Recombinant Newcastle disease virus (NDV/Anh-IL-2) expressing human IL-2 as a potential candidate for suppresses growth of hepatoma therapy

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Abstract
Newcastle disease virus (NDV) have shown oncolytic therapeutic efficacy in preclinical study and are currently approved for clinical trials. NDV Anhinga strain which is a mesogenic strain should be classified as lytic strain and has a therapeutic efficacy in hepatocellular cancer. In this study, we evaluated the capacity of NDV Anhinga strain to elicit immune reaction in vivo and the possibility for using as a vaccine vector for expressing tumor therapeutic factors. Interleukin-2 (IL-2) could boost the immune response against the tumor cells. Therefore, we use NDV Anhinga strain as backbone to construct a recombinant virus (NDV/Anh-IL-2) expressing IL-2. The virus growth curve showed that the production of recombinant NDV/Anh-IL-2 was slightly delayed compared to the wild type. The NDV/Anh-IL-2 strain could express soluble IL-2 and effectively inhibit the growth of hepatocellular carcinoma in vivo. 60 days post-treatment, mice which were completely cured by previous treatment were well protected when rechallenged with the same tumor cell. From the H&E-stained sections, intense infiltration of lymphocyte was observed in the NDV Anhinga strain treated group, especially in NDV/Anh-IL-2 group. The NDV Anhinga strain could not only kill the tumor directly, but could also elicit immune reaction and a potent immunological memory when killing tumor in vivo. In conclusion, the Anhinga strain could be an effective vector for tumor therapy; the recombinant NDV/Anh-IL-2 strain expressing soluble IL-2 is a promising candidate for hepatoma therapy.

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1. Introduction
Oncolytic virus is a promising agent for cancer treatment, employing nature's own agents to identify and destroy malignant cells (1). Newcastle disease virus (NDV) is one of the naturally occurring viruses with inherent oncolytic ability and potential for cancer therapy (2–4). NDV is a single-strand non-segmented negative-sense RNA virus belonging to the Paramyxoviridae family. The genome contains 15186 nucleotides coding for six viral proteins in the order 3'-NP-P-M-F-HN-L-5' separated by non-transcribed

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intergenic (IG) sequences named gene-end (GE), IG and gene-start (GS) (5). NDV causes severe illnesses in avian, but only mild flu-like symptoms in humans (6). The virus specifically replicates in cancer cells rather than in normal cells because of the defective interferon (IFN) signal pathways in cancer cells (2,3,7,8).

Direct mechanism and indirect mechanism mediated NDV-mediated oncolysis (9). The direct oncolytic mechanism is inducing apoptosis through mitochondrial apoptosis pathway which require the viral entry, replication, de novo protein synthesis and activation of caspases (10). The indirect oncolytic mechanism is the immune mediated responses associated with both innate and adaptive immune responses. After infection, NDV induced the expression of immune cytokines (11), and then activated the cytotoxic T-cell, macrophages, NK cells and monocytes for tumor recognizing and tumor killing. But different NDV strains have different effects on tumor therapy and suit for different tumor lineages. Whether the NDV Anhinga strain could elicit a powerful immune response and be a potent vaccine vector is still unknown.

Interleukin-2 (IL-2) has been demonstrated as a powerful drug in clinical tumor therapy over 20 years, which can selectively stimulate human T cells (12). The mature IL-2 consists of 133 amino acids, with a molecular weight of 15.4 kDa. IL-2 are mainly secreted by CD4+ and CD8+ T lymphocytes, it can stimulate proliferation, cytolytic activity and cytokine secretion of T lymphocytes and natural killer cells (13,14).

In the present study, we introduced NDV Anhinga strain as a vaccine vector for expressing IL-2. We evaluated the efficiencies of recombinant virus NDV/Anh-IL-2 for IL-2 expressing and in hepatocellular carcinoma therapy. Our data demonstrated that soluble IL-2 (sIL-2) could be secreted when virus replication. More over the recombinant virus NDV/Anh-IL-2 could significantly enhancing the antitumor capability of NDV in vivo and induces more lymphocyte infiltration than Anhinga wild strain suggesting that NDV Anhinga strain could be used as a vaccine vector and recombinant virus NDV/Anh-IL-2 is a promising candidate for cancer therapy.

2. Materials and methods

2.1. Cell lines and culture

HepG2 tumor cell was obtained from the China Xiehe Medical University, H22 cell was kindly offered by Harbin Pharmaceutical Group Bioengineering Co., LTD. BHK-21 cell was kindly offered by Prof. Karl-Klaus Conzenmann (Max-von-Pettenkofer Institut, Muenchen). Chicken fibroblast cell line DF-1, the human hepatoma cell line HepG2 and the baby hamster kidney cell line BHK-21 were grown in DMEM ( Gibco) with 10% heat-inactivated fetal bovine serum (FBS), 100 µg mL−1 streptomycin, 100 µg mL−1 penicillin. All cell lines were incubated at 37 °C in an atmosphere of 5% CO2.

2.2. Plasmids and virus

The mesogenic NDV Anhinga strain was used to provide a backbone for construction of the recombinant virus. The plasmids pAnh-wt, pTM-N, pTM-P, pTM-L were kept in our lab and have been described by Carlos et al (15,16). To generate the NDV Anhinga strain expressing IL-2 [GenBank: NM_000586.3], the fragment of IL-2 was cloned into BstBI site between the HV and L genes in pAnh-wt. The recombinant plasmid encoding anti-genome of the NDV Anhinga strain and IL-2 gene was named pAnh-IL-2. The recombinant NDV viruses were generated as previously described (17) and sequenced by reverse transcription PCR for fidelity. Recombinant virus NDV/Anh and NDV/Anh-IL-2 which express IL-2 were kept in our lab. The structure of the Recombinant viruses as diagamned in Fig. 1.

2.3. In vitro viral growth in HepG2 cell line

Viral growth was determined in the HepG2 cell line. Cells planted in 24-well plates were infected with recombinant virus at MOI of 10. The supernatants were collected at 24, 48, 72 and 96 h post infection. The viral concentration was measured by end-point dilution on DF-1 cells and calculated as 50% tissue culture infective dose (log10TCID50) per mL.

Fig. 1. Construction of the NDV/Anh-IL-2 virus. This picture displays the genome of recombinant NDV/Anh-IL-2 virus. IL-2 gene fragment was inserted into the BstBI site between HVN and L genes of plasmid pAnh-wt. The gene-start and gene-end sequences were introduced before the ORF of the IL-2 gene by PCR.

2.4. Expression of IL-2 by tumor cells infected with NDV/Anh-IL-2

The expression levels of IL-2 gene in the supernatant of transfected monolayers was measured by means of enzyme immune assay as described by Human IL-2 Quantikine ELISA kit (R&D systems). Cells in six-well plates were infected with recombinant viruses NDV/Anh-IL-2 and NDV/Anh at multiplicities of infection (MOI) of 10. The inoculums were removed 6 h post incubation. The cells were photographed at 24, 48 and 72 h post infection. The OD450 nm of the samples was determined and plotted against a standard curve. The standard curve was generated by serially diluting the stock enzyme IL-2 in dilution buffer supplied by the kit.

2.5. Animal studies

All procedures involving animals followed the guidelines issued by National Institute of Health and the Institutional Animal Care and Use Committee of Northeast Agriculture University. Six-week-old female Kunming mice were housed in a pathogen-free environment and implanted subcutaneously with 5 × 10^7 H22 cells. Tumor volume was measured every other day using a digital caliper in two dimensions. Tumor volume was calculated using the formula: V = 4/3 × π × S²/2 × L/2, where V is the tumor volume, S is the smaller measured diameter and L is the larger diameter (2). When tumor size reached 5–8 mm in diameter (8–10 days), mice were intratumorally injected with 10^7 pfu of NDV/Anh-IL-2 (200 µl) viruses every other day for a total of four injections, phosphate buffered saline (PBS) (200 µl) and 10^7 pfu of NDV/Anh (200 µl) as control. Animals were sacrificed when tumor size reached 18 mm in any dimension or at the termination of the experiment.
2.6. Histopathology examination

Each groups’ Samples of mouse tumor was dissected on day 7 after treatment initiation and fixed in 10% formaldehyde phosphate buffer saline (PBS, pH = 7.4). Then embedded in paraffin, sectioned, stained with hematoxylin/eosin (H&E) (18). Finally were analyzed microscopically.

2.7. Flow cytometry

Cell suspensions from tumors of sacrificed animals were prepared for the presence of CD4⁺ and CD8⁺ cells by fluorescence-activated cell sorting (FACS) analysis. These tumors were dissected and manually dissociated with scissors. Dissociated tissue was then collected and incubated at 37 °C in RPMI 1640. After 15 min of incubation, 120 µl of 0.5 M EDTA were added to the cell homogenates and mixed for 5 min. Cells were then filtered using a cell strainer and stained with CD3⁺, CD4⁺, and CD8⁺ (Miltenyi Biotec) and flow cytometry was done by using Becton Dickinson FACScan flow cytometer.

2.8. Statistical analysis

Statistical analysis was done using one-way analysis of variance (ANOVA) via SPSS 19.0 software (SPSS, Chicago, IL, USA). Data were expressed as means ± standard deviation (SD). p < 0.05 was considered statistically significant.

3. Results

3.1. Generation of the recombinant virus NDV/Anh-IL-2

The IL-2 gene ORF was sub-cloned into Bst B I restriction enzyme site between HN and L genes (Fig. 1). The recombinant virus was generated by cotransfection of the plasmid coding for the virus anti-genome, NP, P and L. Cell fusion could be first observed at 48 h post transfection, which provided evidence of virus production. The viral population was amplified by inoculation of the cell cultures into 10-day-old SPF chicken eggs. Allantoic fluid was collected at 72 h after infection for hemagglutination (HA) and TCID₅₀ tests. The HA titer reached 1:128 and the TCID₅₀ titer was 2 × 10⁸ (data not shown).

3.2. Growth characteristics of the recombinant virus

To analyze the growth characteristic of the recombinant virus NDV/Anh-IL-2 in HepG2 cell line, NDV/Anh strain rescued without foreign gene was used as a control. HepG2 cells in 24-cell plate were infected with recombinant virus at 37 °C in DMEM with 10% fetal calf serum (FCS) in 5% CO₂ incubator. The supernatant was harvested at the time 24, 48, 72, 96 h post infections. The TCID₅₀ was calculated by plating 10-fold dilutions of virus supernatant on DF-1 cells. As shown in (Fig. 2), there was no significant difference between the growth kinetics of the two viruses. The viral yield of NDV/Anh-IL-2 was slightly lower than that of NDV/Anh.

3.3. Expression of IL-2 by tumor cells infected with NDV/Anh-IL-2

Time course of IL-2 expression in HepG2 cells in response to the NDV/Anh-IL-2 infection was further investigated. The IL-2 expression has already shown at 24 h after infection, and it appeared to reach the maximum at 48 h after infection. At 48 h, the expression of IL-2 in NDV/Anh-IL-2 is 19.80 ng mL⁻¹, NDV/Anh and the mock cells were 1.37 ng mL⁻¹ and 1.20 ng mL⁻¹, respectively (p < 0.001). Afterwards, the concentration of IL-2 appeared almost constant after 72 h post infection (Fig. 3). This observation can be important for future applications in clinical, as the foreign gene product induced by the infection with such recombinant NDV will be secreted at an early stage in a large quantity.

3.4. In vivo treatment of hepatoma with NDV/Anh-IL-2

To evaluate the tumor inhibitory efficacy of NDV/Anh-IL-2 in vivo, 10⁷ H22 cells were s.c. implanted in the right inguen. Tumors developed for 8–10 days, when a touchable tumor was formed. When tumors volume reached 50–100 mm³, mice were intratumorally (i.t.) injected with 10³ pfu virus every other day for a total of four times, with PBS and NDV/Anh for control. NDV/Anh-IL-2 virus exhibited obvious inhibitory efficacy compared with PBS and NDV/Anh. In 3 out of 6 mice treated with NDV/Anh-IL-2 underwent complete regression in 2–3 weeks after the first injection. Two way ANOVA analyses revealed that there are no interactions between time and drug treatment. In the fourteenth day, the average volume of PBS group is 3401.25 mm³, the average of NDV/Anh-IL-2 is 1489.97 mm³ and the average of NDV/Anh-IL-2 is 420.677 mm³. The tumor volume growth of both NDV/Anh (p < 0.01) and NDV/Anh-IL-2 (p < 0.01) groups exhibited significant suppression when compared with that of PBS group (Fig. 4). Furthermore, there was significant difference in tumor volume...
growth between NDV/Anh and NDV/Anh-IL-2 treated groups on the fourteenth day (p < 0.05).

More importantly, the three mice which had undergone complete tumor regression exhibited a persistent protection when rechallenged with $10^5$ H22 cell (the same tumor cells they bore before, in opposite inguen, 60 days after complete regression).

3.5. Tumors treated with NDV/Anh-IL-2 showed marked T-cell infiltration in vivo

To illustrate the efficacy of IL-2 recombinant in NDV, H&E stain were used to monitor the lymphocytes infiltration in tumor. Tumors from mice treated with PBS, NDV/Anh and NDV/Anh-IL-2, as previously described, were isolated on day 7 after treatment initiation and stained with H&E. The intense H&E staining pattern of the NDV/Anh-IL-2 treated animals exhibit an increase in tumor-infiltrating lymphocytes compared with NDV/Anh and PBS controls (Fig. 5A). To specifically identify cells within the infiltrate as CD4+ and CD8+ T cell, tumors were removed on day 7th and dissociated into single-cell suspension. These cells were then stained with anti-CD4+ and anti-CD8+ antibody, then analyzed by FACS analysis. The rate of CD4+ infiltrate T cell in PBS, NDV/Anh and NDV/Anh-IL-2 treated group was 0.13%, 2.7% and 4%, respectively. While the rate of CD8+ infiltrate T cell in PBS, NDV/Anh and NDV/Anh-IL-2 treated group was 0.3%, 2.2%, and 5.7%, respectively. Tumors from the animals treated with NDV/Anh enhanced the infiltration rate of both CD4+ and CD8+ T cells obviously; animals treated with NDV/Anh-IL-2 exhibited a better performance than NDV/Anh in hepatoma model. There is significant difference in CD4+ and CD8+ infiltrate T cell number between NDV/Anh group and NDV/Anh-IL-2 treated group (CD4+, p < 0.05; CD8+, p < 0.01) (Fig. 5B and C).

4. Discussion

Cancer remains a major cause of death in humans. However, the present therapeutics has not much improved the survival rate of the patients especially with metastases. In addition to the limitation of resection and side effects of chemo- and radiotherapy, it is necessary to develop new systematic treatments for cancer therapy (19–21).

Newcastle disease virus has been applied in clinical studies of cancer for more than half a century. In the previous studies of utilizing NDV strains as anticancer agent, encouraging results were obtained, because of its tumor selectivity (22). Many tumor cell types exhibit deficient IFN response to viral infection, which is suitable for oncolytic viruses such as NDV replicating selectively in tumor cells but not in normal cells (6). Recent study suggested that the observed oncolytic specificity of NDV in IFN-competent cancer cells might be secondary to defects in apoptotic pathways or overexpression of antiapoptotic proteins, which help to support productive viral infection and multicycle (23). NDV could specifically replicate up to 10,000 times in tumor cells causing only mild side effect in humans (3), making it an attractive candidate of oncolytic agent.

NDV could be classified as lytic strains and non-lytic strains, based on the different oncolytic capabilities. Lytic strain usually includes 73-T (24), MTH-68/H (25) and PV 701 (26) can kill the
tumor cells directly and prevent metastasis by specifically inducing cells fusion to form syncytia (27). Non-lytic strains (e.g., Ulster strain) (27,28) can gradually induce tumor regression by affecting the metabolism of the host cell and enhance the immune response in vivo (27). But different NDV strains have different effects on tumor therapy, for instance, NDV classic 73-T strain, which has an outstanding performance in malignant melanoma therapy, could not damage daudi cells (29,30). So it is significant to discover new effective NDV strain as a vaccine vector for tumor therapy. Our previous study proved that NDV Anhinga strain could be classified as a lytic strain which has an effective oncolytic role on hepatoma (15). But the NDV Anhinga strain has never been used as a vaccine vector for expressing tumor therapeutic factors before. In this study, we proved that NDV Anhinga strain could successfully express tumor therapeutic factor to enhance the antitumor capability.

Numerous reports had shown that NDV is also an immunostimulatory agent, as it can induce antitumor activities of a variety of effector cells, including NK cells, macrophages, and CTL (11,31–33). The immune system could respond to virus-infected cancer cells (or fragments of cancer cells), then better recognition of tumor-specific antigens may occur, and an increased ability to kill uninfected cancer cells may be acquired (9). The immune system would use the same approaches to kill uninfected cancer cells that it uses to kill virus-infected cells. From the H&E-stained sections, the NDV Anhinga strain treated group showed an enhancement of lymphocyte infiltration compared to the PBS group. We can demonstrate that NDV Anhinga strain could boost the immune system and increase the lymphocyte infiltration.

For over 20 years, IL-2 has been widely used in clinical tumor therapy and received a wonderful result. It has been reported that 52 pancreatic carcinoma stage G3 cancer patients were treated with NDV vaccine adding IL-2, followed up experiment demonstrated that 22% were survived for more than 5 years free of tumor, 33% alive for 24, 21 and 18 months separately, while 45% survived for 9–10 months (34). Previous study demonstrated that NDV La Sota strain which is a non-lytic strain recombinanted with IL-2 has a great result in hepatic carcinoma and malignant melanoma therapy (35), which could become a potential candidate for tumor therapy (5).

Adam Vigil et al (4) reported that lytic strain has a better tumor killing capability than non-lytic strain. They proved that lytic strain virus could not only kill the tumor directly, but could also elicit a potent immunological memory in therapeutic process, which has been proved by non-lytic strain virus. They used NDV Hitchner B1 (B1 strain) lentogenic strain as a backbone to construct recombinant virus NDV/F3aa which contain a multibasic cleavage site in the F protein of the virus. Because highly virulent strain of NDV in birds are classified as velogenic, intermediate strains as mesogenic, and nonvirulent strains as lentogenic based on the mean death time in chicken eggs (36). The precursor F0 protein is only fusogenic after it is cleaved into F1 and F2 polypeptides. The different F protein cleavage sequences of NDV strains are recognized by distinct cellular protease (37). The F0 proteins from lentogenic viruses are only cleaved by trypsin-like proteases found in the respiratory and intestinal tracts of birds, whereas the F proteins of velogenic strains can be cleaved by a broad range of protease found in a wide range of tissue.

So after the modification of the F protein, the NDV/F3aa strain has an increased antitumor efficacy compared with the wild-type parental NDV (B1 strain) by increasing the fusion capability of

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**Fig. 5.** Representative sections of H&E-stained mouse tumors. A. Mouse tumors were dissected on day 7 after treatment initiation. Intense infiltration was observed in the NDV/Anh-IL-2 treated tumors compared with both PBS and NDV/Anh treated tumors. B and C, recombinant viruses increased CD4+ and CD8+ T cells infiltration. The tumors from the sacrificed mice dissociated into single-cell suspension. The tumor cells were analyzed for the infiltration of CD4+ and CD8+ by FACS (n = 3 mice per group).
tumor cell membrane (4), which indicated that tumor killing effect of NDV is directly related with its virulence. Therefore, we firstly used the lytic and mesogenic strain Anhinga as backbone to construct the recombinant virus which could secrete human IL-2. From the H&E stain and FACS results, NDV/Anh-II-2 treated animals demonstrated an obvious raise in tumor-infiltrating lymphocytes number compared with NDV/Anh and PBS controls. The results of CD8+ T cell elevation demonstrate that IL-2 had a synergistic action with NDV Anhinga strain. Importantly, the results of CD4+ T cell elevation in the tumor could explain that all of the mice which underwent complete regressions of tumors exhibited a long-lasting protection from tumor. 60 days post-treatment, mice which were completely cured by previous therapy were also well protected when rechallenged with the same tumor cell. All the facts concluded that the increased number of CD4+ and CD8+ T cells in the tumor lead to the enhanced tumor reduction and long lasting tumor memory. We also observed that animals experienced with multiple injections didn’t show any behavioral changes nor any malformations or carcinogenesis signs in their descendants.

5. Conclusion

In our previous study, we have proved the antitumor effect of NDV Anhinga strain in hepatocarcinoma and classified the strain as a lytic strain. In this study, we constructed NDV Anhinga strain recombinant with IL-2 (NDV/Anh-IL-2), which exerts an obvious result in hepatocellular carcinoma treatment in vivo. We demonstrated that NDV Anhinga strain could not only kill tumor directly but also could elicit immune reaction and a potent immunological memory in vivo which could become an effectual vector for tumor treatment. Based on the result of expression of the cytokine human IL-2, NDV/Anh-IL-2 enhanced the antitumor properties by increasing infiltration of lymphocyte in vivo, which could become a potent candidate for clinical cancer therapy especially for hepatocarcinoma. Further studies remain necessary to investigate more suitable tumor lineages for Anhinga strain for clinical application.

Conflict of interest

The authors have no conflicts of interest.

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