Host mediated anti-tumor effect of oncolytic Newcastle disease virus after locoregional application

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Abstract. Several strains of the Newcastle disease virus (NDV) have raised considerable interest in recent years for clinical application because of their oncolytic properties. In this study we characterized virological, immunological and anti-tumor properties of some NDV strains. The oncolytic strain MTH-68/H was the most potent interferon-α inducer and, after UV light inactivation, it was the only tested NDV strain which induced in human PBMC anti-tumor activity in vitro. Upon systemic application to mice bearing a virus susceptible intradermal tumor, no significant anti-tumor effects were observed with the two oncolytic strains Italian and MTH-68/H while the treatment had significant side effects as seen by loss of body weight. In contrast, when using a locoregional application model for treatment of liver metastases of luciferase transfected CT26 colon carcinoma cells, MTH-68/H showed a significant delay in tumor growth, as well as prolonged survival but no effects on body weight. Surprisingly, this CT26 murine tumor cell transfectant was resistant in vitro to virus infection and oncolysis. These results suggest: i) that locoregional application of oncolytic NDV is more effective than systemic i.v. application; and ii) that oncolytic NDV can mediate effects even against a virus-resistant tumor line. The involvement of host anti-tumor immune responses as an important mechanism in therapies based on oncolytic NDV will be discussed.

Introduction

Oncolytic viruses have raised considerable interest as vectors for treatment of human tumors in recent years (1,2). One of these viruses, the avian paramyxovirus NDV has been reported to have anti-neoplastic (3) as well as immune stimulating properties (4,5). Depending on their virulence, different NDV strains are classified as either lentogenic, mesogenic or velogenic but they can not be distinguished by serotypes (6). An important factor that influences the virulence is determined by the cleavage site of the F-gene (7). Some virulent NDV strains are also oncolytic (8-10) and attenuated strains thereof have been applied to cancer patients. While all strains of NDV appear to show tumor selectivity of replication in mouse and human cells (11,12), lentogenic strains show a monocyclic replication behaviour while the more virulent strains show multicyclic replication (13). We used the lentogenic strain Ulster to infect human tumor cells for preparation of the autologous virus modified tumor vaccine ATV-NDV (14). Postoperative vaccination of cancer patients with this vaccine had beneficial effects on long-term survival (15). This was true even for very aggressive tumors refractory to standard treatments, such as glioblastoma and head and neck squamous cell carcinoma (16,17).

Much interest has recently been raised in the NDV variant MTH-68/H that was generated by several passages in chicken embryos of the original Hertfordshire strain of NDV designated Herz33 (18). It was reported to have oncolytic capacity and in vivo anti-tumor activity (19). A recent in vitro study provided evidence that MTH-68/H kills tumor cells by inducing endoplasmic reticulum stress leading to p53 independent apoptotic cell death (20). In view of our positive results with the ATV-NDV vaccine and the known immune stimulatory properties of NDV (21,22), the question arose whether the antitumor effects of oncolytic NDV strains are entirely due to direct antitumor effects of the virus as suggested (20) or may also involve host-mediated anti-tumor mechanisms.

To investigate this question we have performed a comparative analysis of various NDV strains and tested in vitro and in vivo direct and host mediated anti-tumor effects. We will show that the oncolytic strain MTH-68/H induces the strongest interferon-α response in human PBMC. Host mediated anti-tumor effects were tested with a mouse tumor cell line that was resistant to the oncolytic effects of this virus. Our findings suggest that oncolytic NDV strains can induce strong host mediated anti-tumor activity.

Materials and methods

Cells and viruses. Q5M cells (kindly provided by Dr P. Antin, University of Arizona, AZ) were propagated in medium 199 supplemented with 10% fetal calf serum (FCS) and 10%
After a 6-day incubation in 5% CO2 atmosphere at 37˚C the cells were serially diluted in culture medium and added to the wells. When they reached confluency, NDV strains were diluted into 96-well plates at a concentration of 50,000 Vero cells were seeded at a concentration leading to visible agglutination of sheep red blood cells. Aliquots were cryopreserved at -70˚C. Quantification was performed in a multicyclic fashion in tumor cells, was kindly provided by Dr B. Peeters (Animal Sciences Group, Wageningen University and Research Centre, Division of Infectious Diseases, Lelystad, The Netherlands). Peripheral blood mononuclear cells (PBMC) were purified from buffy coats of healthy donors via Ficoll centrifugation.

NDV Ulster was obtained in 1984 from Dr P.H. Russel (University London, UK). NDV Italian was obtained in 1986 from Dr H.D. Klenk (University Giessen, Germany). Recombinant NDV L-AEGFP (23) that was generated from a cDNA clone of NDV LaSota by inserting the EGFP gene as reporter gene and by modifying the sequence of the cleavage site of the F protein so that the virus could replicate as reporter gene and by modifying the sequence of the cleavage site (27). For these 2 primers, 25 μl reaction mixture for the RRT-PCR contained 19 μl master mix (qPCR Core Kit, Eurogentec, Liege, Belgium), 300 nM of each primer for the M gene (sense 5'-TGG CAG CAT TCT GGT TGG CT-3', antisense 5'-AGT GAT CCA CTA TGA TGC-3') and 100 nM probe for the M gene [5'-FAM-TTC TCT AGC AGT GGG ACA GCC GCG (TMARA)-3'] (as described in ref. 26). The samples were dilutions of viral cDNA or of a plasmid containing the M gene, named PCI-Neo-M (kindly provided by Dr B. Peeters). A common threshold was set during the exponential amplification period to determine a specific threshold cycle (Ct) for each sample. By correlating the Ct values with the HU titer initially used for RNA-extraction on the one hand and with the copy number of the M-plasmid on the other hand, M gene copy number per HU could be determined.

For melting curve analysis of the F gene a pair of unlabeled primers (sense 5'-AAC AGG ACA CTG ACC ACT-3', antisense 5'-TGG CAG CAT TCT GGT TGG CT-3') was used to amplify a 196 bp fragment, which contains the region encoding the F cleavage site (27). For these 2 primers, 25 μl reaction mixture for the RRT-PCR contained 19 μl master mix (qPCR Core kit for SYBR® green I, Eurogentec) and 300 nM of each primer according to the manufacturer's recommendation. The GeneAmp software automatically calculates the melting temperature peak (Tm) out of the melting curve.

NDV binding and infection of tumor cells. NDV was added to cells in FCS-free medium and incubated for 1 h in a final volume of 1 ml at 37˚C in a CO2 incubator. During incubation time the cells were stirred each 15 min. Afterwards the cells were washed twice and used for further experiments.

Flow cytometry. Antibodies were diluted in FACS buffer (PBS containing 5% FCS and 0.1% NaN3). Cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min. Subsequently the cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min. Subsequently the cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min. Subsequently the cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min. Subsequently the cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min. Subsequently the cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min. Subsequently the cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min. Subsequently the cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min. Subsequently the cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min. Subsequently the cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min. Subsequently the cells were washed twice with FACS buffer and then incubated with mouse anti-HN mAb (IgG2a) (kindly provided by Dr R. Iorio, Department of Molecular Genetics and Microbiology, University of Massachusetts, Medical School Massachusetts, MA) for 30 min. Subsequently the cells were washed and incubated with goat F(ab')2 anti-mouse Ig-RPE (Southern MA) for 30 min.
from yellow to brown, reaction was stopped by addition of 20 μl 10% SDS and the plates were analyzed in an ELISA reader (Perkin-Elmer Wallac, Freiburg, Germany) at 490 nm. Percent tumor growth inhibition (TGI) for each sample was calculated from the absorbance values (A_{490}) according to 

\[ \text{TGI} = \left(1 - \frac{(A_{490_{\text{sample}}} - A_{490_{\text{background}}})}{(A_{490_{\text{negative control}}} - A_{490_{\text{background}}})}\right) \times 100 \]

Interferon ELISA. PBMC were put into 96-well plates at a concentration of 1x10^5 cells per well and different NDV solutions were added. After 48-h incubation at 37°C, the supernatants were removed, freed from cells by centrifugation and ELISA for interferon-α was performed with an interferon-α module set (Bender MedSystems, Vienna, Austria) as recommended by the manufacturer. Reactions were carried out in duplicates.

Tumor neutralisation assay. PBMC were infected with different doses of UV-inactivated NDV strains and incubated in 6-well plates for 3 days containing DMEM supplemented with 5% FCS. Afterwards the PBMC were put as triplicates in 48-well plates on a MCF7-monolayer of 2.8x10^4 per well at 3.8x10^5 PBMC per well. After incubation at 37°C for 4 days these plates were developed with MTS and TGI was determined as described for the MTS-assay.

Animal experiments. All animals were purchased from Charles River WIMA, Sulzfeld, Germany. For systemic treatment, 6-week-old DBA/2 mice were irradiated with 4.5 Gray 1 day before the inoculation before the intradermal inoculation of 5x10^6 Eb-M7 syngeneic tumor cells. After desensitisation with i.p. injection of 500 HU NDV Ulster on day 1, the animals received 3 i.v. injections of different NDV strains. Each day, body weight and tumor growth was monitored. Tumor volume was calculated according to 

\[ V = \frac{4}{3} \pi d_1 d_2 \]

For locoregional therapy we used a subcutaneous split-spleen reservoir model for multiple portal venous injections (28). On day 1, 8-week-old BALB/c mice underwent surgery under anesthesia with i.p. injection of 150 μl solution containing 600 μg xylazine (Rompun®, Bayer, Leverkusen, Germany) and 1250 μg ketamine (Ketanest®, S. Pfizer, Karlsruhe, Germany) in PBS. The mouse was placed on its right flank, the left flank was shaven and a small cutaneous incision was made. After preparation of a small subcutaneous pocket and incision of the peritoneum, the spleen was carefully mobilized on its vascular pedicle and ligated between the two splenic vascular bundles. Cells (5x10^5) CT26-luc were injected into the upper pole. After 10 min the vascular bundle of the upper pole was ligated and the hemi-spleen was removed. The lower pole was transposed subcutaneously while preserving its vascular bundle, and held in place with a suture closing the peritoneum, followed by a suture closing the skin. All ligatures and sutures were performed with Prolene 5-0 (Ethicon, Norderstedt, Germany). Every 3 days beginning with day 1 the mice were weighed and tumor load was determined by in vivo bioluminescence imaging on an IVIS® Imaging System 100 (Xenogen Corporation, Hopkinton, USA). Therefore, mice were anesthetized with 400 μg xylazine and 1000 μg ketamine, received an i.p. injection of 4.5 mg luciferine and were measured with high resolution binning. A ROI was set on the upper abdomen covering the liver.

Serum levels of GPT and GPT liver enzymes and interferon after regional NDV application. BALB/c mice were anesthetized with 600 μg xylazine and 1250 μg ketamine and received a single intrasplenic injection of 1,000 HU NDV MTH-68/H or PBS. Control mice received an i.v. injection of the same virus dose or PBS. After 12 h mice were sacrificed by heart puncture under anesthesia. Serum levels of glutamate oxalacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were determined by LaboKlin, Bad Kissingen, Germany. Interferon bioactivity was kindly determined by Dr R. Zawatzky (German Cancer Research Center, Heidelberg, Germany) with a VSV infection inhibition assay.

Results

Virological characterization of different NDV strains. In order to determine the number of infective particles, we performed a serial dilution endpoint assay on a Vero cell monolayer. The cells were infected with NDV of the strains MTH-68/H, Italian, NDFLtag-EGFP and Ulster for 6 days and then stained with crystal violet (Fig. 1A). The 2 oncolytic strains MTH-68/H and Italian had similar lytic capacity whereas NDFLtag-EGFP lysed the cells only at a much higher concentration. No lysis was seen with the non-oncolytic strain Ulster. Table I lists the tissue culture infectivity dose of 50% (TCID50) for 1,000 HU as determined by the formula of Reed and Munch (25).

To look at the F protein cleavage site of MTH-68/H, of which no sequence is published yet, we analysed the F cDNAs of NDV strains.

**In vitro anti-tumoral activity.** To compare the anti-tumoral activity of the NDV strains in vitro, we added serial dilutions of NDV MTH-68/H, Italian and Ulster to monolayers of human HeLa cervix and MCF7 breast carcinoma cells, to murine Eb-M7 and ESb-MP lymphoma cell lines and to the luciferase gene transfected murine colon carcinoma CT26-luc. For comparison we used avian QM5 cells. After 48 h, MTS was added and tumor growth inhibition (TGI) determined as a percentage of living cells after addition of virus compared to living cells without virus (Fig. 2A). The oncolytic behaviour of MTH-68/H and Italian was very similar. The TGI_{50} was at 2-3 logs lower for Italian than for the non-oncolytic strains Ulster and NDFLtag-EGFP (Table I). Additionally we quantified the M genes by RT-PCR. Here all viruses showed very similar copy numbers ranging from 450,000 to 800,000 per HU (Table I).

In vitro anti-tumoral activity. To compare the anti-tumoral activity of the NDV strains in vitro, we added serial dilutions of NDV MTH-68/H, Italian and Ulster to monolayers of human HeLa cervix and MCF7 breast carcinoma cells, to murine Eb-M7 and ESb-MP lymphoma cell lines and to the luciferase gene transfected murine colon carcinoma CT26-luc. For comparison we used avian QM5 cells. After 48 h, MTS was added and tumor growth inhibition (TGI) determined as a percentage of living cells after addition of virus compared to living cells without virus (Fig. 2A). The oncolytic behaviour of MTH-68/H and Italian was very similar. The TGI_{50} was at the dilutions 1:1,000 to 1:10,000 for HeLa, 1:1 for MCF7 and 1:100 to 1:1,000 for Eb-M7. No TGI_{50} could be determined for the murine tumor lines ESb-MP and CT26-luc. NDV Ulster showed much lower TGI values. For Eb-M7 the TGI_{50} of Ulster was at 2-3 logs lower dilutions than for the two oncolytic strains.
by staining the cells with anti-HN and performing FACS at different doses (Fig. 2B). After 1 h we tested virus adsorption and entry into the cells. QM5 and HeLa cells were infected with both viruses at an infection dose of 10 HU per 10^7 cells. With UV-inactivated virus, the interferon-α response of PBMC was greatly reduced.

To determine if the interferon response might correlate with an anti-tumor effect, we performed tumor neutralisation (TNA) assays. For this purpose NDV-infected irradiated MCF7 vaccine cells were coincubated with PBMC on a MCF7 monolayer. After 6 days, viable cell staining with MTS was performed and tumor growth inhibition (TGI) determined. When using live virus of strains Italian and MTH-68/H for vaccine production a high anti-tumor effect with TGI up to 90% was observed irrespective of the presence of PBMC (data not shown). To exclude direct oncolytic effects of the virus in the TNA, we performed further experiments with UV-inactivated virus. After 4 days of co-incubation of PBMC with virus (UV) on top of MCF7 monolayers, the % TGI was determined. MTH-68/H (UV) treated PBMC (1,000 and 100 HU per 10^7 cells) showed a significant elevation of the % TGI compared to PBMC without virus. Italian (UV) and Ulster (UV) did not induce a significant TGI effect in vitro.

**Anti-tumor effects after systemic i.v. virus application.** Subsequently we evaluated the anti-tumor effects of systemically applied oncolytic NDV strains. Irradiated 6-week-old DBA/2 mice were inoculated with 5x10^6 syngeneic Eb-M7 tumor cells intradermally at day 0. After desensitisation by intraperitoneal injection of 500 HU NDV Ulster (day 1), treatment was performed on day 2-4 with three i.v. injections of 1,000 HU MTH-68/H or Italian. Body weight and tumor volume were monitored each day. On days 4 to 12 there were several significant body weight drops in the MTH-68/H group and only one drop in the Italian group (Fig. 4A). During this period also a slight delay of tumor growth could be observed in the MTH-68/H group, but this difference was not significant (Fig. 4B).

**Anti-tumor effects after locoregional virus application.** In order to evaluate anti-tumor effects on liver metastases by locoregional application, we used a subcutaneous split-spleen reservoir model (28,29) and combined it with in vivo bioluminescence imaging (30). The experiments were performed in BALB/c mice with the syngeneic tumor line.
Figure 2. *In vitro* anti-tumor activity of different NDV strains. (A) The cell lines indicated (50,000 cells per well) were transferred into 96-well plates and the serially diluted viruses were added in triplicates starting at a dose of 5 HU per well. After 48 h, tumor growth inhibition was determined by development with MTS. For calculation of % tumor growth inhibition (TGI), the negative control was set to zero. (B) QM5 and HeLa cells were infected with the indicated NDV strains. After 1 and 24 h FACS-analysis for HN was performed, in order to test for cells surface absorbed (1 h) and for virus replication (24 h). (B) QM5 and HeLa cells were infected with the indicated NDV strains. After 1 and 24 h FACS-analysis for HN was performed, in order to test for cells surface adsorbed virus (1 h) and for virus replication (24 h). Black bars, *MTH-68/H*; grey bars, *Italian*; white bars, *Ulster*. 
CT26-luc. Fig. 5A illustrates the treatment protocol. First, tumor cells are inoculated through a separated part of a spleen through which they can enter the liver. Seven to nine days later, MTH-68/H virus is applied through s.c. transposed hemi-spleen. From day 9 to day 15 there was a reduction in tumor growth as shown by representative images of the treated mice (Fig. 5B). While this delay in tumor growth was significant (Fig. 6A), there were no significant difference in body weights (Fig. 6B). The MTH-68/H treated mice also showed a significantly longer median survival than the control (23.5 vs. 18.0 days) (Fig. 6C).

Resistance of CT26-luc cells to NDV MTH-68/H infection. When the CT26-luc cells were examined for NDV susceptibility no TGI50 effect was achieved in the MTS-assay (Fig. 2A). The FACS analysis for HN expression 1 h after NDV infection revealed a dose-dependent binding behaviour similar to the previously depicted QM5 and HeLa cell lines (Fig. 7A). HN-staining after 24 h, a test for NDV-replication, showed almost no staining for MTH-68/H and Ulster and only very low mean fluorescence for Italian, thus indicating resistance of this line to NDV infection.

Serum levels of liver transaminases and interferon after MTH-68/H administration. In order to evaluate a possible mechanism of the in vivo effects of NDV MTH-68/H, we administered 1,000 HU of MTH-68/H to BALB/c mice intravenously or by intrasplenic injection. After 24 h the mice were anaesthetized and sacrificed by heart puncture. The activity of the liver enzymes glutamate-oxalacetate-transaminase (GOT) and glutamate-pyruvate-transaminase (GPT) as well as interferon bioactivity was determined in the serum (Fig. 7B). Mice treated with intrasplenic injection of MTH-68/H showed a significant elevation of GPT serum activity in comparison to all other groups. The GOT elevation was only significant in comparison to the group treated with PBS intravenously. Interferon bioactivity was significantly elevated in both groups treated with MTH-68/H with higher levels in the intravenously treated mice.

Figure 3. Immune modulatory properties of different NDV strains. (A) Human PBMC were tested with NDV (live) or UV-inactivated NDV [(NDV (UV)] and placed in 96-well plates at 1x10^6 cells per well. After 24 h, the interferon-α concentration in the supernatants was measured by ELISA. When the experiment was repeated with PBMCs from another donor, the same trend was seen although the absolute interferon concentrations were different. (B) Human PBMC were treated with NDV (UV) at different doses and placed in 6-well plates at 5x10^5 cells per well. After 3 days, the PBMC were transferred in triplicates onto MCT7 monolayers at a concentration of 3.8x10^5 cells per well. Tumor growth inhibition was determined after 4 additional days by development with MTS. p<0.05.

Figure 4. Systemic treatment of intradermal tumors with NDV. DBA/2 mice, pre-irradiated at d-1 with 5 Gy, were inoculated with 5x10^6 Eb-M7 tumor cells intradermally at day 0. At day 1 the treatment groups (for each of them, n=9) were desensitized by intraperitoneal injection of 500 HU NDV Ulster. At days 2-4 they received 1,000 HU of NDV Italian or MTH-68/H via tail vein injection. The control mice (n=9) received PBS instead. Body weights with bars indicating standard deviation (A) and tumor volumes with bars indicating standard error of the mean (SEM) (B) were determined.

Figure 5. Immune modulatory properties of different NDV strains. (A) Human PBMC were tested with NDV (live) or UV-inactivated NDV [(NDV (UV)] and placed in 96-well plates at 1x10^6 cells per well. After 24 h, the interferon-α concentration in the supernatants was measured by ELISA. When the experiment was repeated with PBMCs from another donor, the same trend was seen although the absolute interferon concentrations were different. (B) Human PBMC were treated with NDV (UV) at different doses and placed in 6-well plates at 5x10^5 cells per well. After 3 days, the PBMC were transferred in triplicates onto MCT7 monolayers at a concentration of 3.8x10^5 cells per well. Tumor growth inhibition was determined after 4 additional days by development with MTS. p<0.05.
Discussion

RNA viruses have received considerable attention recently as virotherapy agents (31,32) and as oncolytic viral therapeutics (32,34,35). In this study we have investigated anti-tumor effects of an oncolytic RNA virus (NDV) after short-term systemic or locoregional application to tumor-bearing mice. One tumor model, the mouse lymphoma Eb-M7 (13), consists of cells which are sensitive to NDV replication and toxicity while the other tumor model, the luciferase transfected murine colon-carcinoma CT26-luc (36), was resistant to NDV replication and toxicity. The virus was given either into the tail vein for systemic treatment of Eb-M7 tumors or it was given into a subcutaneously transposed spleen segment for locoregional treatment of liver metastases from CT26-luc tumor cells. For locoregional treatment of liver malignancies, injection of viruses into the hepatic artery has been described for rats (29). For mice, however, because of the size difference, only the intraportal route is feasible. We used the subcutaneous split-spleen reservoir model for multiple portal venous injections (28) for three purposes: i) for production of diffuse artificial liver metastases after locoregional tumor cell application into a separated spleen segment; ii) for independent intrasplenic application of virus so that there can be no contact between virus and tumor cells before they enter the liver; and iii) for multiple intrasplenic injections of virus without the need of performing further laparotomies. In order to assess tumor growth continuously, we combined this model with in vivo bioluminescence imaging which has been shown to correlate well with hepatic tumor burden in mice (30). The experiments revealed that there was no significant antitumor effect after i.v. treatment of virus-sensitive Eb-M7 tumors while in the virus resistant CT26-luc tumor model locoregional treatment resulted in a significant retardation of tumor growth and prolongation of survival. The latter result suggests that the oncolytic NDV strain can exert a loco-

Figure 5. Locoregional treatment of liver metastases: tumor growth reduction. (A) Scheme of the treatment protocol. On day 0, BALB/c mice underwent surgery: after anesthesia the spleen was mobilized on its vascular pedicle and ligated. The two hemi-spleens are shown in the upper photograph. 1x10^7 CT26-luc cells were injected into the upper hemi-spleen (white arrowhead). After 10 min, the upper hemi-spleen was removed and the lower hemi-spleen was transposed subcutaneously. On days 7-9 the mice were treated by 3 injections of 1,000 HU MTH-68/H into the subcutaneously transposed hemi-spleen (black arrowheads in lower photograph). (B) In vivo bioluminescence imaging was performed at the days 9-15 for the group of mice treated with PBS and for the other one that corresponds to mice treated with MTH-68/H.
The attenuated, purified \textit{MTH-68/H} strain was reported to have beneficial effects in patients with advanced cancer (19,37) and since this virus exerted direct cytotoxicity \textit{in vitro} against several tumor cell lines (20) it was concluded that direct cytotoxicity and oncolysis is the key factor of anti-tumor activity of this virus strain (20). This assumption, however, is not corroborated by data from \textit{in vivo} tumor models or from clinical studies. The results obtained in this study, where a virus resistant tumor cell line could be successfully treated argues against oncolysis as the main mechanism of \textit{in vivo} activity of this virus.

We analyzed in the serum of virus treated mice the liver enzymes GPT and GOT. Both enzymes were elevated after intrasplenic virus application and the elevation of GPT was significantly higher than that after intravenous virus application. These data demonstrate a locoregional effect of the virus on the target organ. We also analyzed serum levels of murine interferon-\(\alpha\). While there was a slight increase after intrasplenic inoculation, intravenous application resulted in a significantly stronger serum interferon titer. These results demonstrate that \textit{MTH-68/H} induces interferon-\(\alpha\) not only in human PBMC but also in mice. Virulent strains of NDV can be pathogenic in chicken. They can grow in interferon responsive chicken cells because this avian virus encodes an interferon antagonist, the viral (V) protein (38). This viral protein can disrupt interferon-signalling in chicken cells, but not in mammalian cells, by binding to one of the signal transducer and activation of transcription (STAT) proteins (39). We described before that in human cells the NDV induced interferon-\(\alpha\) causes upregulation of MHC molecules on tumor cells (40). This can lead to potentiation of cytolytic T cell activity (41). Furthermore, we showed that NDV can activate in monocytes NF-\(\kappa\)B (42), nitric oxide (NO) production (42) and cell surface expression of TRAIL (43). NDV activated mouse (4) and human (43) macrophages/ monocytes were able to kill tumor cells. Upon adoptive transfer into tumor-bearing mice they transferred anti-tumor activity (4). We also demonstrated before in mouse (45) and human cells (46) that NDV derived HN molecules on antigen-presenting cells confer T cell costimulatory activity and augment their anti-tumor cytotoxic activity. Oncolytic NDV strains produce oncolysates upon tumor cell infection. We have shown that NDV derived oncolysates are processed by dendritic cells which then potently stimulate autologous T cells from cancer patients (47). Together, these findings provide examples of host mechanisms through which also oncolytic NDV could exert anti-tumor activity.

In contrast to these host mediated immune mechanisms, direct oncolytic effects after systemic virus application would require successful targeting of the virus to the tumor. When we examined this in previous studies, we found that some viruses can indeed target the tumors. This, however, is by no means a highly efficient system. A large amount of virus is found in non-target tissues (13). Anti-tumor effects of NDV are usually stronger when the virus is applied locally than when given systemically (48,49).

A Hungarian group studied strain \textit{MTH-68/H} as a treatment for patients with various cancers. They reported on a case series of 4 patients (37) and even a phase II trial (50). It was used for instance for treatment of a 14-year-old boy...
diagnosed with glioblastoma (GBM) that was debulked, irradiated and treated with chemotherapy but recurred over a year later and caused a great decline in functional and neurological status (51). This patient’s recurrence has been treated intravenously with NDV daily since April 1996. Between November 1996 and September 1998 the recurrence showed progressive shrinking. At the last report the patient was tapered off dexamethasone and chemotherapy and received no medication other than NDV treatment. From a phase I/II study of anti-tumor vaccination of 23 GBM patients with the ATV-NDV tumor vaccine we reported on improvements of anti-tumor immunity and overall survival (16). These findings offer much hope for the use of NDV in the treatment of primary brain tumors. Of course, much more research has to be performed regarding the mode of application and mechanism of in vivo action of this virus.

Another lytic NDV strain, named PV701, which is mesogenic, has been applied systemically in 3 phase I clinical studies with late stage cancer patients (see ref. 52 for the results about the last optimizations). In a dose-escalation study, PV701 was well tolerated in doses of at least 3x10⁶ infectious units by the i.v. route and at least 4x10¹² infectious units by the intratumoral route. Efficient distribution of an oncolytic virus to the tumor site seems to be critical (53).

We characterized in this study an oncolytic NDV strain which is capable to selectively destroy tumor cells and which kills them in a way that differs from that of most cytotoxic chemical drugs. While such a replication competent virological agent may become an important adjunct to standard cancer treatment, its optimal mode of application and its in vivo mechanisms of anti-tumor activity need to be better understood.

The role of host immune responses in the efficacy or toxicity of oncolytic virus therapy is poorly defined. To optimize oncolytic effects, it may be of advantage to transiently suppress innate immune responses, for instance by cyclophosphamide (54), but care should be taken that oncolysis stimulates adaptive immunity, setting up an anti-cancer vaccination effect.

We describe here that MTH-68/H upon locoregional application can exert anti-tumor activity even against an oncolysis resistant tumor line. Our interpretation that this virus can exert not only direct but also host immunity mediated effects is supported by the observation that the effects observed in patients (16,51) often develop only slowly and are sometimes long-lasting. This is typical for immune mediated effects as seen in immunotherapy trials (4,5,15).

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References


