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ORIGINAL ARTICLE

Two Avirulent, Lentogenic Strains of Newcastle Disease Virus Are Cytotoxic for Some Human Pancreatic Tumor Lines *In Vitro*

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ABSTRACT

Context Pancreatic cancer is the fourth leading cause of cancer death in the U.S. Highly infectious Newcastle disease virus (NDV) strains are known to be very cytotoxic for an array of human tumor cell types *in vitro* and *in vivo* but the effects of these and avirulent NDV strains on pancreatic neoplasms are little known. **Objective** Here, the direct cytolytic effects of the avirulent Hitchner-B1 (B1) and Ulster (U) NDV strains on 7 human pancreatic tumor cell lines and 4 normal human cell lines were studied. **Methods** Cytotoxicity assays used serially diluted NDV to determine minimum cytotoxic plaque forming unit (PFU) doses. **Results** For NDV-B1, normal human cells were killed only by relatively high doses (range: 471-3,724 PFU) whereas NDV-U killed these cells at low PFU (range: 0.32-1.60 PFU). Most pancreatic cancer cell types were killed by much lower NDV-B1 doses (range: 0.40-2.60 PFU) while NDV-U killed Capan-1 and SU.86.86 cultures at very low doses (0.00041 PFU and 0.0034 PFU, respectively). **Conclusions** On average, 1,555 times more NDV-B1 was needed to kill normal cells than most pancreatic tumor cells and 558 times more NDV-U to kill the two most sensitive pancreatic cancer lines. These innately-targeted lentogenic viruses may have meaningful potential in treating pancreatic cancer.

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the United States [1]. It has proven to be highly resistant to treatment. At present, the 5-year survival after diagnosis of pancreatic cancer is very low, about 4% [2]. In 2007, a multicenter trial involving 368 patients treated with surgical resection and gemcitabine reported no significant difference in overall survival between the gemcitabine plus surgery and the surgery only groups with median survival of only 22.1 and 20.2 months, respectively [3]. Sadly, even the most advanced and effective treatments for pancreatic cancer (surgery, radiation, and either 5-fluorouracil or gemcitabine chemotherapy) provide only a median of 17 to 19 months of overall survival [4, 5, 6]. From this, it is clear that more effective, entirely novel treatment methods for pancreatic neoplasms are badly 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 [7, 8, 9, 10, 11]. However, very little is known about the direct cytotoxic effects of virulent or avirulent NDV strains on human pancreatic tumors.

NDV is an enveloped negative-sense single-strand RNA virus in the family Paramyxoviridae and genus *Rubulavirus* [12]. Its genomic RNA contains 6 genes which encode 8 proteins [13]. It has been studied for many years due to its ability to kill a variety of human tumor types 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 [14, 15], but NDV also acts as an immune stimulatory adjuvant *in vivo*. Although it can cause devastating mortality in avian species, NDV has few harmful effects in humans except for self-limiting conjunctivitis and mild to moderate flu-like symptoms [15]. 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 [16, 17]. These features make NDV a particularly promising candidate for tumor therapy.

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Abbreviations AT: acetylated trypsin; B1: Hitchner-B1 strain; FCS: fetal calf serum; HA: hemagglutinin; HPDE: human pancreatic ductal epithelial cell; HuFb: human fibroblast; HUJ: Hebrew University Jerusalem strain; HuKC: human keratinocyte; HUVEC: human vascular endothelial cell; LS: LaSota strain; NDV: Newcastle disease virus; PFU: plaque forming unit; U: Ulster strain

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Native or naturally occurring 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) administration of 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 (B1), or Ulster (U) 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*. [18] showed that NDV-LS replicated efficiently in four different tumor cell types *in vitro* but did not describe any cytolytic or cytopathic effects. Apostolidis *et al*. [19] showed that NDV-U did not induce Vero monkey kidney cell cytolysis and NDV-LS induced oncolysis, but only at high titers and only if it had been previously transfected with a more virulent F protein construct. Yaakov *et al*. [20] isolated an attenuated NDV strain derived from B1, NDV-Hebrew University Jerusalem (NDV-HUJ) strain, which showed direct cytopathic effects on a range of tumor cells. As a vaccine, lentogenic strains such as NDV-LS or NDV-U are being actively investigated as adjuvants intended to stimulate an immune response against whole tumor cells or tumor antigens. This method does not require NDV replication in tumor cells or viral induced lysis of target cells [21, 22].

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 cytotoxicity appears to hinge upon the formation of multinucleated syncytia [11]. NDV fusogenicity, both for virus entry into the cell and for syncytia formation, involves both the fusion (F) and hemagglutinin-neuraminidase (HN) viral trans-membrane proteins [23, 24, 25]. However, NDV virulence in chicken, which is highly dependent on F protein primary structure, may not be 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 be unrelated to direct oncolytic potential of NDV strains in human tumors [26].

Little data are available regarding the direct cytolytic effects of NDV in pancreatic cancer. Recently, Fabian *et al*. [27] showed that the mesogenic NDV strain MTH-68/H was highly cytotoxic for PANC-1 cells. Zamarin *et al*. [28] showed that lentogenic NDV-B1 decreased cell survival in PANC-1 cells by 50% after 3 days *in vitro*, but had no effect on the pancreatic cancer line, MIA PaCa-2. Jarahian *et al*. [29] mentioned that PANC-1 cells infected with NDV-U were killed more efficiently by natural killer cells *in vitro*. Schirrmacher *et al*. [21] also showed that NDV-U could infect and replicate in two established human pancreatic cancer cell lines and in more than ten primary tumor explants *in vitro*, but no cytotoxicity data were given. In a phase I clinical trial using mesogenic PV701, Pecora *et al*. [30] studied nine primary pancreatic carcinoma patients of which one or two showed measurable tumor size reductions. In 2003, Liang *et al*. [31] reported disease stabilization in one patient with pancreatic head cancer treated with NDV-LaSota IV strain employed as a vaccine. These scattered reports, often lacking appropriate controls, together with our recent systematic study examining cytotoxicity of lentogenic NDV-LS in cultured pancreatic cells, suggest that some strains of NDV may effectively kill pancreatic cancer cells or tumors [32].

In the present study, we evaluated the direct cytotoxicity of two lentogenic NDV strains, B1-Hitchner and Ulster toward a group of four normal, non-tumorigenic human cell types including primary keratinocytes (HuKC), fibroblasts (HuFbs), vascular endothelial cells (HUVEC), and immortalized human pancreatic ductal epithelial cells (HPDE). We compared this to their cytolytic effects on a panel of seven different established human pancreatic tumor cell lines (PANC-1, PL45, Panc 10.05, CFPAC-1, Capan-1, SU.86.86 and BxPC3). Normal cells were killed only by high doses of NDV-B1. The cytotoxic doses for normal diploid human vascular endothelial cells, keratinocytes, pancreatic ductal cells, and fibroblasts were 1,146, 3,724, 471, and 967 plaque forming unit (PFU), respectively. In contrast, most pancreatic cancer cells were killed by much lower NDV-B1 doses. The doses for Panc 10.05, PL45, PANC-1, SU.86.86, BxPC3, Capan-1 and CFPAC-1 were 2.60, 0.67, 0.60, 0.80, 0.40, 14, and 220 PFU, respectively. NDV-U was cytotoxic for normal diploid human cells at low PFU doses and for Capan-1 and SU.86.86 cells at very low doses (0.00041 and 0.0034 PFU, respectively). Exogenous protease increased NDV-B1 cytotoxicity modestly but not NDV-U cytotoxicity toward pancreatic tumor cells. Pancreatic cancer cell lines derived from cystic fibrosis patients (Capan-1 and CFPAC-1) were significantly less sensitive to killing by NDV-B1 but not with NDV-U. Pancreatic cancer cells need not be actively dividing during NDV treatment for effective tumor selective killing to occur and sensitivity to cytotoxicity was unrelated to culture doubling times seen in actively dividing cultures. Thus, NDV-B1 exhibited high selectivity and high cytotoxicity for most, and NDV-U for some, human pancreatic tumor cells *in vitro*. An average of 1,555 times more NDV-B1 was needed to kill normal cells than most pancreatic tumor cells and 558 times more NDV-U to kill the two most sensitive pancreatic cancer lines than normal cells. These innately-targeted, naturally occurring lentogenic viruses may have meaningful therapeutic potential in the treatment of pancreatic cancers.

MATERIALS AND METHODS

Virus Preparation and Cell Lines

NDV Hitchner-B1 and Ulster strains were kind gifts from Dr. M. Peeples (Children's Research Institute, Columbus, OH, USA) and Dr. R. Iorio (University of Massachusetts, Worcester, MA, USA). These stocks were amplified by passage through 10-day-old chick embryos. Three to four days after inoculation with 10,000 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 [7, 8] and by hemagglutination (HA) assays. For plaque assays, monolayers of spontaneously transformed embryonic chicken fibroblasts (UMNSAH/DF-1, ATCC, Manassas, VA, USA) were cultured in 48-well plates with DMEM plus 10% fetal calf serum until confluence was attained. Serial 5- or 10-fold dilutions of virus-containing stocks were added to monolayers and, after 45 minutes at room temperature to permit virus adsorption, the medium was replaced with fresh DMEM supplemented with 2.5% tryptose phosphate broth (Sigma Chemical, St. Louis, MO, USA) and 0.25% fetal calf serum (FCS). Due to the low serum concentration (serum starvation), cell division ceased for the remainder of the experiment. After 4 days of incubation at 37°C in a 5% CO_2 incubator, cell monolayers were fixed with 100% methanol and stained with 0.2% crystal violet [7, 8]. 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. This amount differed depending on the strain of NDV used.

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 $5x10^7$ cells/mL. Briefly, PBS (25 µL) was added to duplicate sets of wells in 96-well round bottom microtiter plates, NDV samples in allantoic fluid (25 µL) were 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 erythrocytes were added, plates were incubated at either room temperature or 4°C for 60 min. Plates were then assessed for hemagglutination and photographed [33, 34].

Cell Lines and Culture Conditions

Spontaneously transformed embryonic chicken fibroblasts (UMNSAH/DF-1, ATCC, Manassas, VA, USA) were grown in DMEM + 10% fetal calf serum (FCS). Normal primary human keratinocytes derived from preputial skin of adult males (HuKC; 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 (HuFbs, Clonetics, San Diego, CA, USA) were used at passages ranging from 7 to 20 and were grown in DMEM plus 10% FCS. Normal human umbilical vascular endothelial cells (HUVEC; Life Technologies, Grand Island, NY, USA) 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 the 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 16 effectively immortalizing the cell line. The derivation and culture of these cells have been described in detail previously where they 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 [35, 36].

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 U/mL penicillin and 50 µg/mL streptomycin sulfate.

Cytotoxicity Assays

To determine the optimal duration for cytotoxicity assays using human cells, the time course for the effect was evaluated. Cells were exposed to NDV-B1 or NDV-U 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 [7]. 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, medium 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% CO_2 plus

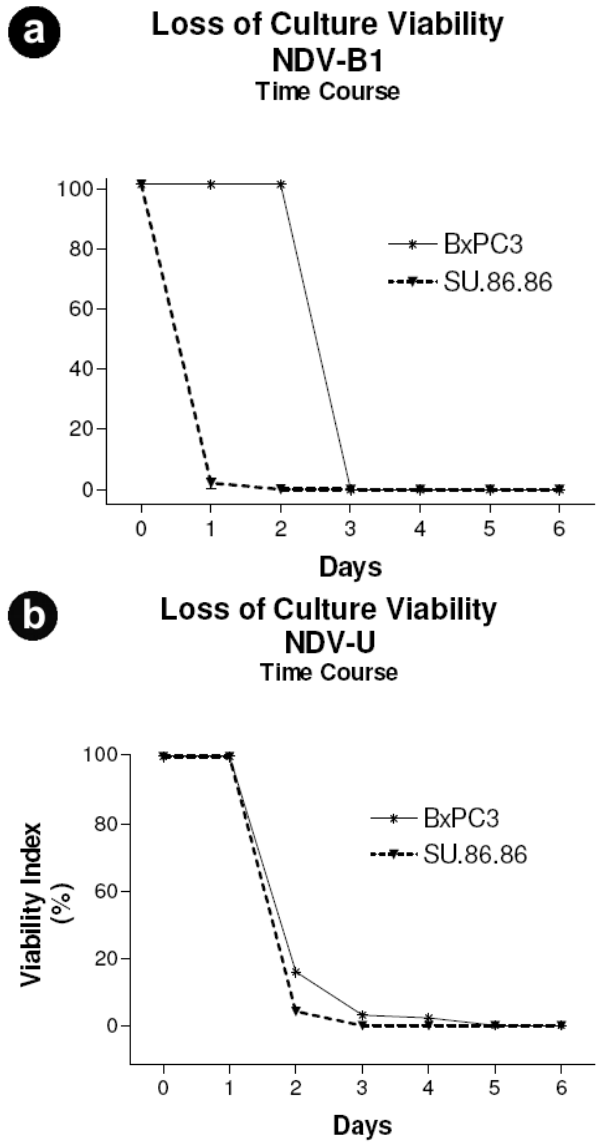

Figure 1. Time course for cytotoxicity after Newcastle disease virus (NDV) infection. Cytotoxicity in SU.86.86 and BxPC3 pancreatic cancer cells was observed over a 6-day time course. Most cell killing induced by NDV Hitchner-B1 strain (NDV-B1) occurred by day 4 post-infection in both cell lines (**a.**) and by day 5 with NDV Ulster strain (NDV-U) (**b.**). Viability index represents the percent of the initial NDV dose yielding complete culture cytotoxicity at each time point. Means from 3 separate experiments are shown.

95% air, culture medium was removed, cell monolayers were fixed with 100% methanol and stained with 0.2% crystal violet in 50% methanol. All human cell lines used here were of epithelial origin, grew substrate-adherent, and attained similar cell size and density at confluence (±15%, unpublished data). As a result, NDV dose per cell was very similar from cell type to cell type at each virus dilution used.

Acetylated trypsin (2.5 µg/mL, final) or 10% allantoic fluid was used to supplement the culture medium for some 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-B1 or NDV-U virus progeny that may be released from infected cells [34]. 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. Instead, keratinocyte cultures were supplemented with 10% allantoic fluid. This supplement did not affect cell viability but introduced chicken proteases that could activate released viral progeny [18, 33].

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. For NDV-B1 and NDV-U these cytotoxicity determinations were repeated 6-17 and 8-20 times for each cell type, respectively. Non-parametric statistics were used. Wilcoxon matched-pairs tests and Friedman test with repeated measures followed by Dunn's comparisons as post-hoc tests were used to compare the effects of acetylated trypsin (AT) on cytotoxicity. Mann-Whitney tests were used to evaluate the differences between cystic fibrosis and non-cystic fibrosis patient derived pancreatic cancer cell lines. Descriptive statistics (mean±SEM) were obtained and groups were compared by using the Kruskal-Wallis ANOVA with Dunn's multiple comparison tests or linear regression using Prism 3.03 software (GraphPad, San Diego, CA, USA). Two-tailed 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-B1 and NDV-U stock suspensions contained 2.56 E3 and 4.09 E5 HA U/mL, respectively. Confluent chicken fibroblasts exposed to serially diluted NDV-B1 or NDV-U 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=13 for B1, n=20 for U). This dilution corresponded to 0.0054 and 8.6 HA units of NDV-B1 and NDV-U, respectively.

Time Course of NDV Cytotoxicity

Time course studies (n=6) showed that more than 99% of cell killing had occurred by day 4 of exposure to NDV-B1 for SU.86.86 and BxPC3 cells (Figure 1). Other cell lines responded similarly (data not shown), so 4-day post-exposure was used as the terminus for all subsequent experiments with this strain. For NDV-U, cell killing occurred somewhat more gradually over the 6-day time course, but extensive cytotoxicity had

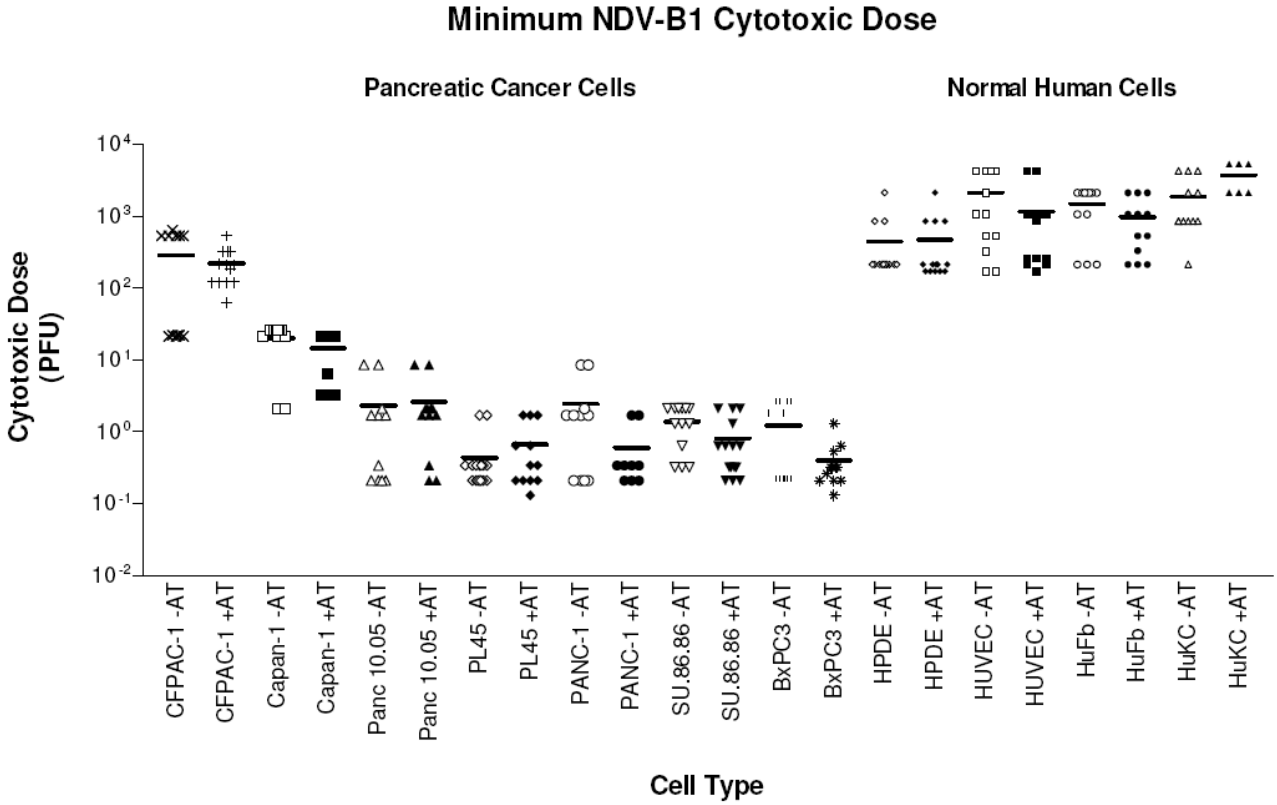

Figure 2. Scatterplot of Newcastle disease virus Hitchner-B1 strain (NDV-B1) cytotoxicity in normal and pancreatic cancer human cells. The minimum cytotoxic doses of NDV-B1 required for cells cultured with (+) or without (-) acetylated trypsin or allantoic fluid (AT) are shown. Mean minimum cytotoxic doses for each cell type are shown as horizontal lines. For all normal control lines, exogenous protease decreased the minimum cytotoxic dose of NDV-B1 by 32% as compared to all normal cells without protease (P=0.033, all pancreatic cancer cells +AT *vs*. -AT, Wilcoxon matched-pairs test). Without AT (n=12 to 14), the mean minimum cytotoxic doses for each normal cell type were high (ranging from 441 to 2,097). With or without AT, human pancreatic ductal epithelial (HPDE) or human fibroblast (HuFb) or human vascular endothelial (HUVEC) or human keratinocyte (HuKC) cells (n=14, 12, 13, 12, respectively) were significantly less sensitive to NDV-B1 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; n=12, 12, 9, 15, 12, respectively). Tumor lines derived from patients with cystic fibrosis (CFPAC-1 and Capan-1) were significantly less sensitive (P<0.001, Mann-Whitney test) to killing by NDV-B1 than were other pancreatic tumor lines either with or without AT.

occurred by day 5, so this was used as the terminus for NDV-U experiments.

Exogenous Protease Increased NDV-B1 but not NDV-U Cytotoxicity

For most pancreatic cancer cell lines treated with NDV-B1, the inclusion of acetylated trypsin or allantoic fluid in culture media decreased the amount of NDV required for cytotoxicity by 21-75% although extensive killing occurred in the absence of added acetylated trypsin (Figure 2). On average, the addition of acetylated trypsin decreased the minimum cytotoxic dose of NDV-B1 in all pancreatic cancer lines studied by 25% as compared to all pancreatic cancer cells without protease (P=0.001, all normal control cells +AT *vs*. -AT, Wilcoxon matched-pairs test). For all normal control lines, exogenous protease decreased the minimum cytotoxic dose of NDV-B1 by 32% as compared to all normal cells without protease (P=0.033, all pancreatic cancer cells +AT *vs*. -AT, Wilcoxon matched-pairs test). However, no significant effect of AT was seen for each cell type within these groups (e.g., HUVEC +AT *vs*. HUVEC -AT; PANC-1 +AT *vs*. PANC-1 -AT, etc.). No significant differences in the cytotoxic doses of NDV-U were seen with or without exogenous protease in normal or pancreatic cancer cell lines (Figure 3; P=0.851 and P=0.291, respectively; Wilcoxon matched-pairs tests).

NDV-B1 was Cytotoxic for Normal Diploid Human Cells Only at High Doses

NDV-B1 was cytotoxic toward normal human cells (Figure 2) but only at relatively high PFU levels (with acetylated trypsin: 471 to 3,724; without acetylated trypsin: 441 to 2,097). Without exogenous protease, normal diploid HUVEC, HuKC, HPDE, and HuFb cultures required high levels of NDV-B1 (mean PFU±SEM: 2.097±512, 1,862±445, 441±144, and 1,472±248, respectively) to induce complete cytotoxicity. With exogenous protease, complete cytotoxicity was seen in HUVEC, HuKC, HPDE, and HuFb cultures with 1,146±367, 3,724±714, 471±148, and 967±223 PFU, respectively. UV-irradiated NDV-B1 was cytotoxic for normal and pancreatic cancer lines only at PFU levels exceeding 50,000 (Figure 4).

NDV-U was Cytotoxic for Normal Diploid Human Cells at Low PFU Doses

NDV-U was consistently cytotoxic toward normal human cells (Figure 3) even at relatively low PFU

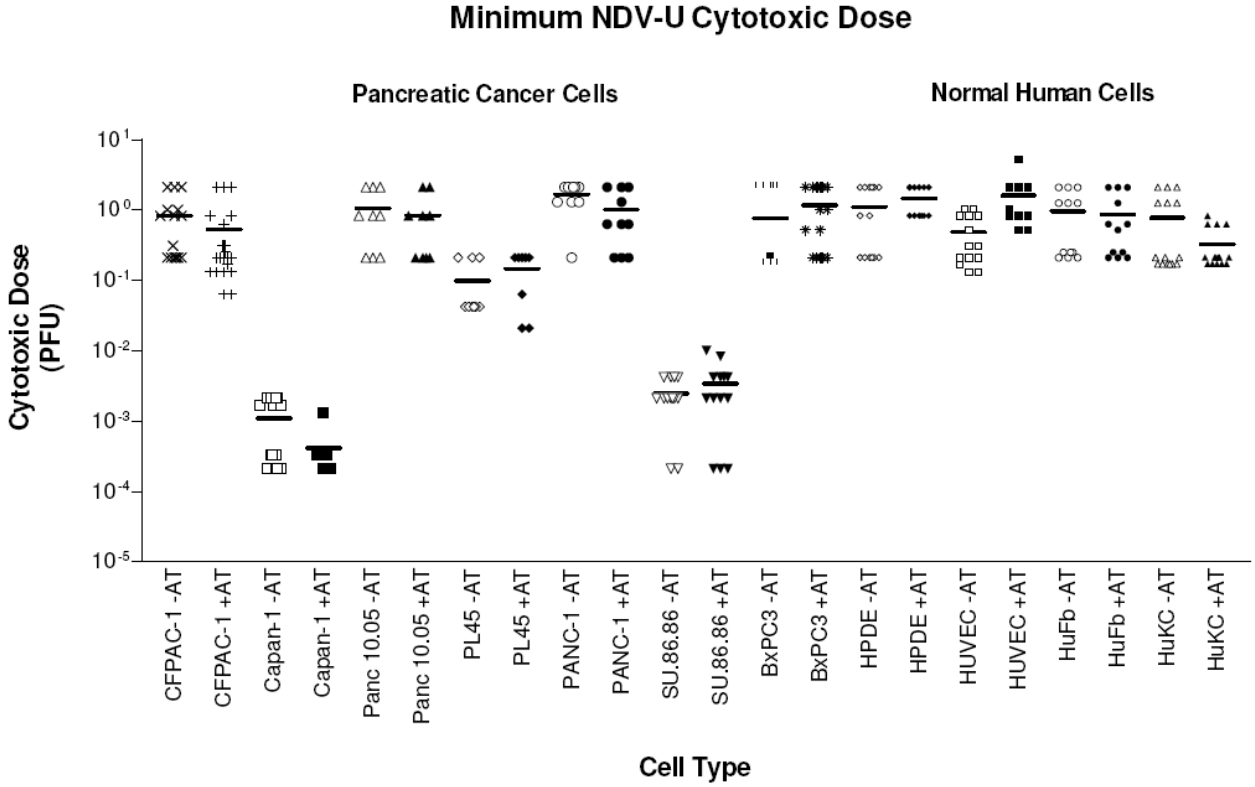

Figure 3. Scatterplot of Newcastle disease virus Ulster strain (NDV-U) cytotoxicity in normal and pancreatic cancer human cells. The minimum cytotoxic doses of NDV-B1 required for cells cultured with (+) or without (-) acetylated trypsin or allantoic fluid (AT) are shown. In the presence of acetylated trypsin or allantoic fluid (n=10 to 15), the mean minimum cytotoxic doses for each normal cell type were low (ranging from 0.32 to 1.60) and are shown as horizontal lines here. In the presence of acetylated trypsin (n=8 to 22), the mean minimum cytotoxic doses for each pancreatic cancer cell type were very low (ranging from 0.0004 to 1.2). For pancreatic cancer cell lines, exogenous protease did not significantly affect the minimum cytotoxic dose of NDV-U (P=0.851, Wilcoxon matched-pairs test) as compared to tumor cells without protease. With or without acetylated trypsin, human pancreatic ductal epithelial (HPDE), human vascular endothelial (HUVEC), and human fibroblast (HuFb) cells were significantly less sensitive (P<0.001, Kruskal-Wallis with Dunn's multiple comparison tests) to killing than were Capan-1 or SU.86.86 cells.

levels (with acetylated trypsin: 0.32 to 1.60; without exogenous protease: 0.48 to 1.10). Exogenous protease had little effect on the cytotoxic dose required. Without exogenous protease, complete cytotoxicity was seen in HUVEC, HuKC, HPDE, and HuFb cultures with 0.48±0.09, 0.78±0.21, 1.10±0.24, and 0.95±0.23 PFU, respectively. UV-irradiated NDV-U was cytotoxic for normal and pancreatic cancer lines only at PFU levels exceeding 50,000 (Figure 5).

NDV-B1 was Cytotoxic for Most Pancreatic Tumor Cell Lines at Low Doses

NDV-B1 was cytotoxic toward most pancreatic cancer cell lines at much lower PFU levels (with acetylated trypsin, ranging from 0.40 to 2.61) than those seen for normal cells (Figure 2). With acetylated trypsin, means±SEM for minimum cytotoxic NDV-B1 dose with Panc 10.05, PL45, PANC-1, SU.86.86, and BxPC3 were 2.60±0.82, 0.67±0.19, 0.60±0.21, 0.80±0.19, and 0.40±0.09, respectively. Without acetylated trypsin, these values were usually somewhat higher at 2.31±0.95, 0.44±0.12, 2.43±0.93, 1.40±0.21, and 1.22±0.32, respectively.

In the presence of acetylated trypsin or allantoic fluid, PL45, PANC-1, SU.86.86, or BxPC3 cells were significantly more sensitive (P<0.001, Kruskal-Wallis with Dunn's multiple comparison tests) to killing than were HPDE, HuFb, HUVEC or HuKC cultures. In addition, Panc 10.05 cultures were significantly more sensitive (P<0.05, Kruskal-Wallis with Dunn's multiple comparison tests) to killing than were all normal cultures except HPDE. Without exogenous protease, Panc 10.05, PL45, PANC-1, SU.86.86, or BxPC3 cultures were significantly more sensitive (P<0.001, Kruskal-Wallis with Dunn's multiple comparison tests) to NDV-B1 cytotoxicity than were HUVEC, HuFb or HuKC cultures. Without acetylated trypsin, Panc 10.05, PANC-1, BxPC3 (P<0.05) or PL45 (P<0.001) cultures were significantly more sensitive (Kruskal-Wallis with Dunn's multiple comparison tests) to NDV-B1 cytotoxicity than were HPDE cultures.

Pancreatic cancer cell lines derived from cystic fibrosis patients (Capan-1 and CFPAC-1) were noticeably less sensitive to killing by NDV-B1. The mean minimum PFU±SEM cytotoxic dose for these cell types was 14.0±2.8 and 220±35, respectively with acetylated trypsin and 20.0±2.7 and 286±80, respectively without acetylated trypsin. Tumor lines derived from patients with cystic fibrosis were significantly less sensitive (P<0.001, Mann-Whitney test) to killing by NDV-B1 than were other pancreatic tumor lines either with or without acetylated trypsin.

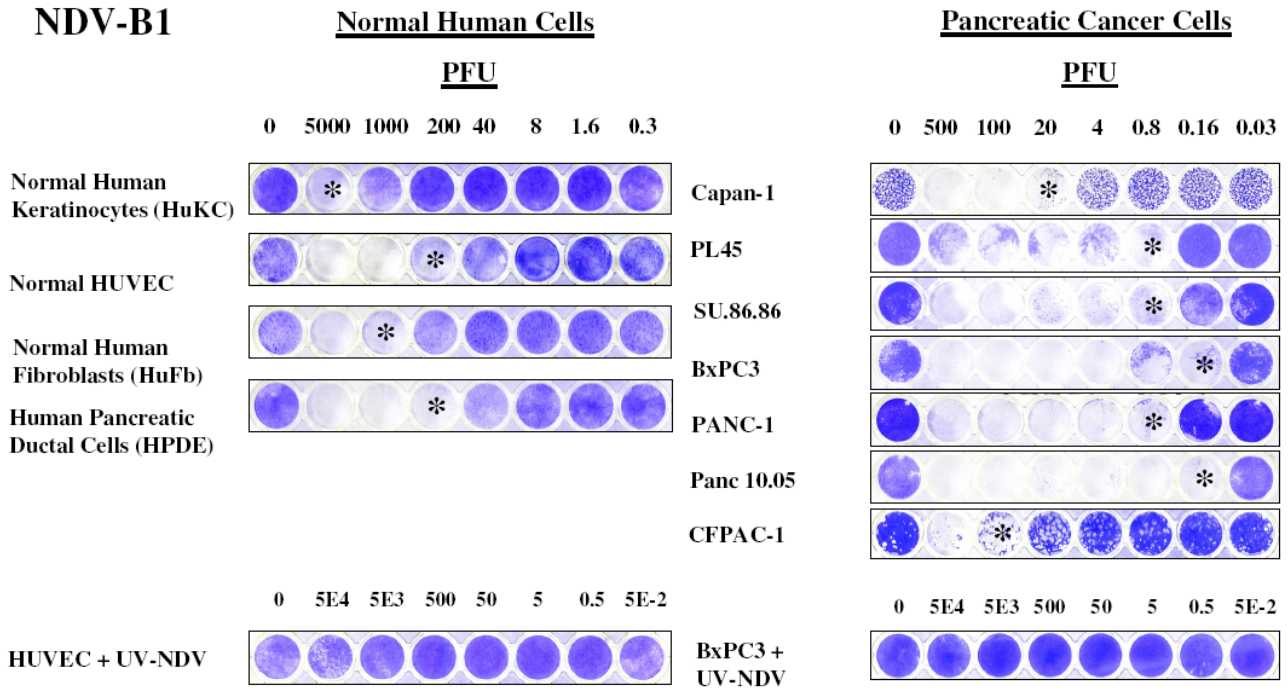

Figure 4. Photographs of multiwell plates containing normal or pancreatic cancer cells stained with crystal violet showing Newcastle disease virus Hitchner-B1 strain (NDV-B1) cytotoxicity. Unstained wells represent those in which total cytotoxicity and lysis had occurred. The far left well in each row was a negative control that received no NDV. Asterisks (*) mark the wells where the minimum cytotoxic dose of NDV was seen for human keratinocyte (HuKC), human vascular endothelial (HUVEC), human fibroblast (HuFb), human pancreatic ductal epithelial (HPDE), Capan-1, PL45, SU.86.86, BxPC3, PANC-1, Panc 10.05, and CFPAC-1 cultures. Acetylated trypsin had been present in all wells shown except those containing HuKC cells which contained 10% allantoic fluid. At 50,000 plaque forming unit (PFU), UV-inactivated NDV-B1 had little effect on HUVEC or BxPC3 cell viability.

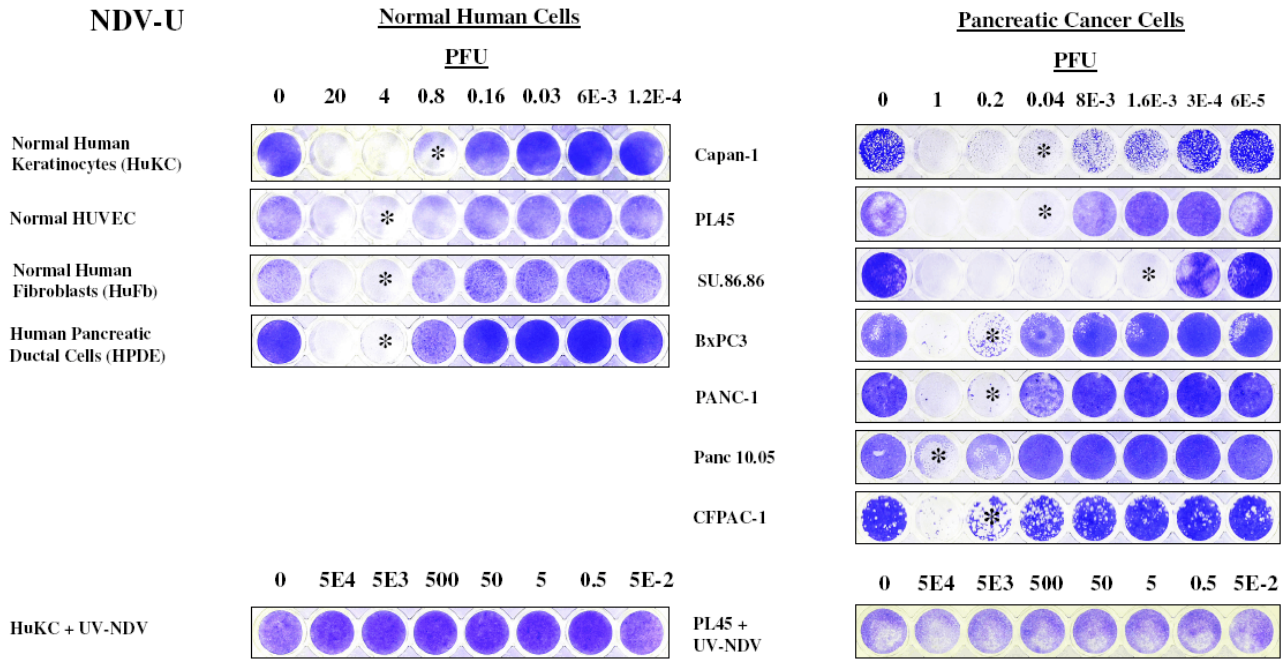

Figure 5. Photographs of multiwell plates containing normal or pancreatic cancer cells stained with crystal violet showing Newcastle disease virus Ulster strain (NDV-U) cytotoxicity. Unstained wells represent those in which total cytotoxicity and lysis had occurred. The far left well in each row was a negative control that received no NDV. Asterisks (*) mark the wells where the minimum cytotoxic dose of NDV was seen for human keratinocyte (HuKC), human vascular endothelial (HUVEC), human fibroblast (HuFb), human pancreatic ductal epithelial (HPDE), Capan-1, PL45, SU.86.86, BxPC3, PANC-1, Panc 10.05, and CFPAC-1 cultures. Acetylated trypsin had been present in all wells shown except those containing HuKC cells which contained 10% allantoic fluid. At 50,000 plaque forming unit (PFU), UV-inactivated NDV-U had little effect on HuKC or PL45 cell viability.

NDV-U was Cytotoxic for Capan-1 and SU.86.86 Cultures at Very Low Doses

With acetylated trypsin, NDV-U was cytotoxic toward Capan-1 and SU.86.86 cultures (Figure 3) at relatively low doses (0.00041 and 0.0034 PFU, respectively). In contrast, even with acetylated trypsin, NDV-U was cytotoxic toward other pancreatic cancer cell lines at much higher doses (ranging from 0.14 to 1.17 PFU, respectively) similar to those seen with normal cells (Figure 3). With acetylated trypsin, means±SEM for minimum cytotoxic NDV-U doses with CFPAC-1, Capan-1, Panc 10.05, PL45, PANC-1, SU.86.86, and BxPC3 were 0.53±0.14, 0.00041±0.00011, 0.84±0.26, 0.14±0.03, 1.01±0.26, 0.0034±0.0008, and 1.17±0.21, respectively. Without acetylated trypsin, these values were similar at 0.82±0.19, 0.0011±0.0002, 1.05±0.28, 0.098±0.028, 1.67±0.20, 0.0025±0.0004, and 0.76±0.22, respectively.

With or without acetylated trypsin or allantoic fluid, Capan-1 or SU.86.86 cells were significantly more sensitive ($P<0.001$, Kruskal-Wallis with Dunn's multiple comparison tests) to killing than were HPDE, HuFb, or HUVEC cultures. The cytotoxic doses required for the other pancreatic cell lines were not significantly different from those seen with any of the normal cells lines studied here ($P>0.05$, Kruskal-Wallis with Dunn's multiple comparison tests.

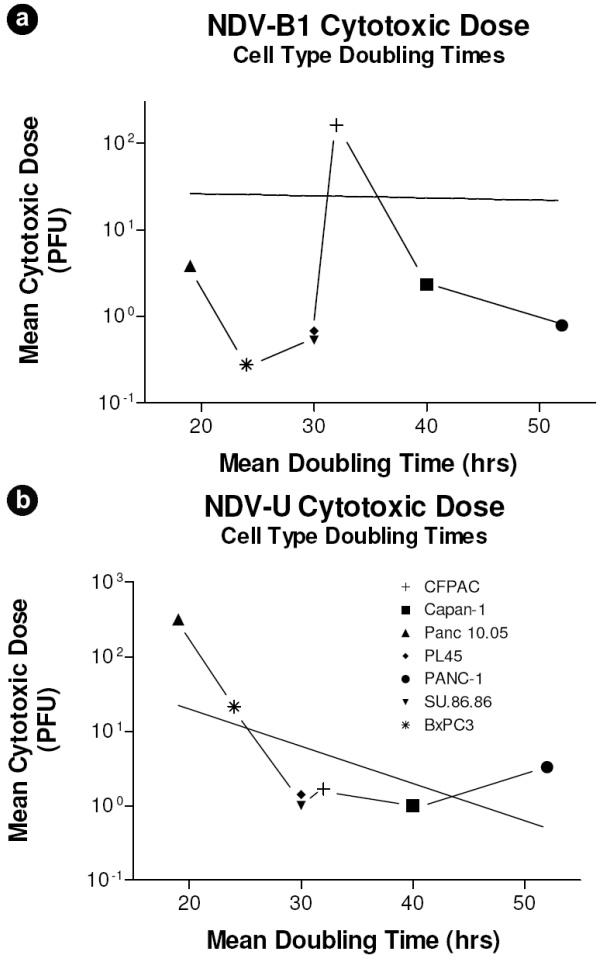

Figure 6. Minimum cytotoxic dose of Newcastle disease virus Hitchner-B1 (NDV-B1) or NDV Ulster (NDV-U) strain for pancreatic cancer cells versus cell doubling time. **a.** NDV-B1 treated cultures. **b.** NDV-U treated cultures. By linear regression analysis, there was no apparent relationship between the doubling time and the sensitivity of these cell types to NDV killing.

Tumor Cell Doubling Time Was not Related to NDV Cytotoxicity

The rate of cell proliferation or doubling time for each tumor cell line was not linearly related to the amount of NDV-B1 or NDV-U required for cytotoxicity (Figure 6). By linear regression analysis, there was no apparent relationship between the doubling time and the sensitivity of these cell types to NDV killing. For NDV-B1 treated cultures, the slope of the regression line was -0.132, $r^2=0.0006$, $P=0.960$ indicating poor goodness-of-fit and a non-significant deviation from a zero slope. For NDV-U treated cultures, the slope of the regression line was -0.050, $r^2=0.339$, $P=0.170$ indicating poor goodness-of-fit and a non-significant deviation from a zero slope.

DISCUSSION

Normal human cells were killed only by relatively high doses of NDV-B1. This was seen in normal diploid human keratinocytes, fibroblasts, and vascular endothelial cells as well as immortalized pancreatic ductal epithelial cells. In contrast, both non-cystic fibrosis pancreatic cancer cell types studied here were killed by much lower NDV-B1 doses ranging from 181- to 5,558-fold less virus than were normal cells. Since the average cytotoxic dose for non-cystic fibrosis pancreatic cancer cell types was 1.01 PFU for NDV-B1 and this represented only 0.0055 HA units of virus, there were exceedingly low levels of virus required for cytotoxicity. For NDV-U, all pancreatic cancer cell types studied here (PFU range: 0.00041 to 1.2) were killed by doses similar to those seen for normal cells (PFU range: 0.32 to 1.6). However, CFPAC-1 and SU.86.86 cells were highly sensitive to NDV-U cytotoxicity and were killed by much lower doses ranging from 470- to 3,902-fold less virus than were normal cells.

Exceedingly low levels of NDV-B1 were required for tumor cell cytotoxicity. The average cytotoxic dose for non-cystic fibrosis pancreatic cancer cell types was 1.01 PFU for NDV-B1 and this represented only 0.0055 HA units of virus. In contrast, the average cytotoxic NDV-U dose for pancreatic cancer cell types was 1.06 PFU which represented 8.6 HA units of virus, more than 1,500-fold more HA units than needed for NDV-B1 cytotoxicity. Thus, NDV-B1 was highly cytotoxic to a wider range of pancreatic tumor cell types at much lower doses than was NDV-U. However, NDV-B1 was poorly effective at killing Capan-1 cells whereas NDV-U was highly cytotoxic to this cell type. Pancreatic tumor cell lines derived from patients with cystic fibrosis showed significantly elevated resistance to NDV-B1 cytotoxicity (ranging from 2.1- to 259-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 cystic fibrosis transmembrane conductance regulator gene or alterations in the intracellular ionic milieu induced by the regulator. CFPAC-1 cells exhibit the most common cystic fibrosis mutation, a deletion resulting in the absence of phenylalanine at position 508 (ΔF508). These cells are homozygous for this mutation and show defective ion transport as a result [37, 38]. This mutation may, in some way, contribute to the unusual resistance of this tumor line to NDV-B1, but the mechanism for such an effect is not evident. Capan-1 is the only human pancreatic tumor line that expresses a functional, cAMP-sensitive, confluence-dependent CFTR and this CFTR lacks any known mutations [39]. Capan-1 is weakly resistant to NDV-B1 unlike most of the other pancreatic lines studied here (Panc 10.05, PL45, PANC-1, SU.86.86, and BxPC3) that lack functional CFTR and are highly sensitive to NDV-B1 killing. In contrast, Capan-1 is highly sensitive to NDV-U killing whereas most of the other pancreatic lines that lack functional CFTR are highly resistant to NDV-U. Thus, any role for this functional CFTR in the differential susceptibility of different types of pancreatic tumor cells to NDV cytotoxicity is unclear. Nonetheless, this differential resistance/sensitivity to NDV killing exhibited by cystic fibrosis patient-derived tumors may be an important consideration in future clinical trials involving NDV. We have seen similar resistance to NDV-LS cytotoxicity in CFPAC-1 and Capan-1 cells [32].

UV-inactivated NDV was mildly cytotoxic for tumor cells, but only at very high doses (PFU>50,000). A similar effect has been described previously for 73-T and other strains of NDV [7, 8, 9, 11, 40]. 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 or allantoic fluid increased the cytotoxicity of NDV-B1 by a modest 25%, but very high levels of cytotoxicity for pancreatic tumor cells were seen in their absence. In contrast, NDV-U killing was not significantly altered in the presence of exogenous protease. These data suggest that NDV-B1 may replicate to yield infectious virus in the absence of exogenously added protease 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 might cleave the viral F protein to generate the highly infectious form of the F protein, although F protein activation was not measured here. Finally, the rate of cell proliferation or doubling time for each tumor cell line was not linearly related to the amount of NDV-B1 or NDV-U required for cytotoxicity. For most forms of chemotherapy, tumor cells must be in specific stages of the cell cycle (particularly in mitosis) for the therapy to be effective. This is not required with NDV, which effectively kills pancreatic tumor cells even if they are in stationary phase. None of the cell cultures used here was undergoing mitosis during NDV infection or during the subsequent 4 or 5 day incubations. They were all in stationary phase due to the extent of culture confluence at the time of infection and to the very low levels of FCS in the media after NDV infection.

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 [41, 42, 43], 73-T in sarcomas, carcinomas, and melanomas [7, 8, 9, 10, 11, 22, 44, 45, 46, 47]; PV701 in various advanced solid tumors [15, 30, 48, 49], HUJ in glioblastoma and lung tumors [20, 50, 51], and Ulster strain in melanoma, breast, and gastrointestinal tumors [52, 53, 54, 55]. Some of these NDV strains have been used primarily as immune adjuvants (Ulster by Schirrmacher *et al*. [52, 53, 54, 56]; 73-T by Cassel *et al*. [46, 47, 57]; MTH-68 by Csatary *et al*. [42, 58]). On the other hand, some of these strains can exert effects via direct cytolytic activity toward tumor cells (e.g., 73-T [7, 8, 9, 11, 45, 59, 60]; PV701 [15, 30, 48]; HUJ [20, 51]; MTH-68 [61, 62, 63]).

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*. [30] studied nine primary pancreatic carcinoma patients of which one or two showed measurable tumor size reductions. Recently, Fabian *et al*. [27] showed that the mesogenic NDV strain MTH-68/H was highly cytotoxic for PANC-1 cells. Zamarin *et al*. [28] showed that lentogenic NDV-B1 decreased cell survival in PANC-1 cells by 50% after 3 days *in vitro*, but had no effect on the pancreatic cancer line, MIA PaCa-2. Jarahian *et al*. [29] reported that PANC-1 cells infected with NDV-U were killed more efficiently by natural killer cells *in vitro*. Schirrmacher *et al*. [21] also showed that NDV-U could infect and replicate in two established human pancreatic cancer cell lines and in more than ten primary tumor explants *in vitro*, but cytotoxicity data were not presented. In 2003, Liang *et al*. [31] reported disease stabilization in one patient with pancreatic head cancer treated with NDV-LS IV strain employed as a vaccine. These reports offer some optimism regarding the potential for the usefulness of NDV in treating pancreatic neoplasms.

Lentogenic NDV strains such as NDV-B1, NDV-U, and NDV-LS have been studied mainly as immune adjuvants in infected tumor cell vaccines or oncolysates rather than for any direct tumor cytotoxicity [31, 52, 53, 54, 55, 56, 64, 65, 66]. It is often assumed that these lentogenic strains would have poor tumor cytotoxicity due to their low infectivity and lysogenicity 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. NDV-B1 and NDV-U differ from one another in a number of respects. The intracerebral pathogenicity index in day-old chicks (ICPI) is 0.20 and 0.00, the intravenous pathogenicity index (IVPI) in 6-week-old chickens is 0.0 and 0.0, the mean death time for chicken embryos infected with one minimum lethal dose of virus (MDT) is 104-120 hours and >150 hours for NDV-B1 and NDV-U, respectively. The amino acid sequence of the F protein cleavage site (109-119) also differs somewhat such that it is SGGGRQGRFIG for the B1 strain and SGGGKQGRFIG for Ulster strain [67, 68, 69]. Finally, Ulster strain is classified as a lentogenic or apathogenic, enterotropic strain in chicken whereas Hitchner-B1 is lentogenic and pneumotropic [70, 71]. Such lentogenic strains must be activated by exogenous trypsin-like proteases such as those found in allantoic fluid, in the lungs, or in the gastrointestinal tract to obtain infectious virus in chickens [71, 72]. Thus, NDV-B1 or NDV-U, which are initially activated and infectious, are expected to undergo only a single round of infection (monocyclic) in human tumor cells unless appropriate protease activation of progeny virus particles occurs. If this were to occur, increased virus infectivity of otherwise weakly infectious virus would likely be elicited.

We have shown that even naturally occurring, lentogenic NDV-B1 and in some cases NDV-U, which are poorly infectious in chicken, are highly cytolytic for human pancreatic tumor cells and highly specific for tumor versus normal human cells. Further, the inclusion of acetylated trypsin or other exogenous protease together with NDV-B1 resulted in a modest but significant increase in cytotoxicity showing that NDV-B1 is highly cytotoxic to pancreatic tumor cells even in the absence of F protein activation by exogenously added proteases. In chicken, NDV is known to be viscerotropic, pneumotropic, or neurotropic depending on the strain [70, 73, 74]. Lentogenic strains are often viscerotropic having a marked propensity to infect enteric organs. The Hitchner-B1 and LaSota strains are used as bird vaccines to protect against velogenic strains of NDV and typically proliferate most efficiently in the respiratory and gastrointestinal tracts [26, 75] where proteases may exist that cleave the NDV fusion protein thereby increasing the weak infectivity of the virus [76, 77]. This tropism for the gastrointestinal tract, the potential for NDV activation there, and their high level of differential cytotoxicity (particularly NDV-B1) toward pancreatic tumor cells *in vitro* may make these lentogenic strains of NDV useful in the treatment of pancreatic cancer. In addition, the low risk to avian species from these lentogenic strains make them even more appealing for clinical use in humans. Further studies, particularly animal studies examining the effects of these strains of NDV on xenogenic human tumors, are needed.

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