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Full paper

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.

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 Conzenlmann (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% CO_2.

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 HN 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 diagramed 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 titration on DF-1 cells and calculated as 50% tissue culture infective dose ($log_{10}TCID_{50}$) per mL.

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 $OD_{450\ nm}$ 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 5×10^5 H22 cells. Tumor volume was measured every other day using a digital caliper in two dimensions. Tumor volume was calculated using the formula: $V = 4/3 \times \pi \times S^2/2 \times L/2$, 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.

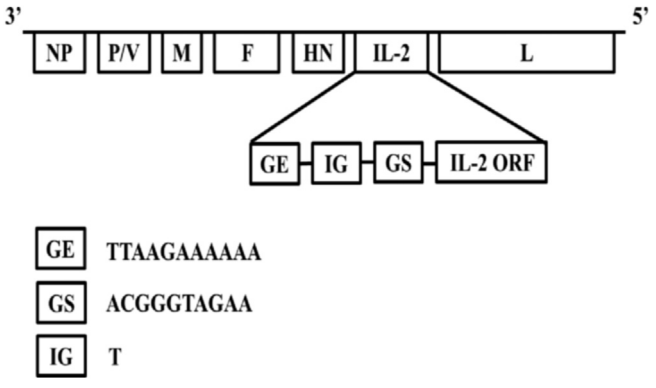

Fig. 1. Construction of the NDV/Anh-IL-2 virus. This picture displays the genome of recombinant NDV/Anh-IL-2 virus. IL-2 gene fragment was inserted into the BstBI site between HN and L genes of plasmid pAnh-wt. The gene-start and gene-end sequences were introduced before the ORF of the IL-2 gene by PCR.

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