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Commentary

Influenza virus hemagglutinin cleavage into HA_1, HA_2: No laughing matter

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What do an odd lab-derived strain of influenza, fowl plague virus, and the 1997 Hong Kong chicken flu have in common? The answer lies in how, and more specifically by what protease, the hemagglutinin protein of these various influenza strains is cleaved for viral activation. What does this have to do with the most devastating influenza virus of them all, the 1918 "Spanish" influenza? The connection may be a new and interesting mechanism for hemagglutinin cleavage proposed by Goto and Kawaoka in an article in this issue of *Proceedings* (1).

In a series of experiments confirming and synthesizing 25 years of experimental data (2–4), Goto and Kawaoka (1) demonstrate specifically how the neuraminidase (NA) protein of influenza A/WSN/33 (H1N1), a curious variant of the first human influenza virus ever isolated, mediates hemagglutinin (HA) cleavage. Goto and Kawaoka (1) provide evidence for a model whereby the NA of WSN/33 directly binds plasminogen, sequestering it for cleavage activation. Subsequently, active plasmin cleaves and activates influenza hemagglutinin. Being able to sequester plasminogen for hemagglutinin cleavage allows the influenza virus to infect cells other than its usual targets.

With the exception of WSN/33 and the few influenza strains named above, most influenza viruses replicate in a strictly limited subset of cells. Influenza A viruses are negative-stranded RNA viruses. Like many other enveloped viruses, they code for a surface glycoprotein that must be cleaved by cellular proteases for activation. HA, a major influenza surface glycoprotein, is translated as a single protein, HA_0. For viral activation, HA_0 (assembled as trimers) must be cleaved by a trypsin-like serine endoprotease at a specific site, normally coded for by a single basic amino acid (usually arginine) between the HA_1 and HA_2 domains of the protein. After cleavage, the two disulfide-bonded protein domains produce the mature form of the protein subunits as a prerequisite for the conformational change necessary for fusion and hence viral infectivity (5).

Influenza is a zoonotic disease, infecting a wide variety of warm-blooded animals, including birds and mammals. In aquatic birds, normal influenza replication takes place in the intestinal tract and tends not to cause symptoms. In mammals like humans and swine, influenza replication is limited to epithelial cells of the upper and lower respiratory tract. This tissue tropism is controlled to some extent by the limited expression of the appropriate protease for viral activation (6). In mammals, the suspected protease in the respiratory tract is tryptase Clara, a serine protease produced by nonciliated Clara cells of the bronchial and bronchiolar epithelia (6).

Occasional avian influenza strains have been described with an insertion mutation at the cleavage site of HA, allowing HA to be cleaved by ubiquitously expressed proteases (furin and other subtilisin family proteases) (6). As a consequence, the virus can replicate throughout the bird's body, producing necrotic foci in spleen, liver, lung, and kidney and encephalitic lesions in brain (7). These highly virulent strains have been observed in only two of the 14 described HA subtypes in birds (6) and include those influenza strains previously described as fowl plague viruses. They emerge only occasionally but can cause devastating mortality in poultry flocks (8).

The insertion responsible for the ubiquitous cleavage adds additional basic amino acids at the cleavage site (9), with a minimal motif of R/L-X-R/L-R. Until recently, this mutation had been found only in avian viruses of the H5 and H7 subtypes, subtypes that were not thought to infect humans. This barrier was broken dramatically in 1997 in Hong Kong when 16 people were infected with an avian H5N1 influenza virus (10). Five people died of complications of infection, including the index case, a 3-year-old child who died with Reye's syndrome (11). The A/Hong Kong/156/97 (H5N1) virus isolated from the 3-year-old child possessed the cleavage site mutation typical of virulent avian influenza viruses (12, 13). Of the 12 patients clinically described (11), seven had pneumonia and/or acute respiratory distress syndrome. Gastrointestinal symptoms and impaired hepatic and renal function also were described. Whether the cleavage site mutation in these cases gave the virus the tissue pantropicity it showed experimentally in chickens (12) is not known, and definitive viral replication outside the respiratory tree in these patients was not observed (11). Nevertheless, it may be that the cleavage site mutation in these cases contributed to the lethality of the virus.

Although the HA cleavage site mutation had not been found previously in humans, an influenza strain with the ability to replicate outside its normal host cells was described >50 years ago. WSN/33 was produced in 1940 by forcing the parent strain, WS/33, to replicate in mouse brain (14) to develop an animal model for the observed neurologic complications associated with the 1918 influenza. The strain was passaged extensively in ferrets, in chicken eggs, in mouse lung, and finally in mouse brain. Although this strain was believed initially to be specifically pneumotropic and neurotropic, producing a lethal encephalitis in mice, it recently has been shown to be pantropic or capable of systemic infection in mice (15).

This leads us back to the paper by Goto and Kawaoka in this issue of *Proceedings* (1). They describe a functional model of how the HA protein of WSN/33 is cleaved more readily, synthesizing data from experiments going back over 25 years. One of the unusual properties of WSN/33 is its ability to undergo HA cleavage activation in tissue culture without the addition of exogenous trypsin. As early as 1973, Lazarowitz, *et al*. (2) were able to explain this observation with the finding that WSN/33 had its HA cleaved by serum plasmin. They

Abbreviations: NA, neuraminidase; HA, hemagglutinin.

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demonstrated that, in the absence of serum or in plasminogen-free serum, no HA cleavage occurred. Schulman and Palese (3) showed that the NA protein of WSN/33 was a necessary component for HA cleavage, and in 1993, Li *et al*. (4) found that the NA of WSN/33 lacked a crucial glycosylation site at residue 146 (residue 130 for WSN/33, which has an in-frame 48 base deletion). Restoration of this glycosylation site by reverse genetics yielded a mutant WSN/33 virus that could not undergo replication in tissue culture without exogenous trypsin. What the above papers lacked was a coherent model to synthesize these observations. The current study describes a model whereby plasminogen binds specifically to the NA of WSN/33. In so doing, the WSN/33 NA sequesters plasminogen on the cell surface so that it can be activated. Once activated, plasmin, also a serine protease, recognizes the single arginine motif at the cleavage site and cleaves HA_0 into HA_1 and HA_2.

The NA of WSN/33 differs from the NA of its parent strain, WS/33, by >1 dozen amino acids. However, Goto and Kawaoka show convincingly that one key difference accounts for the ability of WSN/33 NA to bind plasminogen. The change is at the previously identified loss of a glycosylation site (4) at residue 146 (N2 numbering). The N146R change in WSN/33 alters the N-X-S/T motif necessary for posttranslational glycosylation of NA (4). In a revealing experiment, they produced a mutant NA of the parent virus, WS/33, lacking the conserved glycosylation site by substituting an arginine for asparagine (recreating the WSN/33 sequence at that site). Significantly, this NA also bound plasminogen.

Goto and Karaoka (1) also show that an amino acid shared between WS/33 and WSN/33, the carboxyl-terminal lysine at residue 453, is also necessary for plasminogen binding. The carboxyl-terminal lysine is a feature common not only to WS/33 and WSN/33 but is a general feature of NAs of the N1 subtype. In their study, K453R or K453L mutants of WSN/33 also lost the ability to bind plasminogen. Finally, they were able to demonstrate plasminogen binding to WSN/33 NA by flow cytometry. WSN/33 NA was expressed on the surface of 293T cells. The cells were incubated with plasminogen and detected with an anti-human plasminogen antibody. Plasminogen binding was not observed with the K453R mutant of WSN/33 nor with cells expressing the NA of A/Hong Kong/68 (H3N2).

WSN/33 initially was developed in a series of experiments designed to produce a neurovirulent influenza virus, and it is now known that enhanced HA cleavability conferred by WSN/33 NA is related to its neurovirulence. However, convincing *in vivo* and *in vitro* experiments by Sugiura and Ueda (16) and Nakajima and Sugiura (17) demonstrated that WSN/33 neurovirulence required the matrix (M) and nonstructural (NS) segments in addition to NA. Reassortant viruses containing only NA derived from WSN/33 could not produce lethal encephalitis in immunocompetent mice. Genes from WSN/33 M and NS segments seemed to act as accessory virulence factors to enable efficient viral replication. Reassortants without WSN/33 NA demonstrated uncleaved HA as would be expected given the model of Goto and Kawaoka (1). What these experiments show, however, is that the biological behavior of neurovirulence is polygenic and cannot be fully explained by potentially pantropic HA cleavage alone.

Whereas Goto and Kawaoka's model (1) is based on a laboratory-derived influenza strain, the broader question raised by their work is the role enhanced HA cleavability or altered tissue tropism may play in the virulence of naturally arising human influenza strains. The 1918 "Spanish" influenza virus caused a pandemic, killing 20–40 million people. It killed an unusually high number of young healthy adults (18). Although most deaths were the consequence of secondary bacterial pneumonias (there were no antibiotics available in 1918), a subset died in just a few days with massive pulmonary hemorrhage or edema. Was the lethality of the 1918 influenza a result of enhanced hemagglutinin cleavability? After the cleavage site mutations were characterized in lethal avian H5 and H7 strains, it was suggested that the 1918 strain may have possessed a similar HA cleavage site mutation (19). Because no viral isolates were made during the pandemic, this virus was thought to be lost for direct analysis. In 1997, however, small fragments of viral RNA were obtained for sequence analysis from an autopsy sample of a victim of the 1918 influenza. The initial characterization of the virus confirmed the H1N1 subtype and demonstrated that the 1918 HA did not possess the cleavage site mutation seen in the lethal H5 and H7 viruses (20). This finding eliminated the HA cleavage site mutation as an appealing explanation for the virulent behavior of the 1918 virus.

Perhaps, however, as Goto and Kawaoka (1) suggest, the WSN/33 model of influenza viral activation will help explain the virulence of the 1918 strain. Intriguingly for this hypothesis, the small fragment of the 1918 NA reported last year (20) was a perfect match for WSN/33. Because the 1918 HA did not have the cleavage site mutation, an examination of its NA gene for the N146R change of WSN/33 and for the carboxyl-terminal lysine residue important in plasminogen binding will be very interesting.

The feature described by Goto and Kawaoka (1) only has been observed in a laboratory-derived influenza strain and not in a wild-type virus, so it remains to be seen whether their model of HA cleavage would indeed confer enhanced pathogenicity or tissue pantropicity to a virus other than WSN/33. Does the NA change confer plasmin cleavability on all HAs or just the HA of WSN/33? Conformational differences other than the basic amino acid(s) in the cleavage site can effect protease activity. The glycosylation pattern of HA already has been shown to be critical for cleavage activation in at least one case (19). Furthermore, NA-mediated HA cleavability is necessary but not sufficient for neurovirulence. What other gene changes are present in WSN/33 that give it the ability to cause lethal murine encephalitis? The reassortant viruses described by Sugiura and Ueda (16) suggest that the WSN/33 M and NS segments also are needed to confer neurovirulence. What are the specific changes in these genes? Of interest, Francis and Moore (14) were only able to produce this neurovirulent strain in 1940 by using the parent virus WS/33. All attempts to produce neurovirulent variants of Sw/Iowa/15/30 and PR/8/34 were unsuccessful (14). What accounts for the failure of these related strains to adapt to mouse brain? Finally, is WSN/33 just an unusual laboratory-derived strain, or does it recapitulate genetic and functional features of the 1918 virus?

WSN/33 and another independently derived neurovirulent derivative of WS/33, NWS/33 (21), were produced almost 60 years ago to model the unusual neurologic sequelae reported during the 1918 pandemic. Most notable among these was the coincident pandemic of encephalitis lethargica and postencephalitic Parkinsonism described by von Economo (22). Despite intensive efforts, no causal link between influenza and encephalitis lethargica can be made. Jordan, in a review on epidemic influenza (23), wrote: Until a coincidence or close sequence of the two diseases has been more convincingly demonstrated, it seems premature to discuss the existence of a neurotropic influenza virus or to speculate on the activation of an encephalitis virus by an influenza virus, or on the nature of the encephalitic component of a complex grippe virus. Although written in 1927, the intriguing questions he raised still cannot be adequately addressed (24, 25).

Clinical symptoms and pathological findings during the 1918 influenza pandemic were predominantly respiratory ones. Necrotic lesions in systemic organs like those seen in virulent avian influenza infections were not observed. From a histopathologic standpoint, this suggests that the 1918 virus, even if pantropic, did not behave like a fowl plague virus does in infected birds. On the other hand, a subset of 1918 flu victims

died quite suddenly with massive pulmonary edema or hemorrhage. These changes were not unique to the 1918 pandemic but have been observed, on a much lesser scale, in the 1957 pandemic and other flu outbreaks. In my view, the histologic changes suggest a direct cytopathic effect, perhaps with damage to vascular endothelium of the respiratory system. Could this be the result of enhanced HA cleavability allowing, if not pantropicity, at least a relaxation of the usual exclusive pneumotropism? Only by sequence analysis of the 1918 virus can we begin to address these questions. Thanks to the model of Goto and Kawaoka (1), another potential explanation of the virulence of the 1918 flu can be explored.

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