African Journal of Biotechnology Vol. 11(47), pp. 10675-10681, 12 June, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4103
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Cloning of neuraminidase (NA) gene and identification of its antiviral activity

Yani Zhang, Liwei Ren, Wei Li, Qi Xu, Guobin Chang, Xiaoyan Wang and Bichun Li*

College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, Peoples' Republic of China.

Accepted 1 June, 2012

Neuraminidase not only works as an antigen, inducing target-specific antibodies, but also plays a role of enzyme activity and destroys the sialic acid receptor required by virus infection of the host cell surface which protects the host from virus damage. In order to explore a new idea to use neuraminidase (NA) gene and produce disease-resistant transgenic poultry, prokaryotic expression vector pGEX-NA was constructed to make NA polyclone antibody. Eukaryotic expression vector pcDNA3.0-NA and pcDNA3.0/EGFP-NA was constructed to reveal its subcelluar location by immunofluorescence and enhanced green fluorescent fusion protein (EGFP). Chicken embryonic fibroblast (CEF) cells were transfected with pcDNA3.0-NA and selected by G418 for two weeks, the transfected cells were challenged by Newcastle disease virus (NDV), the morphology of CEF cells were observed to detect the antiviral ability of NA gene. CEF cells were incubated by the cell lysates extracted from the NIH 3T3 cells, which were transfected with pcDNA3.0-NA. The results show that pGEX-NA could express NA protein *in vitro* and NA polyclone antibody worked very well; immunofluorescence and EGFP fusion protein revealed that NA protein located at the cytoplasm near the membrane; NDV-CEF inhibition experiment showed the NA protein could resist and delayed CEF cells from NDV infection.

Key words: Neuraminidase (NA), newcastle disease virus (NDV), antiviral activity, chicken embryonic fibroblast (CEF).

INTRODUCTION

Avian influenza A viruses (AIV) are enveloped, segmented and negative-stranded RNA viruses that cause one of the most serious avian diseases called Bird flu, with severe economic losses to the poultry industry (Christenen et al., 1998). AIV are divided into different subtypes based on its surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA accounts for 90% of enveloped protein, which could induce the animal body to produce the major antigens and antibodies with subtype-specific. NA could not only induce the specific antibody producing, but also stimulate the body to produce cytotoxic T lymphocyte (CTL) response (Ni et al., 2004; Nan et al., 2009); NA protein as a humoral immune target antigens, could induce specific antibodies and inhibit virus to be released from infected cells, thereby reducing the viruses proliferation and increasing the immune protection function (Cong and Liu, 2008). In all, NA not only works as an antigen inducing target-specific antibodies, but also plays a role of enzyme activity and destroys the sialic acid receptor required for virus infection of the host cell surface, which protect the host from virus damage.

So far, there were so many researches focusing on antiviral activity study of NA gene. Johansson et al. (1998) had shown that NA antibodies did not have the ability to neutralize virus infection, but only inhibited the ce specific antibodies; co-expression of NA and HA could protect the chicken from the subtypes HPAIV lethal attacking of H5N1 and H7N1. Based on the previous study, the purpose of this study was to evaluate the role of the antiviral avirus released from the cell surface and infected other cells, thus reducing the propagation of the

*Corresponding author. E-mail: yubcli@yzu.edu.cn.

virus. Zhao et al. (2003) constructed the adenovirus expression vector of NA gene, indicating that recombinant adenovirus could produce a good immune effect; Zhou et al. (2001) also focused on immune effect of neuraminidase gene in mice, in which the results showed that the survival rate of mouse immunized by the NA gene against influenza viruses was 75%, and the survival rate of mouse immunized together by the NA and HA genes was 100%. Chen (2003) showed that HA, NA vaccine could provide mice effectively from flu infection; Co-expression of HA, NA and matrix proteins could produce the best immune protection for animals. Qiao et al. (2003) constructed the recombinant expression fowlpox virus vector of HA-NA gene and studied its immune effects, the results showed that recombinant fowlpox virus in chickens could inductivities of NA protein. pcDNA3.0-NA and pcDNA3.0/EGFP-NA was constructed to reveal its sub-celluar location. The CEF cells were transfected with pcDNA-NA and selected by G418 for two weeks, the transfected cells were challenged with NDV and the antiviral activities of NA were detected.

MATERIALS AND METHODS

Cells, vectors and viruses

NIH 3T3 cells were kept by our laboratory. Primary CEF cells were prepared from 9 to 10 days embryos of SPF chickens and cultured at Dulbecco's modified eagle's medium (Gibco) supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL ampicillin and 100 μg/ml streptomycin. All cultures were maintained in a 5% CO_2 humidified atmosphere at 37°C and passaged every 2 to 3 days. pMD19-T-NA with chicken H5N1 avian influenza virus NA genes was provided by Dr. Wenbo Liu at the College of Veterinary Medicine, Yangzhou University. Eukaryotic expression vector pcDNA3.0-NA was used to express the NA protein under the control of cytomegalovirus (CMV) promoter. Pathogenic NDV F48E8 strain was provided by Dr. Guoqiang Zhu at the College of Veterinary Medicine, Yangzhou University.

Media and reagents

They include: DMEM (Gibco), Ficoll-400 (Pharmacia), FBS and Chicken serum (Gibco), Mitomycin-C (Pharmacia), hSCF (Sigma), mLIF (Sigma), DMSO (Sigma), Glycol (Sigma), Polyethlene glycol (Sigma), bFGF (Sigma), and Hil-11 (Sigma). The others were imported or domestic analytical reagents.

Vector construction

NA gene was amplified from pMD19-T-NA by the forward primer P1 5'-CCGCCGGAATTCATGAATCCAAATCAAAAG-3' (underlined part is *Eco*R I restriction site) and the downstream primer P2 5'-ATTTGCGGCCGCCTACTTGTCAATGGTGAAT-3' (underlined part is *Xho* I restriction site). The primer was synthesized by Sangon Biotech Company (Shanghai, China). The amplification was performed with the thermal profile: initial activation at 94°C for 5 min, 35 cycles of 94°C for 50 s, 60°C for 45 s, 72°C for 1.5 min and 72°C for 10 min. The amplified fragments were separated by electrophoresis on a 0.8% agarose gel, excised and purified with DNA purification kit, and subsequently sequence- verified by Sangon Biotech Company (Shanghai, China). Purified Polymerase chain reaction (PCR) fragments were digested with *Eco*R I and *Xho* I, cloned into the pGEX-6P-1 vector, and named pGEX-NA. The pGEX-NA was transformed into *E. coli* DH5α, and cultured at LB (Amp+) solid medium for 12 to 16 h. Positive clones were picked out and cultured at LB (Amp+) liquid medium for 12 to 16 h, and the plasmid extracted using Plasmid minispin Kit (Dingguo Biotech Company, China). Then, double enzyme digestion was carried out to confirm pGEX-NA. The same method was used to construct eukaryotic expression vector pcDNA3.0-NA and pcDNA3.0/EGFP-NA.

The induced expression of pGEX-NA recombinant plasmid

Ten microliter (10 μl) *E.coli* DH5α bacterium containing the recombinant plasmid pGEX-NA was inoculated to 1 ml LB (Amp+), shaking overnight at 37°C with 160 r/min. One hundred microliter (100 μl) overnight cultures were inoculated to 10 ml LB (Amp+), shaking for 3 h at 37°C with 160 r/min; when the bacteria reached the logarithmic phase (OD600 = 0.6), it was aliquoted with 1 ml bacilli into 1.5 ml tube. There were five groups, with three repeats for each group. Each group was induced by different concentrations of IPTG (0, 0.25, 0.5, 0.75 and 1.0 mmol/L), cultured at 37°C, 160 r/min for 4, 6 and 8 h, respectively. Precipitation was collected by centrifugation and sedimentated for 30 min at -70°C, then rapidly thawed with pre-heated PBS and the precipitation suspended. The suspension was sonicated and centrifuged to collect the supernatant and sediment, 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to confirm the expression of NA protein, respectively.

The preparation of anti-NA serum

The anti-NA serum was produced by the healthy mice which were injected by the linear plasmid pcDNA-NA. The ELISA assay kit was used to detect the antibody titer of anti-NA serum, the OD450 value was measured with a microplate reader.

Western blot analysis of NA recombinant protein

According to the optimal inducing conditions, 0.75 mmol/L IPTG was used to induce the pGEX-NA expression at 37°C for 6 h, protein samples were processed by the sodium dodecyl sulphate-polyacrylamide gel electrophresis (SDS-PAGE) gel electrophoresis and then transferred to Polyvinylidene fluoride (PVDF) membrane. The protein on PVDF membrane was hybridized with anti-NA serum (1:1000), and goat anti-mouse IgG (1:2000) was used as secondary antibody. At last, the target protein NA was colored by DAB.

Cell transfection

NIH-3T3 cells were plated in 24-well dishes, grown into 80 to 90% confluency and transfected with 1 μg/plate plasmid, mediated by 1.5 μg/plate polyethyleneimine (PEI) (Sigma). For the experimental group, NIH-3T3 cells were transfected with pcDNA3.0-NA. For the negative control group, NIH-3T3 cells were transfected with pcDNA3.0. At 48 h post-transfection, the transfected cells were observed under fluorescence inverted microscope.

Immunofluorescence

At 48 h after transfection, transfected cells were fixed with pre-cold methanol for 10 min, washed with PBS, permeabilized for 5 min in

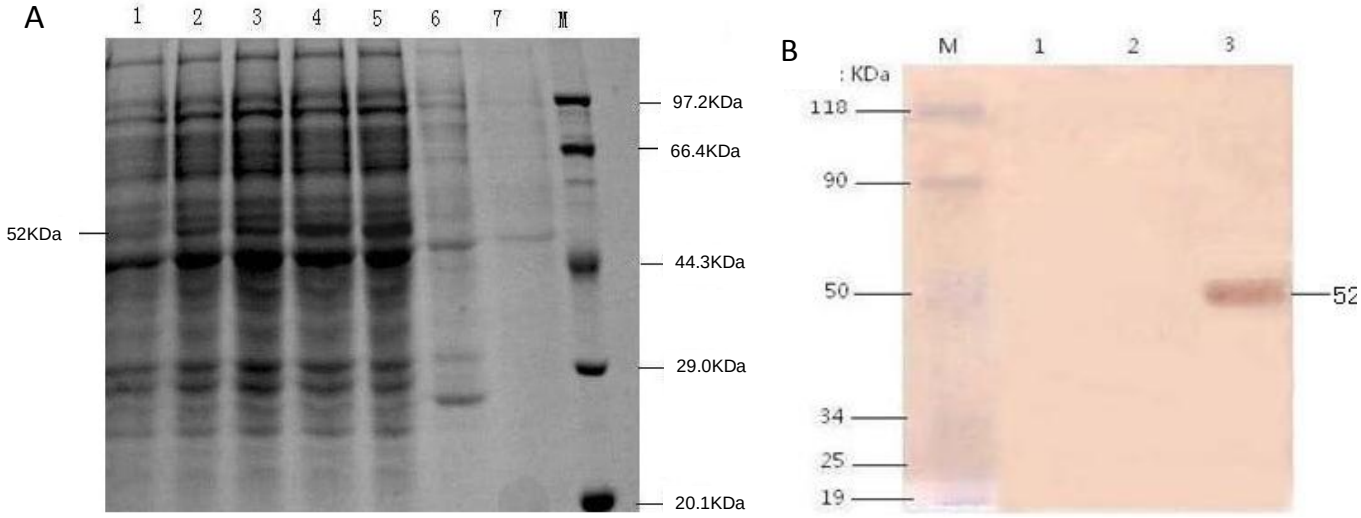

Figure 1. The analysis of pGEX-NA expression product. (A) The results of coomasie blue staining, lane M: protein molecular weight marker; lanes 1 to 5 indicates the IPTG induction expression results of 0, 0.25, 0.5, 0.75, 1.0 mmol/L; lane 6: the IPTG induction expression results of pGEX-6p-1; lane 7: the IPTG induction expression results of *E. coli* DH5α. (B) The results of DAB staining, lane M: molecular weight marker; lane 1: *E. coli* DH5α induced by IPTG; lane 2: pGEX-6p-1 induced IPTG; lane 3: pGEX-NA induced by 0.75 mmol/L IPTG.

1% Triton X-100 and blocked for 60 min in 10% goat serum at room temperature. The primary polyclonal antibody anti-NA serum (1:800) raised against the NA was incubated for 1 h. Secondary antibody fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (GeneTimes Technology, Inc, Shanghai, China) was added at a 1:400 dilution and incubated for 1 h. pcDNA3.0 was used as the negative control. The transfected cells were observed under fluorescence inverted microscope

Reverse transcription polymerase chain reaction (RT-PCR)

After selection with G418 for two weeks, total RNA was extracted from transfected cells using TRIzol Reagent (invitrogen Company Limited) according to the manufacturer's instructions. RT-PCR was performed using PrimeScript® RT reagent kit (Perfect Real Time) (TaKaRa Biotechnology (Dalian) Company Limited) according to the manufacturer's instructions. Briefly, 10 μl reverse transcription was performed for 15 min at 37°C using Oligo dT primer and 1 μg RNA. Twenty-nine (29) cycles of PCR was performed using the forward primer (5'-CCGCCGGAATTCATGAATCCAAATCAAAAG-3') and the reverse primer (5'-ATTTGCGGCCGCCTACTTGTCAATGGTGAAT-3') and the following program: 10 min at 94°C, 90 s at 94°C, 90 s at 55°C, 2 min 30 s at 72°C, followed by 10 min at 72°C. Five microliter (5 μL) RT-PCR products were run on 0.8% agarose gel. pGEM-T-NA was used as the positive control, pcDNA was used as the negative control and β-actin was used as internal control. The amplified fragments were separated by electrophoresis on a 0.8% agarose gel.

The detection of cytopathic effect inhibition

NIH 3T3 cells were transfected with pcDNA-NA, at 48 h post-transfection, the cells were lysed and sonicated, the supernatant was purified, diluted according to 1:2, 1:4, 1:8.1:16, 1:32, 1:64, 1:128 and 1:256, then was added to 96-well plate, growing with monolayer CEF cells, incubated for 2 h, at 37°C, and the supernatant was removed. 100 TCID50 NDV was added to each well (in triplicates). Normal CEF cells transfected with pcDNA3.0 and inoculated NDV was used as positive control; normal CEF cells transfected with pcDNA3.0 and uninoculated NDV was used negative control. Cell cultures were observed under microscope for cytopathic effect (CPE) and 50% CPE inhibition was recorded as described (Qi et al., 2004).

RESULTS

The analysis of pGEX-NA expression product

The supernatant and pellet induced by IPTG for 6 h was harvested for SDS-PAGE analysis. The results showed there was no NA protein expression in the supernatant, and NA protein only expressed in the pellet had the size of 52 kDa; while there was no NA protein expression for the empty vector (pGEX-6p-1) (Figure 1A and Line 6) or *E*.coli DH5$_{\alpha}$ with IPTG induction (Figure 1A and Line 7). Gel image software BandScan5.0 was used to do grayscale analysis and revealed that there was significant difference for different IPTG induction concentrations, especially for the 0.75 mmol/L, which expressed the highest amount (23.7%) of the total bacterial protein, indicating that the recombinant protein was obtained preliminarily (Figure 1A and Line 4).

The detection of pGEX-NA expression product by western blotting

According to the best inducing conditions, the pGEX-NA was induced for 6 h with 0.75 mmol/L IPTG at 37°C,

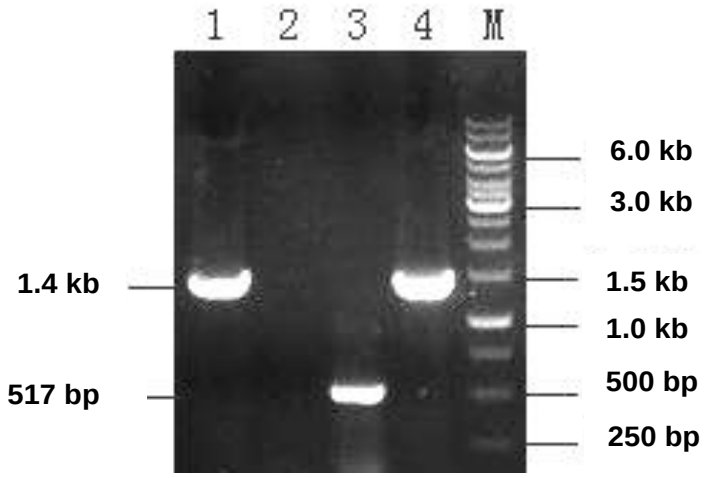

Figure 2. Expression of NA mRNA in NIH 3T3 cells. Lane 1: pGEM-T-NA and used as positive control; lane 2: pcDNA was used as negative control; lane 3: β-actin was used as internal control; lane 3: The NIH 3T3 cells were transfected with pcDNA-NA.

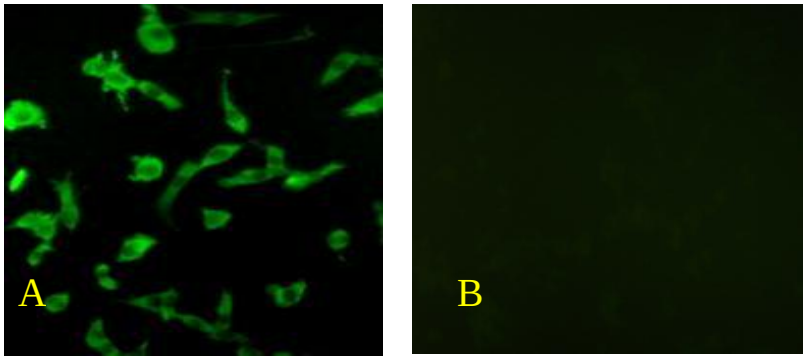

Figure 3. Expression analysis of NA gene in transfected NIH-3T3 cells by IFA (×200). (A) NIH-3T3 cells transfected with pcDNA3.0-NA; (B) NIH-3T3 cells transfected with pcDNA3.0.

SDS-PAGE electrophoresis was performed and then transferred to PVDF membrane. The polyclonal antibody of NA gene was used for Western blot analysis. The results showed that there was NA protein expression after being induced for 6 h (Figure 1B), while there was no NA protein expression for the empty vector or the empty bacterial, it was further confirmed that recombinant NA proteins was expressed.

Expression of NA mRNA in NIH 3T3 cells

To confirm expression of the NA mRNA in eukaryotic cells, the expression vector pcDNA-NA or pGEM-T-NA was transfected into NIH 3T3 cells. After selection with G418 for two weeks, total RNA was extracted for RT-PCR using the primer for amplifying the full-length NA cDNA. As shown in Figure 2, the expected 1.4 kb transcript was amplified from the transfected cells (Figure 2 and Lane 4), but not in the control vector pcDNA3.0 transfected cells (Figure 2 and Lane 2). Meanwhile, GAPDH was amplified (Figure 2 and Lane 3).

The identification of recombination neuraminidase (NA) protein by immunofluorescence

To test whether NA cDNAs could express correct proteins in eukaryotic cells, the NA gene was subcloned into the pcDNA3.0 vector and the expression vectors were transfected into NIH 3T3 cells. By using the antiserum against NA protein, immunofluorescence

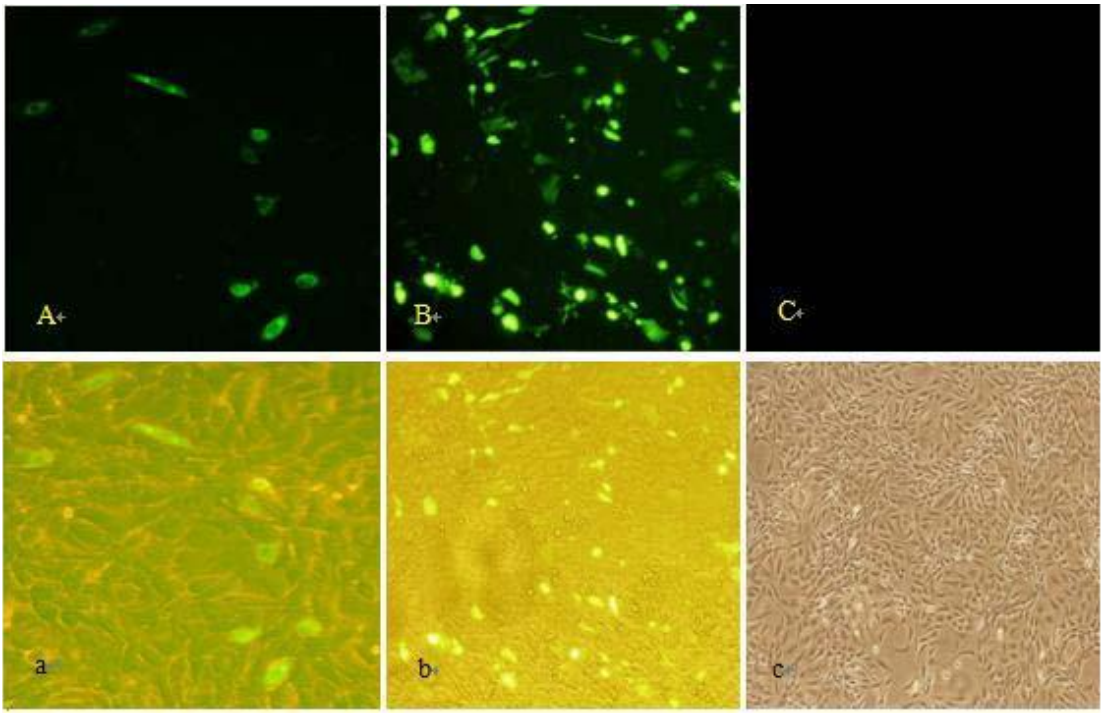

Figure 4. Subcellular location analysis of NA gene (×200). (A) NIH-3T3 cells were transfected with pcDNA3.0/EGFP-NA under bright and dark field; (B) NIH-3T3 cells were transfected with pEGFP-N1 under bright and dark field; (C) NIH-3T3 cells were transfected with pcDNA3.0 under bright and dark field.

Table 1. The antiviral results of NA protein.

Group	Challenged by NDV	CPE50							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
NA protein	100TCID50	-	-	+	+	+	+	+	+
Control	100TCID50	+	+	+	+	+	+	+	+

+: CPE50 positive, -: CPE50 negative.

Revealed typical green fluorescence in the cell cultures transfected with pcDNA-NA (Figure 3A), but not in the cell culture transfected with the control vector pcDNA3.0 (Figure 3B). Meanwhile, it was clear to see that NA protein mainly distributed at cytoplasm near the membrane (Figure 3A).

The sub-cellular localization of neuraminidase (NA) protein

Since there was no endogenous NA protein expression at the NIH 3T3 cells, then NIH 3T3 cells were transfected with pcDNA3.0/EGFP-NA and expressed the fusion protein EGFP-NA. The sub-cellular localization of NA protein could be revealed by the EGFP tag. At 48 h post-transfection, green fluorescence was observed mainly at cytoplasm near the membrane (Figure 4A); combined with the results shown in Figure 4, we got the conclusion that NA protein is located at cytoplasm near the membrane. pEGFP-N1 was used a positive control (Figure 4B), pcDNA3.0 was used a negative contol (Figure 4C).

Antiviral activities of NA protein from NIH 3T3 cells extraction against NDV infection

NIH 3T3 cells were transfected with pcDNA-NA and expressed NA protein, at 48 h post-transfection, the cells were lysed and sonicated, the supernatant was purified, diluted according to 1:2, 1:4, 1:8, 1:16,1:32,1:64,1:128 and 1:256, then it was used to incubate CEF cells for 24 h, respectively followed by challenging with NDV. It can be seen from Tables 1 that 2 and 4-fold dilution could protect CEF cells against NDV, the CEF cells were still closed to grow with fibrous morphology. However, pathological changes appeared at the CEF cells without

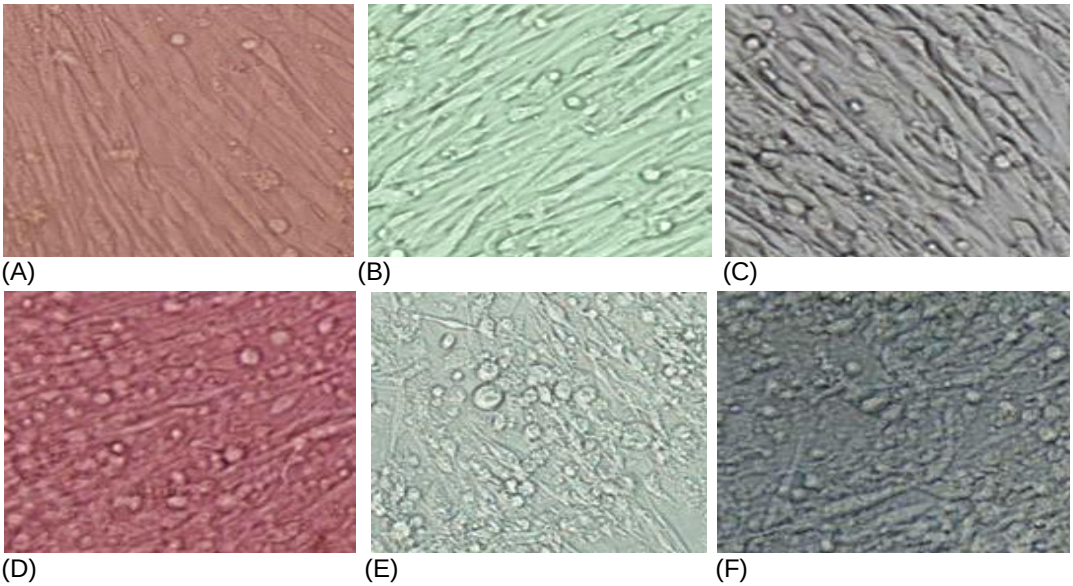

Figure 5. Antiviral Activities of CEF cells transfected with pcDNA-NA against NDV infection (×200). A: Normal CEF cells; B: Normal CEF cells were transfected with pcDNA3.0-NA and infected by NDV at 12 h; C: Normal CEF cells were transfected with pcDNA3.0-NA and infected by NDV at 24 h; D: CEF cells were challenged by NDV directly; E: Normal CEF cells were transfected with pcDNA3.0 and infected by NDV at 12 h; F: Normal CEF cells were transfected with pcDNA3.0 and infected by NDV at 24 h.

incubation by the lysate from the NIH 3T3 cells, the cells became round, dropped and died. This data indicated that CEF cells incubated by the cell lysates from NIH 3T3 could resist and delay NDV infection which were transfected with pcDNA-NA, revealing that NA protein played an important role for this delay.

Antiviral activities of CEF cells transfected with pcDNA-NA against NDV infection

To compare quantitatively the antiviral activities of NA proteins against NDV, the supernatants of the transfected or virus-infected cell cultures were collected at different time points for NDV titration on CEF cells, TCID50 values were $10^{-5.4}$/0.1 ml. To determine the antiviral activities of NA proteins, the expression vector pcDNA-NA, as well as the control vector pcDNA3.0 was transfected into CEF cells. After selection with G418 for two weeks, the transfected cells were challenged with NDV and observed under microscope at different time points. Similarly, the typical CPE was visible in the pcDNA transfected cells as early as 12 h postinfection which was comparable to that in the mock- or pcDNA-NA transfected cell culture. The results could be seen from Figure 5 that the CEF cells transfected with pcDNA-NA showed no pathological changes, which were still close to grow with fibrous morphology at 12 and 24 h post-transfection (Figure 5B and C), then after 24 h transfection, pathological changes emerged. However, the cells transfected with pcDNA3.0 revealed pathological changes earlier than 12 h (Figure 5E), the cells became round, dropped and died after 24 h transfection (Figure 5F). This indicated that NA protein could protect CEF cells against NDV infection.

DISCUSSION

NA gene encoding the neuraminidase protein is the major surface antigen of AIV, and belong to type II glycoprotein, with its N-terminal locating at the capsule membrane, while the C-terminal locating at the outside of capsule. One of the functions of NA is to remove sialic acid from the glycosidic bond of the cell surface, prevent aggregation of virus particles and supply releasing channel for new virus particles (Chen et al., 2000; Yang et al., 2006). Some studies have shown that the virus could adapt to the new hosts by point mutations, insertions or deletions of NA gene sequence, changing its activity and affecting viral replication, which play an important role for the influenza virus transmission among different species. NA is one of the important immunogenic of influenza virus, can stimulate the body to produce specific antibodies and suppress viral replication. To a certain extent, it can accelerate the clearance of the virus in the body.

In this study, eukaryotic expression vector pcDNA-NA was constructed and transfected NIH3T3 cells, 48 h post-transfection, the cell lysates was prepared and the purified NA protein used to incubate CEF cells and then

infected with NDV. The results show that the NA protein could delay the CEF cells from NDV infection. Meanwhile, the CEF cells transfected with pcDNA-NA revealed no pathological changes, and still close to grow with fibrous morphology at 48 h post-transfection, pathological changes emerged slowly after 48 h transfection (Figure 5B and C). However, the cell transfected with pcDNA cannot protect them from NDV infection as early as 12 h post-tranfection (data not shown). These data demonstrated the importance of NA protein against NDV infection at early stage of viral infection. In addition, the prokaryotic expression vector pGEX-NA was constructed to express NA protein and make polyclonal antibody to detect the sub-cellular location of NA protein. The subcellular of NA gene may be closely related to its function; therefore, it is importmant to do subcellular localization of the NA gene.

This study not only constructed a fluorescent expression plasmid (pcDNA3.0/EGFP-NA) to detect NA subcellular localization by EGFP expression, but also obtained a non-fluorescent expression plasmid (pcDNA3.0-NA) to find NA subcellular localization by anti-NA serum, both of the results indicated NA protein is located at the cytoplasm. Moreover, the C-terminus of EGFP was fused with N-terminal of NA protein and formed the fusion protein EGFP-NA. NIH-3T3 cells were transfected with pcDNA3.0/EGFP-NA, observed under a fluorescence inverted microscope after 48 h transfection, the results showed green fluorescence (EGFP) located concentrately at the cytoplasm near the membrane, while there was no green fluorescence in the nucleus. Green fluorescence was full of the cells transfected with pEGFP-N1 for the positive control; there was no green fluorescence for the negative control group. NIH-3T3 cells were transfected with pcDNA3.0-NA for 48 h, immunocytochemical was detected by specific fluorescent antibody (aiti-NA serum), the only green fluorescence was shown at the cytoplasm near the membrane, while there was no green fluorescence for the control group transfected with pcDNA3.0. Therefore, this study further confirmed NA protein located at the cytoplasm near the membrane *in vitro*, which has a great significance for the biological function of NA protein. The precise location of NA genes in the cell would supply strong basis for its anti-viral infection research from single cell level *in vivo* and preparation of disease-resistant genetically modified chicken. The NA gene was used as resistance genes and has unique advantages for disease resistance breeding. Once integration NA gene is expressed, neuraminidase not only works as an antigen inducing target-specific antibodies, but also plays a role of enzyme activity and destroys the sialic acid receptor required for virus infection of the host cell surface, which protect the host from virus damage.

In conclusion, we demonstrated the function of NA in determination of the antiviral activities against NDV at early stage of viral infection, but the antiviral activities were not sufficient to inhibit virus replication at late stage of viral infection. This will lay the foundation for the preparation of disease-resistant transgenic chicken and provide a new idea for poultry breeding.

ACKNOWLEDGEMENTS

This work was supported by Research and Innovation Program for Graduate Cultivation of Jiangsu Province in 2010, National Natural Science Foundation of China (30871791), Natural Science major basic research projects of Jiangsu University (08KJA230001), Specialized Research Grant for Doctoral program (20103250110006) and “Six personal peak” grant. A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions and Jiangsu Province

REFERENCES

Chen Z (2003). Protection and Response Against Influenza a virus infection by hemagglutinin, neuraminidase DNA vaccine. J. microbiol., 23(05): 1-4.

Chen Z, Kadowaki S, Hagiwara Y, Yoshikawa T, Matsuo K, Kurata T, Tamura S (2000). Crossprotection againsta lethal influenza virus infection by DNA vaccine to neuraminidase. Vaccine, 18(28): 3214 - 3222.

Christenen JP, Dietz HH, Bisgaard M (1998). Phenotypic and genotypic characters of isolates of *Pasteurella multocida* obtained from back - yard poultry and from two outbreaks of avian cholera in avifauna in Denmark. Avian Pathol., 27(4): 373-381.

Cong YL, Liu JH (2008). Molecular mechanism of the role of hemagglutinin of influenza virus in interspecies transmission. Chin. J. Vet. Sci., 28(6): 737-742.

Johansson BE, Matthews JT, Kilbourne E D (1998). Supplementation of conventional influenza A vaccine with purified viral neuraminidase results in a balanced and broadened immune response. Vaccine, 16(9): 1009-1015.

Nan WL, Jin NY, Lu HJ, Zhao CQ, Tian XM, Tan L, Zhang JS, Bai L (2009). Prediction of the Th/B cell epitopes on HA of influenza virus (H1N1) and antigenicity analysis. Chin. J. Immunol., 7(25): 630-637.

Ni JQ, Zhang CL, Li HY, Cui SJ Qiu HJ, Wang YF, Tian ZJ, Tong GZ (2004). Primary application of swine influenza virus nucleoprotein expressed in prokaryotic expression system. Chinese J. Preventive Vet. Med., 26(1): 18-21.

Qi L, Wu KY, Li Z, Zhang Y, Dong J, Zhang GL, Zhang ZQ (2004). Screening of the High-yield Influenza B Virus on MDCK Cell and Cloning of Its Whole Genome. Chinese J. Virol., 20(3): 195-199.

Qiao CL, Jiang YJ, Li CJ, Tian GB, Meng QW, Deng GH, Wang XR, Yu KZ, Tang XY (2003). Studies on live fowlpox virus vector-based vaccine of avian influenza rFPV-HA-NA. Immunol. J., 19(01): 46-49.

Yang RM, Li MY, Zhao XL, Zhang Z (2006). The recombinant construction of H5N1 subtype avian influenza virus HA, NA gene in the baculovirus. Shanghai J. Anim. Husb. Vert. Med., 1: 22-23.

Zhao XD, Han F, Wang Y, Yang GB, Ying G, Lin YP, Wang X, Wu SH (2003). Construction of recombinant adenovirus expressing influenza virus neuraminidase gene. Virologica Sinica, 18(3): 213-216.

Zhou X, Li Y M, Hou L, Wang R Y, Dai X (2001). Immune effect of hemaglutinin (HA) gene and Neuraminidase (NA) gene of Influenza virus. Progress In Microbiol. Immunol,. 29(01): 15-19.