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Newcastle disease virus chimeras expressing the Hemagglutinin-Neuraminidase protein of mesogenic strain exhibits an enhanced anti-hepatoma efficacy

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ABSTRACT

Newcastle disease virus (NDV) is an intrinsically tumor-specific virus, many researchers have reported that lentogenic NDV is a safe and effective agent for human cancer therapy. It had been demonstrated that the amino acid sequence of the fusion protein cleavage site is a major factor in the pathogenicity and anti-tumor efficacy of rNDV. However, the role of Hemagglutinin-Neuraminidase (HN) gene that contributes to virulence and anti-tumor efficacy remains undefined. To assess the role of HN gene in virus pathogenicity and anti-tumor efficacy, a reverse genetic system was developed using the lentogenic NDV Clone30 strain to provide backbone for gene exchange. Chimeric virus (rClone30-Anh(HN)) created by exchange of the HN gene of lentogenic strain Clone30 with HN gene of mesogenic strain produce no significant changes in virus pathogenicity as assessed by conducting the mean death time (MDT) and intracerebral pathogenicity index (ICPI) assays. *In vitro*, infection with chimeras could induce the formation of syncytium relative significantly in HepG2 cells. Furthermore, chimeras was shown to induce the cell apoptosis via MTT and Annexin V-PI assays, reduce mitochondrial membrane potential and increase the mRNA transcription level of caspase 3. *In vivo*, ICR mice carrying tumor of hepatoma H22 cells were treated via intratumoral injection of chimeric virus. The treatment of chimera shows an obvious suppression in tumor volume. These results suggest that it could be an ideal approach to enhance the antitumor ability of Newcastle disease virus and highlighted the potential therapeutic application of rClone30-Anh(HN) as a viral vector to deliver foreign genes for treatment of cancers.

1. Introduction

Oncolytic viruses have been used as a potential agent for cancer therapy. Over the past century, researchers have discovered a wide variety of oncolytic viruses, such as mumps, measles, adenovirus, reovirus, Newcastle disease virus (NDV) and others (Lam et al., 2011; Liu et al., 2007). NDV is an avian paramyxovirus, which has shown promising oncolytic activity against human cancers. As a vector for gene therapy, NDV is under clinical evaluation (Zamarin and Palese, 2012). All known strains of the virus are of a single serotype, but three different pathotypes have been described according to standard pathotyping assays. Basing on the mean death time (MDT) in chick embryo, intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI), NDV have been classified into three main pathotypes: velogenic, mesogenic and lentogenic (Alexander, 1998). NDV strains in birds have been classified pathogenically in a relation to their oncolytic properties in cancer cells, in human malignancies. NDV strains have been categorized as either being lytic or non-lytic strains, where velogenic and mesogenic strains were classified as lytic, while lentogenic strains were classified under the non-lytic group (Zamarin and Palese, 2012). Although NDV clone30 strain is lentogenic, it had been approved as an oncolytic virus (Ren et al., 2015).

The HN of NDV is type II intergral membrane protein, which mediates receptor recognition of sialic acid at the end of host-cell surface proteins and possesses neuraminidase activity, necessary

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to prevent virus progeny self-aggregation during budding (Lamb and Parks, 2007). Fusion promotion is a third function of HN protein, which is thought to be mediate by interactions of HN with F protein (Palermo et al., 2007; Porotto et al., 2005). Further studies have been performed to elucidate the molecular determinants for viral pathogenicity (Huang et al., 2003; Huang et al., 2004; Peeters et al., 1999; Wakamatsu et al., 2006). Many researches have concentrated on the role of F and HN proteins in pathogenicity, and consensus has been that the major determinant of virulence correlates with the sequence of the cleavage site of the F protein. These results have been demonstrated that the cleavage site sequence of F protein in lentogenic strain of NDV is mutated to the consensus sequence of mesogenic strain, resulting in significant increases in the standard pathotyping assays results (Peeters et al., 1999; Wakamatsu et al., 2006; de Leeuw et al., 2005). However, results of the research performed to elucidate the contribution of the HN protein to virulence are less conclusive (Huang et al., 2004; Estevez et al., 2007; Wakamatsu et al., 2006).

Hepatocellular carcinoma (HCC) is occupying the third rank among the leading cause of cancer mortality worldwide, with nearly one million new cases reported annually; the currently used conventional treatments include basically surgical removal of the tumor supported by adjunctive therapy like chemotherapy or radiation, but these treatments cannot solve the metastases problem plus having many undesirable side effect (Mor et al., 1998). For these reasons, virotherapy which among many other ways like hyperthermia and immunotherapy, has become strongly suggested to be an alternative treatment with more targeting towards tumor cells and less harm to the normal ones.

In this essay, we constructed and rescued a chimeric virus NDV/rClone30-Anh(HN). The virulent contribution of the HN gene from mesogenic strain in the context of a lentogenic vector backbone was assessed by the standard pathotyping assays. Furthermore, the chimeric virus shows enhanced antitumor efficacy both *in vitro* and *in vivo*. In HepG2 cells infection assay, the chimeric virus shows a marked increase in fusogenic activity by DAPI staining and more efficient apoptosis-inducing ability in tumor cells by MTT, Annexin V-PI and Rhodamine 123 assays compared with parental Clone30 strain. The results of animal experiment shows that chimeric virus treatment could obviously suppresses the volume increasing of H22 transplant tumor in ICR mice.

Our results show that the replacement of HN gene from mesogenic strain does not change the virulence of chimeric virus but enhances the antitumor efficacy of parental strain. The chimeric virus without pathotype change is an optimal agent for hepatoma carcinoma therapy.

2. Material and methods

2.1. Plasmids and viruses

The lentogenic NDV clone30 strain was used to provide a backbone for construction of the chimeric virus. The plasmids pClone30 (containing the whole cDNA of Clone30 genome), pAnh, pBL-NP, pBL-P, pBL-L were kept in our lab (Ren et al., 2015; Wu et al., 2014). The recombinant viruses rClone30 and rClone30-Anh(HN) were grown in embryonated SPF eggs.

2.2. Cell lines and culture

Primary chicken embryo fibroblasts were prepared from 9 to 11 day SPF embryos. DF-1 cells (chicken embryo fibroblast cells) were maintained in DMEM (GIBCO) supplemented with 10% (v/v) FBS and 1% (v/v) pencillin/streptomycin. All cell lines were grown at 37 °C under 5% CO_2 atmosphere. HepG2 cells which are perpetual cell line consisting of human liver carcinoma cells were purchased from China Peking Union Medical College (ATCC No. HB-8065). Murine H22 hepatocarcinoma cell lines which is normally used to build a transplant-tumor mice model were kindly offered by Harbin Pharnaceutical Group Bioengineering Co., LTD. BHK-21 cells were kindly offered by Prof. Karl-Klaus Conzenlmann (Max-von-Pettenkofer Institut, Muenchen). HepG2 and BHK-21 cells were maintained in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin.

2.3. Generation of infectious clone pclone30-Anh(HN)

To replace the HN gene of NDV Clone30 strain by the HN gene from Anhinga strain, HN fragment from pAnh was subcloned into Sfi I and BsiwIsites in pClone30. The recombinant plasmid encoding anti-genome of the NDV clone30 strain and HN gene was named pclone30-Anh(HN). No nucleotide changes were introduced into the non-coding regions flanking these open reading frames.

2.4. Rescue of chimeric virus NDV/rClone30-Anh(HN)

The chimera NDV virus was rescued as described by Fuliang Bai (Bai et al., 2014a). BHK-21 cell monolayers in six-well format were cotransfected with plasmid prClone30-Anh(HN), pBL-NP, pBL-P and pBL-L (1, 0.5, 0.25, 0.1 μg, respectively) by Lipofectamine 2000 reagent, following the instructive procedures of Invitrogen. The six-well plate was frozen at −80 °C and thawed in room temperature for three times after cultivation for 72 h in the incubator. The culture supernatants were harvested and inoculated into the allantoic cavities of 10-day embryonated SPF chicken eggs. After 3 days, the allantoic fluids were harvested for assessment of hemagglutination test. Viruses in HA-positive allantoic fluid were propagated and stored at −80 °C. The rescued virus was identified as rClone30-Anh(HN).

2.5. Virus titration, ICPI and MDT assays

Virus titration were performed by HA test and the 50% tissue infection dose ($TCID_{50}$) test on DF-1 cell monolayers as described by Alexander (1998). Viral titer in cell culture was calculated by the Reed and Muench (1938).

To characterize the chimeric virus and parental virus's pathotypes, two of the standard pathogenicity assays were performed: the intracerebral pathogenicity index (ICPI) and the mean death time (MDT) in embryonated chicken eggs (Alexander, 1998). The ICPI test was performed by inoculation of infective allantoic fluid directly into the brain of 1 day SPF chickens. The EID50 (50% egg infectious dose) and MDT were performed by inoculating serial 10-fold dilutions of the chimeras or parental virus stocks into 9 day embryonated chicken eggs. Calculation of the ICPI, EID_{50} and MDT were performed as previous study (Alexander, 1998).

2.6. Recombinant virus growth curves

Viral growth was determined in the DF-1 cell line. Cells in six-well plates were infected with 1 MOI chimeric virus. After incubation for 1 h, the supernatants were removed and 2 ml DMEM supplemented with 1% antibiotics and 10% allantoic fluid were added. The supernatants were collected at 0 h, 24 h, 48 h, 72 h 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.