovary tissue at any time point (data not shown). In contrast, virus was detected in tumors at all time points, up to and including 13 days postdosing, which was consistent with the 73T-R-198-luc imaging data. Virus levels increased in the tumors and reached a peak titer of $6 \log_{10}$ PFU/g tissue on day 4. Consistent with the viral replication and biodistribution data, the level of hGM-CSF expressed by 73T-R-198 was highest in the tumor on day 4 and it could be detected for up to 13 days postdosing (Fig. 7B). These data confirmed that 73T-R-198 selectively replicated and expressed the transgene in the tumor after systemic delivery. Immune cell infiltration induced by virus infection of the tumor tissues was examined by flow cytometry (Fig. 7C). 73T-R-198 induced neutrophil, NK cell, and macrophage infiltration into the tumors. In addition to the GM-CSF protein expression from the transgene, 73T-R-198 induced expression of IFN- β , CCL3, CCL5, interleukin-6 (IL-6), CCL2, CXCL10, IFN- γ , IL-10, tumor necrosis factor alpha (TNF- α), CXCL9, and IL-12 in the tumors at levels that ranged from 3-fold to 38-fold higher than the levels in the PBS-treated tumors (data not shown).

FIG 7 Viral distribution and immune cell infiltration in mouse organs and tissues. (A and B) Three mice from the PBS- and 73T-R-198-treated groups were sacrificed at 2 h or day 1, 4, or 13 after a single i.v. dosing of 1×10^8 PFU virus. Tissue and blood samples were collected and processed for infectious virus titer by plaque assay (A) and for GM-CSF production by ELISA (B). (C) The tumor tissues were harvested on day 4 postdosing and processed for staining of neutrophils, NK cells, and macrophages and fluorescence-activated cell sorter (FACS) analysis.

DISCUSSION

Among the members of a panel of mesogenic NDV 73T-derived variants that we generated in this study, we selected 73T-R-198 virus with FPCS modification and an intergenic sequence extension of 198 nt with the hGM-CSF transgene to be our lead oncolytic NDV for clinical development. This virus is not expected to cause Newcastle disease in chickens based on its ICPI value of <0.4 , which mitigates its potential environmental impact and facilitates its development as a novel oncolytic virus. The two major attenuation mechanisms introduced into this virus make reversion to the 73T-wt pathogenic phenotype difficult. 73T-R-198 is efficient in selectively replicating in and killing tumor cells *in vitro* and suppresses tumor growth in a mouse tumor model as previously reported for other NDV strains (30–33).

Reverse genetics has provided a powerful tool to manipulate viral genomes. NDV 73T-R's FPCS $^{111}\text{HNRTKR-FI}^{118}$ sequence derived from the human cytomegalovirus (CMV) gB protein cleavage site ($^{111}\text{HNRTKS-TD}^{118}$) is different from the 73T-wt FPCS sequence of $^{111}\text{GRRQKR-FI}^{118}$. The authentic gB cleavage site introduced to the NDV FPCS was not functional unless the $\text{T}^{117}\text{D}^{118}$ residues next to the cleavage site were converted to $\text{F}^{117}\text{I}^{118}$. 73T-S-km was derived from 73T-S ($^{111}\text{HNRTKS-FI}^{118}$) during *in vitro* passage, and it lost a potential N-linked glycosylation site (NRT) at the FPCS that might improve the accessibility of the FPCS to host protease, resulting in improved F protein cleavability. 73T-S-km is genetically stable during *in vitro* passage. The R116 residue in the F protein of 73T-R is preferred by furin protease and is commonly present in viral glycoproteins, bacterial toxins, and other host proteins (34), which likely explains the higher F protein cleavage efficiency of 73T-R than 73T-S-km.

To meet the lower pathotype definition value ($\text{ICPI} < 0.4$) used by the Department for Environment, Food & Rural Affairs (DEFRA), United Kingdom, and to enable worldwide use of 73T-R in virotherapy, we adjusted the avian virulence of 73T-R by insertion at the HN-L junction of 198 nt or 318 nt to further reduce viral pathogenicity to an ICPI value of <0.4 (Table 1). Yan and Samal (20) reported an inability to recover recombinant NDV Beaudette C virus with random nucleotide sequences inserted at the HN and L junction but could recover virus with sequence derived from another paramyxovirus, suggesting that the viral RNA-dependent RNA polymerase recognizes the inserted paramyxovirus sequence more efficiently than random sequence. Consistent with what has been reported (20), the 73T-R virus with an insertion of a 198-nt random sequence or a 318-nt viral sequence replicated to lower titers in embryonated chicken eggs and had slower growth kinetics in chicken DF-1 cells. We therefore chose the 198-nt viral sequence insertion as our lead candidate. We found that the intergenic sequence insertion had a more negative impact on viral transcription and replication in avian cells than in human cells. Unexpectedly, the intergenic sequence insertion in the HN-L junction increased transcription of the upstream viral genes in mammalian cells despite reduced L polymerase protein production (Fig. 3). The extended intergenic sequence prior to the L gene may result in the viral polymerase leaving the RNA template prematurely and cycling back to the 3' end to reinitiate transcription, resulting in increased expression of the upstream genes. The recycling of the viral RNA-dependent RNA polymerase appears to be less efficient in avian cells, leading to a reduction of overall viral RNA transcription. Since the RNA viruses lack re-

combination mechanisms, the intergenic sequence insertion cannot be deleted naturally, making this attenuation approach desirable. The genetic stability of the 73T R-198 candidate in poultry has yet to be determined.

NDV is a strong inducer of type I IFN in many types of cells (35). The V protein produced via a P mRNA editing mechanism has IFN antagonistic activities (15, 36), targeting MDA-5 protein (27) and inhibiting interferon regulatory factor 3 (IRF-3) activation (28). The virulent NDV Beaudette C strain has greater interferon antagonistic activities than the avirulent LaSota strain due to V protein sequence differences (14). The reduced V protein level in 73T-R-198-infected avian cells should make this virus more sensitive to innate immune responses, resulting in low chicken pathogenicity. On the other hand, upregulation of the V protein in 73T-R-198-infected human cells downregulates the IFN-mediated antiviral response, which would be beneficial for viral growth. Indeed, 73T-R-198 grows well in HeLa cells, making it possible to manufacture this oncolytic virus in a cell culture system. NDV produced in HeLa cells is more resistant to complement-mediated viral inactivation than virus produced in eggs because of incorporation of human regulators of complement proteins (our unpublished data and reference 37), which may delay viral clearance when the virus is delivered by systemic administration.

NDV 73T-R-198 maintains its potent oncolytic activities as demonstrated by its selective replication in and killing of tumor cells *in vitro* and *in vivo*. Among more than 150 human cancer cell lines tested, approximately 58% of the cells were sensitive to NDV-mediated killing, defined as $>40\%$ cell death 3 days postinfection with 0.1 PFU/cell (data not shown). NDV 73T-R-198 administered intratumorally or intravenously selectively replicated in tumors and suppressed tumor growth (Fig. 7), as also demonstrated by other investigators using different NDV variants (30–33). In our studies, 73T-R-198 exhibited greater oncolytic activity *in vitro* than 73T-S-km and was more potent in a xenograft mouse tumor model. However, the delayed tumor cell killing activity observed with 73T-S-km may allow antitumor immune responses to be elicited before the infected tumor cells become lysed in immunocompetent animals. Therefore, the utility of 73T-S-km-like recombinant NDV as oncolytic agent has yet to be determined.

We showed that NDV 73T-R-198 delivered systemically enhanced immune cell infiltration as shown by increased numbers of neutrophils, NK cells, and macrophages in the tumors. The immune stimulatory effect of NDV is particularly pronounced when localized NDV infection is combined with anti-CTLA4 immune checkpoint blockade immunotherapy in an immunocompetent mouse tumor model (38). The therapeutic effect was associated with tumor-infiltrating lymphocytes (TILs) not only in the injected tumor but also in distal tumors, and tumor-specific responses were mediated by activated CD8^+ T cells, NK cells, and type I IFN (38). Here, we have inserted an hGM-CSF transgene into the NDV and showed that the hGM-CSF protein is highly expressed by the 73T-R198 virus *in vitro* and in a HT1080 xenograft tumor model. GM-CSF is a potent immune stimulator promoting the differentiation of progenitor cells into dendritic cells. It also increases the number and function of white blood cells in patients (39). Tumor cell death caused by virus replication is expected to result in the release of tumor antigens and may elicit an *in situ*, patient-specific antitumor response which could be enhanced by hGM-CSF expressed by NDV. Clinical studies with engineered HSV-1 expressing hGM-CSF (talimogene laher-

parepvec) administered intratumorally showed responses in local and distal tumors with evidence of tumor regression and enhanced TILs (40, 41). The hGM-CSF transgene expressed by NDV has been previously shown to increase antitumor bystander effects *in vitro* (31). The therapeutic effect of a NDV oncolytic agent expressing a hGM-CSF transgene has yet to be evaluated in clinical studies. We look forward to the initiation of such studies and to the generation of clinical data that may be useful to guide the design of a next generation of NDV oncolytic agents expressing different therapeutic genes.

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