Original Article Emergence of HA mutants during influenza virus pneumonia

Maria Eugenia Vázquez Manríquez¹, Akiko Makino², Motoko Tanaka², Yasuhisa Abe³, Hiroyuki Yoshida³, Ichiro Morioka³, Soichi Arakawa³, Yasuhiro Takeshima⁴, Kentaro Iwata⁵, Jin Takasaki⁶, Toshie Manabe⁶, Takaaki Nakaya⁷, Shota Nakamura⁷, Anjarath Lorena Higuera Iglesias¹, Rosa Maria Rivera Rossales¹, Erika Pena Mirabal¹, Tateki Ito⁶, Toshio Kitazawa⁶, Teruaki Oka⁸, Makoto Yamashita⁹, Koichiro Kudo⁶, Kyoko Shinya²

¹National Institute of Respiratory Diseases, Mexico City, Mexico; ²Division of Zoonosis, Kobe University, Japan; ³Department of Infection Control and Prevention, Kobe University Hospital, Japan; ⁴Department of Pediatrics, Kobe University Hospital, Japan; ⁵Division of Infectious Diseases, Kobe University Hospital, Japan; ⁶National Center for Global Health and Medicine, Tokyo, Japan; ⁷Research Institute for Microbial Diseases, Osaka University, Japan; ⁸Kanto Central Hospital, Tokyo, Japan; ⁹Biological Research Laboratories, Daiichi Sankyo Co., Ltd, Tokyo, Japan

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Abstract: During the influenza pandemic of 2009, the number of viral pneumonia cases showed a marked increase in comparison with seasonal influenza viruses. Mutations at amino acid 222 (D222G mutations) in the virus hemagglutinin (HA) molecule, known to alter the receptor-recognition properties of the virus, were detected in a number of the more severely-affected patients in the early phases of the pandemic. To understand the background for the emergence of the mutant amino acid D222G in human lungs, we conducted histological examinations on lung specimens of patients from Mexico who had succumbed in the pandemic. Prominent regenerative and hyperplastic changes in the alveolar type II pneumocytes, which express avian-type sialoglycan receptors in the respiratory tract of severely affected individuals, were observed in the Mexican patients. An infection model utilizing guinea pigs, which was chosen in order to best simulate the sialic acid distribution of severe pneumonia in human patients, demonstrated an increase of D222G mutants and a delay in the diminution of mutants in the lower respiratory tract in comparison to the upper respiratory tract. Our data suggests that the predominance of avian-type sialoglycan receptors in the pneumonic lungs may contribute to the emergence of viral HA mutants. This data comprehensively illustrates the mechanisms for the emergence of mutants in the clinical samples.

Keywords: Influenza, mutant, hemagglutinin, pneumonia, hyperplastic pneumocytes, syaloglycan receptor

Background

The genetic background of the A(H1N1)pdm09 virus that emerged in 2009 possessed notable differences when compared with the Asian (H2N2) and Hong Kong flu (H3N2) due to the reassortment of certain genes between both seasonal and animal influenza viruses [1, 2]. With the exception of the polymerase basic protein 1 (PB1) gene, the A(H1N1)pdm09 virus was shown to possess segmented viral RNA genomes that had been newly introduced from animal flu viruses [3]. This A(H1N1)pdm09 virus is similar to seasonal influenza viruses in being easily transmitted between humans. However, the amino acid substitutions of the A(H1N1)

pdm09 virus, which are usually indicators of human-adaptation in seasonal influenza viruses, were more similar to those found in avianderived viruses [4, 5]. In fact, the pathogenicity of this A(H1N1)pdm09 virus was higher than that of seasonal influenza viruses, although still lower than that of highly-pathogenic avian influenza viruses. In other words, the A(H1N1) pdm09 virus was the causal factor in cases of viral pneumonia more frequently than were seasonal influenza viruses, indicating potential differences in its pathogenicity [6-8].

A recent study, which utilized A(H1N1)pdm09 virus samples of patients from Norway infected with the virus, showed that a D to G substitu-

tion at amino acid 222 (D222G) in the virus hemagglutinin (HA) molecule correlated with the severity of the disease [9]. Analyses carried out in other countries further supported this finding [10-12]. Baldanti et al. found that the viral load in clinical specimens from patients with severe symptoms was higher in the lower respiratory tract, and subsequently uncovered a prevalence of D222G/N mutant viruses in the viral load of the lower respiratory tract compared to those in the upper respiratory tract [12]. A similar tendency was also reported in a separate study [13].

Because D222G HA mutations can alter the receptor-recognition properties of the virus, substitutions at the amino acid 222 position have been the focus of intense investigation. For example, two 1918 pandemic influenza strains, wild-type (WT) or D222G HA mutant, have been shown to possess different receptor recognition properties. The A/New York/1/1918 virus possessing a D222G HA mutation has been found to bind less intensely to the classical alpha-2,6 (human-type) sialic acid, but was seen to bind with a higher specificity to alpha-2,3 (avian-type) sialic acid, when compared to the A/South Carolina/1/1918 WT virus [14]. Because avian-type sialoglycans are found in the lower respiratory tract of human lungs [15, 16], viral growth in this region of the lung may accelerate the emergence of and/or selection for D222G HA mutants.

To understand the pathological mechanisms involved in the emergence of mutant strains, we analyzed autopsy specimens from cases in Mexico as well as samples obtained from infected animals.

Methods

Pathological analysis

All autopsies and necropsies were conducted at the Instituto Nacional de Enfermedades Respiratorias (INER) in Mexico. Paraffinembedded lung specimens from two autopsy cases, previously confirmed to be positive for A(H1N1)pdm09 virus infection and without concomitant bacterial infection, were stained with hematoxylin and eosin. Another section from each paraffin specimen was processed for immunohistological staining using an anti-influenza virus rabbit antibody (R309 used at a 1:2000 dilution). Antigen-antibody reactions were developed with 3,3'-diaminobenzidine tetrahydrochloride substrate (Dako) and visualized using the DakoEnVision system (Dako Co. Ltd). Paraffin-embedded respiratory tissue specimens from two other autopsy cases and four necropsy cases were selected for sialic acid examination using lectin staining. To detect sialyloligosaccharides reactive to SAα2,3Gal- or SAα2,6Gal-specific lectins, we used fluorescein isothiocyanate (FITC)-labeled SNA lectin and biotinylated MAL II lectin (Vector Laboratories, Burlingame, CA), and Alexa Fluor 594-conjugated streptavidin (Molecular Probes, Inc., Eugene, OR). The sections were counterstained with 4',6-diamino-2-phenylindole dihydrochloride (DAPI; Dojindo Molecular Technologies, Inc., Kumamoto). Ethical approval for the histological analysis of the human tissues was obtained from the Ethics Committee of the National Center for Global Health and Medical School of Kobe University.

Viruses

Three A(H1N1)pdm09 (A/ strains Kobe/24/2009, A/Kobe/30/2009, and A/ Kobe/31/2009), were isolated from patients who either visited or were hospitalized at the Kobe University Hospital between August 2009 and March 2010. Clinical samples were initially screened by employing a conventional one-step reverse transcription polymerase chain reaction (RT-PCR; SuperScript[™] III One-Step RT-PCR System with Platinum® Taq High Fidelity, Takara), using primer pairs for the matrix gene of the influenza A virus (244 base pairs, forward primer: ATGATYCTTYTAACCGAGGTCGA-AACG; reverse primer: TGGACAAANCGTCTA-CGCTGCAG) and the HA gene of the A(H1N1) pdm09 virus (313 base pairs, forward primer: TACCGAGATATGCATTCGCAATGG; reverse primer: TATCCTGACCCCTGCTCATTTTGATGG).

Sequencing of the viral genome

Viral RNA was extracted from clinical samples using the QIAamp Viral RNA Mini kit (Qiagen, Maryland, USA). RT-PCR (Prime Script, Takara; Phusion, Finnzymes) was performed using primer pairs specific to each viral RNA segment. PCR products were directly sequenced using specific primers for each segment. Sequencing data was collected with a 3730x/ DNA Analyzer (Applied Biosystems) and ana-

Case No.	A^*/N^\dagger	Date of onset	Disease duration	Bacterial infection ^{\$}	Underlying diseases, superinfection			
Ι	А	9/22/2009	8 days	-	Penicillin allergy, Obesity, Hypertension			
ii	А	3/27/2009	13 days	-	Hypertension			
а	А	8/29/2009	9 days	S. pyogenes	$\text{COPD}^{\delta},$ Diabetes mellitus, $\text{CHF}^{\text{S}},$ Hypertension			
b	Ν	n/a‡	n/a	n/a	n/a			
С	А	6/13/2009	11 days	n/e⁰	Alcoholism, Cocaine, Thinner			
d	Ν	n/a	n/a	n/a	n/a			
е	Ν	n/a	n/a	n/a	n/a			
f	Ν	n/a	n/a	n/a	Fungal infection			

 Table 1. Clinical characteristics of Mexican patients

*autopsy sample; [†]Necropsy sample; [‡]not available; [@]not examined; ⁸Chronic obstructive pulmonary disease; [§]Congestive heart failure. [§]Bacterial infection was assessed by performing a sputum culture using sheep blood agar, chocolate (heated blood) agar, and MacConkey agar, and by performing a blood culture test using a fully automated microbiology growth and detection system (BD BACTECTM instrumented blood culture system).

lyzed by SeqMan Pro (DNASTAR, Inc., Madison, WI). To examine the genetic variability of the virus population in each clinical sample, next generation sequencing was performed. RT-PCR products of viral HA were prepared from clinical samples using tagged primers (380 base pairs, forward primer: tag-AATTCATACCCAAAGCTCAG-CAAATCC; reverse primer: tag-GGGTGTCT-GACAAGTTGTATTGCAATCG [tag sequences available through Roche Diagnostics K.K.]). PCR products were subsequently processed and analyzed for variants using the GS Junior System (Roche).

Assessment of the emergence of mutants

Three A(H1N1)pdm09 strains (A/Kobe/24/-2009, A/Kobe/30/2009, and A/Kobe/31/-2009) were grown and titrated in Madin-Darby canine kidney (MDCK) cells with 3 µg/ml of L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington, Lakewood, NJ). Cells were cultured at 35°C in 5% CO₂. To understand the mechanisms involved in the emergence of viral mutants, six female Hartley guinea pigs (mean weight, 340 g) were anesthetized with an intramuscular injection of ketamine (15 mg/kg) and xylazine (1 mg/kg), and intranasally inoculated with 250 µl of corresponding viral fluid (103-104 plaque forming units/animal). Animals were euthanized at day 3 and 6 post-infection (p.i.) and both nasal washes and BALFs were collected. In order to minimize the number of animals used for this experiment, we used only two animals to test the tendency of mutant emergence of each viral strain. The genetic variability of the virus population in each sample was assessed using RT-PCR (380 base pairs, forward primer: AATTCATACCCAAAGCTCAGCAAATCC; reverse primer: GGGTGTCTGACAAGTTGTATTGCAATCG), followed by cloning and sequencing the HA gene. A minimum of 10 clones per sample were examined and analyzed for mutations. All animal experiments were done in so as to conform to the Guide for the Care and Use of Laboratory Animals of Daiichi Sankyo Co., Ltd.

Results

Histological features of influenza pneumonia

The tendency towards an increase in the mutant population in samples of severe cases indicated a possible selection for variants in the host's respiratory tract [10-12]. To assess if conditions in the lung were conducive to such mutant selection, autopsied lungs from two laboratory-confirmed cases were examined histologically.

Clinical records showed that, although these patients had suffered from other complications, their sputum and blood were otherwise free of bacteria (**Table 1**). The first case had an 8-day disease course, with the right upper lung lobe showing typical signs of diffuse alveolar damage (DAD): acute bronchitis, severe alveolar edema, and hemorrhaging (**Figure 1**(i)). The most prominent finding was massive hemorrhaging in the alveoli (**Figure 1**(i)A), accompanied by a distribution of severe alveolar edema with mild to moderate cellular infiltration (**Figure 1**(i)B and (i)C). Scattered thrombi were also observed (**Figure 1**(i)D), and the edema extend-



Figure 1. Histological characteristics of influenza pneumonia in Mexican patients. i. Coronal plane showing the right lower lung lobe. A. Massive hemorrhaging into the alveoli, B, C. severe alveolar edema with moderate to mild cellular infiltration, D. thrombi, E. edema in the connective tissue septa, and F. desquamation of bronchiolar mucosal epithelium, neutrophilic infiltration, and presence of proteinaceous exudates and coagulative debris. ii. Coronal plane showing the right upper lung lobe. A. Massive hemorrhaging into the alveoli, and B. thrombi with fibrin-like material and minor infiltration of lymphocytes, macrophages, and neutrophils into alveolar spaces. The accumulated exudates were surrounded by hyperplastic alveolar cells, C. thrombus, D. hyaline-membrane formation, E. focal alveolar edema, and F. terminal bronchi. Regenerative hyperplastic epithelium shows accumulation of fibrin-like material and cell debris, and infiltration of inflammatory cells and red blood cells into the lumen.



Figure 2. Distribution of sialoglycans in patients with severe pneumonia. A. Bronchus. Increased mucin secretion was evident in goblet cells. Cell surface and mucin were stained by Sambucus nigra (SNA) lectin (green). B. Bronchial epithelia with neutrophil and lymphocyte exocytosis. Cell surface was stained by SNA lectin. C. Mild exfoliation of alveolar cells into alveolar spaces. Type II alveolar cells were stained by MAL II lectin (red). D. Severe accumulation of fibrin and cell debris in alveolar spaces, and presence of scattered regenerative alveolar cells. Large regenerative alveolar cells were stained by MAL II lectin. E. Scattered type I alveolar cells (arrow heads) and many regenerative alveolar cells. Cluster of regenerative cells were stained by MAL II lectin. F. Hyperplastic alveolar cells linearly lined along most of the alveolar wall. Hyperplastic alveolar cells were stained by MAL II lectin. Left panel; lectin staining. Right panel; hematoxylin and eosin staining. Bar = 50 μ m.

ed into the connective tissue septa (Figure 1(i) E). Severe desquamation of the bronchiolar mucosal epithelium was observed, as well as neutrophilic infiltration, proteinaceous exudates, and coagulative debris (Figure 1(i)F). Focal erosion, mononuclear cell infiltration into the lamina propria mucosae, and excessive mucus production from mucus glands was also observed in the bronchi. Finally, the tracheobronchial lymph node contained massive numbers of red blood cells, macrophages, and mast cells.

The second case corresponded to a 13-day disease course (Figure 1(ii)) and, similar to the first case, massive hemorrhaging in the alveoli was observed (Figure 1(ii)A). However, in contrast to the aqueous alveolar material present in the first case, the second case revealed coagulated fibrin-like material as well as minor infiltration of lymphocytes, macrophages, and neutrophils in the alveolar spaces. In addition, the accumulated exudates were surrounded by hyperplastic alveolar cells (Figure 1(ii)B). Thrombi were scattered throughout the section examined (Figure 1(ii)C), and hyaline-membrane formation (Figure 1(ii)D) and alveolar edema (Figure 1(ii)E) both showed focal distribution. Moreover, the terminal bronchi possessed regenerative and hyperplastic epithelium with fibrin accumulation, as well as small amounts of cell debris, neutrophils, macrophages, lymphocytes, and red blood cells in the lumen (Figure 1(ii)F). Severe erosion, partial mucosal epithelial ulceration, suppurative inflammation in the lamina propria mucosae, submucosal duct rupture, mucus material leakage into the lamina propria mucosae, and focal lymphocyte and plasma cell infiltration in the submucosal gland were observed in the bronchi.

In summary, alveolar hemorrhaging and thrombi were the two most common findings in each case. However, there were differences between the main histological characteristics of DAD in these two cases, which may reflect differences in the duration of the course of the disease, as lung histology in the second case revealed prominent regenerative and hyperplastic changes in both alveolar type II pneumocytes and terminal bronchiolar epithelium. We did not

Virus strain	Sample	Symptom	a.a.*	D222G/N mutation rate in the population (%)					
			at position	Patient	CK p1§	Guinea pig d3 p.i. ^{\$}		Guinea pig d6 p.i.	
			222			NW [†]	BALF [‡]	NW	BALF
A/Kobe/K24/09	tracheal aspirate	severe	Ν	0.11	6.7	6.3	43.8	0	12.5
			G	0.02	0	6.3	18.8	0	6.3
A/Kobe/K30/09	nasal swab	mild	Ν	0.00	0	0	0	0	0
			G	0.00	0	0	0	0	0
A/Kobe/K31/09	tracheal aspirate	severe	Ν	0.00	0	0	0	0	0
			G	0.07	0	0	0	0	0

Table 2. Percentage of D222G/N HA mutants in patient, isolated, and animal sample populations

*amino acid; ^{\$}virus passaged in MDCK once; [®]post-infection; [†]nasal wash; [‡]bronchoalveolar lavage fluid.

find any specific immunohistological reactions in those samples.

Distribution of sialoglycans in severe cases

We previously demonstrated that type II pneumocytes and Clara cells at terminal bronchioles express alpha-2,3-linked (avian-type) sialoglycans on their cell surfaces [15]. Therefore, several autopsy/necropsy samples from pandemic flu patients in Mexico were selected and screened for sialic acid distribution, using lectin staining, to assess whether severe pneumonic lesions contained a different sialoglycan distribution when compared to normal lungs. Table 1 lists the patients' background information. Goblet cells in the large bronchus showed active mucin production as assessed by Sambucus nigra (SNA) lectin staining (green; Figure 2A). Neutrophil and lymphocyte exocytosis in the epithelium was apparent in the small bronchi; however, sialoglycan distribution, as assessed by SNA lectin binding, was similar to that found in normal lungs (Figure 2B). In the mild pneumonic lesions, which showed only mild exfoliation of alveolar cells into alveolar spaces, scattered type II alveolar cells exhibited a normal appearance and were stained by Maackia amurensis (MAL) II lectin (red; Figure 2C). As the pneumonic lesions increased in severity, regenerative alveolar cells became more prominent, forming clusters that could be seen using MAL II lectin staining (Figure 2D and 2E). In cases of fungal superinfection, hyperplastic alveolar cells were linearly lined along the alveolar wall and stained by MAL II lectin (Figure 2F). Our findings show that prolonged pneumonia causes hyperplasia of type II pneumocytes, and that this condition results in increased avian-type sialoglycans in the host lungs.

Predominance of avian-type sialoglycan in the lungs promotes the emergence of viral mutants

Due to the unavailability of samples, it was impossible to perform virological examinations on the Mexican virus. In place of these, animal experiments using Japanese clinical isolates were conducted in order to assess whether the predominance of avian-type sialoglycan in the lung was conducive to the selection of mutations. Guinea pigs were selected as appropriate experimental animals for this study, as prior studies have shown that human-type sialoglycans predominate in the guinea pig nasal mucosa, whereas avian-type sialoglycans predominate in the lung, so the distribution of its sialoglycans mimics that of severe human pneumonia [17]. Three strains were selected for the tests, two obtained from severe cases (A/Kobe/24/2009 and A/Kobe/31/2009), and one from a mild case (A/Kobe/30/2009). Using general direct sequencing, no strain harboring the D222G/N mutation was found, even in the two severe cases. Moreover, next generation sequencing analysis revealed only a small mutant population in these clinical samples (Table 2).

The viruses were intranasally inoculated into the guinea pigs. Nasal washes and bronchoalveolar lavage fluid (BALFs) were collected at Day 3 and 6 p.i., and the genetic variability of the virus population in each sample was assessed using partial virus genome cloning and sequencing. No emergence of viral mutants was detected in two of three viral strains tested. However, one strain that had been isolated from a severe case did show an increased number of mutants in the tested isolates (**Table 2**). In summary, our results demonstrate that the predominance of avian-type sialoglycans in the lungs promotes the emergence of HA mutants in a viral strain.

Discussion

Despite being unable to obtain direct virological proof through the use of samples collected from influenza cases in Mexico, the results reported here support the hypothesis that viral HA mutants are more likely to emerge in the lungs of patients with severe pneumonia. In contrast to Boonarkart et al's report, which demonstrated an increase of human type sialoglycans in pneumonic lesions [18], the cases presented here show that regenerative alveolar cells express avian-type sialoglycans. Previous reports documenting cases of severe influenza pneumonia and experimental primate infection model have shown that viral infection in the lower lung was frequently detected in type II pneumocytes [13, 19-22]. These findings suggest that the regenerative cells (i.e., type II alveolar cells) tend to be a target for viral infection in seriously ill patients suffering from prolonged cases of viral pneumonia, and may provide a conducive environment for the emergence of viral HA mutants in alveoli. Furthermore, animal experiments using guinea pigs, chosen to simulate the sialic acid distribution of severe pneumonia in human patients, demonstrated both an increase of D222G mutants and a delay in the diminution of mutants in the lower respiratory tract in comparison to the upper respiratory tract. Although the necropsy of a single animal per strain per point in time does not permit statistical analysis, this data comprehensively identifies mechanisms which resulted in the emergence of mutants in the clinical samples [9-12].

However, the emergence of HA mutants was likely a conditional characteristic of the viruses, as the mutation was certified in only one of the three strains tested in the guinea pig model. In other words, the mutation emergence rate also depends upon the type of viral strain involved. The results obtained from our animal studies suggest that there may be mechanisms which favor the emergence of mutant strains, not only in host factors but also in viral factors. However, further analysis will be needed to fully elucidate the viral factors involved. Nevertheless, the correlation between the mutation and pathogenicity is so certain that it cannot be ignored, due to the fact that the D222G mutation in the HA molecule is wellknown to cause changes in the receptor-binding properties of the virus as well as having been shown to influence the infection of target cells and the efficiency of the infection and its pathogenicity [23-26]. In fact, the guinea pig infection model in our study showed differences in the severity of the pneumonia depending on the viral strains used. On Day 6 p.i., only the animal inoculated with the strain that resulting in the emergence of HA mutants developed severe, widely-distributed hemorrhagic pneumonia. In contrast, the two other animals, each inoculated with different strains, developed only partially-consolidated non-hemorrhagic pneumonia (data not shown). Therefore, the HA mutation can be described as both of the cause and result of the severe pneumonia.

In past pandemics, the highest number of cases of severe pneumonia have typically been seen during the early phase of the pandemic [27, 28]. Recent surveillance of Japanese patients also demonstrated the presence of HA mutations in a minor subpopulation of isolated viruses in the early phase of the 2009 pandemic [25]. The emergence of this HA variant may well be related to the increase in the number of severe pneumonia patients in the earlier phase of pandemics. It will thus be necessary to accumulate and share such knowledge for future pandemic preparedness.

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