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Special Topic **Article**

Influenza A virus H5N1 entry into host cells is through clathrin-dependent endocytosis

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Influenza A virus H5N1 presents a major threat to human health. The entry of influenza virus into host cells is believed to be mediated by hemagglutinin (HA), a virus surface glycoprotein that can bind terminal sialic acid residues on host cell glycoproteins and glycolipids. In this study, we elucidated the pathways through which H5N1 enters human lung carcinoma cell line A549. We first proved that H5N1 can enter A549 cells via endocytosis, as lysosomotropic agents, such as bafilomycin A1 and chloroquine, can rescue H5N1-induced A549 cell death. By using specific inhibitors, and siRNAs that target the clathrin pathway, we further found that H5N1 could enter A549 cells via clathrin-mediated endocytosis, while inhibitors targeting caveolae-mediated endocytosis could not inhibit H5N1 cell entry. These findings expand our understanding of H5N1 pathogenesis and provide new information for anti-viral drug research.

influenza A, H5N1, endocytosis, lysosomotropic agents, clathrin

Recent outbreaks of highly pathogenic avian influenza H5N1 in poultry and humans have raised the concern that a pandemic will occur in the near future. H5N1 was first isolated from a patient in Hong Kong in 1997[1]. Since then, H5N1 has circulated in birds and spread to large parts of the world, with sporadic human infections. By March 11, 2009, 411 cases of human infection with H5N1 have been reported, 256 of which were fatal, with a cumulative case fatality rate of 62% (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_03_11/en/index.html). Since there are no effective drugs or vaccines for this infection, health professionals from the WHO and the CDC believe the question is not if we are going to have another avian influenza pandemic, but when it will occur[2,3].

Entry of enveloped viruses into target cells can be through either direct membrane fusion at the cell surface or via endocytosis. While some viruses do not need endocytosis for entry, most are likely to use one or more of the various pathways of endocytosis. It has now been proven that the endocytic pathways that viruses can employ include: clathrin-mediated endocytosis, caveolae/lipid rafts-mediated endocytosis, macropinocytosis, and a non-clathrin-, non-caveolae-dependent endocytosis, which is not well defined[4, 5]. Among these pathways, clathrin-mediated endocytosis is the most commonly observed uptake pathway for viruses. After binding to its receptors, the virus is sequestered into clathrin-coated pits, internalized by receptor-mediated endocytosis in clathrin-coated vesicles and delivered to endosomes, where the virus will penetrate in the acidic milieu[6,7].

The virus surface glycoprotein hemagglutinin (HA) is believed to mediate influenza virus entry into host cells by binding to terminal sialic acid residues on the host cell surface and mediating the fusion of the viral membrane with the cellular membrane within acidic compartments within cells. The mechanism of influenza vi-

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rus entry has been widely studied; however, all the studies have been carried out with virus strain H1N1, and most of the studies have used the canine kidney cell line MDCK. It has been reported that the influenza virus can enter cells via clathrin-mediated endocytosis[8,9], caveolae-mediated endocytosis[9,10], or non-clathrin-, non-caveolae-dependent endocytosis[11], depending on the different cell lines or virus strains used. However, since the risk of an H5N1 pandemic is increasing due to the widespread occurrence of birds infected with H5N1 and the lack of effective drugs or vaccines, it is imperative to understand the mechanism of the disease caused by H5N1 in humans. In this study, we used the H5N1 strain and a cell line from human lung tissue, the main target of H5N1 infection, and found that the H5N1 virus can enter a human lung carcinoma cell line via clathrin-dependent endocytosis.

1 Materials and methods

1.1 Cells and virus

The human lung adenocarcinoma A549 cell line was purchased from ATCC and cultured in F-12/HAM'S (Hyclone) medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin at 37℃ in 5% carbon dioxide.

The influenza virus used in this study was the H5N1 virus (A/Jilin/9/2004(H5N1)) from Professor JIN NingYi, the Institute of Military Veterinary Medicine, Academy of Military Medical Sciences (Changchun, China). The virus was inoculated into 10 to 11-day-old SPF embryonated fowls' eggs by the allantoic route. Hemagglutinating allantoic fluid was collected from eggs and the virus was inactivated using formaldehyde.

1.2 MTT assay

A549 cells were seeded in 96-well-plates at 1×10^5 cells/mL one day before they were treated with either the indicated amount of inactivated H5N1 or an equal volume of vehicles for the indicated time at 37℃. The cell viability was then determined with a CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega according to the manufacturer's instructions.

1.3 Drug treatment to block virus entry

Drug inhibition experiments were performed as previously described[12]. Briefly, A549 cells were pretreated with the indicated amount of chloroquine, bafilomycin A1, chlorpromazine (CPZ), methyl-β-cyclodextrin (MβCD), nystatin or filipin (Sigma-Aldrich) for 1 h at 37℃ before they were incubated with inactivated H5N1 for 3 h. Cell viability was then determined as described above.

1.4 siRNA and Western blot

A549 cells were transfected with siRNA against either the clathrin heavy chain (CHC) or control siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. At 24 h, the cells were trypsinized and reseeded in 96-well-plates. After another 24 h, the cells were treated with the inactivated virus and cell viability was determined as described above. To eliminate any non-specific knockdown effect of siRNA, siRNAs from two manufacturers were used: one from Santa Cruz Biotechnology, the other synthesized by Guangzhou Ribobio Co., Ltd. The target sequence was 5′-TAATCCAATTCGAAGACCA AT-3′.

The effect of siRNA knockdown was determined 48 h after siRNA transfection by Western blot using a clathrin heavy chain antibody from Santa Cruz Biotechnology.

1.5 CTB uptake assay

A549 cells were plated on coverslips the day before they were used. Cells were mock treated or pretreated as described above and incubated with 5.0 μg/mL cholera toxin subunit B (CTB) conjugated to Alexa-488 (Invitrogen) for 15 min at 4℃. The cells were then transferred to 37℃ for another 15 min. The cells were washed with 0.1 mol/L glycine, 0.1 mol/L NaCl at pH 3.0 before they were fixed and analyzed with a confocal microscope (Leica TCS SP2). Specific PMT adjustment for 488 was used. Images were captured using a 100× oil objective (Plan-Apo 1.4) at room temperature with the confocal acquisition software LCS (Leica).

1.6 Statistical analyses

All data were shown as mean ± SD. and statistical analyses were conducted using two-tailed Student's *t*-test.

2 Results

2.1 H5N1 caused A549 cell death

It has been reported that the influenza virus can induce cell death in numerous cell types[13,14], but the degree

and extent of cell toxicity caused by the influenza virus may vary depending on the specific cell type and virus strain. To determine whether our strain of H5N1 can cause A549 cell death, we first incubated A549 cells with the inactivated H5N1 virus then measured cell viability with MTT. We found that this strain of H5N1 can cause A549 cell death in a dose- and time-dependent manner (Figure 1A and B).

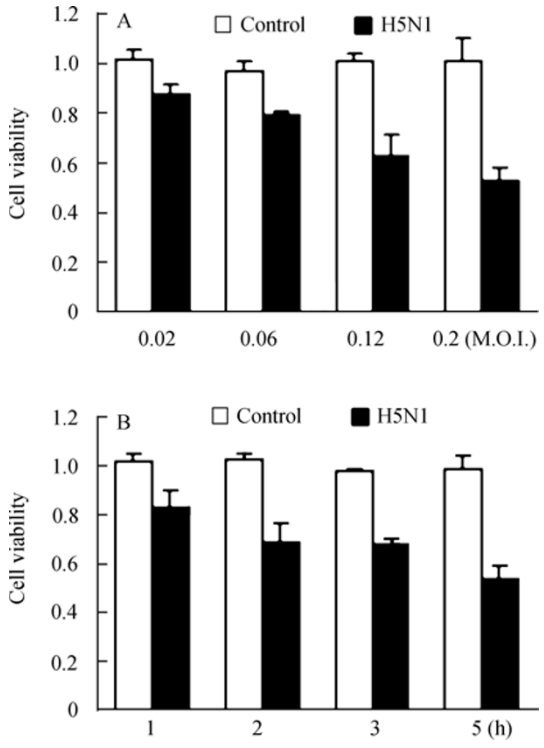

Figure 1 Inactivated H5N1 caused A549 cell death in a dose-dependent, time-dependent manner. A, Cell viability of A549 cells after 3 h treatment of the indicated amount of inactivated H5N1 or the same volume of allantoic fluid; B, Cell viability of A549 cells after 0.12 M.O.I. inactivated H5N1 or the same volume of allantoic fluid treatment for the indicated time.

2.2 H5N1 enters A549 cells via endocytosis

Entry of enveloped viruses into host cells can be through either endocytosis or direct membrane fusion. In the endocytic entry pathway, the endocytosed virions are typically subjected to an acidic pH, which results in fusion of the viral membrane with endosomal membranes, releasing the viral genome into the cytosol. Therefore, the endocytic pathway is usually thought to be pH-sensitive while the direct membrane fusion pathway is usually pH-independent. To determine whether the H5N1-induced cell death is related to endocytosis, we pretreated A549 cells with lysosomotropic agents, which elevated the pH of acidic organelles, thus blocking endocytosis. We found that, compared with mock-treated A549 cells, chloroquine- and bafilomycin A1-pretreated A549 cells had increased cell viability after H5N1 treatment (Figure 2A and B), indicating that endocytosis is involved in this process.

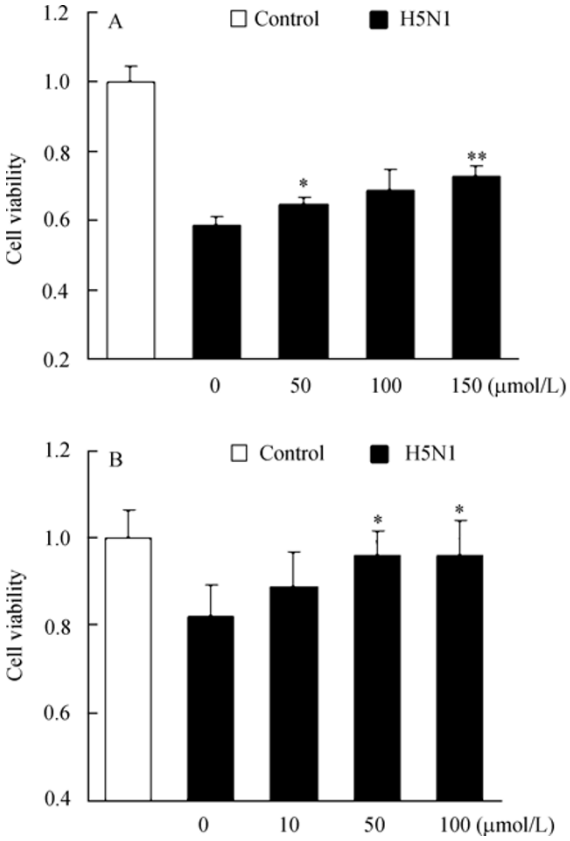

Figure 2 Lysosomotropic agents rescue H5N1-induced A549 cell death. A. Cell viability of A549 cells mock treated or pretreated with the indicated amount of chloroquine before they were treated with 0.1 M.O.I. inactivated H5N1 for 3 h. B. Cell viability of A549 cells mock treated or pretreated with the indicated amount of bafilomycin A1 before they were treated with 0.05 M.O.I. inactivated H5N1 for 3 h. *P<0.05 and **P<0.01, compared with mock-treated (0) cells.

2.3 The role of clathrin-mediated endocytosis in virus entry

After determining that H5N1 can cause cell death via endocytosis, we next attempted to elucidate the specific pathway. The endocytic pathways that viruses use can be clathrin-dependent or -independent. We first tested the role of clathrin-mediated endocytosis.

In the classic clathrin-mediated endocytic pathway, soluble clathrins from the cytoplasm are recruited to the plasma membrane where the clathrin triskelia assembles into a polygonal lattice to form coated pits that bud and

pinch off from the membrane and give rise to clathrin-coated vesicles[15,16]. CPZ can cause clathrin to assemble on endosomal membranes and prevent the assembly of coated pits at the cell surface, thus inhibiting clathrin-mediated endocytosis[17]. We found that pretreatment of A549 cells with CPZ could rescue H5N1-induced cell death (Figure 3A), which suggests that clathrin may be involved in this process.

However, it has been reported that drugs targeting specific endocytic pathways may have pleiotropic effects and may not be specific[4]. To further confirm the role of clathrin-mediated endocytosis, we next used a more specific siRNA approach. As mentioned above, clathrin functions as a triskelion, a three-legged structure, that is assembled by three heavy chains each with an associated light chain[18]. Knockdown of the heavy chain with siRNA has been proven to be an effective and specific method to assay the role of clathrin-mediated endocytosis[12,19,20]. We used two siRNAs against CHC to determine the role of clathrin-mediated endocytosis. Western blots showed that both siRNAs can efficiently knockdown the CHC protein (Figure 3C and D). Additionally, both siRNA treatments significantly enhanced cell viability after treatment with inactivated H5N1 (Figure 3B). All of these results suggest that H5N1 can enter A549 cells via clathrin-mediated endocytosis.

2.4 The role of caveolae-mediated endocytosis in virus entry

It has been reported that in addition to clathrin-mediated endocytosis, the influenza H1N1 virus can also enter cells via caveolae-mediated endocytosis[9]. Caveolae are flask-shaped invaginations of the cell membrane composed of high levels of cholesterol as well as an integral membrane protein, caveolin[21]. Together with lipid rafts, caveolae are cholesterol-rich microdomains. Therefore, sequestering cholesterol with sterol-binding drugs, such as nystatin or filipin, or depletion of cholesterol from cell membranes with MβCD will inhibit caveolae-mediated endocytosis[4,22].

To analyze whether H5N1 can enter A549 cells via caveolae-mediated endocytosis, we used the above three drugs to specifically inhibit this pathway. The effects of these drugs on caveolae-mediated endocytosis were

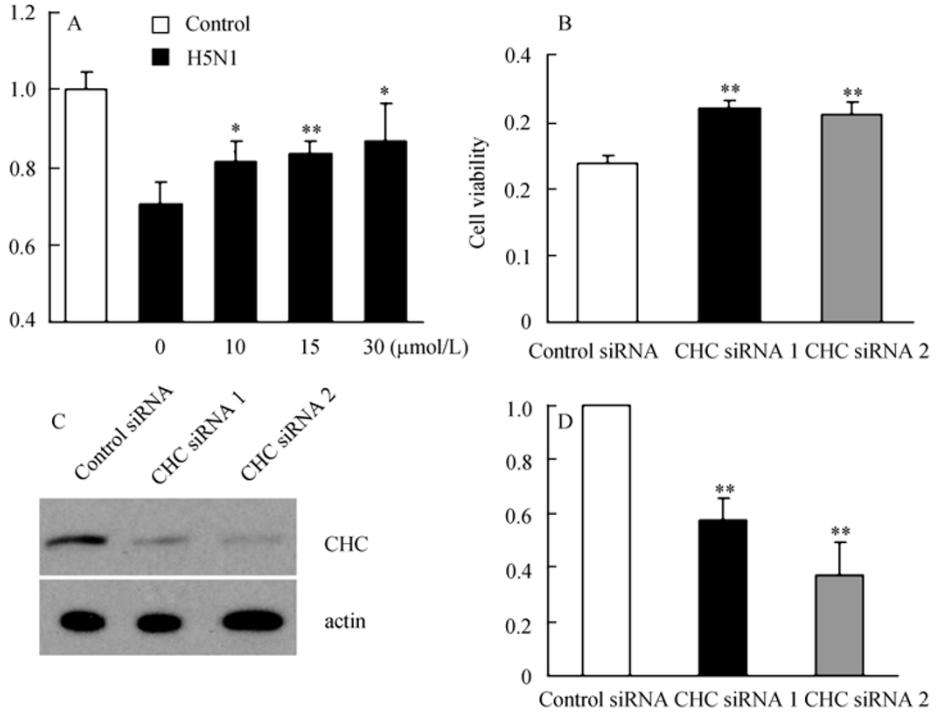

Figure 3 Inhibitors and siRNAs targeting the clathrin pathway can rescue H5N1-induced cell death. A, Cell viability of A549 cells mock treated or pretreated with the indicated amount of chlorpromazine before they were treated with 0.1 M.O.I. inactivated H5N1 for 3 h. B, Cell viability of 0.1 M.O.I. inactivated H5N1-treated A549 cells (6 h) transfected with control siRNA or siRNA against clathrin heavy chain. C, Western blot of control siRNA or siRNA against clathrin heavy chain-transfected A549 cells probed with anti-clathrin heavy chain and actin. D, The relative band density of clathrin to actin was analyzed with Bio-Rad Quantity One software. **P*<0.05 and **P<0.01, compared with mock treated (0), or control siRNA treated cells.

examined by measuring the uptake of CTB since it is targeted to caveolae and its uptake can be blocked by these kinds of drugs[23,24]. Figure 4A shows that, compared with mock-treated A549 cells in which the CTB was internalized within cells, all the drug-treated cells showed inhibited CTB uptake in which the CTBs were clustered on the cell surface, indicating that these drugs work very well on A549 cells. However, these drugs did not rescue H5N1-induced cell death (Figure 4B—D), which suggested that caveolae-mediated endocytosis may not be involved in H5N1 entry into A549 cells.

3 Discussion

A virus must enter into host cells before it initiates its life cycle. Entry of enveloped virus can be through endocytosis or direct membrane fusion, which are pH-dependent or -independent, respectively. The entry of influenza virus was previously studied primarily using the H1N1 strain. Due to the increasing risk of H5N1 outbreaks, it is imperative to elucidate the specific pathway through which H5N1 enters human host cells. In this study, we used a human lung carcinoma cell line and found that H5N1 can enter A549 cells via clathrin-mediated endocytosis.

After determining that H5N1 can induce A549 cell death, we attempted to determine what could rescue H5N1-induced cell death. Lysosomotropic agents were proven to increase cell viability, indicating that H5N1 may cause cell death through endocytosis, as an acidic milieu is an important factor for endocytosis and lysosomotropic agents can elevate the pH of endosomes, thus inhibiting endocytosis. However, it should be noted that although there is no such report as yet, this result does not exclude the possibility that influenza can enter cells via direct membrane fusion, as a virus may employ different pathways to enter different cell types[11,12].

There are many factors that can influence whether a virus uses clathrin-dependent or clathrin-independent endocytosis. Among them, the nature of the virus, no doubt, is the most important factor. A recent study

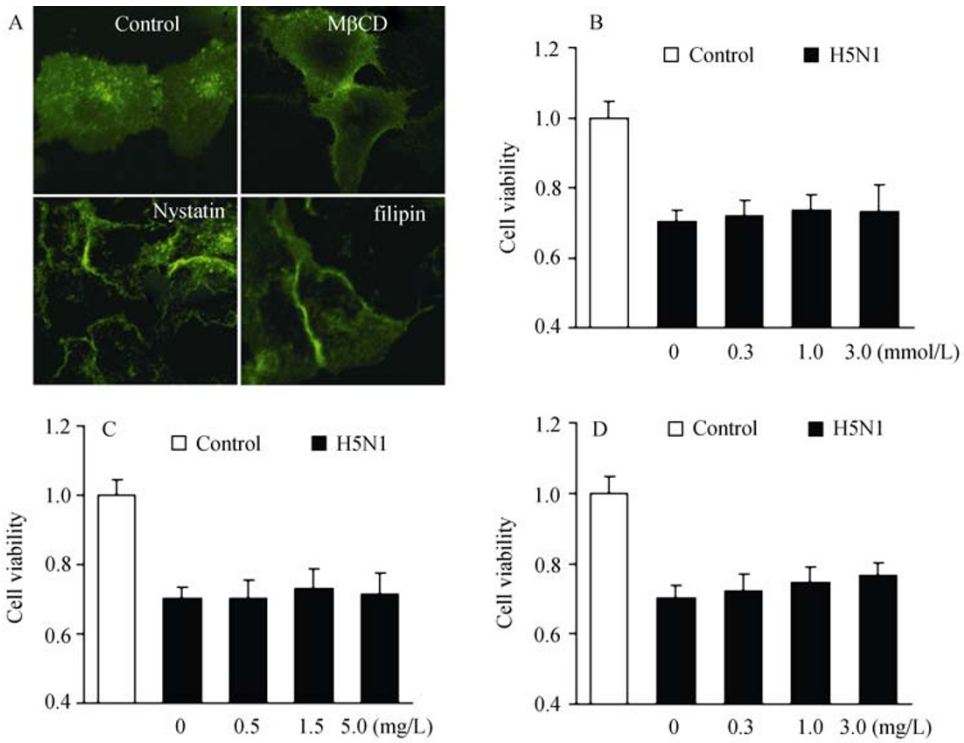

Figure 4 Inhibitors targeting caveolae- or lipid raft-mediated endocytic pathways do not rescue H5N1-induced cell death. A, Uptake of Alexa-488 CTB by A549 cells mock treated or pretreated with 3 mM MβCD, 5 mg/L nystatin or 3 mg/L filipin; B, Cell viability of A549 cells mock treated or pretreated with the indicated amount of MβCD before they were treated with 0.1 M.O.I. inactivated H5N1 for 3 hours; C, Cell viability of A549 cells mock treated or pretreated with the indicated amount of nystatin before they were treated with 0.1 M.O.I. inactivated H5N1 for 3 h; D, Cell viability of A549 cells mock treated or pretreated with the indicated amount of filipin before they were treated with 0.1 M.O.I. inactivated H5N1 for 3 hours.

showed that the size of the endocytosed particle is also an important factor in determining whether clathrin- or caveolae-mediated endocytosis is used[25]. In our study, we found that inhibitors and siRNAs targeting the clathrin pathway could rescue H5N1-induced cell death while drugs inhibiting the caveolae pathway could not, suggesting that H5N1 can enter A549 cells via clathrin-mediated endocytosis.

Recently, it has been reported that the fusion peptide of HA was not associated with cholesterol rich microdomains[26], which supports our data that neither cholesterol-binding nor depletion drugs inhibit H5N1 entry. However, as has been pointed out by Sara et al., there is the possibility that a novel, an uncharacterized type of lipid raft may be involved in virus uptake[11]. The particular relationship between influenza virus and lipid rafts needs further investigation.

Although some researchers have pointed out that specific host factors may be required for H5N1 entry into host cells[27], those factors have still not been identified. It is suggested that the lack of a specific influenza virus receptor is responsible for the many different entry pathways that influenza viruses use to enter different cells[11]. For this reason, it is possible that H5N1, like H1N1, can enter different cell lines via different endocytic pathways.

Taken together, our data indicate that H5N1 can enter A549 cells via clathrin-mediated endocytosis. This finding is relevant for the development of novel therapeutic drugs targeting influenza virus H5N1.

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