Increase in the Neuraminidase Activity of a Nonpathogenic Newcastle Disease Virus Isolate during Passaging in Chickens

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ABSTRACT. A pathogenic mutant of the Newcastle disease virus (NDV) was previously generated by passaging a non-pathogenic isolate from wild waterfowl. Velogenic mutant 9a5b (IVPI=2.67) contains three amino acid substitutions (128H, 495K and 573stop) in the hemagglutinin-neuraminidase (HN) protein, as compared with nonpathogenic waterfowl isolate 415/91 strain, and two of these (128H and 495K) were introduced after mesogenic 9a3b (IVPI=1.88). To investigate the role of the HN protein in NDV virulence, the function of HN protein such as neuraminidase (NA), Hemadsorption (HAD) and fusion promotion activities was examined by introducing the point mutations observed in passaged mutants into the HN gene cDNAs. *In vitro* functional assay using mutant protein expression demonstrated that the 128H substitution markedly increases NA activity and 573stop substitution increase NA and HAD activities. On the other hand, 495K substitution had little effect on any activities. These results indicate that a single amino acid substitution (128P to H) in the NDV HN protein affects the neuraminidase activity and is possibly correlated with the virulence.

KEY WORDS: hemagglutinin-neuraminidase protein, IVPI, Newcastle disease virus, passage, pathogenicity.

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Newcastle disease virus (NDV) is classified as a member of the genus *Avulavirus*, belonging to the family *Paramyxoviridae* [12, 16]. It causes a highly contagious respiratory, neurological or enteric disease in all species of birds, and is responsible for significant economic losses in the commercial poultry industry worldwide. Strains of NDV can be differentiated on the basis of their pathogenicity in chickens into strains of high (velogenic), intermediate (mesogenic) and low (lentogenic) virulence [1, 2, 24].

NDV has two surface glycoproteins, a fusion (F) protein and a hemagglutinin-neuraminidase (HN) protein [11]. The F protein mediates fusion of the virion envelope with the cellular plasma membrane. It is synthesized as an inactive precursor, F₀, which must be proteolytically cleaved to form a disulfide-linked heterodimer of F₁ and F₂ in order to activate membrane fusion activity. The F proteins of virulent strains differ from those of avirulent strains with regard to the presence of a pair of dibasic amino acids at the carboxyl (C) terminus of F₂ [5, 20, 22]. Whereas the F protein of virulent strains is cleaved by furin or other ubiquitous intracellular host cell proteases, the F protein of avirulent strains is cleaved by trypsin-like enzymes found in limited tissue. As a result, avirulent strains can only replicate in areas with trypsin-like enzymes, such as the respiratory and intestinal tracts, whereas virulent strains can replicate in a range of tissues and organs, resulting in fatal systemic infection [20, 21].

The HN protein possesses both the receptor recognition

and neuraminidase (NA) activities associated with the virus. It is responsible for the attachment of virus particles to sialic acid-containing receptors of the host cell, it promotes the fusion activity of the F protein and acts as NA by removing the sialic acid from progeny virus particles in order to prevent self-agglutination [10, 11, 27]. Thus, the HN protein plays an important role in viral infection. Chimeric experiments using recombinant viruses with the HN genes of virulent and avirulent strains demonstrated changes in virulence [8, 14]. Therefore, the HN protein of NDV is thought to affect pathogenicity in chickens.

In a previous study, we reported the generation of the mesogenic NDV 9a3b and velogenic 9a5b strains [25] from a nonpathogenic waterfowl isolate, Goose/Alaska/415/91 (415/91) strain [26], by passaging in chicken air sac and brain. Sequence analysis of the F protein cleavage site showed that the increase in pathogenicity was due to changes in the cleavability of the F protein. However, pathogenicity was further increased with passage in chicken brain, following a change in the cleavability of the F protein [25]. Genetic comparisons between 415/91 and 9a5b demonstrated three amino acid substitutions (128P to H, 495E to K, 573E to Stop codon) in the HN proteins (GenBank accession number: AB524405 and AB524406). Two of these substitutions were detected between 9a3b and 9a5b and no amino acid substitution in other proteins, thus suggesting that these amino acids are related to the pathogenicity of NDV in chickens (GenBank accession number: AB534205). In the present study, the function of HN protein and its contribution to virulence were examined by *in vitro* functional assays using mutant HN proteins.

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MATERIALS AND METHODS

Cells and viruses: Human embryonic kidney (HEK)-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Baby hamster kidney (BHK)-21 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum. Virus-infected cells were maintained in MEM containing 0.3% bovine serum albumin. The NDV strains used in this study were Goose/Alaska/415/91 (415/91), and viruses passaged through air sacs and brains; 2a, 9a3b, 9a4b and 9a5b [25]. Viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs for 48 hr at 35°C, and the allantoic fluid was harvested and stored at -80°C.

Cloning of HN and F genes: In order to clone the HN and F genes, two primer sets, HN:415HNstart-*EcoRI* (5'-GAATTCGCCGCCACCATGGAGAGAGGGTCAGC-CAAGTTG-3') and 415HNstop-*XhoI* (5'-CTCGAGCGGAGCTCGCCATGCCTACC-3'), F:415Fstart-*BglII* (5'-AGATCTGCCGCCACCATGGATCCCAAGCCTTC-TACCAG-3') and NDHR1 (5'-GGCCTCTTCGACCGT-TCTACCCGT-3'), were used for PCR with KOD Plus DNA polymerase (TOYOBO, Tokyo, Japan). The amplified DNA fragments were cloned into pT7Blue using Perfectly Blunt Cloning Kits (Novagen, Darmstadt, Germany). Plasmids were digested with restriction enzyme and cloned in pCAGGS/MCS. The obtained clones were designated pC415HN, pC2aHN, pC9a3bHN, pC9a5bHN and pC9a5bF. pC9a4bHN was constructed from pC9a3bHN using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.).

Construction of mutant HN genes: Mutant HN proteins were also generated by introducing mutations using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The three amino acid residues at positions 128, 495 and 573 in the mutated HN proteins were as follows: clone 415-128H (128H, 495E, 573E), clone 415-495K (128P, 495K, 573E), clone 9a5b-128P (128P, 495K, 573Stop), clone 9a5b-573E (128H, 495K, 573E). The mutant HN clones in the pT7Blue plasmid were subcloned into pCAGGS and the obtained mutants were designated pC415-128H, pC415-495K, pC9a5b-128P and pC9a5b-573E, respectively (Table 1). Sequence analysis showed no unexpected mutations in those plasmids.

Transient expression systems: Mutated HN and F proteins were expressed in HEK-293 or BHK-21 cells. All experiments were carried out in 6-well plates seeded a day earlier at 2.0×10^5 cells/cm². Plasmids (0.5 µg/cm²) were transfected into cells using the TransIT LT1 kit (Panvera, Madison, WI, U.S.A.) according to the manufacturer's protocol. Assays were performed at 48 or 50 hr post-transfection.

Enzyme-linked immunosorbent assay for measurement of cell-expression level of HN proteins: Enzyme-linked immunosorbent assay (ELISA) was performed in order to determine expression levels of HN proteins. Cells expressing HN proteins were fixed overnight in formalin at 4°C, and

Table 1. Amino acid residues coded in the constructed HN expression plasmids

Plasmid	Amino acid residues		
	128 ^{a)}	495	573
pC415HN	P	E	E
pC2aHN	P	E	Stop
pC9a3bHN	P	E	Stop
pC9a4bHN	H	E	Stop
pC9a5bHN	H	K	Stop
pC415-128H	H	E	E
pC415-495K	P	K	E
pC9a5b-128P	P	K	Stop
pC9a5b-573E	H	K	E

a) Amino acid position.

were then washed three times with phosphate-buffered saline (PBS), containing CaCl₂ and MgCl₂. Subsequently, cells were incubated in PBS containing 1% bovine serum albumin at room temperature for 1 hr, followed by anti-Goose/Alaska/415/91 chicken serum as a primary antibody for 1 hr at room temperature. After washing three times with PBS, goat anti-chicken immunoglobulin G labeled with horseradish peroxidase as a secondary antibody was added for 1 hr at room temperature. After washing three times, the enzyme reaction with 150 µl of ABTS solution (Roche Diagnostics, Tokyo, Japan) was carried out for 5 min at room temperature. Cell contents were then collected and centrifuged at $8,000 \times g$ for 1 min and 100-µl aliquots of supernatant were added to the wells of a 96-well plate. Absorbance was measured with a Model 3550 microplate reader (BIO-RAD, Tokyo, Japan) at a wavelength of 450 nm.

Sequencing: Viral RNA was extracted directly from infected allantoic fluid using the QIAamp Viral RNA Mini-kit (Qiagen, Tokyo, Japan), and reverse transcription was carried out with M-MLV reverse transcriptase (TaKaRa, Otsu, Japan) using random 6-mers, and cDNA was amplified by PCR. Nucleotide sequences were analyzed using an autosequencer (Gene Rapid, Amersham Pharmacia Biotech, Uppsala, Sweden).

NA assay: NA assay was carried out as described previously [3, 18]. HEK-293 cells transfected with HN protein expression plasmids were washed twice in phosphate-buffered saline and once in phosphate buffer (PB, pH 5.9). The reaction was initiated by the addition of 170 µl of substrate mix [12.5 mg/ml fetuin in PB] per well. After incubation for 30 min at 37°C, the reaction mixture was harvested and centrifuged at $15,000 \times g$ for 1 min, and 100 µl of supernatant was incubated with 50 µl of 200 mM sodium periodate for 20 min at 20°C. After excess periodate was blocked with 500 µl of NaAsO₂ solution [100 mg/ml NaAsO₂, 500 mM Na₂SO₄, 0.3% H₂SO₄], 1.25 ml of TBA solution [6 mg/ml 2-thiobarbituric acid, 500 mM Na₂SO₄] was added, followed by incubation for 15 min at 100°C. Specific pigment was extracted into 2 ml of acid butanol and the optical density at 549 nm was measured by photospectrometry.

Fig. 1. Functional assays of mutant HN proteins. HAd activity was determined by the ability of expressed HN proteins to absorb chicken erythrocytes. NA activity was determined by the ability of expressed proteins to catalyze the release of sialic acid from fetuin. The ability to complement 9a5b F in the promotion of fusion was determined by calculating as the ratio of the total number of nuclei in multinuclear cells to the total number of nuclei in the field. The expression levels of HN proteins were determined by ELISA. All data are expressed relative to the amount for the velogenic 9a5b protein and represent the mean of at least four independent determinations.

Hemadsorption (HAd) assay: HEK-293 cells transfected with each HN protein expression plasmid were washed three times in PBS, and were overlaid with 0.75% chicken erythrocytes (RBCs) in PBS. Plates were kept at 4°C for 15 min. Unbound RBCs were removed by washing with PBS three times. The RBCs bound to HN-expressing cells were lysed with 0.05 M ammonium chloride, and the released hemoglobin was measured at 549 nm in a spectrophotometer.

Fusion promotion (FP) assay: BHK-21 cells transfected with each HN protein and F protein plasmids were washed three times with PBS, fixed with cold methanol for 1 hr, and stained with Giemsa. The FP index was calculated as the ratio of total nuclei to the number of cells in which nuclei were present [9].

RESULTS

In order to clarify the contribution of HN protein to the pathogenicity of NDV, HN proteins from a series of passaged viruses, 415/91, 2a, 9a3b, 9a4b and 9a5b, as well as several mutant proteins, were expressed in cell cultures (Table 1). Expression levels of each mutated HN protein

were comparable to those of the 415/91 HN protein. The cells expressing each mutant HN protein were assayed for NA, HAd and FP activities in order to explore the effects of amino acid substitutions.

The NA activity of the 415/91 HN protein was only 3% that of the 9a5b HN protein. The 2a HN protein had the E573stop substitution, and the 9a3b HN protein had 25–28% NA activity when compared to 9a5b. Furthermore, the 9a4b HN protein, which had the P128H substitution, showed a conspicuous increase in NA activity, and reached a similar titer to that of the 9a5b. The NA activity of the mutant HN 415-128H protein was about 7-fold higher than that of 415, and that of mutant HN 9a5b-128P and 415-495K proteins showed similar activity to that of the 415. The mutant HN 9a5b-573E protein showed 60% lower NA activity than 9a5b (Fig. 1). These results indicate that two amino acid substitutions, 128P to H and 573E to Stop, markedly increase NA activity.

The HAd activity of the 415/91 HN protein was 56% that of the 9a5b HN protein. However, the HAd activity of the 2a and 9a3b HN proteins were similar to that of 9a5b (102–114%), although 9a4b showed lower (71%) activity. More-

over, the HAd activity of the mutant HN proteins also indicated that the amino acid substitution of 573E to a stop codon is important for HAd activity (Fig. 1).

The FP activity of the 415/91 HN protein was higher than that of 9a5b (by approximately 250%). The FP activities of the 2a and 9a3b HN proteins were similar to those of the 9a5b, and 9a4b HN protein was lower (65%). The activities of mutant HN 415-495K protein and 415-128H protein were almost equal to that of 9a5b HN protein. Moreover, the mutant HN 9a5b-128P protein and 9a5b-573stop protein also had high FP activities (Fig. 1). There was no correlation between FP activity and amino acid substitutions.

DISCUSSION

In this report, we examined the functional role of the HN protein of NDV in virulence. The HN protein of NDV is a multifunctional protein and has NA, HAd and FP activities [10, 11, 27]. Therefore, we examined the effects of three amino acid substitutions (128P to H, 495E to K, and 573E to stop codon) introduced into the HN protein of 9a5b on these biological activities. The results showed that the 128H substitution markedly increases NA activity. The residue at amino acid 128 is distant from the NA catalytic site [4]; thus, substitution of this residue may alter the conformation of HN protein and affect NA activity.

Based on differences in stop codon position, NDV HN proteins are classified into three types, with 616, 577 or 571 amino acids [23]. The largest type, with 616 amino acids, is the precursor-type, designated HN₀616, and is proteolytically converted into the biologically active HN protein [19]. Almost all lentogenic waterfowl isolates contain this type of precursor HN₀. The translation products with 577 and 571 amino acids are already in an active form, and are designated HN577 and HN571, respectively. Velogenic strains contain these active HN proteins. In the present study, it was found that the original lentogenic waterfowl isolate 415/91 contains the precursor-type HN₀616, and the chicken-passaged virus 2a converts this to an active HN protein of 572 amino acids. These results demonstrate that the prototype HN₀616 protein from waterfowl isolates is converted to active HN by a point mutation to change the position of the stop codon (573E to stop codon) during passage in chickens. Furthermore, such mutations may occur at the earlier stage of passaging when compared to F cleavage site changes in nature. The fact that there are no field isolates containing highly cleavable F and precursor HN₀ support this notion [28].

On the other hand, the 495K substitution had little effect on any activities. Takimoto *et al.* [27] reported that the FP activity a mutant HN protein of NDV, with a Glu to Ala change at residue 495, was lower when compared to the wild-type HN protein. The residue at 495 is distant from the site of interaction between the F protein and the receptor binding site. The functional significance of the 495K substitution thus requires further investigation.

The NA activity of 9a5b HN protein was much higher

than that of 9a3b, while the HAd and FP activities were comparable. As the pathogenicity of 9a5b was higher than that of 9a3b, this change in NA activity was thought to affect its pathogenicity in chickens. The pathogenicity of 9a4b was not determined because 9a4b appears to include a mixed population and the IVPI values were uncertain. Further investigation using cloned 9a4b virus is necessary for comparative analysis of pathogenicity.

The role of the HN gene in NDV virulence has been reported [8]. Through the use of reverse genetics procedures, the HN genes of virulent and avirulent recombinant NDV strains were exchanged. The chimeric viruses with reciprocal HN proteins either gained or lost virulence, indicating that virulence is associated with the amino acid differences in the HN protein. The present study demonstrated that a single amino acid substitution (128P to H) in the HN protein has an effect on the NA activity and is possibly correlated with the virulence. These findings support the hypothesis that development of virulence in NDV is a multigenic event. However, the exact mechanism responsible for the effects of NA activity on virulence is still unknown. Further studies using reverse genetics technique are therefore necessary to clarify this issue.

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