Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells

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The process of cell death caused by influenza virus infection in cultured MDCK and HeLa cells was analysed. This infection gave rise to nuclear fragmentation and chromatin condensation accompanied by chromosomal DNA fragmentation into oligonucleosomes. Chromosomal DNA fragmentation progressed concomitantly with cell lysis of MDCK cells and HeLa cells, producing high and low yields of virus particles, respectively, indicating that the extent of cell lysis was not proportional to the virus production. The endonuclease inhibitor zinc blocked DNA fragmentation in MDCK cells. Cycloheximide inhibited DNA fragmentation as well as cell lysis. Inhibition occurred when the drug was added to the medium within 2 h after infection but not efficiently at 4 h or later. Infection induced the Fas Ag gene, which encodes a possible apoptosis-mediating molecule, in the early infectious stage followed by the expression of Fas Ag on the cell surface. These results suggested that influenza virus infection causes apoptotic death of cultured cells, and their fate might be determined at an early stage of the infection by induction of an apoptotic gene.

Introduction

Influenza virus damages epithelial cells of the human respiratory tract predominantly (Sweet & Smith, 1980; Murphy & Webster, 1990). It also causes cell death following infection of tissue culture cells. However, the mechanisms of virus-induced cell damage that eventually lead to cell death are not well understood (Sweet & Smith, 1980). Massive budding of the virus particles from the membrane of the infected cells may cause cellular damage. However, cellular degeneration can occur even with incomplete replication of the virus in tissue culture cells, such as HeLa cells (Henle et al., 1955).

There are two major morphologically and biochemically distinct modes of death in eukaryotic cells: apoptosis and necrosis (Wyllie et al., 1980; Duvall & Wyllie, 1986; Kerr & Harmon, 1991). Necrosis is considered to be a pathological reaction occurring in response to perturbations in the cellular environment such as complement attack, severe hypoxia, hyperthermia etc. These stimuli increase the the permeability of the plasma membrane resulting in irreversible swelling of the cells (Wyllie et al., 1980). On the other hand, apoptosis is considered to be a physiological process involved in normal tissue turnover which occurs during embryogenesis, ageing and tumour regression (Wyllie et al., 1980). Apoptotic cells undergo several dramatic morphological changes, including development of fragmented nuclei, extensive chromatin condensation and loss of cell volume. In most cases, apoptosis requires protein synthesis (Wyllie et al., 1984), followed by an increase in cytosolic Ca2+ concentration (McConkey et al., 1989; Zheng et al., 1991). A Ca2+-dependent endonuclease has been suggested to cause chromosomal DNA fragmentation into oligonucleosomes (Jones et al., 1989; Gaido & Cidlowski, 1991).

In this paper, we characterized the process of cell damage caused by influenza virus infection in cultured cells, and found that it induced fragmentation of chromosomal DNA into oligonucleosomes and also caused nuclear fragmentation due to chromatin condensation. These events were blocked by protein synthesis inhibition, suggesting that the cell death induced by influenza virus infection occurred through apoptosis.

Methods

Virus and cell culture. MDCK and HeLa cells, obtained initially from Dr K. Shimizu (Nihon University, Tokyo, Japan), and Dr K. Nagata (National Institute of Genetics, Mishima, Japan), respectively, were cultivated in Eagle's minimal essential medium (MEM) containing 10% (v/v) bovine calf serum. A wild-type strain of influenza
A/UDorn/72 (H3N2) virus, SP626, was grown in the allantoic cavity of 10-day-old embryonic chicken eggs which were incubated at 34 °C as described previously (Hatada et al., 1989). Subconfluent monolayers of the cells were infected with virus at an m.o.i. of 10 for 60 min at 4 °C. The infected cells were washed twice with PBS and then incubated in Eagle's MEM without serum at 34 °C for the times indicated. Haemagglutinin (HA) titres were determined as previously described (Minor & Dimmock, 1975). Virus plaque assay was performed on confluent monolayers of MDCK cells that were infected with virus solution for 1 h at room temperature, overlaid with 0.6% agarose containing 2.5 μg/ml of trypsin, and incubated for 3 days at 34 °C. Cells were stained with 1% crystal violet in 20% ethanol. For isotopic labelling of proteins, the infected cells were incubated for 30 min at 34 °C in methionine-free Eagle's MEM supplemented with 10 μCi/ml [35S]methionine (1000 Ci/mmol, Amersham). After incubation, the cells were washed with PBS and lysed in a lysis buffer (Laemmli, 1970). The lysates were heated for 3 min at 100 °C and were analysed by SDS–PAGE followed by fluorography as described previously (Hatada et al., 1989). For u.v.-irradiation of virus, 0.2 ml virus solution in a 3.5 cm Petri dish was exposed to u.v. light for 10 min at a distance of 10 cm from a 5 W lamp at a wavelength of 253-6 um (Manaslu-light).

**Analysis of cell lysis.** For the cytolysis assay, 2 × 10⁴ monolayer cells were infected for 60 min at 34 °C with 20 μCi of Na₂¹⁴CrO₄ (400 to 1200 Ci/g, New England Nuclear) before influenza virus infection. The cells were washed three times with PBS, infected with the virus and then incubated in Eagle's MEM without serum, as described above. During incubation at 34 °C, 100 μl samples of the medium, taken at the times shown, were centrifuged at 1000 g for 5 min, and the supernatant was collected for determination of radioactivity using a gamma counter. The percentage of specific ¹⁴Cr release was determined as 100 × [experimental release (c.p.m.) – spontaneous release (c.p.m.)/total release (c.p.m.) – spontaneous release (c.p.m.)] (Blakely et al., 1987). The counts obtained from cells lysed by freezing and thawing treatment were used as the total release values. Alternatively, cell viability was determined by trypan blue dye exclusion with a haemocytometer.

**DNA preparation and gel electrophoresis.** Monolayer cells were rinsed twice with PBS, trypsinized and scraped from the dishes. The cells were resuspended in PBS at a density of 2 × 10⁶ cells per ml and mixed with an equal volume of 1% low melting-point agarose in PBS at 37 °C. The agarose–cell mixture was dispensed into a 100 μl-insert mould (Pharmacia LKB Biotechnology) and was solidified by placing the mould on ice. The agarose block was then placed in 250 μl of digestion mixture containing 1% (w/v) N-sarkosyl, 0.5 M-EDTA (pH 9) and 1 mg/ml proteinase K, and was incubated for 48 h at 50 °C. The supernatant of the digestion mixture was diluted twice with distilled water and then extracted with phenol–chloroform solution and with chloroform. After ethanol precipitation and washing with 70% ethanol, DNA was resuspended in 10 mM-Tris–HCl pH 8, 1 mM-EDTA, treated with RNase A, and run on a 1.6% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and was photographed.

**Morphological observation.** The scraped cells were collected by centrifugation, washed twice with PBS and then fixed in 2.5% glutaraldehyde in 0.1 M-sodium cacodylate buffer pH 7.2 for 24 h. After a wash with the sodium cacodylate buffer, the cells were post-fixed in 1% aqueous osmium tetroxide for 2 h, followed by a wash in the same buffer. They were then dehydrated in a series of ethanol solutions of decreasing dilution, cleared in propylene oxide, and embedded in an Epon–Araldite mixture. Semi-thin sections were stained with toluidine blue for light microscopy. Ultrathin sections were stained with lead and uranyl acetate, and then observed using an electron microscope (Hitachi H-600) as described previously (Uehara et al., 1992).

Flow cytometry. Cells were washed with PBS, incubated with PBS containing 0.04% EDTA at 4 °C for 10 min and then were removed from their dishes using a pipette. They were then fixed with 1% paraformaldehyde in PBS for 20 min at room temperature and washed twice with PBS. The cells were labelled with anti-Fas monoclonal antibody (provided by Dr S. Yonehara, Japan Tobacco Company Life Science Research Laboratory), which was diluted to a final concentration of 2.5 μg/ml with PBS containing 1% BSA, washed with PBS, and treated with phycoerythrin (PE)-conjugated goat anti-mouse IgM antibody (Southern Biotechnology Associates). The stained cells were analysed on a Cytron Absolute flow cytometer (Ortho Diagnostic Systems) in which a single 488 nm argon laser was used to excite PE (orange) as previously described (Miyawaki et al., 1992).

![Graph](image_url)

**Fig. 1.** Effect of influenza virus infection on the lysis of MDCK (a) and HeLa cells (b). Cytolysis was monitored by ¹⁴Cr release from the cells (○). For the assay, cells were labelled with Na₂¹⁴CrO₄ for 60 min, infected or mock-infected with virus, and then incubated in Eagle's MEM without serum as described in Methods. At various times p.i. the radioactivity in the medium was determined using a gamma counter. The percentage of specific ¹⁴Cr release was calculated as described in Methods. The HA titre (×) of the medium was measured at the same time points as for ¹⁴Cr release. The c.p.e. is denoted as follows: ±, slight morphological changes without cell rounding; +, less than 50% of cells rounded; + +, more than 50% of cells rounded and many cells afloat; + + +, almost total destruction of monolayers.
Results

Fragmentation of the chromosomal DNA of virus-infected cells

We first followed the time course of cytolysis in cells infected with influenza virus by monitoring the release of $^5$Cr-labelled proteins from the cells, which seemed to correlate well with c.p.e. Cytolysis of virus-infected MDCK cells began at 12 h post-infection (p.i.) and increased to a level of 80% at 36 h p.i. concomitant with the increase in HA titre (Fig. 1a) as well as the virus p.f.u. titre of the medium (3.6 x 10$^7$/ml at 36 h p.i.). Cytolysis of the virus-infected HeLa cells proceeded with a similar time course but with a slight delay (60% lysis at 48 h p.i.), and the HA and p.f.u. titres in the medium were much lower than those of MDCK cells throughout the infectious stage (Fig. 1b) (p.f.u. titre at 36 h p.i. was 4 x 10$^6$/ml). This result suggested that cytolysis is not in proportion to the extent to which virus components were generated. Although HeLa cells were reported as incapable of supporting the production of fully infectious virus (Henle et al., 1955), small amounts of infectious particles were detected in our experiment. This may be due to differences between the strains of influenza virus.

Fig. 2. Effect of influenza virus infection on DNA fragmentation in MDCK cells (a) or HeLa cells (b). DNAs were prepared from mock-infected (a) and virus-infected (b) cells at 0, 6, 12, 18, 24, 30, 36 h p.i. (lanes 1 to 7, respectively) for MDCK cells, or at 0, 4, 8, 12, 24, 36, 48 h p.i. (lanes 1 to 7, respectively) for HeLa cells. DNAs were separated by 1.6% agarose gel electrophoresis followed by staining with ethidium bromide. Lane M contains pUC19 plasmid DNA digested with Bsp1286I to provide markers. Their sizes are indicated on the left (bp).

Fig. 3. Effect of ZnSO$_4$ on the fragmentation of DNA into oligonucleosomes in influenza virus-infected MDCK cells. (a) Cells were mock-infected (a) or infected with virus (b), and then ZnSO$_4$ was added to the medium at concentrations of 0, 0.04, 0.1, 0.5 mM (lanes 1 to 4, respectively) in mock-infected cells, and 0, 0.04, 0.06, 0.08, 0.1, 0.25, 0.5 mM (lanes 1 to 7, respectively) in virus-infected cells. (b) Cells were infected with virus, and ZnSO$_4$ (0.1 mM) was added to the medium at 0, 2, 4, 6, 8 h p.i. (lanes 2 to 6, respectively). Lane 1 contains the DNA from virus-infected cells without ZnSO$_4$ treatment. DNAs were prepared from the cells at 12 h p.i. and were analysed by 1.6% agarose gel electrophoresis followed by staining with ethidium bromide. Lane M contains the same DNA markers as in Fig. 2.
Fig. 4. Effect of CHX (100 μg/ml) on influenza virus-induced cytolysis and DNA fragmentation in HeLa cells. (a) Cytolysis was monitored by \[^{51}\text{Cr}\] release from the cells as described in Fig. 1 in the presence (○) and absence (□) of CHX in virus-infected cells or in the presence (■) of CHX in mock-infected cells. (b) DNAs were prepared from mock-infected (a and b) and virus-infected (c and d) HeLa cells in the absence (a and c) or presence (b and d) of CHX at 0, 12, 24, 36, 48 h p.i. (lanes 1 to 5, respectively), and were analysed by 1.6% agarose gel electrophoresis followed by staining with ethidium bromide. Lane M contains the same DNA markers as in Fig. 2.

or to altered characteristics of HeLa cells. Cell death as monitored by trypan blue dye exclusion showed a similar time course to that observed by \[^{51}\text{Cr}\] release analysis (data not shown). Chromosomal DNA fragmentation of infected cells was then examined by agarose gel electrophoresis at various times p.i. Chromosomal DNA in virus-infected MDCK cells produced oligonucleosomes which appeared by 6 h p.i. (Fig. 2a). This also occurred in virus-infected HeLa cells by 24 h p.i. (Fig. 2b). The delayed appearance in HeLa cells may reflect a slower progression and lower degree of cell lysis than that of MDCK cells (Fig. 1a, b). In mock-infected HeLa cells, a low level of DNA fragmentation was observed due to incubation of the cells in the serum-free medium (Fig. 2b). We examined the effect of zinc, which is known to inhibit the internucleosomal DNA fragmentation oc-

Fig. 5. (a) Effect of the time of CHX addition on cell death of virus-infected HeLa cells. Cells were mock-infected (mock) or virus-infected (infection), and incubated for 30 h. For the virus-infected cells, 100 μg/ml CHX was added to the medium at 0, 2, 4, 6 h p.i. (0, 2, 4 and 6, respectively). In mock-treated cells, CHX was added to the medium at 0 h p.i. (+); (−) indicates samples without CHX treatment. Cell viability was determined by a trypan blue dye exclusion test. (b) The time course of protein synthesis in virus-infected HeLa cells. The virus-infected cells were pulse-labelled with \[^{35}\text{S}\]methionine at 0, 1.5, 3.5, 5.5 h p.i. (lanes 2 to 5, respectively) for 30 min and lysed. Mock-infected cells (lane 1) were pulse-labelled from 5-5 to 6 h p.i. and lysed. The lysates were electrophoresed on 18% polyacrylamide gels containing 3 M-urea, followed by fluorography. The positions of the HA, nucleoprotein (NP), non-structural viral proteins (NS1, NS2) and matrix (M1) viral protein are shown on the right.
Influenza virus-induced apoptosis

Fig. 6. Morphological changes of virus-infected (a) or mock-infected (b) HeLa cells. Cells were fixed at 30 h p.i. in glutaraldehyde solution as described in Methods. Semi-thin sections stained with toluidine blue were observed under a light microscope at a magnification of ×1000. Arrows indicate the apoptotic nuclei. Large arrowheads indicate the cytoplasmic changes of apoptotic cells, and small arrowheads indicate the non-apoptotic nuclei.

Effect of cycloheximide on virus-infected cells

Since inhibitors of protein or RNA synthesis reportedly block apoptosis, this process is thought to require active cellular metabolism (Wyllie et al., 1984; Cohen & Duke, 1984). As shown in Fig. 4(a), addition of 100 μg/ml cycloheximide (CHX), which completely inhibited both host and virus protein synthesis (data not shown), to the medium just after virus infection effectively blocked the cytolysis of HeLa cells to the level of non-infected-cells. The internucleosomal cleavage of chromosomal DNA in the virus-infected cells was also reduced by CHX to the control level (Fig. 4b), which was also observed in the mock-infected cells similarly treated. Because CHX itself induced significant cell degeneration of mock-treated MDCK cells, its effect on MDCK cells could not be examined. In further experiments (Fig. 5a), we examined the effect of the time of CHX addition on cell death. Cell
viability was followed quantitatively by trypan blue dye exclusion. Treatment with CHX at or before 2 h p.i. efficiently blocked cell death (only about 20% of the infected cells died), whereas the treatment at 4 h.p.i. or later did not (60 to 70% of the infected cells died, which was the same level as that found for the infected cells without CHX treatment). This result indicated that there was a critical period for the induction of cell death during the early stage of infection, occurring from 2 to 4 h p.i. We examined the time course of protein synthesis in virus-infected HeLa cells by pulse-labelling with [35S]-methionine. The host protein synthesis continued during the early stage of the infection, whereas viral protein synthesis was observed at 3.5 to 4 h p.i., and became dominant at 5.5 to 6 h p.i. (Fig. 5b). These observations suggest that small amounts of viral proteins which had been produced before the addition of CHX at 4 h p.i. might directly induce apoptosis or, alternatively, that viral infection might induce a host protein(s) that triggers the apoptosis, during the early phase of virus growth (see Discussion).

Morphological changes in HeLa cells due to influenza virus infection

Apoptosis induces characteristic morphological changes in cells such as condensation and fragmentation of the nucleus as well as loss of cytoplasm. To confirm that influenza virus infection induces such changes, we examined virus-infected HeLa cells 30 h p.i. by light and electron microscopy. Two types of change were seen in the infected cells (Fig. 6a). One was an extensive condensation of chromatin into several dense masses and compaction of cytoplasm associated with cytoplasmic vacuolization, characteristic features of apoptosis. Another was a slightly swollen cytoplasm with less staining than that of mock-infected cells but without remarkable chromatin condensation. The latter feature might be

Fig. 7. Expression of Fas Ag on virus-infected HeLa cells. (a) Flow cytometric analysis of Fas Ag on virus- (i) or mock- (ii) infected HeLa cells at 12 h p.i. Cells were stained with anti-Fas monoclonal antibody, followed by PE-conjugated goat anti-mouse IgM antibody, and analysed by flow cytometry. Solid lines indicate the profiles of the virus- or mock-infected HeLa cells. Broken lines indicate the profiles of the control with irrelevant fluorochrome-labelled monoclonal antibodies. (b) Effect of u.v.-irradiated virus on Fas Ag expression. Flow cytometric analysis of Fas Ag on virus- (solid line), u.v.-irradiated virus- (broken line) or mock- (dotted line) infected HeLa cells at 12 h p.i. (c) Effect of u.v.-irradiated virus on chromosomal DNA fragmentation. DNAs were prepared from MDCK cells infected with virus (lane 1) or u.v.-irradiated virus (lane 2) at 12 h p.i., and separated by 1-6% agarose gel electrophoresis, followed by staining with ethidium bromide. Lane M contains the same DNA markers as in Fig. 2.
Expression of Fas Ag in virus-infected HeLa cells

To confirm that virus infection causes apoptosis, we examined whether a putative apoptosis-mediating molecule, Fas Ag, was present in virus-infected HeLa cells using flow cytometry. Fas Ag was detected in appreciable amounts on the surface of virus-infected HeLa cells 12 h p.i. (the mean fluorescence intensity of Fas Ag was 106.5), whereas there was a lower staining level of Fas Ag in mock-infected cells (mean fluorescence intensity 64.1) (Fig. 7a). Virus infection caused an increase in Fas Ag mRNA to about 14-fold the control level at 3 h and 4 h p.i., and this then rapidly decreased to an undetectable level (unpublished). These findings suggest that the expression of the Fas Ag-encoding gene in virus-infected HeLa cells is activated at a transcriptional level, and may explain why the inhibitory effect of CHX on cell death occurs when added to the medium within 2 h p.i. but not when added 4 h p.i. or later (Fig. 5a). We then examined the effect of u.v.-inactivated virus on the cell death and Fas Ag expression. U.v.-irradiation markedly suppressed the Fas Ag expression in HeLa cells (Fig. 7b). U.v.-irradiation also lowered the HA titre of infected MDCK cells by more than 25, and effectively prevented the chromosomal DNA fragmentation (Fig. 7c) as well as the cytology of virus-infected cells (data not shown).

Discussion

Much remains to be discovered about the mechanisms whereby influenza virus exerts cytopathic damage not only in infected animals, but also in tissue culture cells. In this report, we analysed the damage caused by virus infection in cultured MDCK and HeLa cells, which permitted high and low levels of replication of A/Udorn virus, respectively. We observed that both cells exhibited several features characteristic of programmed cell death (apoptosis), suggesting that virus replication is not a direct cause of the cell lysis.

In many cases, apoptosis can be prevented by inhibitors of protein or RNA synthesis. However, in some instances apoptosis does not require protein synthesis (Duke et al., 1983). Moreover, cell death caused by tumour necrosis factor was facilitated by inhibition of protein or RNA synthesis (Rubin et al., 1988). It is thus likely that there are two groups of apoptosis-related proteins in a cell. One group comprises positive factors necessary to induce apoptosis, whereas the other is their antagonists (Umansky, 1991). The effect of a protein synthesis inhibitor on apoptosis might be dependent on the cell type, the stage of the cell cycle or cell differentiation. As described here, CHX added within 2 h p.i., but not at 4 h p.i. or later, inhibited the cell death of influenza virus-infected HeLa cells, suggesting that virus infection induces positive factors for programmed cell death between 2 and 4 h p.i. On the other hand, induction of DNA fragmentation by CHX alone in HeLa or MDCK cells may suggest the presence of antagonists in these cells. Although several viral proteins have been implicated in pathogenicity (Sweet & Smith, 1980; Webster & Rott, 1987), they are not the sole determinants. Participation of a host factor(s) has to be also considered. Recently, a putative apoptosis-mediating molecule, termed Fas Ag or APO-1, has been isolated (Yonehara et al., 1989; Trauth et al., 1989; Itoh et al., 1991; Oehm et al., 1992). Sequence analysis showed that it belongs to the tumour necrosis factor/nerve growth factor receptor superfamily. Fas Ag was found to be expressed on some Epstein–Barr virus-infected T or B cell populations (Uehara et al., 1992; Falk et al., 1992). Supporting these findings, we found that influenza virus infection elicited a significant increase in the Fas Ag mRNA 3 and 4 h p.i., followed by the expression of Fas Ag on the surface of HeLa cells 12 h p.i. Because the virus infection had shut off the host protein synthesis 6 h p.i., Fas Ag may be synthesized within at least 6 h (or 4 h judging from the result of Fig. 5a) p.i. but is not immediately delivered to the cell surface. The delay in antigen expression on the cell surface and cell lysis suggests that either the HeLa cell or the influenza virus has a mechanism to retard the process of apoptosis. Several viruses reportedly encode proteins to overcome infection-induced apoptosis (for review see Gooding, 1992). It is unclear, however, whether influenza virus has such a mechanism.

One possible mechanism by which influenza virus infection elicits Fas Ag gene expression is through formation of dsRNA, which was previously shown to induce c-fos gene expression in HeLa cells (Zinn et al., 1988), and to activate binding of NF-κB to an inducible element in the human β-interferon promoter (Visvanathan & Goodbourn, 1989). Double-stranded RNA was also shown to induce numerous cytokines (Majde et al., 1991), and has been suggested to trigger the acute-phase response in influenza virus infection in vivo (Kimura-Takeuchi et al., 1992). This possibility is supported by our observation that synthetic dsRNA, poly(I)-poly(C), transiently induced Fas Ag mRNA (T. Takizawa, unpublished).
Nucleosomes have been shown to have mitogenic effects on lymphoid cells in vitro (Bell et al., 1990), and a considerable degree of polyclonal B cell activation has been reported in AIDS patients (Clifford et al., 1983). Consistent with these observations are the reports of the presence of autoantibodies against nucleosomal constituents such as histone H2B or DNA being found in AIDS patients (Stricker et al., 1987; Laurent-Crawford et al., 1991). It is therefore important to examine the presence of these autoantibodies in influenza virus-infected patients in order to consider the pathological relevance of apoptosis in vivo.

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