Newcastle Disease Virus LaSota Strain Kills Human Pancreatic Cancer Cells in Vitro with High Selectivity

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ABSTRACT
Context Pancreatic cancer is highly resistant to treatment. Previously, we showed that Newcastle disease virus (NDV) strain 73-T was highly cytotoxic to a variety of tumor cells both in vitro and in vivo but the effects of NDV on pancreatic tumors are unknown. We determined the cytotoxicity of the lentogenic LaSota strain of NDV (NDV-LS) toward 7 different human pancreatic tumor cell lines and 4 normal human cell lines (keratinocytes, fibroblasts, pancreatic ductal cells, and vascular endothelial cells).

Methods Cytotoxicity assays used serially diluted NDV incubated for 96 hours post-infection. Cells were fixed, stained, and minimum cytotoxic plaque forming unit (PFU) doses were determined (n=10-24/cell line).

Results Normal cells were killed only by high doses of NDV-LS. The cytotoxic doses for pancreatic ductal cells, fibroblasts, and vascular endothelial cells were 729, 626, and 1,217 plaque forming units, respectively. In contrast, most pancreatic cancer cells were killed by much lower doses. The doses for PL 45, Panc 10.05, PANC-1, BxPC3, SU.86.86, Capan-1 and CFPAC-1 were 0.15, 0.41, 0.43, 0.55, 1.30, 17.1 and 153 plaque forming units, respectively.

Conclusions Most pancreatic tumor cells were more than 700 times more sensitive to NDV-LS killing than normal cells. Such avirulent, lentogenic NDV strains may have therapeutic potential in the treatment of pancreatic cancers.

INTRODUCTION
Pancreatic cancer has proven to be highly resistant to treatment. At present, the 5-year survival after diagnosis of pancreatic cancer is very low, about 4%. Clearly, novel treatment methods are needed. In previous studies, we showed that the mesogenic Newcastle disease virus (NDV) strain 73-T was highly cytotoxic to a variety of tumor cells both in vitro and in vivo but caused relatively little damage to normal cells [1, 2, 3, 4, 5]. However, very little is known about the direct effects of virulent or avirulent NDV strains on human pancreatic tumors.

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Abbreviations AT acetylated trypsin; FCS: fetal calf serum; HA: hemagglutinin; HEKn: human keratinocyte; HN: hemagglutinin-neuraminidase; HPDE: human pancreatic ductal epithelial cell; HUfB: human fibroblast; HUVEC: human vascular endothelial cell; NDV: Newcastle disease virus; NDV-LS, NDV-La Sota strain; PFU, plaque forming unit

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NDV is an enveloped negative-sense single-strand RNA virus in the family Paramyxoviridae and genus Rubulavirus [6]. Its genomic RNA contains 6 genes which encode 8 proteins [7]. It has been studied for many years due to its ability to kill a variety of types of human tumors with high potency and specificity. This selectivity is thought to arise from the weak endogenous interferon response in tumor cells as compared to normal cells [8, 9], but NDV also acts as an immune stimulatory adjuvant in vivo. Although it can cause high morbidity and mortality in avian species, NDV has few harmful effects in humans except for self-limiting conjunctivitis and mild to moderate flu-like symptoms [9]. The virus also exhibits a low rate of spontaneous mutation, low levels of recombination or antigenic drift, and does not become integrated into host DNA [10, 11]. These features make NDV a particularly promising candidate for tumor therapy.

Native NDV strains have been employed in animals and in patients as anti-tumor agents in 3 different modalities: 1) injection of infectious virus; 2) administration of virus infected oncolysate; or 3) infected whole cell vaccines. For direct cytolysis, live native mesogenic or velogenic NDV is added to cell cultures or injected into subjects where it infects and replicates in tumor cells which subsequently undergo apoptosis and lysis. Lentogenic NDV strains such as LaSota (LS), Hitchner-B1, or Ulster are seldom used in
this way because they are considered to be non-lytic and thus less likely to have direct cytolytic effects. Krishnamurthy et al. [12] showed that NDV-LS replicated efficiently in 4 different tumor cell types in vitro but did not describe any cytopathic effects. As a vaccine, lentogenic strains such as NDV-Ulster are often used as adjuvants to stimulate an immune response against tumor cells or antigens. This method does not require NDV replication in tumor cells or direct viral induced lysis of target cells [13, 14].

The binding of NDV to target cells does not seem to require a specific receptor, instead ubiquitous sialic acid moieties on the cell surface serve as binding sites. NDV cytopathicity appears to hinge upon the formation of multinucleated syncytia [5]. NDV fusogenicity, both for virus entry into the cell and for syncytia formation, involves both the fusion (F) and hemagglutinin-neuraminidase (HN) viral transmembrane proteins [15, 16, 17]. However, NDV virulence in chicken, which is highly dependent on F protein primary structure, is not the only or even the main determinant of NDV fusogenicity and cytotoxicity in mammalian tumor cells. As a result, the range of viral strain infectivity in chicken (i.e., lentogenic, mesogenic, velogenic) may not be as relevant to direct oncolytic potential of NDV strains in human tumors [18].

The direct cytolitic effects of mesogenic and velogenic NDV strains (e.g., 73-T, Beaudette C, Italien, Roakin, MTH-68) have been reported previously [5, 14, 19, 20, 21, 22, 23] but such highly infectious strains are problematic in clinical use due to possible unintentional release of highly infectious virus into the environment. As a result, avirulent lentogenic strains may be better alternatives for future clinical use. We evaluated the direct cytototoxicity of the lentogenic LaSota (LS) strain of NDV toward normal human cell lines including primary keratinocytes (HEKn), fibroblasts (HuFbs), immortalized pancreatic ductal cells (HPDE), and vascular endothelial cells (HUVEC). We compared this to their cytolytic effects on several different human pancreatic tumor cell lines (PANC-1, PL45, Panc 10.05, CFPAC-1, Capan-1, SU.86.86 and BxPC3).

**MATERIALS AND METHODS**

**Virus Preparation and Cell Lines**

NDV LaSota strain was a kind gift from Dr. R Iorio (University of Massachusetts, Worcester, MA, USA). This stock was amplified by passage through 10-day-old chick embryos. Three to four days after inoculation with 10,000 plaque forming units (PFU) of NDV, allantoic fluid was removed from the eggs aseptically and centrifuged at 13,000 g for 10 min to remove debris. Supernatants were divided and stored frozen at -80°C until use.

NDV stock was quantified using plaque assays as described previously [1, 2] and by hemagglutination (HA) assays. For plaque assays, monolayers of spontaneously transformed embryonic chicken fibroblasts (UMNSAH/DF-1) were cultured in 48-well plates with DMEM plus 10% fetal calf serum until confluence was attained. Serial 5-fold or 10-fold dilutions of virus-containing stocks were added to monolayers and, after 4 days of incubation at 37°C in a 5% CO2 incubator, cell monolayers were fixed with 100% methanol and stained with 0.2% crystal violet [1, 2]. For time course studies of cytotoxicity, cells were incubated with virus for 1, 2, 3, 4, 5, or 6 days after which time monolayers were fixed and stained. One plaque forming unit (PFU) was defined as the amount of NDV required to kill all cells in a well containing confluent chicken fibroblasts after 4 days of incubation.

**Hemagglutination (HA) Assay**

The HA titer of NDV suspensions was determined by end point dilution of erythrocyte agglutination. Chicken erythrocytes (Rockland Biologicals, Gilbertsville, PA, USA) in Alsever’s solution were washed 3 times in PBS and resuspended at a concentration of 5x10⁷ cells/mL. Briefly, PBS (25 µL) was added to duplicate sets of wells in 96-well round bottom microtiter plates and 1/10 diluted NDV-LS in allantoic fluid (25 µL) was placed into the first pair of wells and then diluted by 2-fold serial dilutions. Next, 25 µL of PBS were added to each well. Finally, 25 µL of RBCs were added, plates were incubated at either room temperature or 4°C for 60 min. Plates were then assessed for hemagglutination and photographed [24, 25].

**Cell Lines and Culture Conditions**

Spontaneously transformed embryonic chicken fibroblasts (UMNSAH/DF-1, ATCC, Manassas, VA, USA) were grown in DMEM plus 10% fetal calf serum (FCS). Normal primary human keratinocytes derived from preputial skin of neonatal males (HEKn; Clonetics, San Diego, CA, USA) were used at passages ranging from 4 to 15. They were grown in Epilife with calcium, bovine pituitary extract, and EGF. Normal primary human fibroblasts derived from preputial skin of adolescent males (HuFb), were adapted to culture, used at passages ranging from 7 to 20, and grown in DMEM plus 10% FCS. Normal human vascular endothelial cells (HUVEC) were maintained in Medium 200 plus low serum growth supplement (LSGS; Invitrogen, Carlsbad, CA, USA) and used at early passages. The immortalized human pancreatic ductal cell line, HPDE6-E6E7-c7 (HPDE), was a kind gift of Dr. MS Tsao (Ontario Cancer Institute, Toronto, Canada). This cell line was originally derived from human pancreatic ductal epithelium that had been transfected in vitro with the E6 and E7 genes from human papilloma virus and effectively immortalizing the cell line. These cells were shown to be phenotypically and functionally very similar to normal pancreatic ductal epithelium and, in terms of gene expression, were also similar to normal pancreatic epithelium [26, 27].

PANC-1 and SU.86.86 pancreatic epithelial carcinoma, PL45 and CFPAC-1 pancreatic ductal adenocarcinoma,
and BxPC3, Capan-1, and Panc 10.05 pancreatic adenocarcinoma were obtained from ATCC (Manassas, VA, USA). PANC-1 and PL45 were cultured in DMEM with 10% FCS. SU.86.86 and BxPC3 were grown in RPMI-1640 with 10% FCS. Panc 10.05 cells were grown in RPMI-1640 containing 1 mM pyruvate, 0.23 U/mL human insulin, and 15% FCS. CFPAC-1 and Capan-1, both of which express CFTR, the cystic fibrosis transmembrane regulator, were grown in Iscove DMEM with 10% or 20% FCS, respectively. All media contained 50 units/mL penicillin and 50 µg/mL streptomycin sulfate.

Cytotoxicity Assays

To determine the optimal duration for cytotoxicity assays using mammalian cells, the time course for the effect was evaluated. Cells were exposed to NDV-LS for 1 to 6 days and cytotoxicity was assessed each day as described below. Simple cytotoxicity assays were performed as described previously with minor variations [1]. Briefly, each cell line was plated into 48-well plates with fully supplemented media. When the cells had grown to confluence, medium was aspirated and DMEM was added to all wells. NDV was then added and serial 5-fold or 10-fold dilutions were performed. After allowing 60 min for virus adhesion at 37°C, media containing non-adherent virus was aspirated and replaced with fresh DMEM supplemented with antibiotics. After 1-6 days of incubation at 37°C in an atmosphere of 5% CO₂ plus 95% air, culture medium was removed, cell monolayers were fixed with 100% methanol and stained with 0.2% crystal violet in 50% methanol. Acetylated trypsin (2.5 µg/mL, final) was added to the culture medium for many experiments during the 4 day post-infection incubation period. Acetylated trypsin is stable for extended periods in culture media at physiological temperatures and is capable of activating any NDV-LS virus progeny that may be released from infected cells [25]. The concentration of acetylated trypsin was determined in preliminary experiments using a range of trypsin concentrations. The highest trypsin concentration at which no effects on culture morphology or cell survival occurred was chosen for all subsequent experiments (2.5 µg/mL, final; data not shown). Human keratinocytes were exquisitely sensitive to trypsin such that any trace of this enzyme in the media proved to be cytotoxic.

STATISTICS

For each experiment, the lowest virus dose that still resulted in the lysis of most or all cells in a given well was recorded. This “minimum cytotoxic dose” was then used to characterize each cell line and virus strain. Between 10 and 24 repetitions (n) of these cytotoxicity determinations were performed for each cell type. Non-parametric statistics were applied. Wilcoxon matched-pairs tests were used to compare the effects of acetylated trypsin (AT) on cytotoxicity and Kruskal-Wallis ANOVA was used to evaluate the differences between CF and non-CF patient derived pancreatic cancer cell lines.

Due to the prevailing hypothesis that the presence of AT or some other protease is necessary for full activity or cytotoxicity of NDV, one-tailed tests were used for evaluating the effects of AT while two-tailed tests were chosen for the other analyses. Descriptive statistics (mean±SEM) were obtained and groups were compared by using the Kruskal-Wallis ANOVA with Dunn’s multiple comparison tests using Prism 3.03 software (GraphPad, San Diego, CA, USA). P values less than 0.05 were considered statistically significant.

RESULTS

Hemagglutination Assays and PFU Determination in Diploid Chicken Fibroblasts

Hemagglutination assays showed that the NDV-LS stock suspension contained 102,400 to 204,800 HA units/mL (Figure 1). Confluent chicken fibroblasts exposed to NDV-LS serially diluted by factors of 5 or 10 and then incubated for 4 days showed complete cytotoxicity even at extreme dilutions of stock virus suspension. The greatest dilution at which complete killing occurred was used to establish PFU equal to 1 (n=22). This dilution corresponded to 1.9 to 3.9 E-3 HA units of NDV-LS stock.

Figure 1. Photograph of a hemagglutinin assay plate for NDV-LS. The HA titer was determined as described in the Methods section. Two-fold serial dilutions of NDV-LS stock were added to the wells in duplicate in a microtiter plate. The greatest dilution in which erythrocyte agglutination still occurred was either 1/2,560 or 1/5,120. This represents 102,400-204,800 HA units/mL.
Time Course of NDV-LS Cytotoxicity

Time course studies (n=6) showed that most cell killing occurred by day 4 of exposure to NDV-LS for SU.86.86 and BxPC3 cells (Figure 2). Other cell lines responded similarly (data not shown), so 4 days post-exposure was used as the terminus for all subsequent experiments.

Acetylated Trypsin Increased NDV-LS Cytotoxicity

For most pancreatic cancer cell lines, the inclusion of acetylated trypsin in culture media decreased the amount of NDV required for cytotoxicity by factors of 1.3- to 5.6-fold although extensive killing occurred in the absence of added acetylated trypsin (Figure 3ab). On average, with acetylated trypsin the minimum cytotoxic dose of NDV-LS in all pancreatic cancer lines studied was decreased by half (P<0.001, Wilcoxon matched-pairs test). Similarly, the cytotoxic dose with or without acetylated trypsin in normal control lines was decreased by 64% (P<0.001, Wilcoxon matched-pairs test).

NDV-LS Was Cytotoxic for Normal Diploid Human Cells Only at High Doses

NDV-LS was cytotoxic toward normal human cells (Figures 4 and 5) but only at relatively high PFU levels (with acetylated trypsin: 252 to 729; without acetylated trypsin: 1,058 to 2,173). Without acetylated trypsin, normal diploid HuFb and HEKn cultures required very high levels of NDV-LS (mean PFU±SEM: 2,173±359 and 1,771±439, respectively) to induce complete cytotoxicity. Without acetylated trypsin, HPDE and HUVEC cells also required high NDV-LS doses (PFU±SEM: 1,058±194 and 1,217±209, respectively) for cytotoxicity. With acetylated trypsin, complete cytotoxicity was seen in HPDE, HuFb, and HUVEC cells with 729±255, 626±143, and 252±71 PFU, respectively. UV-irradiated NDV-LS was cytotoxic for normal and pancreatic cancer lines only at PFU levels exceeding 50,000 (Figure 5).

NDV-LS Was Cytotoxic for Most Pancreatic Tumor Cell Lines at Low Doses

NDV-LS was cytotoxic toward most pancreatic cancer cell lines (Figures 6 and 7) at much lower PFU levels.
With acetylated trypsin, ranging from 0.15 to 1.3) than those seen for normal cells. With acetylated trypsin, means±SEM for minimum cytotoxic NDV dose with Panc 10.05, PL45, PANC-1, SU86.86, and BxPC3 were 0.41±0.13, 0.15±0.03, 0.43±0.13, 1.30±0.61, and 0.55±0.24, respectively. Without acetylated trypsin, these values were somewhat higher at 0.70±0.16, 0.19±0.04, 0.74±0.16, 3.4±0.94, and 3.1±1.0, respectively.

With acetylated trypsin, HPDE or HuFb cells were significantly less sensitive (P<0.001, Kruskal-Wallis with Dunn’s multiple comparison tests) to killing than were Panc 10.05, PL45, PANC-1, SU.86.86, or BxPC3 cells. HUVEC cells were also significantly less sensitive (P<0.01 Kruskal-Wallis with Dunn’s multiple comparison tests) to killing than were Panc 10.05, PANC-1, or PL45 cells. Without acetylated trypsin, HPDE or HuFb or HPDE or HEKn cells were significantly less sensitive to NDV-LS cytotoxicity as compared to Panc 10.05, PL45, PANC-1, SU.86.86, or BxPC3 cells (P<0.01, Kruskal-Wallis with Dunn’s multiple comparison tests).

Pancreatic cancer cell lines derived from cystic fibrosis patients (Capan-1 and CFPAC-1) were noticeably less sensitive to killing by NDV-LS. The mean minimum PFU±SEM for these cell types was 17±7 and 153±24, respectively with acetylated trypsin and 15±3 and 422±111, respectively without acetylated trypsin. Tumor lines derived from patients with cystic fibrosis were significantly less sensitive (P<0.001, Kruskal-Wallis test) to killing by NDV-LS than were other pancreatic tumor lines either with or without acetylated trypsin.

**DISCUSSION**

Normal human cells were killed only by relatively high doses of NDV-LS. This was seen in normal diploid human keratinocytes, fibroblasts, and vascular endothelial cells as well as pancreatic ductal epithelial cells. In contrast, all non-CF pancreatic cancer cell types studied here were killed by much lower NDV doses ranging from 194- to 11,437-fold less virus. In contrast, pancreatic tumor cell lines derived from patients with CF showed significantly elevated resistance to NDV-LS cytotoxicity (ranging from 1.6- to 148-fold less virus than normal cells) compared to other pancreatic cancer cell lines. This resistance may reflect membrane related changes due to mutations in the CF transmembrane conductance regulator gene or alterations in the intracellular ionic milieu induced by the regulator. This resistance to NDV killing exhibited by CF patient-derived tumors may be an important consideration in future clinical trials involving NDV.
UV-inactivated NDV was mildly cytotoxic for tumor cells, but only at very high doses (PFU greater than 50,000). A similar effect has been described previously for 73-T and other strains of NDV [1, 2, 3, 5, 28]. UV-inactivated NDV is still capable of binding to cells and, when present in large amounts, will promote cell fusion and the formation of multinucleated cells which subsequently die. Exogenously added acetylated trypsin increased the cytotoxicity of NDV-LS by factors of 1.3- to 5.6-fold, but very high levels of cytotoxicity for pancreatic tumor cells were seen in its absence. This suggests that NDV-LS may replicate to yield infectious virus in the absence of acetylated trypsin or that the fully activated form of the virus is not necessary to achieve potent cytotoxicity in pancreatic tumors. Viral activation could be accomplished by endogenous pancreatic enzymes that cleave the viral F protein to generate the highly infectious form of the F protein. Finally, the rate of cell proliferation or doubling time for each cell line was unrelated to the amount of NDV-LS required for cytotoxicity (Figure 8).

Several NDV strains (MTH-68, 73-T, Ulster, PV701, HUJ) have been shown to be cytotoxic for a range of classes of human tumors and, in clinical studies, some have shown promise for treating a variety of tumor types. Strain MTH-68 has been shown to have beneficial effects in glioma, astrocytoma, and various advanced cancers [29, 30, 31], 73-T in sarcomas, carcinomas, and melanomas [1, 2, 3, 4, 5, 14, 20, 32, 33, 34]; PV701 in various advanced solid tumors [9, 35, 36, 37], HUJ in glioblastoma and lung tumors [38, 39, 40], and Ulster strain in melanoma, breast, and gastrointestinal tumors [41, 42, 43, 44]. Some of these NDV strains have been used primarily as immune adjuvants (Ulster by Schirrmacher et al. [41, 42, 43, 45]; 73-T by Cassel et al. [33, 34, 46]; MTH-68 by Csatary et al. [30, 47]). On the other hand, some of these strains can exert effects via direct cytolytic activity toward tumor cells (e.g., 73-T [1, 2, 3, 5, 19, 20, 48]; PV701 [9, 35, 36]; HUJ [39, 40]; MTH-68 [49, 50, 51]).

However, the susceptibility of pancreatic tumors or tumor cells to NDV has been studied only to a very limited extent. In a phase I clinical trial using PV701, Pecora et al. [35] studied 9 primary pancreatic carcinoma patients of which 1 or 2 showed measurable tumor size reductions. Zamarin et al. [52] showed that the lentogenic Hitchner-B1 strain of NDV could cause a 50% decrease in cell survival in PANC-1 cells but no decrease in MIA PaCa-2 cell survival after 3 days in vitro. In 2007, Fabian et al. [53] showed that the mesogenic NDV strain MTH-68/H was highly cytotoxic for PANC-1 cells. Schirrmacher et al. [13] also reported that Ulster strain could infect and replicate in 2 established human pancreatic cancer cell lines and in more than 10 primary tumor explants in vitro and Jarahian et al. [54] found that PANC-1 cells infected with NDV-Ulster were killed more efficiently by NK cells. In 2003, Liang et al. [55] reported disease stabilization in one patient with pancreatic head cancer treated with NDV-LaSota IV strain as a vaccine. These reports offer some optimism regarding the potential for NDV efficacy in the treatment of pancreatic neoplasms.
Lentogenic NDV strains such as NDV-LS have been studied mainly as immune adjuvants in infected tumor cell vaccines or oncolysates rather than for any direct tumor cytotoxicity [41, 42, 43, 44, 45, 55, 56, 57, 58]. It is often assumed that these lentogenic strains would have poor tumor cytotoxicity due to their low infectivity and lyso geniecity in chickens. This low infectivity is determined by the primary amino acid sequence of the F protein of NDV which contains few basic amino acid residues in the critical 395-403 positions. Such lentogenic strains must be activated by exogenous trypsin-like proteases such as those found in allantoic fluid or in the gastrointestinal tract to obtain infectious virus in chickens [59, 60]. Thus, activated NDV-LS is expected to undergo only a single round of infection in human tumor cells unless inappropriate protease activation of progeny virus particles occurs. If this had been to occur, increased virus infectivity of otherwise weakly infectious virus might be elicited.

We have shown that even lentogenic NDV-LS, which is poorly infectious in chicken, is highly cytolytic for human pancreatic tumor cells and highly specific for tumor versus normal human cells. Further, the inclusion of acetylated trypsin together with the NDV-LS resulted in a modest but significant increase in cytotoxicity suggesting that NDV-LS is highly cytotoxic to pancreatic tumor cells even in the absence of F protein activation by exogenous trypsin. In chicken, NDV may be viscero tropic or neurotropic depending on the strain [61, 62, 63]. Most lentogenic strains are viscero tropic having a marked propensity to infect enteric organs. The Hitchner-B1 and LaSota strains are often used as bird vaccines to protect against velogenic strains of NDV and typically proliferate most efficiently in the respiratory and gastrointestinal tracts [16, 64]. This may be related to the presence of proteases in these locations that cleave the NDV fusion protein thereby increasing the infectivity of the virus [65, 66]. This predilection for the GI tract, the potential for NDV-LS activation there, and its high level of differential cytotoxicity toward pancreatic tumor cells in vitro may make this lentogenic strain of NDV particularly useful in the treatment of pancreatic cancer.

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