

# Avian influenza virus A/chicken/Hubei/489/2004 (H5N1) induces caspase-dependent apoptosis in a cell-specific manner

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**Abstract** The economic damage caused by H5N1 avian influenza virus outbreak in domestic poultry and the threat of this virus to human health make the research of this virus highly significant. During the 2004 outbreak of avian influenza in Hubei province, People's Republic of China, we isolated a new H5N1 subtype avian influenza virus named as A/chicken/Hubei/489/2004(H5N1) (shorten as AIVHubei489 here). In this study, the infectivity and apoptosis-inducing characteristics of AIVHubei489 were studied. We demonstrated that AIVHubei489 could infect MDCK cells and the infection induced apoptosis. Our data also showed that the apoptosis induced by this virus in MDCK cells was caspase activity dependent. Moreover, we proved that caspase 8 but not caspase 9 was involved in this apoptosis. The infectivity and apoptosis-inducing activity of AIVHubei489 in Vero and HeLa cells were also studied. Our results showed that AIVHubei489 could replicate in Vero and HeLa cells, but the infection did not cause apoptosis in either of the two cell lines. Thus, AIVHubei489 induced apoptosis through a caspase-dependent pathway in a cell-specific manner.

**Keywords** Avian influenza virus · Apoptosis · Caspase activity

## Introduction

The opinion of that avian influenza virus can only infect poultry but not human has been changed since the 1997 outbreak of a highly pathogenic H5N1 subtype avian influenza virus in poultry at farms and live animal markets in Hong Kong, during which 18 cases (6 fatal) of human infections with H5N1 avian influenza virus were reported. That was the first known instance of human infection with an avian influenza virus [1]. According to the record of World Health Organization, from 2003 to 2007, there have been 315 persons were infected by H5N1 avian influenza virus and 191 of them were dead [2]. The economic damage caused by H5N1 avian influenza virus outbreak in domestic poultry and the threat of this virus to human health make the research of this virus highly significant.

H5N1 subtype avian influenza viruses belong to type A influenza virus. The genome of type A influenza virus consists of eight segmented negative strand RNA which is highly mutational. It is known that type A influenza viruses replicate with different efficiencies in different cell lines [3]. Some type A influenza viruses are reported to induce apoptosis in infected animals and cultured cells [4–10].

Apoptosis is a kind of gene controlled active cell death which is involved in many physiological processes such as embryogenesis and development etc. It also often occurs when cells are infected by viruses. Though usually apoptosis occurring at early stage of virus infection functions as an effective way to suppress virus replication by the host, the function of type A influenza virus induced apoptosis has not been elucidated yet. Evidence showed that caspase

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3 activation is essential for efficient influenza virus propagation [11].

Apoptosis occurs mainly through two pathways: cell death receptor pathway which involves upstream caspase 8 activation [12] and mitochondria pathway which involves upstream caspase 9 activation [13]. Both the pathways converge at the activation of downstream caspase 3 or 7 [14]. Recent research discovered that there exist caspase-independent apoptotic pathways [15, 16]. Though it has been shown that apoptosis by equine influenza virus infection caspase activation [17], the pathway through which influenza viruses induce apoptosis still remains unclear.

During the 2004 outbreak of avian influenza in Hubei province, People's Republic of China, we isolated a new H5N1 subtype avian influenza virus: AIVHubei489. All the eight genome fragments of AIVHubei489 have been sequenced (GenBank access numbers: gil55233237, gil55233236, gil55233234, gil55233232, gil55233229, gil55233227, gil55233225, and gil55233222.). In this article, we studied the infectivity and apoptosis-inducing characteristics of AIVHubei489 in cultured cells. Our results showed that AIVHubei489 could infect MDCK, Vero and HeLa cells but only caused apoptosis in MDCK cells. Moreover, we demonstrated that apoptosis induced by AIVHubei489 in MDCK cells is caspase activity dependent and might be through death receptor pathway which required caspase 8 activity.

## Materials and methods

### Cell lines and viruses

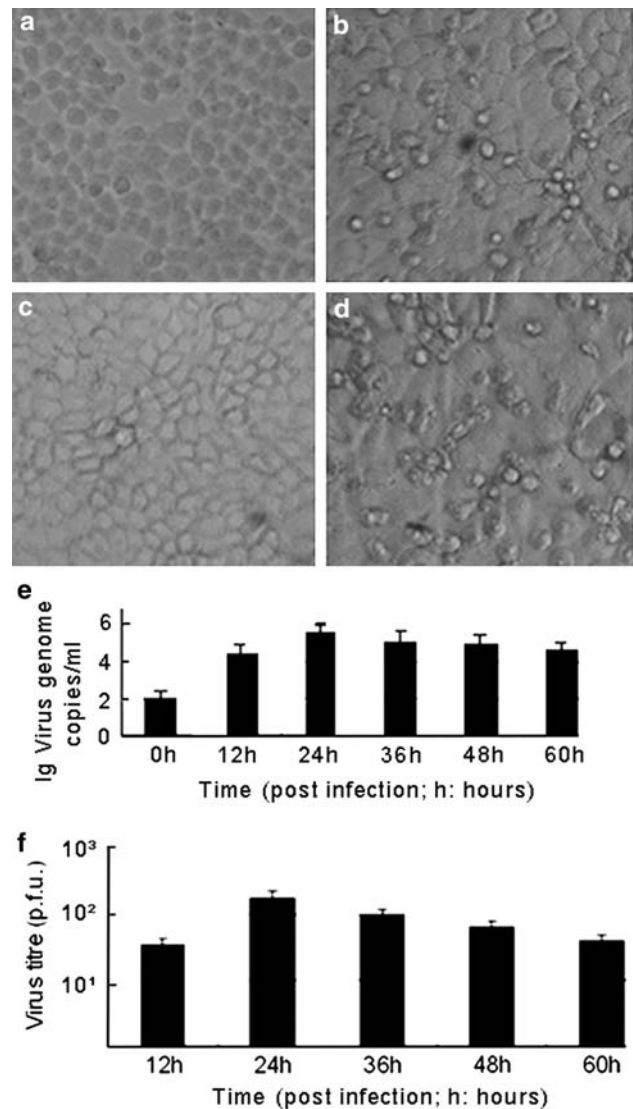
MDCK and HeLa cells were cultured in Eagle's minimal medium (MEM) with 10% (V/V) fetal calf serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Vero cells were cultured in Dulbecco's Modified Eagle medium (DMEM) with 10% (V/V) fetal calf serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Avian influenza virus (AIVHubei489) was propagated in allantoic cavity of 11-day-old embryonated chicken eggs for 48–72 h at 35°C, the allantoic fluid was harvested and the virus titer was determined by end dilution method before use.

### Antibodies and caspase inhibitors

Caspases 3, 7, 8, and 9 polyclonal antibodies were purchased from BioVision, alkaline phosphatase (ALP)-conjugated donkey anti-rabbit immunoglobulin G antibody was purchased from Proteintech Group. The pan-caspase inhibitor Z-VAD-FMK (Alexis Biochemicals) was dissolved in DMSO to 10 mM and stored at -70°C.

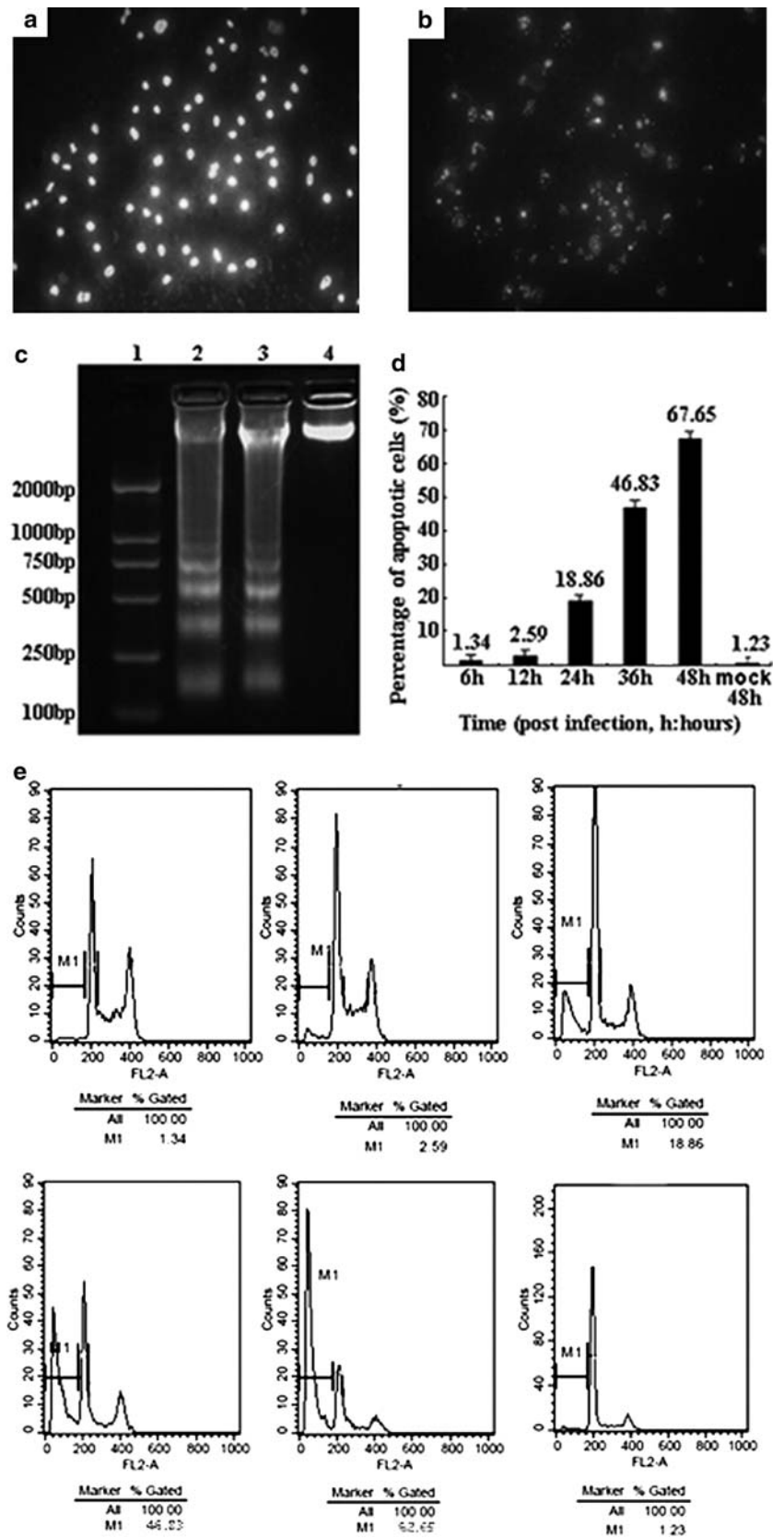
### Caspase activity inhibition treatment by Z-VAD-FMK and assessment of virus replication

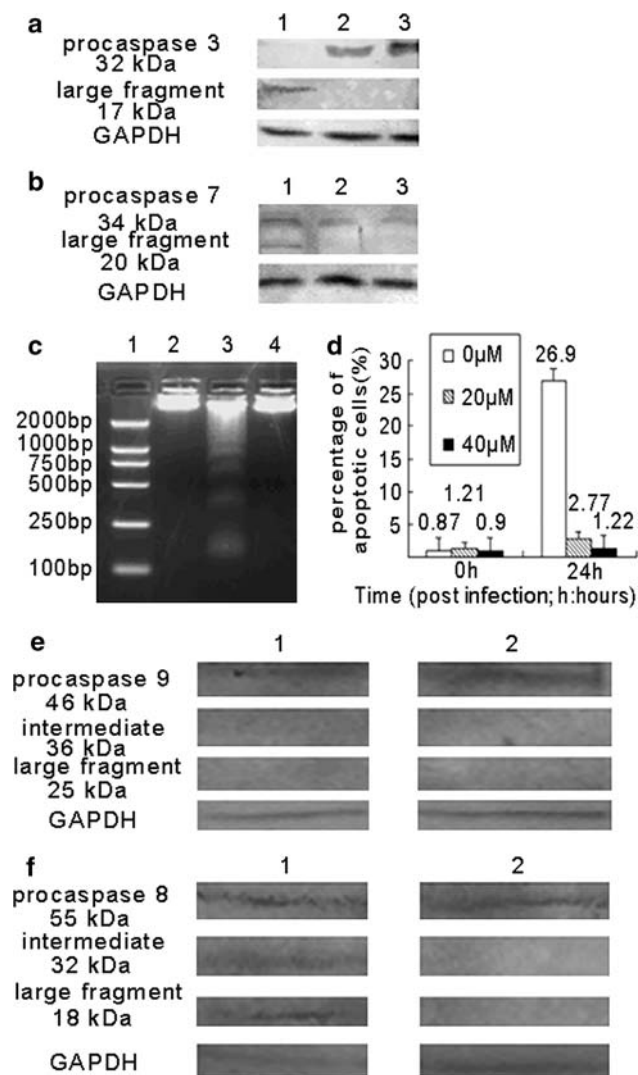
When using Z-VAD-FMK to inhibit the caspase activity in virus-infected cells, Z-VAD-FMK were added to the medium at the final concentration of 20 or 40 µM after virus attachment (MOI = 1) till the time when cells were harvested. Virus replication was assessed by qRT-PCR using avian influenza virus 5 subtype fluorescence quantitative RT-PCR assay kit (Triphill Company, The linear range of this kit is  $10 \times 10^{10}$  copy/ml. This kit specifically



**Fig. 1** AIVHubei489 infection induced cytopathogenic effect in MDCK cells. MDCK cells infected with AIVHubei489 (MOI = 1) for 12 h (b) and 24 h (d) were taken pictures under microscope at a magnification of 20. Mock-infected MDCK cells at 12 h (a) and 24 h (c) were used as control. The copy numbers of viral genome at different time points postinfection in MDCK cell are shown in (e). The infective titer is shown in (f)

**Fig. 2** AIVHubei489 infection induced apoptosis in MDCK cells. AIVHubei489 infection induced nuclei fragmentation (a, b) and DNA fragmentation (c) in MDCK cells and the quantitative analysis of AIVHubei489 induced apoptosis in MDCK cells by flow cytometry (d, e). MDCK cells infected with AIVHubei489 (MOI = 1) for 24 h (b) and mock-infected MDCK cells at 24 h (a) were subjected to Hoechst33258 staining and were detected under BH-2 fluorescence microscope (Olympus) at a magnification of 10. Chromosomal DNA extracted from AIVHubei489-infected MDCK cells (MOI = 1) at 24 h (c, lane 2)- and 48-h (c, lane 3) postinfection and from mock-infected MDCK cells at 48 h (c, lane 4) were detected under UV light after separated by 2% agarose gel electrophoresis (lane 1 was DNA Marker DL2000 from Takara). In (d), MDCK cells infected by AIVHubei489 (MOI = 1) were harvested at 6-, 12-, 24-, 36- and 48-h postinfection, trypsinized and then stained with propidium iodide and examined by FASCort. The percentages of apoptotic cells were determined using the CellQuest software program. Mock-infected MDCK cells at 48 h served as control. Columns represent the mean percentages of apoptotic cells of three replicate samples ( $\pm$ SD). The original flow cytometry data is shown in (e)





**Fig. 3** Apoptosis induced by AIVHubei489 in MDCK cells was caspase activity dependent. Effector caspases 3 and 7 were activated in AIVHubei489-infected MDCK cells (**a, b**). Cell lysates prepared from AIVHubei489-infected MDCK cells (MOI = 1) at 24 h (**a**—lane 1, **b**—lane 1) and 6-h postinfection (**a**—lane 2, **b**—lane 2) or mock-infected MDCK cells at 24 h (**a**—lane 3, **b**—lane 3) were subjected to Western blot analysis against caspase 3 (**a**) or 7 (**b**) polyclonal antibody after separated by 15% SDS-PAGE. Western blot analysis of the lysates against GAPDH antibody was used as loading control. AIVHubei489-induced apoptosis was blocked by pan-caspase inhibitor (**c, d**). Chromosomal DNA extracted from mock-infected MDCK cells (**c**—lane 2), AIVHubei489-infected MDCK cells (24-h postinfection, **c**—lane 3) and AIVHubei489-infected MDCK cells with treatment of 40-μM Z-VAD-FMK (24 h posttreatment, **c**—lane 4) were detected under UV light after separated by 2% agarose gel electrophoresis (**c**, lane 1; DNA Marker DL2000 from Takara). AIVHubei489-infected MDCK cells and MDCK cells infected with AIVHubei489 treated with 20- or 40-μM Z-VAD-FMK were harvested at 24-h postinfection and the percentages of apoptotic cells were counted by flow cytometry analysis (**d**). Columns represent the mean percentages of apoptotic cells of three replicate samples ( $\pm$ SD). Not caspase 9 but caspase 8 was activated in AIVHubei489-infected MDCK cells (**e, f**). Cell lysates prepared from AIVHubei489-infected MDCK cells (MOI = 1) at 24-h postinfection (**e**—lane 1, **f**—lane 1) and mock-infected MDCK cells at 24 h (**e**—lane 2, **f**—lane 2) were subjected to western blot analysis against caspase 9 (**e**) or 8 (**f**) polyclonal antibody after separated by 15% SDS-PAGE. Western blot analysis of the lysates against GAPDH antibody was used as loading control

dark at room temperature, the cover slides were observed under BH-2 fluorescence microscope (Olympus).

#### Purification of cellular DNA and detection of fragmented DNA

Nuclear genomic DNA was extracted from mock or AIVHubei489-infected cells according to the methods described before [18]. Briefly, at the indicated time point after infection, the cells were collected and centrifuged at  $1,000\times g$  for 10 min. Cell pellets were resuspended in lysis buffer (10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 10 mM EDTA, 1 mg/ml proteinase K, 200 μg/ml RNase A and 0.4% SDS) and incubated overnight at 56°C. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated in double volume ethanol and 1/10 volume sodium acetate. The precipitated DNA was resuspended in TE buffer (100 mM Tris-Cl and 10 mM EDTA, pH 8.0) and resolved by 2% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.5) and visualized under UV light after ethidium bromide staining.

#### Preparation of cell lysate and Western blot analysis

The cells were collected and centrifuged at  $1,000\times g$  for 10 min, washed in ice-cold PBS twice, resuspended in lysis buffer [200 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton, 1 mM EDTA, added fresh prepared 10 μg/ml

detects H5/H7/H9 subtype influenza virus) according to the protocol provided by the manufacturer. Briefly, cells were harvested at the indicated time postinfection, total RNA was extracted using Trizol reagent (Invitrogen), RT-PCR was then performed (50°C, 30 min for RT reaction followed by PCR with 94°C/2 min, 60°C/40 s, 45 cycles). The fluorescence signal was measured by Roter Gene3000 real-time rotary analyzer (Corbett Robotics). The positive controls in series dilutions were subject to the same RT-PCR reaction and the Ct values were used to graph the standard curve. The copy number of the virus was measured using the standard curve and the sample Ct value.

#### Hoechst33258 staining

Cells grown on a cover slide were washed by PBS twice, fixed by fixative (methanol: acetic acid = 3:1) for 15 min twice and stained by Hoechst33258 in dark for 45 min. After washed by distilled water three times and dried in



aprotinin (Sigma), 10  $\mu\text{g/ml}$  Leupeptin (Sigma), 10  $\mu\text{g/ml}$  pepstatin (MP Biomedicals) and 1 mM PMSF (Amersco)], after three times freeze–thaw and centrifugation at  $12,000\times g$  for 3 min at  $4^\circ\text{C}$ , the supernatant was transferred to a new tube and stored at  $-70^\circ\text{C}$  for later use. For Western blot analysis, cell lysates were subjected to SDS–polyacrylamide gel electrophoresis in 15% polyacrylamide gel. The separated peptides were transferred to a nitrocellulose membrane (ImBlotter) and followed by Western blot using polyclonal antibody against caspase 3 or 7 or 8 or 9 as the first antibody and Alkaline phosphatase (ALP)-conjugated donkey anti-rabbit immunoglobulin G antibody as the second antibody.

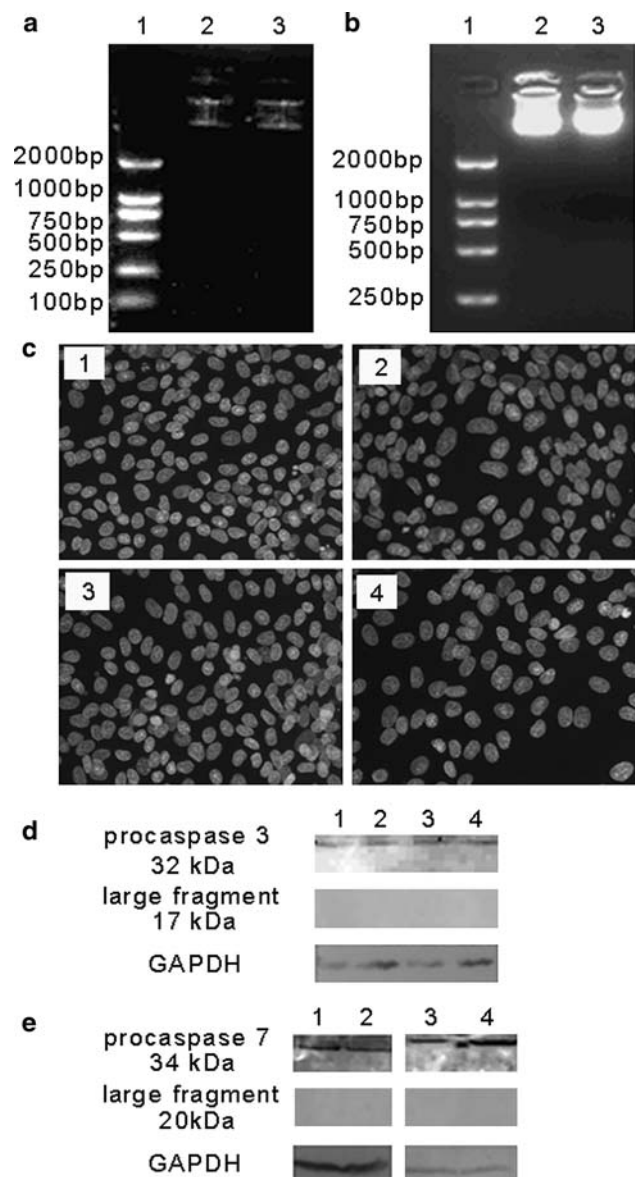
#### Flow cytometry analysis

Cells were harvested and centrifuged at  $1,000\times g$  for 10 min, washed with PBS twice and then fixed with cold 75% ethanol at  $4^\circ\text{C}$  overnight. Cells were centrifuged and washed with PBS (containing 1% BSA) twice, resuspended in 800  $\mu\text{l}$  PBS (containing 1% BSA), 100  $\mu\text{l}$  propidium iodide solution (500  $\mu\text{g/ml}$  in 38 mM trisodium citrate, pH 7.2) and 100  $\mu\text{l}$  RNase A (10 mg/ml), incubated at  $37^\circ\text{C}$  for 30 min, then measured on FACSsort (Becton Dickinson). The percentage of apoptotic cells was determined using the CellQuest software program.

## Results

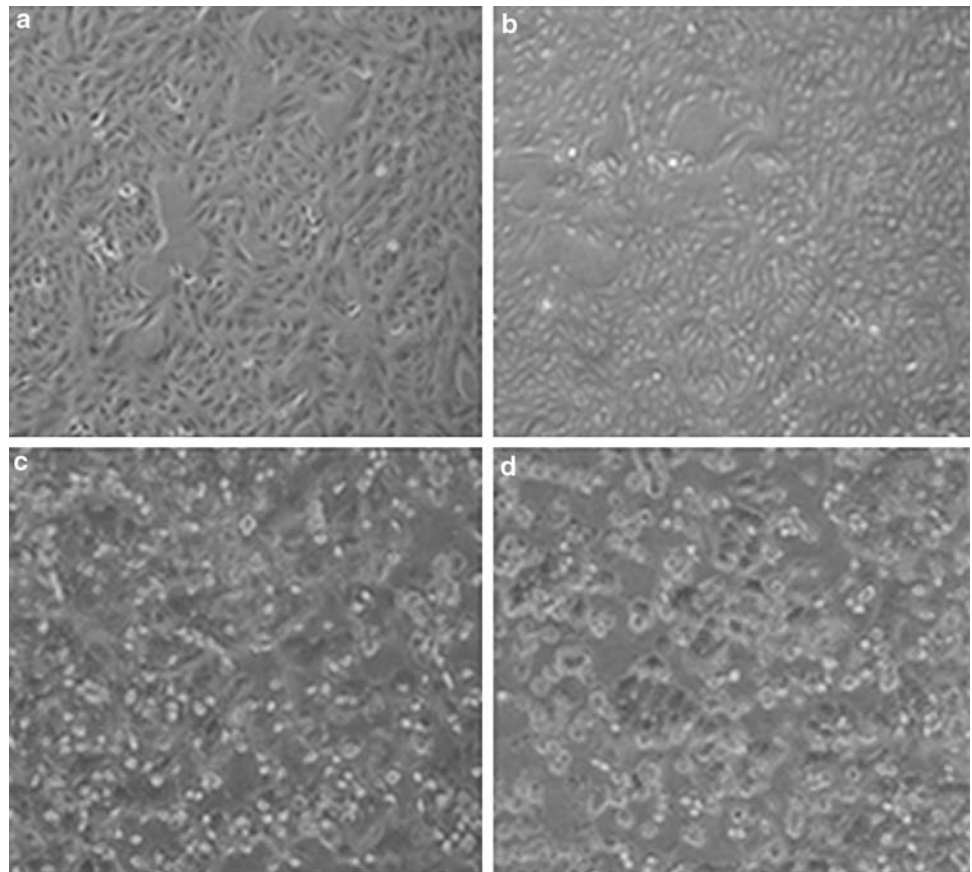
### AIVHubei489 could infect MDCK cells and the infection induced cytopathogenic effect

To find out that whether AIVHubei48 could infect MDCK cells, MDCK cells were infected by AIVHubei489 (MOI = 1) and morphological changes were observed under microscope. The result showed that at 12-h post AIVHubei489 infection, MDCK cells were largely intact (Fig. 1b), but at 24-h postinfection, the cells started to show rounded, shrank, and detached (Fig. 1d). Whereas mock-infected MDCK cells were intact at both 12- and 24-h postinfection (Fig. 1a, c, respectively). To be sure that the observed pathogenic effect was due to virus replication, qRT-PCR analysis of virus replication was performed and the result showed that AIVHubei489 did replicate in MDCK cells and the replication reached the highest level at 24-h postinfection (Fig. 1e). The infective titer is shown in Fig. 1f. Taken together the above data, AIVHubei489 could infect MDCK cells and virus replication induced cytopathogenic effect in MDCK cells.



**Fig. 4** AIVHubei489 infection did not induce nuclei fragmentation or caspase 3 or 7 activate in Vero or HeLa cells. Chromosomal DNA extracted from AIVHubei489-infected Vero or HeLa cells at 24-h postinfection (**a**—lane 2, **b**—lane 2, respectively) and from mock-infected Vero or HeLa cells at 24 h (**a**—lane 3, **b**—lane 3, respectively) were detected under UV light after separated by 2% agarose gel electrophoresis (**a**—lane 1, **b**—lane 1; DNA Marker DL2000 from Takara). Vero or HeLa cells infected with AIVHubei489 (MOI = 1) for 48 h (**c1**, **c2**, respectively) and mock-infected Vero or HeLa cells at 48 h (**c3**, **c4**, respectively) were subjected to Hoechst33258 staining and were detected under BH-2 fluorescence microscope (Olympus) at a magnification of 10. Cell lysates prepared from AIVHubei489-infected Vero cells (**d**—lane 1, **e**—lane 1) and HeLa cells (**d**—lane 3, **e**—lane 3) at 24-h postinfection or mock-infected Vero cells (**d**—lane 2, **e**—lane 2) and HeLa cells (**d**—lane 4, **e**—lane 4) at 24 h were subjected to Western blot analysis against caspase 3 (**d**) or 7 (**e**) polyclonal antibody after separated by 15% SDS-PAGE. Western blot analysis of the lysates against GAPDH antibody was used as loading control

**Fig. 5** AIVHubei489 infection induced cytopathogenic effects in Vero and HeLa cells. Vero and HeLa cells infected with AIVHubei489 for 48 h (**c**, **d**, respectively) were taken pictures under microscope at a magnification of 10. Mock-infected Vero and HeLa cells at 48 h (**a**, **b**, respectively) were used as control



#### AIVHubei489 infection induced apoptosis in MDCK cells

To further study whether the observed cytopathogenic effects induced by AIVHubei489 in MDCK cells indicated apoptosis, MDCK cells infected by AIVHubei489 were harvested at 24-h postinfection and subjected to Hoechst33258 staining. Fluorescopy photos showed nuclei fragmentation in infected cells (Fig. 2b) but not in mock-infected MDCK cells (Fig. 2a), suggesting that AIVHubei489 infection induced apoptosis in MDCK cells.

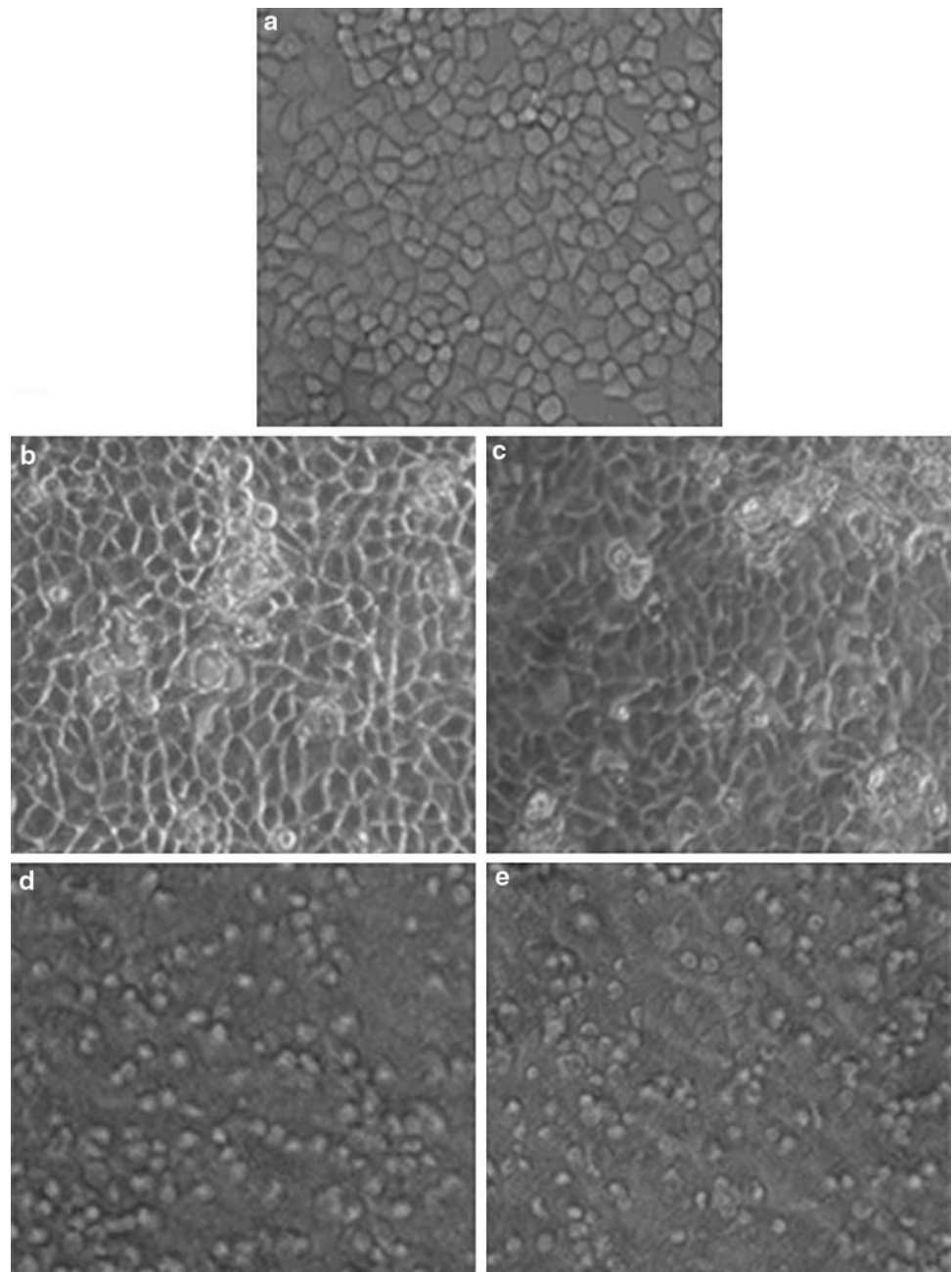
In order to confirm that AIVHubei489 infection did induce apoptosis in MDCK cells, the cellular DNA of infected cells were extracted and the fragmentation of chromosome DNA has been analyzed. Chromosomal DNA of MDCK cells were fragmented at 24- and 48-h post AIVHubei489 infection (Fig. 2c; lanes 2 and 3, respectively), while the DNA of mock-infected MDCK cells were not fragmented at 48 h (Fig. 2c; lane 4). Further more, flow cytometry assay showed that AIVHubei489 infection induced apoptosis in MDCK cells with the percentage of 18.86, 46.83, and 67.65 at 24-, 36- and 48-h postinfection (Fig. 2d, e), respectively. Taken together, AIVHubei489 infection induced apoptosis in MDCK cells.

#### AIVHubei489 infection induced MDCK cell apoptosis is caspase activity dependent

In order to study whether the apoptosis induced by AIVHubei489 infection in MDCK was caspase dependent, we first analyzed the activation of the two effector caspases, caspases 3 and 7, in AIVHubei489- infected MDCK cells. Western blot analysis showed that at 24 h postinfection, procaspases 3 and 7 were cleaved (Fig. 3a, b; lanes 1 and 1, respectively), indicating that caspases 3 and 7 were activated during the apoptosis induced by AIVHubei489 infection.

To confirm the function of caspase activity in AIVHubei489-induced apoptosis, we used a pan-caspase inhibitor Z-VAD-FMK to inhibit the caspase activity in AIVHubei489-infected MDCK cells and then checked the DNA fragmentation of infected cells. The result showed that DNA fragmentation of infected MDCK cells was blocked by 40- $\mu$ M Z-VAD-FMK (Fig. 3c). Moreover, flow cytometry assay result showed the ratio of apoptotic cells was significantly reduced by both 20- and 40- $\mu$ M Z-VAD-FMK (Fig. 3d). Thus, our results proved that AIVHubei489 infection induced apoptosis in MDCK cells was caspase activity dependent.

**Fig. 6** AIVHubei489 replicates in Vero and HeLa cells in a normal speed. Vero or HeLa cells were infected with AIVHubei489 (MOI = 1) and cell culture supernatants were collected at 24-h postinfection, diluted 100-fold, infected MDCK cells, respectively. The infected MDCK cells were observed under microscope at 24 (b, c, respectively)- or 48-h (d, e, respectively) postinfection (a was mock-infected MDCK cells at 48 h) and taken pictures at a magnification of 20



To further study which pathway is involved in AIVHubei489 induced apoptosis in MDCK cells, we analyzed the activation of caspases 8 and 9 in AIVHubei489-infected MDCK cells. Western blot result showed that, at 48-h postinfection, caspase 8 was activated but caspase 9 was not (Fig. 3e, f), indicating that the apoptosis might be through death receptor pathway.

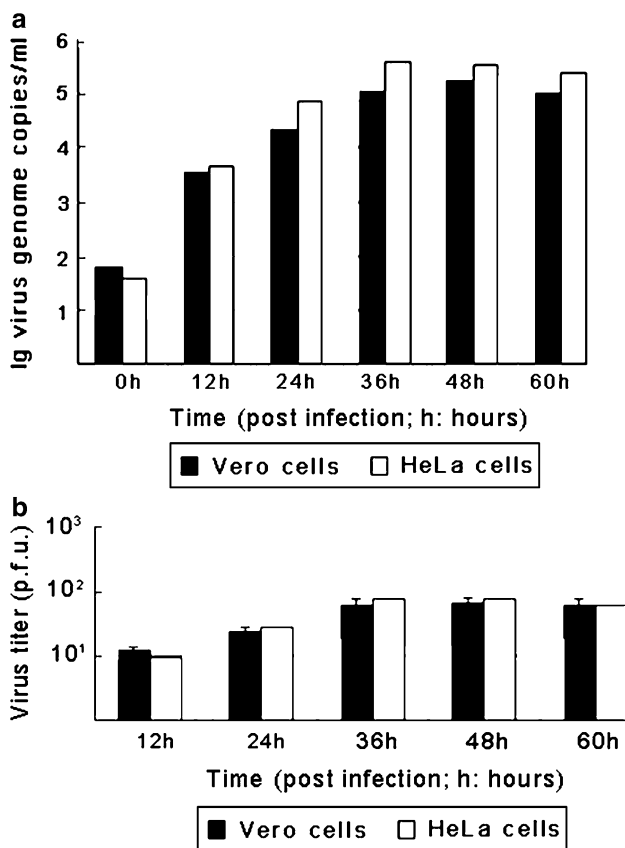
AIVHubei489 could infect Vero or HeLa cells but did not induce apoptosis in either cell lines

To primarily investigate the physiological significance of the apoptosis inducing ability of AIVHubei489, we studied

the apoptosis inducing ability in Vero and HeLa cells. Unlike MDCK cells, Vero and HeLa cells did not show DNA fragmentation or nuclei fragmentation even at 48-h post AIVHubei489 infection (Fig. 4a–c). Caspases 3 and 7 were not activated (Fig. 4d), and suggesting that AIVHubei489 infection did not induce apoptosis in Vero or HeLa cells.

To see if lack of DNA fragmentation and caspase activation were due to inability of AIVHubei489 to replicate in Vero or HeLa cells, we checked the cytopathogenic effects of the infections of AIVHubei489 in Vero and HeLa cells. Cytopathogenic effects could not be detected at 24-h postinfection (data not shown), but at





**Fig. 7** The virus titer shown as genome copies (a) and infectious titer (b) in Vero and HeLa cell lines at different time point postinfection

48-h postinfection obvious cytopathogenic effects were observed (Fig. 5c, d, respectively), while the mock-infected Vero and HeLa cells maintained intact until 48 h (Fig. 5a, b, respectively).

In order to make clear that whether the late appeared cytopathogenic effects detected in Vero and HeLa cells were related to slow replication of AIVHubei489, we further used the Vero and HeLa cell culture supernatants of 24-h post AIVHubei489 infection to infect MDCK cells. Both supernatants induced cytopathogenic effects in MDCK cells at 24-h postinfection (Fig. 6b, c, respectively) and the obvious cytopathogenic effects were detected at 48-h postinfection (Fig. 6d, e, respectively). This result demonstrated that the late appeared cytopathogenic effects detected in Vero and HeLa cells were not due to slow replication of AIVHubei489. qRT-PCR analysis of virus replication showed that AIVHubei489 did replicate in both Vero and HeLa cells and the replication reached the highest level at 48 and 36 h postinfection, respectively (Fig. 7). Taken together, AIVHubei489 could infect Vero and HeLa cells, but the infection did not induce apoptosis in either cells.

## Discussion

AIVHubei489 is a newly isolated avian influenza virus which caused the most serious outbreak of avian influenza in 2004, Hubei province, People's Republic of China. Because the genome of avian influenza virus is highly mutational, to elucidate the biological characteristics of each newly found virus is very necessary for developing effective ways to prevent the virus infection and cure the virus-related disease.

We have proved in this article that though AIVHubei489 could infect MDCK, Vero and HeLa cells, the infection of AIVHubei489 induced apoptosis only in MDCK cells. It is also demonstrated in this article that the apoptosis induced by AIVHubei489 was caspase activity dependent in which caspases 3 and 7 activities were required.

It has been reported that different strains of avian influenza viruses induce apoptosis through different pathways. For instance, different subtypes of swine influenza viruses (H1N1, H3N2, and H1N2) induce apoptosis in PK-15 cells involving mitochondrial pathway in which caspase 9 is activated [19]. While it is also reported that influenza A/WSN virus induces apoptosis via activation of the FADD/caspase-8 pathway [20]. We observed that AIVHubei489 infection induced caspase 8 but not caspase 9 activation, suggesting that the apoptosis induced by AIVHubei489 infection might be through cell death receptor pathway but not through mitochondria pathway.

It is recently reported that the delayed onset of apoptosis in H5N1-infected macrophages was not due to replication of the avian virus [21]. Our data proved that the delayed cytopathogenic effect in AIVHubei489-infected HeLa and Vero cells was not due to slow replication of the virus, neither. The fact that infection induced apoptosis in MDCK but not in Vero or HeLa cells suggested that AIVHubei489 induced apoptosis in a cell specific manner. We deduce that certain cell line has a special immune surveillance mechanism in detecting AIVHubei489 replication and how this mechanism is related to the host-virus relationship is worth of further study.

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