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Expression of Newcastle disease virus (NDV) M protein from a recombinant plasmid prolongs the survival of NDV-infected chicken embryos and enhances the virus replication

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Summary. – To explore the role of M protein in the replication of NDV in chicken embryos, the M gene was cloned and inserted into plasmid pcDNA4.0. Western blot analysis showed that the M protein was expressed in DF-1 cells after transfection with M gene plasmid. Chicken embryonated eggs inoculated with the M gene plasmid and 2 days later infected with NDV showed 10 times higher hemagglutination (HA) titers and an increased survival of the embryos as compared with the embryos inoculated with the empty plasmid. These data indicated that the expression of M protein in the NDV-infected chicken embryos primarily prolonged their survival and consequently enhanced virus replication.

Keywords: Newcastle disease virus; M protein; chicken embryo

Introduction

NDV is one of the major pathogens affecting poultry industry across the globe. It is a member of the genus *Avulavirus,* the family *Paramyxoviridae* (Seal *et al.*, 2000a). The NDV genome contains 15,186 nts with six ORFs encoding structural proteins including nucleocapsid protein, phosphoprotein, polymerase protein, M protein, and two transmembrane glycoproteins hemagglutinin-neuraminidase and fusion protein (Faaberg and Peeples, 1988; Seal *et al.*, 2000a,b; Park *et al.*, 2003). M protein of NDV is considered to be relatively conserved among the isolates of paramyxoviruses (Seal, 1995, 2000b; Zanetti *et al*., 2003). It may play a pivotal role in the virion assembly and release as demonstrated by recent findings that NDV M protein is necessary and sufficient for virus-like particles budding and release *in vitro* (Seal *et al*., 2000a; Takimoto and Portner, 2004; Pantua *et al.*, 2006). In addition, M protein of other paramyxoviruses has been proposed to interact with fusion protein and/or hemagglutinin-neuraminidase and nucleocapsid protein (Sanderson *et al*., 1994; Stricker *et al*., 1994; Ali and Nayak, 2000). Moreover, it inhibits protein synthesis of host cells (Peeples *et al*., 1992). However, its exact role in the replication of NDV is not clear.

To explore the role of NDV M protein in the replication of NDV, we performed delivery of M gene plasmid in the embryonated eggs that were consecutively infected with NDV. Interestingly, we found that the delivery of NDV M gene plasmid prolonged embryo survival and facilitated replication of NDV in the liver of chicken embryos.

Materials and Methods

Viruses and cells. NDV F48E9, a velogenic strain, was kindly provided by Dr. Jinhua Liu. NDV Lasota, a mild strain, was maintained in our lab. Both strains were propagated in the allantoic cavity of 9- to 11-day-old SPF embryonated chicken eggs (Meulemans *et al*., 1987). Viral growth in the allantoic fluid of embryonated eggs was measured by

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Abbreviations: HA = hemagglutination; M = matrix; NDV = Newcastle disease virus; p.i. = post infection; p.t. = post transfection

hemagglutination test and HA titres were calculated according to Reed and Muench (Mast *et al*., 2006). Stock virus was stored at -80^{0}C until used. DF-1 cells (provided by Dr. Sheng Cui) were grown in the medium containing 1 part of DMEM and 1 part of F12 medium (Invitrogen) supplemented with 10% fetal bovine serum.

Embryonated chicken eggs. Fertilized eggs of SPF White Leghorn chickens were purchased from Merial Inc. (Beijing). Eggs were incubated at 37^{0}C with a relative humidity of 55%.

Construction of M gene plasmid. NDV M gene was subcloned from plasmid pEGX-6p-1-M originally prepared from NDV, strain Lasota by RT-PCR (provided by Dr. Haixia Liu) and cloned into expression plasmid vectors pcDNA4.0 (Invitrogen). The M gene plasmid construct, pcDNA4.0-M, was confirmed by PCR using specific primers for (5'-TC CCGGATCCTCATGGACTCATCTA-3', 5'-GCCGCGAAT TCCGACTTATTTCTTA-3') and by restriction enzymatic analysis using *Eco*RI and *Bam*HI (TaKaRa), respectively. The sequence of M gene plasmid was confirmed by sequencing analysis (Shanghai Sangon Biological Engineering & Technology and Service Co.). The plasmid DNA was prepared with Maxiprep Kits (Vigorous Biotech Co.) according to manufacturer's instructions.

Transfection of DF-1 cells with plasmids. DF-1 cells were transfected with pcDNA4.0-M or empty pcDNA4.0 plasmid as a negative control, when the cell confluence was around 80% using cationic transfection reagent VigoFect (Vigorus Biotechnology) (Wang *et al*., 2008). The transfected DF-1 cells were collected 48 hrs post transfection (p.t.) and used as an antigen in Western blot analysis.

Inoculation of embryonated chicken eggs with plasmids. 13-day old embryonated eggs (n = 20 in each group) were inoculated via chorioallantoic route with 60 μg of empty plasmid (pcDNA4.0) or M gene plasmid (pcDNA4.0-M) combined with 20 μl of incomplete Freund adjuvant (IFA) and DMSO mixed in 1:2 ratio. After 24 hrs post inoculation (p.i.) dead embryos were removed and the viable ones were inoculated 2 days later with 10 ELD_{50} of NDV F48E9. The infected embryos were checked every 6 hrs for 36–48 hrs p. i. The allantoic fluid and organs of infected embryos were harvested and stored at -80^{0}C till use. The organs were homogenized and centrifuged at 5,000 rpm for 10 mins and the supernatants were collected for measurement of NDV using HA test. The results were analyzed by Mann-Whitney and ANOVA methods, respectively, and the difference between the two groups was considered statistically significant when $p \leq 0.05$ (Wang *et al*., 2008).

Western blot analysis. The transfected DF-1 cells were washed twice with cold PBS and treated with lysis buffer containing 0.05 mol/l Tris, 0.15 mol/l NaCl, 0.01 mol/l EDTA, 1% NP40, and protease inhibitor, pH 8.0, for 20 mins on ice. The cell lysate was centrifuged at 12,000 rpm for 10 mins at 4^{0}C. The supernatant was subjected to PAGE with 10% gel and separated proteins were transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk, the membrane was incubated with anti-hisG monoclonal antibody (Invitrogen) diluted 1:1000 for detecting M-his tag fusion protein or with anti-NDV positive serum diluted 1:50 (National Institute of Veterinary Drug Control) or anti-β-actin monoclonal antibodies (sc-1616) diluted 1:1000 (Santa Cruz Biotechnology). The blots were subsequently incubated with corresponding horseradish peroxidase-labeled secondary antibodies as goat anti-mouse IgG antibody (DingGuo Biotech), rabbit anti-chicken IgG antibody (Xinjingke Biotech), and donkey anti-goat IgG antibody (Santa Cruz). The blots were developed using chemiluminiscence blot detection reagent (Vigorous Biotech).

HA titration. DF-1 cells were transfected with M gene plasmid or empty plasmid for 48 hrs and infected with 0.5 $TCID_{50}$ of NDV strain F49E9 for 30 mins. The medium was then replaced with fresh growth medium containing 1% fetal bovine serum. The cells were harvested when 50% CPE were observed. The infected cells were 3 times frozen and thawed, centrifuged at 5,000 rpm and HA titers of NDV in the supernatant were determined. For the titration of NDV in the chicken embryos, HA assay was used as previously described (Alexander, 1998).

Immunochemical detection of M protein. The 13-day-old embryonated eggs were inoculated with M gene plasmid or empty plasmid. Two days later, liver tissue samples were collected, fixed in 4% paraformaldehyde and embedded in paraffin. The tissue sections (5 μm) were prepared and subjected to immunohistology using anti-NDV polyclonal chicken serum (National Institute of Veterinary Drug Control) and peroxidase-labeled secondary antibodies against chicken IgG (DingGuo Biotech) or anti-hisG monoclonal antibody (Invitrogen). The sections were examined using a light microscope (Nikon eclipse 80i) after a color was developed with chromogenic peroxidase substrate diaminobenzidine (DAB-H_2O_2) (Zheng *et al.*, 2004).

Results

Expression of M protein in transfected DF-1 cells and in chicken embryos

To determine the expression of NDV M gene in eukaryotic cells *in vitro*, DF-1 cells were transfected with M gene plasmid (pcDNA4.0-M) and examined for the presence of M protein by Western blot using anti-NDV serum and also anti-hisG monoclonal antibody. M protein was detected 24–48 hrs p.t. (Fig. 1). In addition, NDV M protein could also be detected in the liver of chicken embryos 48 hrs after delivery of M gene plasmids *in ovo* using immunochemical

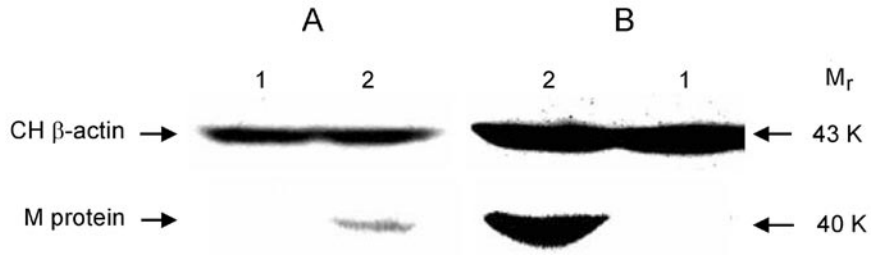

Fig. 1

Expression of M protein from the M gene plasmid in DF-1 cells

Western blot analysis with anti-NDV serum (A) or anti-β-actin monoclonal antibody and anti-hisG monoclonal antibody (B). Transfection with the empty (lanes 1) and M gene plasmid (lanes 2).

examination (data not shown). These results indicated that M gene could be successfully expressed *in vitro* and *in ovo* system as well.

Effects of M protein expression on the infection of DF-1 cells with NDV

DF-1 cells transfected with M gene plasmid and infected with NDV for 45 hrs showed 50% CPE. On the other hand, only 25% of empty plasmid-transfected, NDV-infected DF-1 cells showed CPE (Fig. 2). The average HA titers of NDV in M gene plasmid and empty plasmid-transfected, NDV-infected cells were 5.6 and 9.6, respectively. Hence, there was no significant difference in HA titers between the two groups of transfected cells ($p > 0.05$).

Effects of the M protein expression on the infection of chicken embryos with NDV

Inoculation of the chicken embryos with NDV is one of the major methods for propagation of NDV for the laboratory research, clinical diagnosis, and production of commercial vaccines. To determine the optimal embryonic age for NDV infection, the chicken embryos aged 10, 13, and 16-days were inoculated with 10 ELD_{50} of NDV strain F48E9. The infected embryos were checked every 6 hrs and the dead ones were collected. Consequently, the HA titers in allantoic fluid, brain, liver, and muscles were determined (Fig. 3). The growth of NDV strain F48E9 in the liver reached a maximum in 13-day-old embryos, but slowed down in 16-day-old embryos. The similar patterns of NDV growth were also observed in allantoic fluid and muscles. Therefore, the 13-day-old embryos were used for further experiments.

It was found that the chicken embryos transfected with M gene plasmid were more resistant to NDV infections than those transfected with empty plasmid ($p = 0.0027$) (Fig. 4.). However, all of them died at 36 hrs p.i. On the other hand, HA titers of NDV found in the liver, stomach, lungs, heart, and guts of chicken embryos transfected with M gene plasmids were significantly greater than those transfected with empty plasmid ($p < 0.05$). We did not find any difference in HA titers in the muscle, kidney, and allantoic fluid between the tested

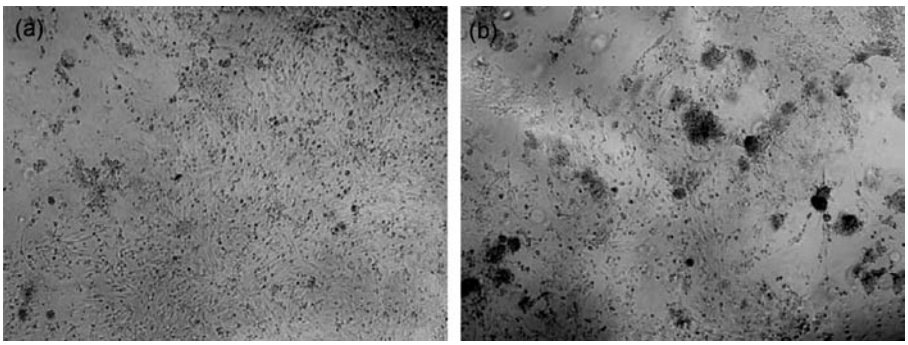

Fig. 2

DF-1 cells transfected with the empty (a) or M gene plasmid (b) and subsequently infected with NDV

Cells were visualized using light microscopy at 45 hrs p.t.

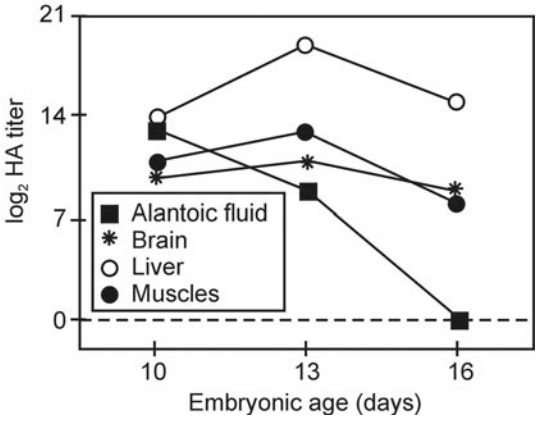

Fig. 3

Replication of NDV in different tissues of chicken embryo

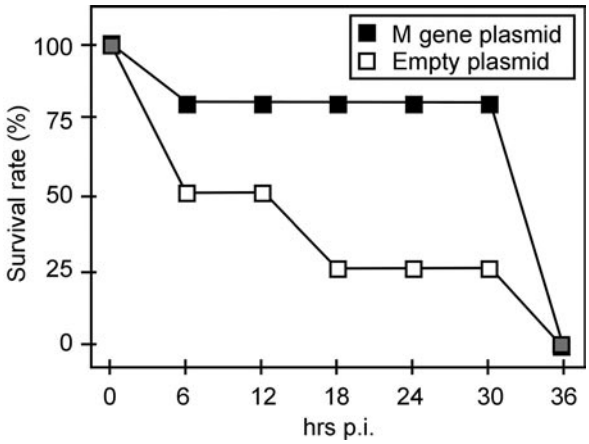

Fig. 4

Survival of chicken embryos transfected with M gene or empty plasmid and infected with NDV

The difference in survival rates between M gene plasmid and empty plasmid transfected groups was statistically significant.

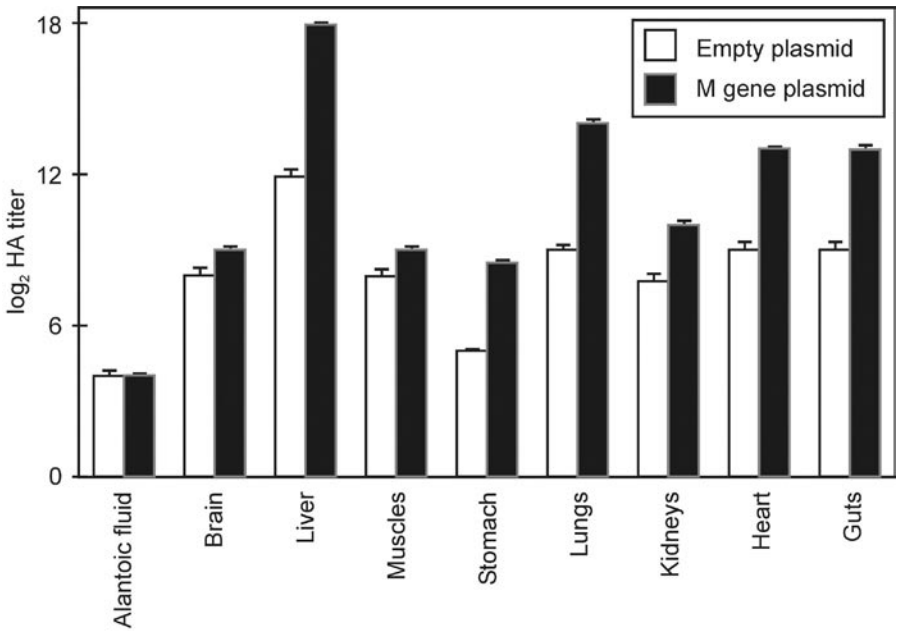

Fig. 5

HA titers in different organs of chicken embryos transfected with M gene or empty plasmids and infected with NDV

The differences of NDV HA titers in the liver, stomach, lungs, heart, and guts of chicken embryos between M gene plasmid and empty plasmid transfected groups were statistically significant.

groups (Fig. 5). These results suggested that *in ovo* delivery of M gene prolonged only partially the survival of NDV-infected chicken embryos, in spite of the fact that virus replication in NDV-infected embryos was enhanced in comparison with empty plasmid transfected NDV-infected embryos.

Discussion

In recent years, the *in ovo* transfection of avian embryos with plasmid vectors has been developed successfully for the expression of exogenous proteins that could be detected

in multiple organs (Oshop *et al*., 2002, 2003; Gomis *et al*., 2004). Using this system, we observed the prolonged survival effect of M gene plasmid transfection in embryos subsequently infected with NDV, which may directly contribute to the enhancement of NDV replication in chicken embryos. The enhanced virus replication was demonstrated by the increased HA titres of NDV in the organs of M gene plasmid-transfected embryos. In addition, another reason may also exist, since M protein could inhibit protein synthesis of host cells, which might suppress the immune response to NDV infection (Peeples *et al*., 1992; Wang *et al*., 2008). Accordingly, the NDV replication was enhanced in M gene plasmid transfected chicken embryos.

Although it sounds possible that the prolonged survival effects of *in ovo* delivery of M gene plasmids-transfected embryos was due to the immunosuppressive effects of M protein, there is also another possibility that *in ovo* delivery of the M gene plasmid may also elicit host response to NDV, which arrests the growth of NDV in embryonated eggs since the host response could be induced in 17-day-old chicken embryos after inoculation with plasmid (Oshop *et al*., 2002, 2003). To avoid a strong host response elicited by M gene plasmids, we used 13-day-old chicken embryos in our experiment. Our result suggested that *in ovo* delivery of plasmid did not induce a strong host response in embryos during earlier developmental stage because inoculation of 13-day old embryos with M gene plasmid dramatically enhanced the NDV HA titers in the liver of embryos ($p < 0.05$). However, several questions remain to be answered. Does M protein interfere directly or indirectly with host response to NDV infection? If yes, what effects of M protein has on key transcription factors in regulation of immune response, such as a NF-κB, activator protein AP-1 and activators of transcription proteins STAT that regulate many aspects of cell growth, survival, and differentiation. Further efforts will be required to investigate the exact role of NDV M protein in the interactions between NDV and the host.

In conclusion, our results demonstrated that *in ovo* delivery of M gene plasmid prolonged the survival of embryos infected with NDV and enhanced virus replication.

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