Differential onset of apoptosis in influenza A virus H5N1- and H1N1-infected human blood macrophages

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Pathogenesis of the highly pathogenic avian influenza virus A/Hong Kong/483/97 (H5N1/97) remains to be investigated. It was demonstrated recently that H5N1 dysregulation of proinflammatory cytokines in human macrophages is a p38-kinase-dependent process. The results indicated that macrophages may play a role in disease severity. To investigate cellular responses to H5N1 infection further, apoptosis and its related pathways were studied in primary blood macrophages. Here, it is shown that the H5N1/97 virus triggered apoptosis, including caspases and PARP activation, in infected macrophages with a delayed onset compared with H1N1 counterparts. Similar results were also found in human macrophages infected by precursors of the H5N1/97 virus. Thus, these results showed that the delay in apoptosis onset in macrophages infected by H5N1/97 and its related precursor subtypes may be a means for the pathogens to have longer survival in the cells; this may contribute to the pathogenesis of H5N1 disease in humans.

The H5N1/97 'bird-flu' incident was the first documented direct transmission of an avian influenza virus to humans, causing devastating infections with severe viral pneumonia and a mortality rate of >30% (Claas et al., 1998; Subbarao et al., 1998; Yuen et al., 1998). The H5N1 virus continued to disseminate among migratory birds and led to a widespread outbreak in domestic poultry around the world. It had caused over 200 human cases as of May 2006 (WHO avian influenza information; http://www.who.int/csr/disease/avian_influenza/en/). The increasing incidence of avian-to-human transmission provides an opportunity for these highly pathogenic avian influenza viruses to adapt to the host environment, which may result in reassortment of genetic materials between avian and human influenza viruses. Generation of such newly reassorted influenza virus may lead to a potential pandemic threat (WHO avian influenza information; http://www.who.int/csr/disease/avian_influenza/en/).

Previously, we demonstrated that H5N1/97 viruses, in contrast to human influenza A virus subtypes including H1N1 and H3N2, induce high levels of proinflammatory cytokines in differentiated primary human blood macrophages (Cheung et al., 2002). It was suggested that this cytokine dysregulation contributes to pathogenesis and severity of the disease (Fisman, 2000; Headley et al., 1997; To et al., 2001). In delineating the mechanisms of cytokine dysregulation, we recently reported that p38K, a mitogen-activated protein kinase (MAPK), plays a significant role in the hyperinduction of tumour necrosis factor alpha (TNF-α) in H5N1-infected macrophages (Lee et al., 2005). In addition, a recent study showed that H5N1-infected macrophages enhance TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in Jurkat T cells (Zhou et al., 2006). These recent reports indicate that human blood macrophages may play a critical role in the pathogenesis of H5N1 infection.

Among cellular responses against invading viruses, induction of apoptosis has been postulated to be one of the most effective host defence mechanisms. The cell-death process results in inhibition of virus replication, limitation of virus dissemination and minimization of uncontrolled inflammatory responses. It has been shown that influenza virus induces apoptosis in vivo and in vitro (Brydon et al., 2005; Fesq et al., 1994; Hinshaw et al., 1994; Takizawa et al., 1993, 1999). However, the functional role of influenza virus-induced apoptosis is still not well defined. To gain insights into the virulence of the highly pathogenic avian influenza virus, together with an understanding of the cellular
responses of macrophages during microbial infection, we studied the mechanisms of apoptosis in primary blood macrophages infected with H5N1 (483/97) or human influenza virus H1N1 (54/98).

Primary blood macrophages were isolated from mononuclear cells of healthy blood donors as described previously (Lee et al., 2005). The cells were mock-treated or infected with influenza viruses (H5N1 or H1N1) at an m.o.i. of 2 for 30 min and harvested at indicated time points for analysis. The infectivity of H5N1 and H1N1 viruses on human macrophages was examined by immunofluorescent staining using monoclonal antibodies specific for the nucleoprotein of influenza viruses (DAKO). Positive staining for the nucleoprotein in influenza virus-infected macrophages was found at 8 h post-infection (data not shown). Additionally, cell death, as demonstrated by nuclear condensation or nuclear fragmentation, was determined by staining the cells with 4,6-diamidine-2-phenylindole dihydrochloride (DAPI) at indicated time points post-infection. As shown, the number of dead cells was lower in those cells infected by the H5N1 virus than in cells infected by its H1N1 counterpart (Fig. 1a).

A previous report showed that replication of influenza virus is necessary to trigger apoptosis in infected cells (Price et al., 1997). We thus measured the viral titres of H5N1 or H1N1 in the infected macrophages at 6, 10, 18 and 24 h post-infection. There was no significant difference between the viral titres in the macrophages infected by H1N1, H5N1 (Fig. 1b) or A/Quail/Hong Kong/G1/97 (H9N2/G1), the precursor of H5N1/97 (see Supplementary Fig. S1, available in JGV Online). Hence, the delayed onset of apoptosis in H5N1-infected macrophages was not due to replication of the avian virus. Furthermore, we performed kinetic studies to measure the level of the viral genes for the polymerase protein (PA) and nucleoprotein (NP) in H5N1- or H1N1-infected macrophages by using a quantitative RT-PCR assay. Consistent with the virus titration results, we did not find any significant differences in the transcription levels of PA and NP in H5N1- or H1N1-infected macrophages at 0, 6 or 10 h post-infection (see Supplementary Fig. S2, available in JGV Online).

We next examined the ultrastructural features of H5N1- and H1N1-infected cells at 12 h post-infection under a transmission electron microscope (Philips EM208S). The cellular morphology of H5N1-infected macrophages (Fig. 1c, middle) was comparable to that of mock-treated cells at 12 h post-infection (Fig. 1c, right). In contrast, H1N1-infected cells showed characteristics of apoptotic cells, including nuclear condensation and chromatin adherence to the nuclear membrane (Fig. 1c, left). Our results demonstrated a differential onset of cell death in macrophages infected with H5N1 compared with those infected with the H1N1 virus.

We investigated the apoptotic pathways in virus-infected macrophages further by measuring the activation of caspase-activated poly(ADP–ribose) polymerase (PARP; Pharmingen), an essential factor in the initiation of apoptosis (Soldani & Scovassi, 2002), at 6, 12 and 18 h post-infection by using Western analysis (Fig. 1d). Over the time course, there was a 4- or 8-fold increase in the levels of the cleaved PARP fragment in H5N1-infected cells at 12 or 18 h post-infection, respectively, compared with the mock-treated cells (Fig. 1d, lanes 6 and 7). In contrast, there was an 18- or 22-fold increase in PARP fragment levels in H1N1-infected cells at the corresponding time point (Fig. 1d, lanes 3 and 4). As PARP is the downstream target of the caspase cascade, we then measured the activity of two important apoptotic markers, caspases 3 and 8, by Western analysis using antibodies from Cell Signaling Technology and Upstate Biotech, respectively. Our results showed that the cleaved fragment of caspase 3 was barely detectable in cells infected with H5N1 (Fig. 2a, lane 9) and was not detected in those infected with H9N2/G1 (data not shown) at 12 h post-infection. In contrast, levels of activated caspase 3 were increased significantly in H1N1-infected cells at 10 h post-infection and persisted at 12 h (Fig. 2a, lanes 4–5). Consistent with the caspase 3 findings, H5N1 did not induce the degradation of procaspase 8 strongly at 12 h post-infection compared with H1N1 (Fig. 2b, lanes 9 and 5).

We further examined the functional role of caspase 8 in influenza virus-induced apoptosis by treating virus-infected cells with a specific inhibitor for caspase 8, Z-IETD-FMK (Takizawa et al., 1999). Following caspase 8 inhibition, our results showed that the level of the cleaved PARP fragment in H5N1- or H1N1-infected macrophages was reduced significantly, by 82 and 86%, respectively, compared with the corresponding samples without inhibitor treatment (Fig. 2c, lanes 3 and 5). Therefore, the differential onset of cell death in H5N1 and H1N1 infection was associated with the delayed activation of the caspase cascade.

To investigate the characteristics of the onset of apoptosis in avian influenza virus infection further, we measured the levels of PARP activation in macrophages infected with precursors of H5N1/97, including H9N2/G1, A/Teal/HK/W312/97 (H6N1) and A/Goose/Guangdong/1/96 (H5N1/437/6) (Guan et al., 1999; Hoffmann et al., 2000; Subbarao & Shaw, 2000; Xu et al., 1999), and human influenza viruses, including H1N1 and H3N2, at 18 h post-infection (Fig. 3a, b). The level of activated PARP in macrophages infected with the H5N1/97 virus or its precursors was lower than that in H1N1- or H3N2-infected cells. Our findings suggested that macrophages infected with H5N1/97 or its precursors undergo slower kinetics of apoptotic responses compared with H1N1- or H3N2-infected cells. In addition, H9N2/G1 induced the cleavage of PARP, but not that of caspase 3, suggesting that H9N2/G1 activates PARP through a caspase 3-independent pathway (Hong et al., 2004).

The detailed mechanisms of delayed onset of apoptosis occurring in avian influenza virus-infected human macrophages remain to be investigated. It is plausible that the infected macrophages cannot recognize the avian influenza...
Delayed onset of apoptosis in H5N1-infected macrophages

Fig. 1. H5N1/97-infected macrophages show less cell death and apoptosis than H1N1-infected macrophages. (a) Primary human blood macrophages (0.5×10⁶) were fixed with 4% paraformaldehyde and stained with DAPI at 18 h post-infection with H5N1 (483/97) or H1N1 (54/98) virus at an m.o.i. of 2. Values represent the mean ± SD of cells from three different donors analysed statistically by the two-tailed, paired t-test. *P<0.1. (b) Replication of the H5N1 (483/97) virus in primary human blood macrophages. Primary human blood macrophages (1×10⁶) were infected with human influenza virus H1N1 (54/98) or avian influenza virus H5N1 (483/97) at an m.o.i. of 2. Culture supernatants were collected at 6, 10, 18 and 24 h post-infection. Viral titres (TCID₅₀) of the samples were measured by titration in Madin–Darby canine kidney (MDCK) cells. The results shown are representative of experiments performed on cells from three different donors. (c) Primary human blood macrophages (2×10⁶) were infected with influenza virus H5N1 (483/97) or H1N1 (54/98) at an m.o.i. of 2. At 12 h post-infection, the cells were collected for ultrastructural examination by transmission electron microscopy. H1N1-infected macrophages (left); H5N1-infected macrophages (middle); mock-treated macrophages (right). Magnification, ×28 000; CC, chromatin condensation. The results shown are representative of experiments performed on cells from three different donors. (d) Primary human blood macrophages (1×10⁶) were harvested at 6, 12 and 18 h after infection with H5N1 (483/97) or H1N1 (54/98) virus at an m.o.i. of 2. The activated level of PARP was assayed by Western analysis using a monoclonal anti-PARP antibody. Equal loading of protein samples was determined with anti-actin antibodies. The results shown are representative of experiments performed on cells from three different donors. The density of the protein band was determined by using Bio-Rad Quantity One imaging software. Numbers in parentheses are density values of activated PARP relative to that of actin. Mock, uninfected cells.

Virus effectively and trigger apoptosis efficiently to fight against the invading avian influenza virus. It has been documented that the sequences of H5N1/97 virus are quite different from those of the human influenza H1N1 or H3N2 viruses (Cooper & Subbarao, 2000). Whether the variation of the viral genome sequences affects the interactions between the host and the virus remains to be determined.
Induction of anti-apoptotic genes and pathways in H5N1-infected macrophages is another possible mechanism leading to the delayed onset of apoptosis. In our previous study, we observed that p38 MAPK is activated differentially in primary human macrophages after infection by H5N1/97 virus. This activation was shown to be associated with superinduction of TNF-α by H5N1 (Lee et al., 2005). Moreover, it has been shown that double-stranded RNA activation of p38 MAPK results in inhibition of the activity of caspases 3, 8 and 9 (Tadlock et al., 2003). This may be analogous to the situation in H5N1 infection of macrophages, in which preferential activation of p38 MAPK may contribute to delayed apoptosis compared with H1N1 infection. However, apoptosis in H5N1-infected macrophages was not decreased by using the p38 MAPK-specific inhibitor SB203580 (see Supplementary Fig. S3, available in JGV Online). Recent findings showed that the interaction of Ccl5 and Ccr5 induces anti-apoptotic signals for macrophages during viral infection (Tyner et al., 2005). As Ccl5 was highly induced in H5N1-infected human macrophages compared with those infected by human influenza viruses (Cheung et al., 2002), further investigation is required to examine whether this signalling cascade plays a role in the delayed onset of apoptosis in H5N1-infected cells.

Our results also provide additional information on the role of macrophages in the pathogenesis of H5N1 infection. H5N1-infected patients present with severe viral pneumonia and lymphopenia. A recent study reported that H5N1-infected macrophages could enhance TRAIL-induced apoptosis in T cells compared with H1N1 infection (Zhou et al., 2006). Therefore, our results suggest that the delayed onset of apoptosis in H5N1-infected macrophages may prolong the interaction of TRAIL-expressing macrophages with T cells to enhance the induction of apoptotic cell death in the T cells.

In conclusion, we have provided experimental evidence that avian influenza viruses, including H5N1/97 and its precursors, trigger an apoptotic response mediated by caspase activation that is similar to, but delayed compared with, that induced by human influenza viruses including H1N1 and H3N2. Our findings on the delay of apoptosis in H5N1 viruses combined with superinduction of proinflammatory cytokines may contribute, in part, to...
understanding how these novel viruses cause fatal disease in humans.

Acknowledgements

This work was supported by research grants to A. S. Y. L. (HKU 7430/03 M) and to M. P. (HKU 7459/03) from the Research Grants Council of Hong Kong and the Research Fund for Control of Infectious Disease to A. S. Y. L. (project no. 05050112), as well as the 2003 Vice Chancellor’s Development Fund from The University of Hong Kong to M. P.

References


