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Special Topic **Review**

Avian influenza H5N1: an update on molecular pathogenesis

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Avian influenza A virus constitutes a large threat to human health. Recent outbreaks of highly pathogenic avian influenza H5N1 virus in poultry and in humans have raised concerns that an influenza pandemic will occur in the near future. Transmission from avian species to humans remains sporadic, but the mortality associated with human infection is very high (about 62%). To date, there are no effective therapeutic drugs or a prophylactic vaccines available, which means that there is still a long way to go before we can eradicate or cure avian influenza. This review focuses on the molecular pathogenesis of avian influenza H5N1 virus infection. An understanding of the viral pathogenesis may facilitate the development of novel treatments or effective eradication of this fatal disease.

avian flu, H5N1, acute lung injury

1 Introduction

In the past decade, two respiratory viruses – Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) and avian influenza H5N1 virus – have emerged and caused mortality among infected individuals. In addition, these viruses have created heavy economic burdens on society. Recently, the outbreak of highly pathogenic avian H5N1 influenza virus in poultry and humans (through direct contact with infected avian species) has raised concerns that an influenza pandemic may occur in the near future. Although it is imperative to develop effective measures to prevent influenza pandemics, neither successful strategies nor effective drugs are currently available. Acute lung injury (ALI) is the major manifestation associated with patients hospitalized with H5N1 influenza infection. Many of these patients progress rapidly to acute respiratory distress syndrome (ARDS) and multi-organ failure[1−3]. According to the most recent World Health Organization data, reported on March 11, 2009, the cumulative mortality attributed to avian influenza H5N1 virus infection is greater than 60% (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_03_11/en/index.html). In order to permit eradication of pathogenic avian influenza viruses and to prevent related diseases, many researchers have emphasized the need for research on pathogenesis, epidemiology, therapy, and vaccines[4]. This review focuses on the molecular pathogenesis of the avian influenza H5N1 virus, which may facilitate the development of novel and effective therapeutic drugs or preventative measures for this fatal disease.

2 Overview of avian influenza H5N1 virus

2.1 Virus structure and biology

Influenza A virus belongs to the *orthomyxovirdae* family. It is an enveloped virus that ranges in size from 80 to 120 nm[5]. Its genome consists of eight segments of single-stranded negative sense RNA, which encode 10 or 11 proteins depending on the isolate[5].

Hemagglutinin (HA) and neuraminidase (NA) are two surface glycoproteins encoded by separate RNA segments. Influenza A viruses can be classified according to

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the antigenicity of HA and NA. Currently, 16 HA subtypes (H1−H16) and nine NA subtypes (N1−N9) have been identified among type A influenza viruses[3,5,6]. The "H" and "N" subtypes seem to be able to assort in any combination, and many of the 144 possible combinations have been found in natural reservoir species; although, some combinations are more common than others[5].

HA is involved in the attachment of viruses to host cells receptors, which are predominantly believed to be the terminal sialic acid residues on host cell glycoproteins and glycolipids. Following viral entry into internal acidic compartments, HA is involved in fusion of the viral and cellular membranes, resulting in the release of virion contents into cells[7]. NA-mediated cleavage of sialic acid from the cell surface is involved in two stages of the viral life cycle: (i) during virus entry, NA promotes virus access to target cells in airways by removing decoy receptors on mucins, cilia, and cellular glycocalix[8]; and (ii) at the final stage of infection, NA cleaves sialic acid from the cell surface and progeny virions, facilitating virus release from infected cells[9].

Other proteins encoded by the viral genome include the polymerase PB1, PB2, and PA; the nucleoprotein NP; the non-structural protein NS1; and the matrix proteins M1 and M2. Most of these proteins also play important roles in virus adaptation and pathogenicity[3,6]. For example, the NS1 from avian influenza H5N1 virus is thought to cause cytokine dysregulation, which may play an important role in the pathogenesis of this viral infection. A recombinant human influenza H1N1 virus expressing the NS1 from the 1997 H5N1 virus showed increased pathogenicity in pigs[10].

Depending on their virulence, avian influenza viruses can be classified as either highly pathogenic avian influenza (HPAI), which usually kills birds within one week, or low pathogenic avian influenza (LPAI), which causes minimal illness in birds[6,11]. In recent years, the most commonly reported HPAI strain has been H5N1. Avian influenza H5N1 virus emerged as an avian pathogen in 1996 and was isolated from a patient in 1997[3,12]. Since then, this strain has circulated in birds and spread to large parts of the world, leading to sporadic human infections, which are thought to be caused by direct contact with infected avian species in most cases. There have been rare reports of human-to-human transmission of this virus. By March 11, 2009, 411 cases of humans infected with avian influenza H5N1 virus had been reported, leading to 256 deaths and a cumulative case fatality rate of 62% (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_03_11/en/index.html).

2.2 Antigenic shift and drift

The segmented nature of the influenza genome facilitates reassortment between different strains and lineages of influenza during concomitant infection. This reassortment can yield major genetic changes, referred to as antigenic shifts[7], generating a virus to which the population may lack immunity. By contrast, antigenic drift is due to the accumulation of point mutations generated during RNA replication by an error-prone RNA-dependent RNA polymerase[7,13]. Both antigenic drift (mutation) and antigenic shift (reassortment) contribute to the high adaptability of the virus, and its ability to evade the immune system[5]. The winter human influenza season, the scattered and sporadic transmission of avian influenza to humans due to the wide-spread distribution of H5N1 in birds and other mammals, and the development of mutations in the avian influenza virus genome under selective pressure all increase the likelihood of a pandemic in the future[11]. Therefore, some experts believe that it is not a question of whether there will be another influenza pandemic, but a question of when it will occur[11,14].

2.3 Host range and tissue tropism

The natural host reservoir for avian influenza virus is waterfowl, but the virus can be transmitted to other species, including humans. It should be noted that isolation of a virus from one species does not mean that this species is a natural host or reservoir for the virus. However, on rare occasions, an avian influenza virus may adapt to that species, which may then serve as a reservoir[5].

The binding specificity of the influenza A HA protein for glycoproteins and glycolipids on the host cell determines the host specificity and tissue tropism of these influenza viruses. Traditionally, it was thought that avian influenza viruses preferentially bind to cell surface receptors that have 2,3-sialic acids linked to a galactose residue (SAα2,3-Gal), while human lineage viruses prefer to bind receptors consisting of terminal sialic acids with 2,6 linkages to galactose residues associated with glycoproteins or glycolipids(SAα2,6-Gal)[15,16]. While the tracheal epitheliums of birds and humans mainly express virus receptors with α2,3 and α2,6 sialic acid linkages, respectively, pigs express both types of recep-

tors[17]. This expression pattern provides solid molecular evidence that pigs serve as "mixing vessels" for human and avian influenza viruses[18,19], which facilitates the reassortment and generation of new pandemic strains that may have the potential for human-to-human transmission. It has recently been suggested that the distribution of terminal SAα2,3-Gal and SAα2,6-Gal within the respiratory tract may not be as clear cut as has been previously reported[20]. Other viral and host components may act as determinants of successful viral replication and transmission[20,21].

3 Clinical manifestations

3.1 Clinical symptoms – not just involving the lung

The clinical spectrum of H5N1 infection has ranged from asymptomatic infection or mild influenza-like illness to severe pneumonia, ARDS, and multi-organ failure[2]. Fever (> 38℃), cough, and dyspnoea are the major symptoms upon presentation[2]. The mortality and morbidity associated with influenza infection are not attributable to respiratory disease alone. For example, gastrointestinal symptoms, such as watery diarrhea, vomiting, and abdominal pain, are common in the early stage of the disease[2]. In addition, viral RNA has been detected in autopsy samples of multiple non-respiratory organs, including the intestines, liver, spleen, and brain, suggesting widespread viral dissemination[3]. Some reports have indicated a correlation between influenza infection and cardiovascular diseases, such as ischemic stroke and myocardial infarction[22]. There have been two cases of fatal avian influenza H5N1 virus infections without apparent respiratory symptoms, but with acute encephalitis[23]. Together, these findings indicate that avian influenza infection is not restricted to the lung and that the infection spectrum of avian influenza H5N1 virus may be wider than had been previously thought.

3.2 Laboratory and radiographic findings – how to diagnose

Laboratory findings of human influenza H5N1 virus infection include leukopenia, lymphopenia, mild-to-moderate thrombocytopenia, and elevated levels of aminotransferases[1]. These findings are common but not universal to influenza H5N1 virus infection. Other reported abnormalities have included elevated levels of creatine phosphokinase, hypoalbuminemia, increased D-dimer levels, and changes indicative of disseminated intravascular coagulopathy[1]. Radiographic abnormalities of influenza H5N1 virus infection include multifocal airspace consolidation, interstitial infiltrates, and patchy or lobar involvement with rapid progression to bilateral and diffuse ground-glass opacities[2], which are typical changes associated with ARDS.

It should be noted that these clinical, laboratory, and radiographic features are not specific to influenza H5N1 virus infection. To confirm influenza H5N1 virus infection, an exposure history, including close contact with sick or dead poultry, wild birds, or other severely ill persons, as well as travel to an area with influenza H5N1 virus activity or work in a laboratory handling samples possibly containing influenza H5N1 virus, is required[2].

3.3 Acute lung injury – the main reason for death

In 1918, many people died of a new and uncharacterized pneumonic illness. In the lungs of individuals who had died of this illness, clinicians and pathologists found "homogeneous structureless non-cellular exudates which fill the bronchioles and…forms as it were a plastering round the inside of the alveoli…and is very similar to that which can be seen in fatal cases of poisoning by chlorine gas[24]". This pattern of injury is termed diffuse alveolar damage (DAD) or ALI[22] and is the histological change associated with ARDS. This illness was caused by avian influenza H1N1 virus. The epidemic, famously known as the "1918 Spanish Flu", killed 50−100 million people[25].

In patients infected with influenza H5N1 virus, diffuse alveolar damage with hyaline membrane formation, patchy interstitial lymphoplasmacytic infiltrates, bronchiolitis with squamous metaplasia, and pulmonary congestion with varying degrees of hemorrhage[1] are often observed, and considered typical ALI changes. ALI is a common reaction to pneumocyte (mainly type I[22]) damage and may be initiated by noxious gases or infective agents, including SARS-CoV and influenza. The mortality rate of ALI is high and there are few therapeutic options other than mechanical ventilation and supportive clinical care.

4 Molecular pathogenesis of acute lung injury

4.1 Innate immunity and the TLR4 pathway

Hallmarks of ALI include acute neurtophilic infiltration and proinflammatory cytokine secretion in the lungs, which suggest that the innate immune response is activated during ALI. However, the role that innate immu-

nity plays in ALI was not clear until recently.

Imai et al.[26] recently reported that, in the gastric acid aspiration model of ALI, TLR4 knockout mice were protected from lung injury. TLR4 is the receptor for Gram-negative bacterial lipopolysaccharide (LPS). Furthermore, the TLR4 signaling pathway in macrophages can be either MyD88-dependent or -independent (TIR-domain-containing adaptor-inducing interferon-β, TRIF pathway), activating early-phase NF-κB or late-phase NF-κB, respectively[27]. In this acid-induced lung injury model, the TLR4-TRIF-TRAF6-NF-κB signaling pathway was found to be the key signaling pathway linking acid aspiration to the severity of ALI[26]. The production of interleukin 6 (IL-6) is likely involved in the phenotype of this model, since IL-6 was found to be upregulated in acid-treated mice and because IL-6-deficient mice exhibited a significant improvement in lung function[26].

TLR4 is the receptor for LPS and it is usually activated during bacterial infection. Under this non-infectious condition, the ligand that activates the TLR4 signaling pathway is not known. Previously, it has been reported that minimally oxidized LDL can activate TLR4 on macrophages[28]. Imai and colleagues observed reactive oxygen species (ROS) in alveolar macrophages following *in vivo* acid challenge and found that ROS led to the production of oxidized phospholipids (OxPLs). They further showed that an OxPL induced cytokine production and acute lung injury via TLR4[26]. This OxPL was found to be oxidized 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (OxPAPC), as revealed by use of a monoclonal antibody[26]. In summary, in this acid aspiration lung injury model, OxPAPC was shown to stimulate IL-6 production and trigger lung injury via the TLR4-TRIF-TRAF6 pathway, in contrast to the LPS-induced signaling pathway, which is dependent on MyD88.

In patients infected with lethal avian influenza H5N1 virus, significant amounts of OxPAPC have been detected, indicating that the pathway described above may play an important role in influenza H5N1 virus-induced ALI. Indeed, mice that were treated with inactivated influenza H5N1 virus exhibited ALI; however, the severity of ALI in TLR4- or TRIF-knockout mice was attenuated. In addition, inactivated influenza H5N1 viruses can induce ROS formation and TLR4 surface expression on primary human monocytes, and loss of NCF1, a key component of the NADPH oxidase complex required for oxidative burst and ROS formulation, improves the severity of H5N1 induced ALI[26]. Together, these data indicate that influenza H5N1 virus can cause ALI through the ROS-TLR4-TRIF pathway.

4.2 Cell death and lung injury

Cell death, largely due to apoptosis and necrosis, has been demonstrated in the lung during the pathogenesis of ALI/ARDS[29,30]. Apoptosis has been observed in the alveolar epithelial cells of influenza H5N1 virus-infected humans, suggesting that apoptosis may play a major role in the pathogenesis of this virus in humans by destroying alveolar epithelial cells[31]. In addition, apoptosis of lymphocytes has been reported in mice infected with lethal influenza H5N1 virus[32]. However, whether the observed apoptotic cell death was a direct result of viral replication or a consequence of cytokine dysfunction is currently not clear.

In addition to necrosis and apoptosis, alternative cell death mechanisms, including autophagy and oncosis, have recently been described. Autophagy and oncosis have been indicated to be involved in ALI caused by other agents[29,33], but whether they are involved in influenza H5N1 virus-induced ALI requires further investigation.

4.3 Other pathways

It has been demonstrated that the renin-angiotensin system (RAS) plays a key role in SARS-CoV-induced ALI. The downregulation of a "good ACE" (ACE2) by SARS-CoV leads to an increase in angiotensin II, which binds to its receptor AT1aR and aggravates lung injury[34,35]. Due to similarities in clinical symptoms and ALI manifestations caused by SARS-CoV and avian influenza H5N1 virus, we suggest that RAS may also be involved in influenza H5N1 virus-induced lung injury.

5 Conclusions

There is a relative consensus among virologists, clinicians, and officers of disease control organizations that an influenza epidemic may occur in the near future, with influenza H5N1 virus being the most probable pathogen. Understanding of the pathogenesis, including the molecular mechanisms of pathogenesis, of this fatal disease is of vital importance, considering the limited availability of anti-viral drugs and vaccines. We believe that findings from future research, especially research focusing on pathogenesis, will lead to more promising therapeutic strategies and prevention methods.

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