The following article appeared in *Oncology Letters, 15: 1246-1254 (2018);* and may be found at: https://doi.org/10.3892/ol.2017.7387

Antitumor and immunostimulatory activities of a genotype V recombinant attenuated veterinary Newcastle disease virus vaccine

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Received June 30, 2017; Accepted October 17, 2017

DOI: 10.3892/ol.2017.7387

Abstract. Antitumor conventional treatments including chemo/radiotherapy result in several side effects and non-specificity. Therapies including the use of oncolytic viruses, particularly the Newcastle disease virus (NDV), have emerged as an attractive alternative due to their capacity to kill cancer cells directly or through stimulation of the immune system. In the present study, a commercial vaccine composed of a recombinant attenuated NDV strain P05 (rNDV-P05) was assessed for antitumor and immunostimulatory activity. Firstly, hemagglutination activity was evaluated at different pH and temperature conditions. Then, cancer cell lines and peripheral blood mononuclear cells (PBMC) were co-cultured with or without rNDV-P05 and cytoplasmic nucleosomes were measured by enzyme-linked immunosorbent assay (ELISA) as an apoptosis indicator. Antitumor cytokines produced by PBMC in response to the virus were analyzed by ELISA and reverse transcription quantitative polymerase chain reaction. Characterization of rNDV-P05 indicates that the virus is slightly sensible to acid and basic pH, and stable at temperatures no greater than 42˚C. The majority of cell lines developed apoptosis in co-culture with rNDV-P05 in a dose-time dependent manner. The highest level of HeLa, HCC1954 and HepG2 cell apoptosis was at 48 h/50 hemagglutination units (HU), and HL-60 was 24 h/50 HU. A549 cell line and PBMC did not show sensitivity to apoptosis by the virus. PBMC from healthy donors stimulated with the rNDV-P05 increased significantly the levels of interferon (IFN)-α, IFN-γ, tumor necrosis factor (TNF)-α and soluble TNF-related apoptosis-inducing ligand in culture supernatants, as well as their mRNA expression. These results demonstrate that the pro-apoptotic effect of rNDV-P05 and its magnitude is specific to particular tumor cell lines and is not induced on PBMC; and the virus stimulates the expression of several key antitumor cytokines. This study promotes the use of rNDV-P05 in an alternate application of different viral strains during virotherapy with NDV.

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Key words: Newcastle disease virus, antitumor activity, immunostimulatory activity, genotype V, strain P05

Introduction

According to the World Health Organization cancer is a broad term for a large group of diseases that can affect any part of the body. It is caused by transformation of normal to malignant cells that behave unusual and grow beyond their usual boundaries. It is the second leading cause of death globally, and was responsible for 8.8 million deaths in 2015 (1). Conventional chemo and radiotherapy have many adverse effects and fail to cure many types of cancer in humans, thus alternative therapies to treat cancer patients have become more popular since late last century (2). Among these therapies the use of oncolytic viruses, which have tropism to malignant cells but not to normal cells, has been gaining field during the last decades (3) and even a FDA-approved virotherapy is already available since 2015 (IMLYGIC™; Amgen, Inc., Thousand Oaks, CA, USA). Oncolysis induced by these viruses is mostly an immunogenic type of cancer cell death that includes immunogenic apoptosis, necrosis and autophagic cell death (4). As consequence, oncolytic virus induces a potent post-oncolytic antitumor activity that is considered crucial for its efficient therapeutic activity (5).

The Newcastle disease virus (NDV) is one of the various species of viruses that are under clinical evaluation as vector for oncolytic tumor, gene and immune stimulation therapies (3). It is member of the *Avulavirus* genus in the Paramyxoviridae family (6) and possesses a 15,186 nucleotide negative single

strand RNA genome that encodes six genes including the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase (L) (7). Phylogenetic comparison of the full-length HN and F proteins shows two major divisions represented by class I and class II. Class II is further divided into at least 8 (I-VIII) genotypes (8) and genotypes I (such as Ulster) and II (such as La Sota) have the NDV strains most used for virotherapy (2,3). Additionally, NDV is classified into three pathotypes depending on the severity of the disease that it causes in birds: lentogenic (avirulent), mesogenic (intermediate), or velogenic (virulent) (7). The cleavage site in the F protein of the NDV has been shown to be a major determinant of virulence (9,10). In this regard, pathogenic classification of NDV strains in birds correlates with their oncolytic properties in cancer cells, which can be categorized as either lytic or non-lytic, with velogenic and mesogenic viruses being lytic and lentogenic viruses in general being non-lytic (11).

NDV has been demonstrated to mediate its oncolytic effect by both intrinsic and extrinsic caspase-dependent pathways of cell death (12). NDV-induced apoptosis is dependent on upregulation of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and caspase activation, which cause opening of mitochondrial permeability transition pores, loss of mitochondrial membrane potential, and subsequent activation of apoptosis process (13). Besides, NDV can also develop additional immune stimulatory mechanisms. It is known that it provokes the release of danger signals during virus replication into the tumor cell cytoplasm (14-16) and stimulates the immune system to produce cytokines such as interferons (IFNs) or TNF (17). In turn, these inflammatory cytokines lead to the activation of natural killer (NK) cells, monocytes, macrophages and sensitized T cells, stimulating both innate and adaptive immune response (17-21).

It is known that in NDV therapy the treatment outcomes are dependent on the kind of tumor and on the used NDV strain (2). Besides, to avoid limiting effects of neutralizing antibodies during virotherapy the use of different NDV strains is considered. In this study, we wanted to explore the capability and specificity of a genotype V recombinant attenuated NDV vaccine, which was made from a velogenic strain obtained from an outbreak in Puebla (Mexico) in 2005 and denominated APMV1/Chicken/Mexico/P05/2005 (22), to induce apoptosis over a panel of tumor cell lines and to stimulate human peripheral blood mononuclear cells (PBMC) *in vitro*.

Materials and methods

Cell lines and culture. Tumor cells were kindly provided by Dr Pablo Ortiz (HL-60 and HCC1954; CIBO, Guadalajara, Mexico), Dr Susana Del Toro (HeLa; CUCS, Guadalajara, Mexico) and Dr Daniel Cervantes (A549 and HepG2; UAA, Aguascalientes, Mexico). The human cell lines HeLa (HPV-18 cervix adenocarcinoma) and HepG2 (hepatoblastoma) (23) were cultured in DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 1 g/l glucose, 4 mM L-alanyl-glutamine, 10% heat-inactivated fetal bovine serum (FBS; Gibco), 50 IU/penicillin and, 50 μg/ml streptomycin (Sigma-Aldrich Israel Ltd., Rehovot, Israel); the human cell lines HCC1954 (breast cancer), HL-60 (promyelocytic leukemia) and A549 (lung carcinoma) were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 g/l glucose, 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated FBS, 50 IU/penicillin and, 50 μg/ml streptomycin (Sigma-Aldrich Israel Ltd.) at 37˚C and 5% CO_2 in a humidified atmosphere. PBMCs were obtained from healthy donors, after obtaining informed consent, by centrifugation in Ficoll-Hypaque (Lymphoprep™; Axis-Shield PoC AS, Oslo, Norway) density gradient at 832 x g for 20 min. Cells from sample/medium interface were collected and washed three times with phosphate buffer saline (PBS) pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Cell suspension was depleted of erythrocytes by incubation in 75 mM NH_4Cl for 5 min, washed twice in PBS and cultured with supplemented RPMI-1640 medium. The trypan blue dye exclusion assay was performed to determine cell number and viability of PBMC.

Recombinant NDV. All experiments were carried out with a recombinant Newcastle disease virus (rNDV-P05), from the Mexican velogenic strain APMV1/Chicken/Mexico/P05/2005 (22,24) obtained from a commercial attenuated live vaccine (Investigación Aplicada, S.A. de C.V., Tehuacan, Mexico). To concentrate and clarify the virus, one vaccine vial was diluted in 50 ml of PBS and centrifuged at 3,300 x g at 4˚C for 20 min (Hermle Z383K; Hermle Labortechnik GmbH, Wehingen, Germany). Then the supernatant was filtered (0.22 μm; Millipore Ireland Ltd., Cork, Ireland) and the virus pelleted by ultracentrifugation at 150,000 x g at 4˚C for 6 h (Beckman Coulter Optima™ max-xp; Beckman Coulter, Inc., Brea, CA, USA) in a 20% sucrose cushion. The virus was suspended in 200 μl of PBS and its concentration and activity were determined by hemagglutination assay. To determine optimal pH for viral activity, PBS solutions with different pH (3, 5, 7.4 and 9) were previously prepared and used to suspend the virus after ultracentrifugation. To evaluate optimal temperature, the virus was diluted in PBS pH 7.4 and incubated at 22, 37, 42, 60 and 80˚C for 2 h.

Hemagglutination assay. Virus concentration and hemagglutinating activity were determined from 25 μl of ultracentrifuged rNDV-P05 sample by incubating two-fold serial dilutions of viral samples in 96-well microtiter plate with 1% human erythrocytes for 45 min at room temperature. Results were expressed as hemagglutination units (HU), where one HU represents the highest virus dilution leading to visible hemagglutination. As positive and negative controls, a polyvalent influenza vaccine (Flu) and PBS were used, respectively.

Viral protein separation and staining. A volume of 5 μl from a 10,000 HU/ml sample of concentrated virus was resolved by 10% sodium dodecyl sulfate-polyacrilamide gel (SDS-PAGE) using a Mini Protean III system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and then stained with Coomassie blue R-250 staining for visual interpretation as previously illustrated (25).

Apoptosis detection. The capacity of virus to induce apoptosis on tested cells was determined following the instructions of a

Table I. Oligonucleotides for gene expression quantification.

Gene	GenBank accession	Oligonucleotides (5'>3')
TNF-α	NM_000594	F: CCTCTCTCTAATCAGCCCTCTG
		R: GAGGACCTGGGAGTAGATGAG
IFN-α	NM_000605	F: GCTTGGGATGAGACCCTCCTA
		R: CCCACCCCCTGTATCACAC
IFN-γ	NM_000619	F: TCGGTAACTGACTTGAATGTCCA
		R: TCGCTTCCCTGTTTTAGCTGC
TRAIL	NM_001190942	F: TGCGTGCTGATCGTGATCTTC
		R: GCTCGTTGGTAAAGTACACGTA
β-actin	NM_001101	F: CATGTACGTTGCTATCCAGGC
		R: CTCCTTAATGTCACGCACGAT

TNF-α, tumor necrosis factor-α; IFN, interferon; TRAIL, tumor necrosis factor related apoptosis inducing ligand; F, forward; R, reverse.

commercial enzyme-linked immunosorbent assay (ELISA) kit (Cell Death Detection ELISA^{PLUS}; Roche Diagnostics GmbH, Mannheim, Germany) for the detection of histone-associated-DNA-fragments (nucleosomes) in cytoplasm as an indicator of late apoptosis. In brief, tumor cell lines and PBMC were seeded in a 96-well plate at 10^4 cells/well in 200 μl for 24 and 48 h in presence (10 and 50 HU) or absence (PBS vehicle, control) of rNDV-P05. After incubation, cells were lysed and centrifuged at 200 x g for 10 min (Eppendorf 5415C; Eppendorf, New York, NY, USA), and supernatants (cytoplasmic fraction) were evaluated for the detection of nucleosomes by ELISA. Apoptosis levels were calculated as the enrichment factor (EF) of nucleosomes released into the cytoplasm as follows: EF = $OD_{405\ nm}$ of treated cells/$OD_{405\ nm}$ non-treated cells (control).

PBMC stimulation assay and cytokine quantification. For human PBMC stimulation, 10^6 cells were seeded in 24-well plates with 1 ml of supplemented RPMI-1640 medium. After 4-6 h acclimatization, cells were co-cultured with rNDV-P05 (10 and 20 HU) or with an equivalent volume of PBS (vehicle control) for 24 h. As positive control, 5 μg/ml of lipopolysaccharide (LPS; Sigma-Aldrich) was added to cells. After stimulation, supernatants from all groups were kept at -80˚C until utilization. Measurement of IFN-α, IFN-γ, TNF-α and TRAIL was determined from supernatants by commercial ELISA kits [human IFN-α platinum (eBioscience, Vienna, Austria); human IFN-γ (Thermo Fisher Scientific, Inc., Waltham, MA, USA); human TNF-α (Life Technologies, Merelbeke, Belgium); human TRAIL (CD253; Abcam, Cambridge, UK)]. Absorbance was read using an iMark™ microplate reader (Bio-Rad Laboratories, Tokyo, Japan) and concentration for each cytokine was determined carrying out a curve with the standards supplied by the kit.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Human PBMC were stimulated as previously described, but co-cultured with rNDV-P05 or positive/negative controls only 4 h. Total RNA was isolated from all groups using a commercial kit Allprep RNA/protein (Qiagen GmbH, Hilden, Germany) following the company's instructions. Purified RNA was quantified with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.) with the A260/280 ratio and only samples with ratio >1.8 were employed. RNA (50 ng) was retrotranscripted to complementary DNA (cDNA) using the kit SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), and then 100 ng of cDNA were used as template for qPCR according to SYBR-Green technology kit (Bio-Rad Laboratories, Inc.). All qPCR experiments were carried out in a 7500 Fast Real Time PCR system Applied Biosystems V2.0. Specific primers for *TNF-α* (ID: 25952110c1), *IFN-α* (ID: 11067751a1), *IFN-γ* (ID: 56786137c1), *TRAIL* (ID: 300193031c1), and *β-actin* (ID: 4501885a1) genes (Table I) were chosen from Primer Bank (https://pga.mgh.harvard.edu/primerbank/) and used at a final concentration of 120 (TNF-α and IFN-α) or 200 nM (IFN-γ, TRAIL, and β-actin). The cycling conditions were: initial denaturation at 95˚C for 3 min, then denaturation at 95˚C for 10 sec, annealing at 60˚C (TNF-α), 59˚C (IFN-α), 60˚C (IFN-γ), 60˚C (TRAIL) and, 60˚C (β-actin) for 30 sec, for 40 cycles. Target gene expression was reported as the fold change in the normalized signal relative to *β-actin* gene through the ΔΔCq method (26).

Statistical analysis. Data represent the mean ± SEM from at least three independent experiments. All analyses were done with the GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Statistical analysis was performed by a two-sided Student's t-test, except for comparing NDV treated against control in antitumor assays, in which one-way ANOVA with Dunnett's post test was employed. A P-value <0.05 was considered significant.

Results

rNDV-P05 characterization. rNDV-P05 proteins from ultracentrifuged samples were resolved and identified through SDS-PAGE (Fig. 1A). Five major bands corresponded to the molecular weights of the structural proteins of NDV, including HN (75 kDa as a monomer and 150 kDa as dimer), F (65 kDa), NP (50-55 kDa), and M (40 kDa) proteins. rNDV-P05 activity was determined by hemagglutination assay (Fig. 1B) and

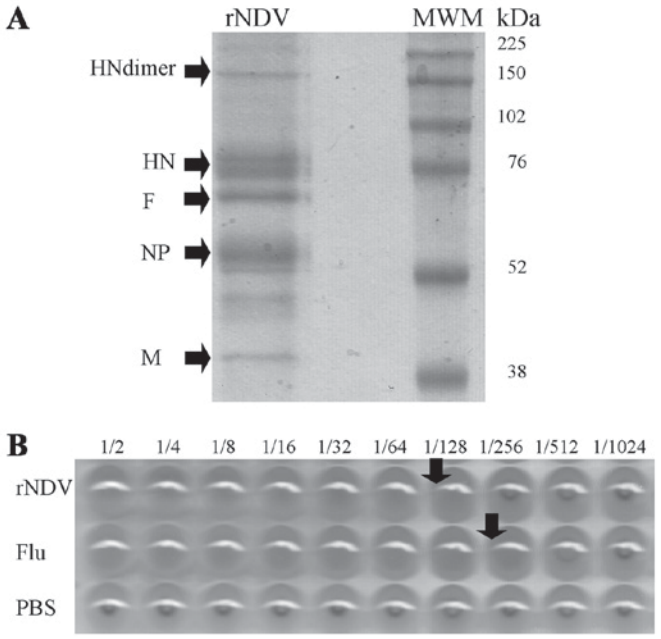

Figure 1. Characterization of proteins and hemagglutinating activity of rNDV. (A) Sodium dodecyl sulfate-polyacrilamide gel from concentrated virus after ultracentrifugation. Arrows indicate molecular weight for the major viral protein: HN as monomer and dimer, F, NP, M. (B) Representative hemagglutination assay for determination of activity and concentration from rNDV-P05 stocks. PBS and Flu vaccine were used as negative and positive controls, respectively. HN, hemagglutinin-neuraminidase; F, fusion protein; NP, nucleocapsid protein; M, matrix protein; MWM, molecular weight marker; kDa, kilo Daltons; rNDV-P05, recombinant attenuated NDV strain P05; PBS, phosphate buffer saline; Flu, polyvalent influenza vaccine.

1 HU of rNDV-P05 sample was titered at 1:128. Furthermore, in order to find out the best conditions for rNDV handling, different pH and temperatures were applied to virus after ultracentrifugation and then hemagglutination assay was developed. Detailed results are summarized in Table II. Hemagglutination titers of rNDV changed according to pH, with the lowest value observed at pH 3 (1:32; P<0.001), and one titer decreased at pH 5 and pH 9 (1:64; P<0.001) compared to pH 7.4 (1:128). On the other hand, temperatures higher than 42˚C totally abolished hemagglutinating activity of the virus.

rNDV-P05 induces apoptosis in HeLa, HCC1954, HL-60 and HepG2 cells, but not in A549 cells and PBMC. Antitumor activity of rNDV-P05 was determined as the capacity of the virus to induce apoptosis on different tumor cell lines. Four of the five tumor cell lines showed sensitivity to the rNDV, mostly in a dose and time dependent way (Fig. 2). For HeLa cells, EF values increased when cells were incubated with 10 or 50 HU of virus at both times of incubation, in relation to control cells (P<0.001 to 10 HU at 48 h, P<0.001 to 50 HU at 24 and 48 h; Fig. 2A). The pro-apoptotic effect of 50 HU of virus was significantly greater than that of 10 HU at 48 h (P<0.01). Incubation time was also relevant in antitumor activity, as 48 h showed greater effect than 24 h, both with 10 (P<0.001) or 50 HU (P<0.05) of the virus. For HCC1954 cells, EF significantly augmented at 24 h (P<0.05 to 10 HU, P<0.001 to 50 HU) and 48 h (P<0.001) when incubated with both doses of rNDV-P05 (Fig. 2B). This pro-apoptotic effect was significantly greater when cells were incubated with the highest dose of virus and during the longest time (P<0.001). For HL-60 cells, as shown in Fig. 2C, EF values augmented when cells were treated with 10 or 50 HU of virus for 24 and 48 h (P<0.01 and P<0.05 to 10 HU at 24 and 48 h, P<0.001 and P<0.01 to 50 HU at 24 and 48 h) and the antiviral effect of 50 HU of the virus was significantly greater than that of 10 HU when cells were exposed during 24 h (P<0.05). Surprisingly, it was observed a significant decrease in EF values as incubation time was prolonged using both 10 HU (P<0.05) and 50 HU (P<0.01) of rNDV. In the case of HepG2 cells, incubations with rNDV-P05 induced a significant increment in values of EF as compared to control conditions (P<0.01 and P<0.001 to 10 HU at 24 and 48 h, P<0.001 to 50 HU at 24 and 48 h; Fig. 2D). The antitumor effect of rNDV was greater when HepG2 cells were incubated with 50 HU of the virus independently of the incubation time (P<0.05), but exposures of 48 h induced a significant increase in pro-apoptotic effect in relation to that of 24 h only when 10 HU of the virus were used (P<0.001). Finally, A549 cells and PBMC were not susceptible to apoptosis induction at any time or with any used concentration of rNDV (Fig. 2E and F). All together, the results showed that the pro-apoptotic effect of rNDV-P05 and its magnitude is specific to particular tumor cell lines because there is not an evident effect on A549 cell line. Besides, no pro-apoptotic effect was observed on PBMC from healthy donors, that is important to study subsequently the immunostimulatory activity of the virus on PBMC.

rNDV-P05 induces cytokine release by PBMC. To investigate whether rNDV-P05 can stimulate immune cells, PBMCs from five healthy donors were incubated with 10 and 20 HU of the virus and several cytokines in supernatants were quantified. Twenty-four h of stimulation with rNDV-P05 induced the secretion of high amounts of TNF-α, IFN-α, TRAIL and IFN-γ from PBMC, as shown in Fig. 3. For TNF-α, both NDV doses significantly increased the secretion of this cytokine by PBMC compared to control cells (P<0.001; Fig. 3A), and it was equivalent to half of the concentration released in response to LPS. Besides, particular values of this cytokine for each donor were very similar. As shown in Fig. 3B, low amounts of TRAIL were secreted by control or LPS-stimulated cells, while incubation with 10 or 20 HU of rNDV-P05 significantly increased the release of TRAIL by PBMC, compared to control conditions (P<0.01). Interestingly, cells from donors four and five recorded values of TRAIL in response to virus that were similar to that of control cells. In relation to IFN-α (Fig. 3C), control and LPS-stimulated cells from all donors did not secrete detectable amount of the cytokine, however both NDV doses considerably induced the release of IFN-α (P<0.05 to 10 HU, P<0.01 to 50 HU). It is noteworthy that again the lowest response to NDV treatment was observed in donors four and five. Finally, both doses of virus and LPS significantly increased the secretion of IFN-γ as compared to control cells (P<0.05 to LPS, P<0.01 to 50 HU, P<0.001 to 10 HU; Fig. 3D). In this case, cells from donor four released the lowest concentration of IFN-γ in response to rNDV stimulation.

Early cytokine response at mRNA level induced by rNDV-P05 in PBMC. As previously described, stimulation of PBMC with

Table II. Physicochemical characterization of rNDV-P05 activity by hemagglutination assay.

	pH				Temperature (˚C)				
Activity	3	5	7.4	9	22	37	42	60	80
Inverse of HU	32[a]	64[a]	128	64[a]	128	128	128	0	0

rNDV-P05, recombinant attenuated NDV strain P05; HU, hemagglutination units. [a]P<0.001 vs. pH 7.4.

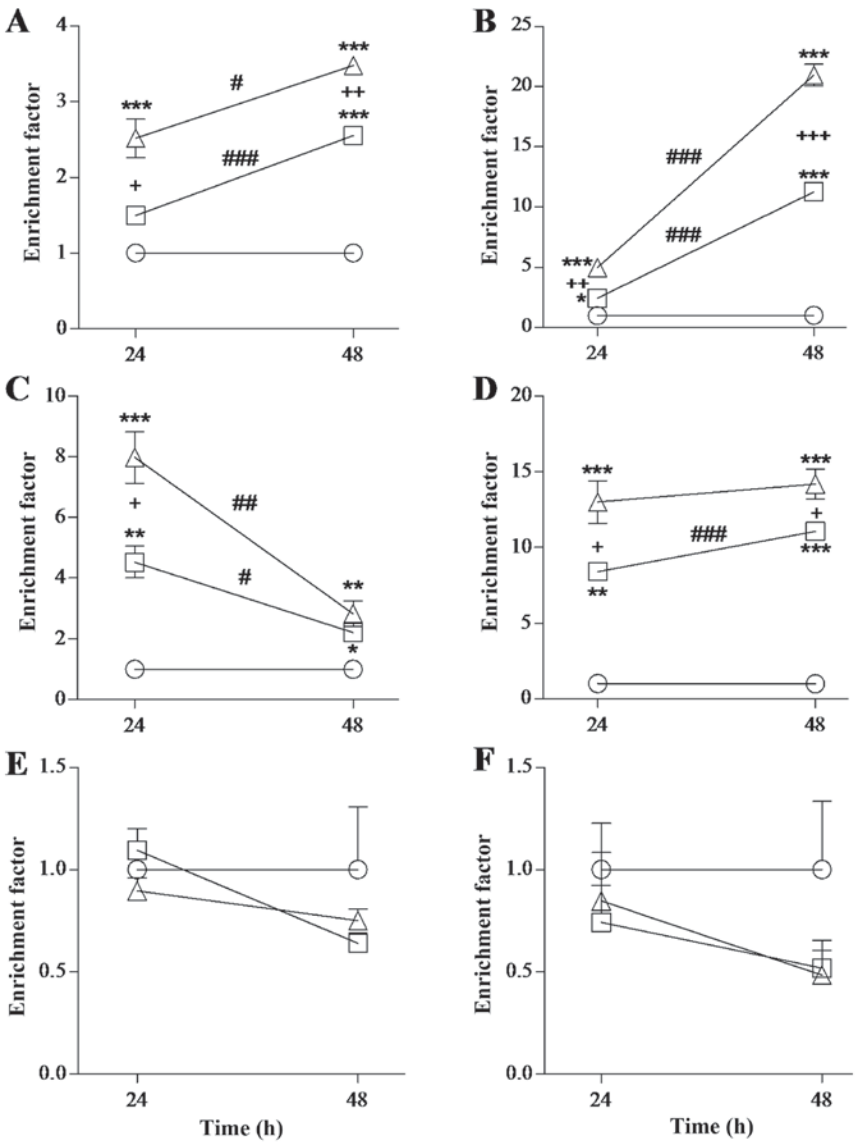

Figure 2. Evaluation of rNDV antitumor activity over different cell lines. (A) HeLa cells, (B) HCC1954 cells, (C) HL-60 cells, (D) HEpG2 cells, (E) A549 cells and (F) PBMC were exposed to different doses of rNDV-P05 [10 (□) and 50 (Δ) HU] at 24 and 48 h. The EF was evaluated. An equal volume of PBS without virus was added to cells for control assay (○). Results are presented as the mean ± standard error of three independent experiments done for each cell line. *P<0.05, **P<0.01, ***P<0.001 compared to control; +P<0.05, ++P<0.01, +++P<0.001, 10 compared to 50 HU; #P<0.05, ##P<0.01, ###P<0.001, 24 compared to 48 h. All conditions tested on A549 and PBMC were not statistically significant. rNDV-P05, recombinant attenuated NDV strain P05; HeLa, HPV-18 cervix adenocarcinoma; HepG2, hepatoblastoma; HCC1954, breast cancer; HL-60, promyelocytic leukemia; A549, lung carcinoma; PBMC, peripheral blood mononuclear cells; EF, enrichment factor; PBS, phosphate buffer saline; HU, hemagglutination units.

rNDV was evaluated in a late stage (24 h) through ELISA for cytokine concentration in culture supernatants. Additionally, an early response was determined by real time qPCR at 4 h after stimulation (Fig. 4). PBMC treated with 20 HU of virus increased the expression of TNF-α 3.06-fold (P<0.01; Fig. 4A), TRAIL 8.64-fold (P<0.01; Fig. 4B), IFN-α 10.80-fold (P<0.01; Fig. 4C), and IFN-γ 11.41-fold (P<0.05; Fig. 4D), compared to unstimulated PBMC. At this time, LPS-treated PBMC only increased significantly the expression of TRAIL compared to control group (P<0.05; Fig. 4B).

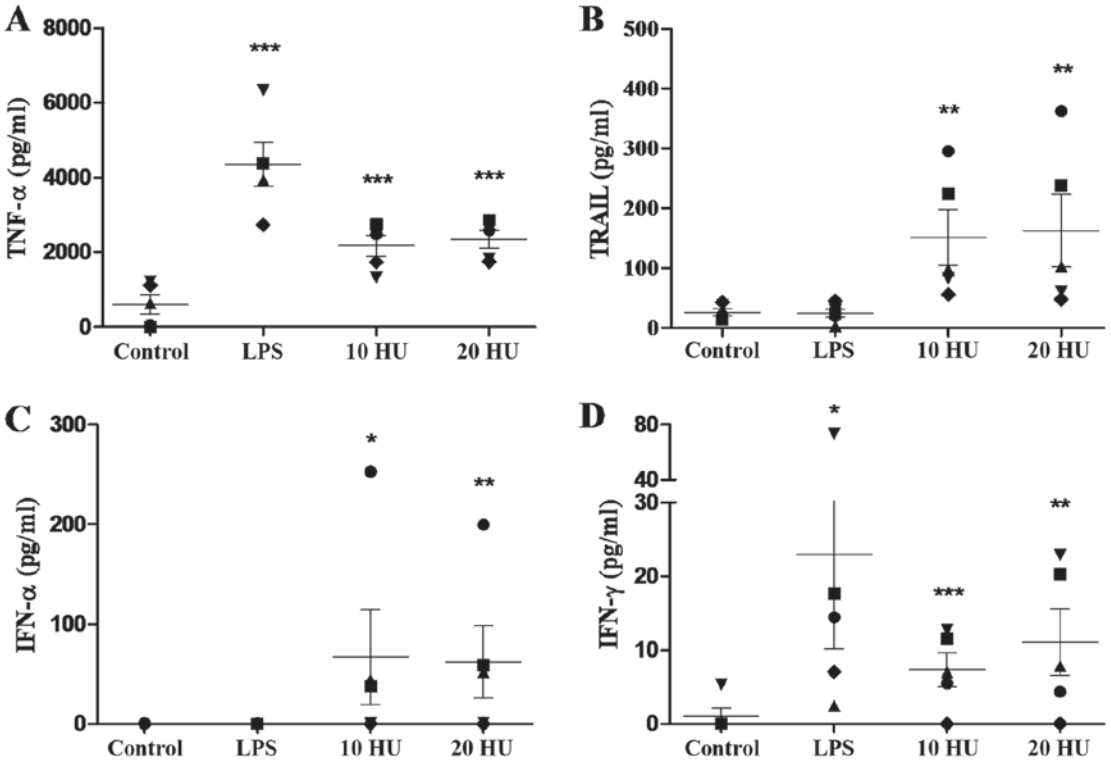

Figure 3. Immunostimulatory activity of rNDV-P05 over PBMC evaluated by ELISA. (A) TNF-α, (B) TRAIL, (C) IFN-α, and (D) IFN-γ release by PBMC after 24 h of co-culture with rNDV-P05 (10 and 20 HU/ml) or LPS (5 μg/ml). Control cells were incubated with PBS. Results are presented as the mean ± standard error. Cytokines were determined by duplicate in PBMC isolated from five healthy donors: donor 1 (●); 2 (■); 3 (▲); 4 (♦); and 5 (▼). *P<0.05, **P<0.01, ***P<0.001 compared to control. rNDV-P05, recombinant attenuated NDV strain P05; PBMC, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor-α; TRAIL, tumor necrosis factor related apoptosis inducing ligand; IFN, interferon; LPS, lipopolysaccharide; PBS, phosphate buffer saline; HU, hemagglutination units.

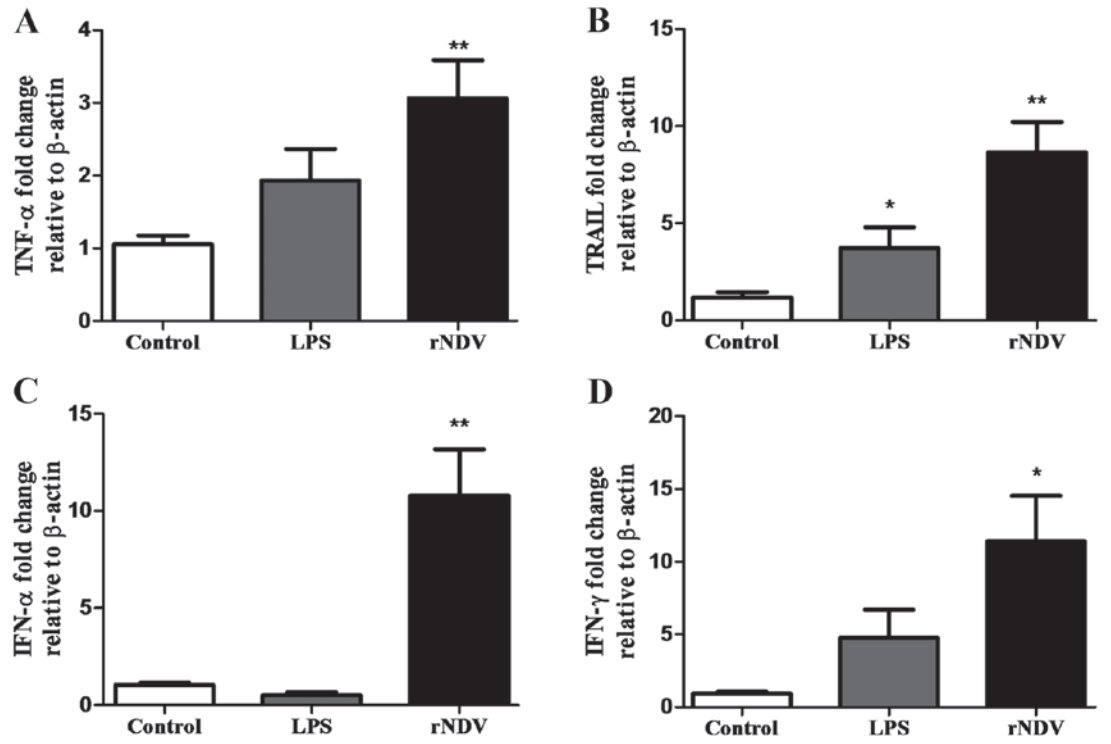

Figure 4. Cytokine expression assessment in PBMC stimulated with rNDV-P05 by quantitative PCR. Changes in the expression of (A) *TNF-α*, (B) *TRAIL*, (C) *IFN-α*, and (D) *IFN-γ* genes were evaluated at 4 h after co-culture with rNDV-P05 20 HU/ml or LPS (5 μg/ml). Control cells were incubated with PBS. *β-actin* gene was used as housekeeping gene. Results are presented as the mean ± standard error. Total RNA from three healthy donors was used and quantified in triplicate. *P<0.05, **P<0.01 compared to control. PBMC, peripheral blood mononuclear cells; rNDV-P05, recombinant attenuated NDV strain P05; TNF-α, tumor necrosis factor-α; TRAIL, tumor necrosis factor related apoptosis inducing ligand; IFN, interferon; LPS, lipopolysaccharide; HU, hemagglutination units; PBS, phosphate buffer saline.

Discussion

Recently, increasing attention has focused on alternative therapies with lesser side effects than current treatments for cancer (chemo and radiotherapy). An approach for this concern is the use of oncolytic viruses, especially NDV, which had demonstrated the capability to kill malignant cells as a result of activation of apoptotic pathways, both *in vitro* and *in vivo* (2,27). However, it has been reported that treatment response is dependent on the used NDV strain (2). In this sense, our goal was to determine whether a recombinant attenuated NDV from a genotype V velogenic strain (22,24) possesses hemagglutinating, antitumor and immunostimulatory activity.

Firstly, we demonstrated that rNDV-P05 has hemagglutinating activity, which was partially lost under acid or basic conditions, and totally at higher temperatures than 42˚C. These data gave us an idea for correct and optimum handling of the virus. Our results are similar to those described to LaSota and R2B strains (28), with the exception that rNDV-P05 remains with the same hemagglutination titter even at 42˚C while LaSota and R2B strains reduced one titer (28). That is important since hemagglutinating activity has been demonstrated to play a crucial role in antitumor and immunostimulatory activities (15).

Regarding to antineoplastic activity of NDV, it is already clear that oncolytic strains are mostly velogenic and that lentogenic strains are more immunostimulatory than antiproliferative (2,27). Although rNDV-P05 expresses HN and F proteins from a velogenic strain, it replicates as a lentogenic strain due to a replacement in the cleavage site in F protein from polybasic to monobasic amino acids (24). Keeping this in mind, our next aim characterizing rNDV-P05 was to explore whether the virus had the capability or not to induce apoptosis over a panel of tumor cell lines from a variety of tissues. In this study, we have demonstrated that rNDV-P05 was able to induce apoptosis over four from five tested cell lines, which had previously shown susceptibility to NDV (29-32). In solid tumors, this is important for virotherapy, given that the cell death induced by the virus in the tumor microenvironment can evoke not only local, but also a systemic tumor-specific immune surveillance against primary and metastatic malignant cells. This effect can be mediated through the release of tumor-associated antigens, damage-associated molecular patterns, pathogen-associated molecular patterns and inflammatory cytokines that can help to develop a local and systemic Th1 response (33-35).

Furthermore, it is worthy of mention that due to the fact that neutralizing antibodies (mostly anti-HN and anti-F) may limit repeated delivery of the virus (36) and therefore decrease its efficacy, approaches such as the use of different viral strains or engineered viruses expressing different surface glycoproteins can be considered (27). It has been demonstrated that neutralizing antibodies are strain specific (37). In this sense, the rNDV proposed here could be considered for virotherapy, not only by the fact that belongs to a different genotype of most used NDV strains for virotherapy (22), but also because it has shown to possess promising antineoplastic action against numerous cancer cell lines and to be safe when co-cultured with normal cells (like PBMC) from healthy donors.

Additionally to the antitumor activity of rNDV-P05, we found that the virus has the capability to upregulate the expression of a group of cytokines involved in tumor clearance, such as TNF-α, TRAIL, IFN-α and IFN-γ. TNF-α is a membrane-integrated protein and a soluble cytokine with inflammatory and antitumor activity (38-41). Interestingly, in this study the attenuated rNDV-P05 triggered a significant increase of TNF-α in culture supernatants and an early upregulation at the mRNA level. Previous studies *in vitro* using the lytic NDV strain 73-T showed that human PBMC upregulate the expression of TNF-α in supernatant after 24 h of stimulation (18,42). Other reports have demonstrated the capability of NDV to stimulate the production of this cytokine, although it was in murine macrophages (19,43). In patients with advanced solid tumors, the administration of a single or multiple doses of the lentogenic NDV PV701 induced the rejection of the tumors with an increased amount in serum of pro-inflammatory cytokines, such as TNF-α (44). Thus, TNF-α overexpression might be involved in the antineoplastic activity of NDV-stimulated PBMC.

As a member of the apoptosis-inducing TNF family, TRAIL has shown its ability to kill a broad range of malignant cells *in vitro* and *in vivo* (45,46). Particularly for NDV, it has been demonstrated the overexpression of TRAIL in membrane of human PBMC stimulated with NDV Ulster, especially in monocytes and T cell populations (15). In another study, NDV Ulster-activated human monocytes enhanced their cytotoxic activity over different cell lines, which was TRAIL-dependent and similar to that for IFN-α-activated monocytes (47). More recently, the NDV strain 7793 was used to active murine NK cells. NDV 7793-activated NK cells overexpressed TRAIL in membrane and in supernatants respect to untreated cells, and the effect was similar to that of IFN-γ-activated NK cells (48). According to our study, human PBMC stimulated with the rNDV increased significantly the levels of TRAIL secreted into the culture supernatants, as well as the mRNA expression. TRAIL expression in LPS-stimulated cells was only significant when it was measured by qPCR; this result could be explained by the fact that the mRNA levels might reflect the total of TRAIL molecules (membrane-attached and soluble) and TRAIL in culture supernatants might reflect only the secreted forms of TRAIL. Due to the fact that we detected a prompt upregulation of TRAIL mRNA in response to rNDV incubation, we can propose that as an early event the increase in TRAIL expression is mostly dependent on direct viral stimulation, as previously described (49,50). However, it is known that TRAIL can be upregulated also by IFN-α/β (51,52) and IFN-γ (52,53). Interestingly, we detected high concentrations of IFN-α/γ in response to rNDV stimulation; thus, we cannot exclude that part of soluble TRAIL measured in PBMC cultures comes from populations of monocytes and/or T-cells stimulated with type I IFNs, as it has been already reported (20,47).

Kinetic expression of IFN-α by PBMC in response to NDV has been previously evaluated (54). In accordance with that study, we found that stimulation of human PBMC with rNDV, but not with LPS, induced an upregulation of *IFN-α* gene compared to control. At protein level, we also found an augmentation of IFN-α in the culture supernatants from rNDV-stimulated cells compared to control. An increment in IFN-α secretion in response to the lytic NDV 73-T and Ulster strains has been previously reported (15,18). However, in other study using NDV Ulster strain the production of

IFN-α was not detected neither in populations of monocytes nor plasmacytoid dendritic cells (DCs) obtained from human PBMC; only a recombinant version of the virus that expressed granulocyte-macrophage colony-stimulating factor was able to increase significantly IFN-α levels (20). In this regard, rNDV-P05 was able to induce IFN-α secretion at the level reported to NDV Ulster (20) and without the requirement to express a foreign protein to trigger it. Increased levels of this cytokine have also been detected in serum from patients treated with NDV (44). The fast production of IFN-α is the first-line defense mechanism after viral contact with innate immune system, as it possesses antiproliferative properties (55-57), remarking the importance of its production during virotherapy.

According with early reports, the role that IFN-γ plays in tumor rejection during NDV therapy is not clear. For instance, stimulation of human PBMC with NDV 73-T for 24 h did not enhance the production of this cytokine, at least not at detectable levels (18). A different approach using MCF-7 mammary cancer cells infected with NDV Ulster as a tumor vaccine was not able to induce the secretion of IFN-γ in human T-cells after five days of co-culture. Only a recombinant version of the virus expressing interleukin (IL)-2 enhanced the concentration of IFN-γ in supernatant, when compared to control groups (21). Recently, employing a genotype VIII velogenic strain (AF2240) over human PBMC, it was demonstrated the capacity of this strain to stimulate IFN-γ production (17). Here, we found that PBMC (except from donor 4) respond to rNDV-P05 and LPS with an upregulation in IFN-γ secretion. An increase at mRNA level was also observed. Our results highlight that although rNDV-P05 is recombinantly attenuated, it triggers a similar effect when compared with a velogenic strain (17), without needing the expression of foreign cytokines, such as IL-2 (21). The upregulation in IFN-γ induced by rNDV on human PBMC could be beneficial *in vivo*, due to IFN-γ is involved in activating anticancer immunity (T cells, NK cells and DCs), in inhibiting the activity of immune-suppressive cells (regulatory T cells and myeloid-derived suppressor cells), as well as in the conversion of tumor-associated macrophages (58,59).

In conclusion, taking together our and previous reports, there is a heterologous response of tumor and immune cells after NDV stimulation and it is noteworthy that the genotype of the strain applied for virotherapy seems to be, at least in part, the reason for this effect. This study shows for the first time that a genotype V NDV possesses antitumor and immunostimulatory activities. Due to rNDV-P05 performed a promising antitumor activity and was able to stimulate the expression of four key antitumor cytokines, the present study opens the path for its use in an alternate application of different viral strains during virotherapy.

Acknowledgements

This study was supported by the National Council of Science and Technology of Mexico (grant no. 207977). Oscar Antonio Ortega-Rivera has a doctoral fellowship from the National Council of Science and Technology of Mexico. The authors wish to thank Dr Carlos Olvera Sandoval and Ms Pamela Gallegos Alcalá for excellent technical assistance and Dr Daniel Cervantes García for his support with PCR analysis.

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