Effect of Brefeldin A on Influenza A Virus-Induced Apoptosis in vitro

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Abstract. Effect of brefeldin A (BFA) on influenza virus-infected cells was examined by measuring cell viability, microscopic examination, and DNA fragmentation analysis. When Madin-Darby canine kidney (MDCK) cells were infected with influenza A virus together with BFA treatment, virus-induced cell death was observed earlier than in the virus-infected cells without BFA treatment. By microscopic examination, the mode of cell death in virus-infected cells with BFA treatment appeared different from that of the cells without BFA treatment. Because influenza virus causes apoptosis in the host cells, extent of DNA fragmentation was compared between virus-infected cells with and without BFA treatment. BFA treatment resulted in inhibition of DNA fragmentation of virus-infected cells. Treatment of virus-infected cells with antioxidant (10 mM N-acetyl-L-cysteine) also inhibited DNA fragmentation of influenza virus-infected cells. A possible mechanism of BFA affecting influenza virus-induced apoptosis is discussed. — Key words: apoptosis, brefeldin A, influenza virus.

Influenza virus infection has been shown to cause apoptosis in the host cells [3, 4, 16, 17]. Although Fas antigen and its ligand are considered to be responsible in influenza virus-induced apoptosis [17], exact mechanism of the virus-induced apoptosis has not been elucidated.

Brefeldin A (BFA) is a fungal metabolite that shows antiviral effect on several viruses [1, 2, 5]. When this compound was used to block the viral glycoprotein transport in Madin-Darby canine kidney (MDCK) cells infected with influenza virus, we found that the virus-infected cells treated with BFA died earlier than those without BFA treatment. The present study was done to understand the effect of this antiviral compound on influenza virus-infected cells in vitro.

First, viability of the influenza virus-infected cells with or without BFA (5 μg/ml) treatment was examined (Fig. 1). MDCK cells in 96-well culture plate were infected with reassortant virus NWS-N8 [12] by m.o.i. (multiplicity of infection) of 0.5, then after 15 or 24 hr later, cells were stained with 0.1% crystal violet for 15 min and washed with distilled water. Dyes were extracted by 0.5% sodium dodecyl sulfate (SDS) and absorbance at 590 nm were measured by microplate reader (Model 450; Bio-Rad) [modified from ref. 6]. After 15 hr infection, no effect of BFA on cell viability was observed either mock infected or virus-infected cells (Fig. 1). After 24 hr, however, decreased viability was apparent in the virus-infected cells treated with BFA. This suggests that BFA may accelerate cell death induced by influenza virus infection.

Influenza virus-induced cell death has been indicated as apoptosis [3, 4, 16, 17]. Apoptosis is characterized morphologically by formation of apoptotic body and biochemically by DNA fragmentation in vitro [15]. Next, we morphologically examined this accelerated cell death caused by BFA treatment by comparing between virus-infected cells treated with and without BFA. MDCK cells were infected with NWS-N8 at m.o.i. of 0.5 for 15.5, 16.5 and 22.5 hr in the presence or absence of BFA (5 μg/ml), then fixed by methanol followed by Giemsa’s staining (Fig. 2).

After 15.5 hr infection (Fig. 2a), apoptotic bodies appeared in the virus-infected cells. On the other hand, only a few apoptotic bodies were observed in the cells with BFA treatment (Fig. 2b). Instead of appearance of apoptotic body, expansion of intercellular space was observed in the presence of BFA. After 22.5 hrs infection period, morphological differences between virus infected cells with (Fig. 2) and without (Fig. 2) BFA treatment were apparent. In the presence of BFA, intercellular space expanded much more, probably because dead cells were removed from the surface of the culture plates indicated by decreased number of nuclei per microscopic field (Figs. 2f and d). No significant difference was observed between mock infected cells treated with and without BFA (data not shown). These observations indicate that BFA affects the mode of cell death after virus infection.

To further confirm alternation of the mode of cell death by BFA treatment, we analyzed DNA fragmentation of influenza virus-infected cells with and without BFA treatment (5 μg/ml). MDCK cells were infected with NWS-N8...
N8 at m.o.i. of 0.5 for 12 and 13 hr with or without BFA treatment, then cellular DNA was extracted with lysis buffer (20 mM Tris-HCl; pH 7.8, 10 mM EDTA, 0.5% sodium-N-lauroylsarcosinate). Cellular DNA was analyzed by 2% agarose in Tris-borate-EDTA buffer (TBE; pH 8.3). In virus-infected cells, DNA fragmentation was detected 12 and 13 hr after virus infection (Fig. 3a). On the other hand, the fragmentation was almost completely inhibited when virus-infected cells were treated with BFA for 12 hr. Slight fragmentation was observed in the virus-infected cells with BFA treatment 13 hr post infection. Together with microscopic analysis, it is apparent that BFA treatment of influenza virus-infected cells inhibited virus-induced apoptosis, leading to another type of cell death, necrosis.

Mechanism by which BFA treatment inhibits influenza virus-induced apoptosis is not clear at this moment. Although BFA treatment does not affect synthesis of neuraminidase (NA), one of the viral glycoproteins [13], it blocks transport of NA to the cellular membrane of the host cells resulting in the accumulation of NA in the ER of the host cells [T. Saito, unpublished data]. Blockade of the viral glycoprotein transport through Golgi apparatus by BFA [7, 8], thus makes the intact virus particle formation at the cellular membrane impossible. There is a possibility that inhibition of the virus particle formation may have something to do with apoptosis inhibition. Treatment of HIV-chronically infected U937 cells by N-acetyl-L-cysteine (NAC), oxidative stress scavenger, inhibits apoptosis and decreased viral particles [9].

We treated influenza virus-infected MDCK cells with NAC, then DNA fragmentation was analyzed (Fig. 3b).

**Fig. 2.** Microscopic examination (<50) of influenza virus-infected cells with (b, d, f) or without (a, c, e) BFA treatment. Virus infected cells were incubated for 15.5 hr (a, b), 16.5 hr (c, d) or 22.5 hr (e, f).

**Fig. 3.** a) DNA fragmentation of virus-infected cells. Virus- (lanes 1, 2, 3, and 4) or mock-infected cells (lanes 5 and 6) were incubated for 12 hr (lanes 1 and 2) or 13 hr (lanes 3, 4, 5, and 6). BFA was added during incubation (lanes 1, 3, and 5). b) Effect of NAC on influenza virus-induced apoptosis. Virus-infected cells were untreated (lane 1) or treated with 0.01 mM (lane 2), 0.1 mM (lane 3), 1 mM (lane 4) or 10 mM (lane 5) of NAC for 12 hr.
Treatment of virus-infected cells with 10 mM NAC resulted in apoptosis inhibition (Fig. 3b, lane 5). NAC treatment of virus-infected cells did not result in necrosis observed in the case of BFA treatment (data not shown). This suggests that oxidative stress on the cellular membrane [10, 11, 14, 18], caused by virus particle formation, may be responsible for influenza virus-induced apoptosis.

Based on the results showing that both BFA and NAC can inhibit influenza virus-induced apoptosis, one possible explanation for the effect of BFA on the influenza virus-infected cells is as follows; BFA blocks transport of the viral membrane proteins, such as hemagglutinin, neuraminidase and matrix protein (M2), resulting in inhibition of virus particle formation. This reduces oxidative stress on the cellular membrane, in turn, prevents apoptotic cell death of virus-infected cells. On the other hand, accumulation of aberrant quantity of viral proteins in the ER leads dysfunction of the host cells, ending up with necrotic cell death. There are, in addition, other possibilities that were not addressed in this study. One of them is that, because Fas antigen appears to play an important role in influenza virus-induced apoptosis [17], BFA treatment also blocks transport of this glycoprotein, then results in inhibition of apoptosis.

In summary, we reported inhibitory effect of BFA on influenza virus-induced apoptosis. BFA not only inhibits apoptosis but leads necrotic death of influenza virus-infected cells in vitro. Precise mechanism by which BFA prevents virus-induced apoptosis remains to be clarified.

REFERENCES