Induction of Apoptosis in Murine Coronavirus-Infected 17Cl-1 Cells

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1. INTRODUCTION

Apoptosis is an important process in the development and homeostasis of multicellular organisms (Jacobson *et al* 1997). In most cases, apoptosis is executed by activating a proteolytic system involving a family of caspases. Caspases participate in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in cell death (Cohen 1997). Apoptosis also represents a highly efficient defence mechanism against virus infection; apoptosis aids in removal of viral proteins and nucleic acids by the infected host.

Eleouet *et al* (1998) demonstrated that infection of coronavirus transmissible gastroenteritis virus (TGEV) induces caspase-dependent apoptosis in several cell lines. Belyavskyi *et al* (1998) showed that mouse hepatitis virus (MHV) strain 3 (MHV-3) infection of cultured macrophages induces apoptosis, while it is not clear whether MHV-3-induced apoptosis is caspase-dependent. In the present study we demonstrated that MHV infection in an established cultured cell line also induced caspase-dependent apoptosis.

The Nidoviruses (Coronaviruses and Arteriviruses).
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2. MATERIALS AND METHODS

2.1 Viruses and cells

Plaque-cloned A59 strain of MHV was used. Mouse 17Cl-1 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (with sodium pyruvate, JRH Biosciences) containing 10% fetal calf serum (FCS). After MHV infection, FCS concentration was reduced to 2%.

2.2 DNA fragmentation assay

Low-molecular weight DNA was extracted as described by Hinshaw *et al* (1994).

2.3 Hoechst dye staining

At various times postinfection (p.i.) of MHV, cells were collected, washed with phosphate buffered saline (PBS), and resuspended in 100 μl PBS. Cells were fixed by first slowly adding 200 μl of fixing solution (methanol: acetic acid = 3: 1) and then adding additional fixing solution up to 2 ml. After incubation at room temperature for at least 1 h, the cells were collected by centrifugation and resuspended in 400 μl of fixing solution. Cell suspensions were deposited on microscopic slides and air-dried. Cells were stained with a drop of staining solution (50% glycerol, 50% 0.1 M Tris-HCl [pH 7.4], 1 μg/ml Hoechst 33342), coversliped, and observed under a fluorescent microscope.

2.4 Effect of caspase inhibitors on apoptosis induction

17Cl-1 cells were incubated for 2 h with 80 μM Z-Asp-Glu-Val-Asp-fmk (Z-DEVD-fmk) (Enzyme Systems Products, Livermore, CA) prior to MHV infection. After MHV infection, cells were incubated in the presence of 80 μM of Z-DEVD-fmk. At 24 h p.i., the Z-DEVD-fmk was replenished. At 48 h p.i., apoptosis was measured by DNA fragmentation assay.

3. RESULTS

To investigate whether MHV infection in established cell lines induced apoptosis, we examined MHV-infected 17Cl-1 cells for the presence of internucleosomal DNA cleavage, an event commonly observed in apoptotic

cells. At various times p.i., cells attached to the plates were scraped by using a rubber policeman and combined with those floating in the medium. Low-molecular-weight apoptotic DNA fragments were extracted and then separated by agarose gel electrophoresis (Fig. 1A). A sign of internucleosomal DNA cleavage, which appeared as a low level of smearing of DNA in agarose gel electrophoresis, was first detected at 36 h p.i. At 62 h p.i., a DNA ladder was evident in MHV-infected cells but not in the mock-infected cells. DNA ladder was also detected in MHV-JHM-infected cells (data not shown). We used the terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay (ApoAlert DNA Fragmentation Assay Kit, Clontech) to further confirm DNA fragmentation in MHV-infected 17Cl-1 cells. At 36 h p.i., approximately one-quarter of the infected cells were TUNEL positive. Very few TUNEL positive cells were observed in the mock-infected cultures (data not shown). To further confirm that MHV-infected 17Cl-1 cells were undergoing apoptosis, MHV-infected 17Cl-1 cells were stained with the fluorescent dye Hoechst 33342. In the mock-infected culture, the majority of cells contained intact nuclei at 68 h p.i., while at the same time MHV-infected 17Cl-1 cultures showed chromatin condensation (Fig. 2). These data demonstrated that apoptosis was induced in MHV-infected 17Cl-1 cells, and the strong apoptotic signs were evident late in infection.

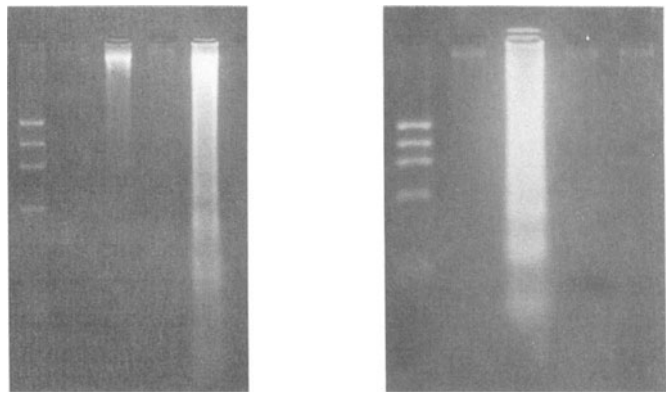

Figure 1. DNA fragmentation analysis. (A) 17Cl-1 cells were mock-infected or infected with MHV-A59. Low-molecular-weight DNA was extracted from cells at indicated time after infection. (B) Culture fluid containing MHV-A59 was UV-irradiated for indicated time at 0°C, and then inoculated into 17Cl-1 cells. Low-molecular-weight DNA was isolated at 46 h p.i. Samples were electrophoresed in 2% agarose gels and visualized with ethidium bromide.

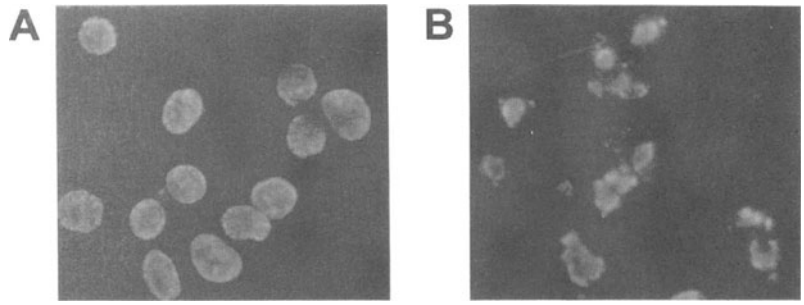

Figure 2. Chromatin condensation in 17Cl-1 cells infected with MHV. 17Cl-1 cells were mock-infected (A) or infected with MHV-A59 (B). At 68 h p.i., mock-infected cells and floating cells in MHV-infected culture media were collected and stained with Hoechst 33342.

To test whether binding of MHV to MHV receptors or some unidentified substances, other than MHV, present in the inoculum induced apoptosis, MHV was inactivated by irradiation of inoculum with UV-light and then added to 17Cl-1 cells. The infectivity of UV-irradiated samples was less than 1 PFU. After incubation for 1 h at 37°C, the inoculum was removed and the cells were incubated for up to 46 h. Neither CPE (data not shown) nor internucleosomal DNA cleavage (Fig. 1B) was observed in the cells that underwent this treatment, demonstrating that binding of MHV to MHV receptors alone or unidentified substances, which may be present in the inoculum, did not induce apoptosis. Replication of MHV was necessary for inducing apoptosis.

In the very late stage of this study we noticed that changing cell culture condition significantly affected the induction of apoptosis. When MHV-infected 17Cl-1 cells were incubated in a medium containing DMEM from a different vendor (Gibco/BRL), DNA ladder was clearly detected as early as 24 h p.i. (data not shown). There were several differences in the contents of two batches of DMEMs used in this study; one major difference was that DMEM from Gibco/BRL lacked sodium pyruvate, while DMEM used in most of our other experiments contained sodium pyruvate.

We examined whether apoptosis was induced in another MHV-susceptible cell line, DBT. Floating fused MHV-A59-infected DBT cells and the small number of cells that were still attached to the plates were collected at 12 h, 36 h, and 48 h p.i. Gel electrophoresis of low-molecular-weight DNA extract from these samples and TUNEL assay at 12 h p.i. showed no sign of DNA fragmentation (data not shown), demonstrating that MHV infection in DBT cells did not induce apoptosis.

To determine whether MHV-induced apoptosis was caspase-dependent, we studied the effect of Z-DEVD-fmk, an irreversible and cell-permeable

inhibitor of caspase-3, on the induction of apoptosis in MHV-A59-infected 17Cl-1 cells. DNA fragmentation assay showed that addition of Z-DEVD-fmk, at a final concentration of 80 μM, strongly inhibited internucleosomal DNA cleavage (Fig. 3A), demonstrating that MHV-induced apoptosis in 17Cl-1 cells was caspase-dependent. One step MHV growth curves in 17Cl-1 cells in the presence and absence of Z-DEVD-fmk showed no significant difference, indicting that suppression of caspase-3 activity did not affect MHV growth (Fig. 3B).

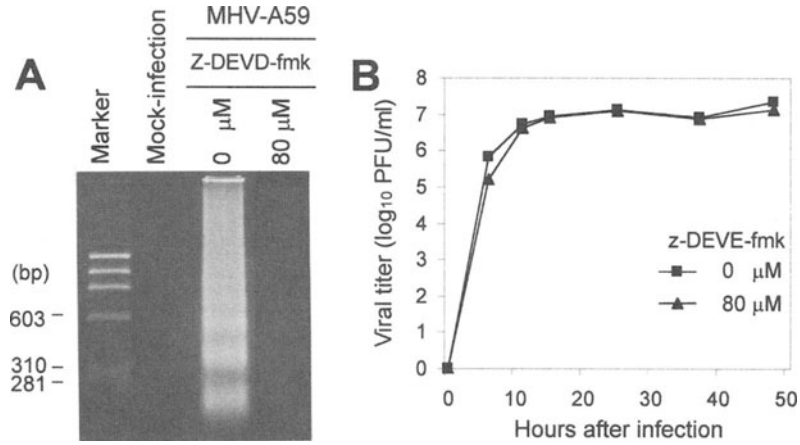

Figure 3. Effect of Z-DEVD-fmk on MHV-induced apoptosis. (A) 17Cl-1 cells were mock-infected or incubated with medium lacking Z-DEVD-fmk or that containing Z-DEVD-fmk at a concentration of 80 μM. At 48 h p.i., low-molecular-weight DNA was extracted and separated in a 2% agarose gel. (B) Effect of Z-DEVD-fmk on one-step growth curve of MHV-A59 in 17Cl-1 cells.

4. DISCUSSION

Induction of apoptosis in MHV-infected 17Cl-1 cells was demonstrated by agarose gel electrophoresis of small DNA fragments, TUNEL assay, and Hoechst staining. Our present data and previous findings of apoptosis in TGEV-infected cultured cells (Eleouet *et al* 1998) and in MHV-3-infected cultured macrophages (Belyavskyi *et al* 1998) established that apoptosis is one mechanism of cell death in coronavirus-infected cells. MHV-induced apoptosis was suppressed by caspase-3 inhibitor Z-DEVD-fmk, demonstrating that MHV-induced apoptosis was caspase-dependent. TGEV-mediated apoptosis is also caspase-dependent (Eleouet *et al* 1998). These data indicate that coronavirus infection induces caspase-dependent apoptosis (Cohen 1997).

MHV-infected DBT cells did not show any significant sign of apoptosis throughout infection, indicating that apoptosis was not a universal event among MHV-infected established cell lines. DBT cells are derived from an astrocytoma isolated from CDF1 mice (Hirano *et al* 1974), while 17Cl-1 cells are a line of spontaneously transformed mouse BALB/c 3T3 cells (Sturman and Takemoto 1972); DBT cells and 17Cl-1 cells are derived from different organs of different strains of mice. The difference in the apoptotic response to MHV infection between DBT cells and 17Cl-1 cells may be determined by the difference in the origin of organs and/or difference in the strain of mice.

ACKNOWLEDGMENTS

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