Increased Induction of Apoptosis by a Sendai Virus Mutant Is Associated with Attenuation of Mouse Pathogenicity

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An avirulent mutant of Sendai virus, Ohita-MVC11 (MVC11), was generated from a highly virulent field strain, Ohita-M1 (M1), through successive passages in LLC-MK2 cell cultures (M. Itoh, Y. Isegawa, H. Hotta, and M. Homma, J. Gen. Virol. 78:3207-3215, 1997). In LLC-MK2 cells, MVC11 induced a high degree of apoptotic cell death that was demonstrated by chromatin condensation of the nucleus and DNA fragmentation, and production of MVC11 declined markedly after prolonged culture. On the other hand, M1 did not induce prominent apoptosis and maintained high virus titers. In primary mouse pulmonary epithelial cell cultures, M1 replicated rather slowly to reach maximum level of virus production at 3 days postinfection, and high levels of virus production were maintained thereafter without causing apoptosis. In contrast, MVC11, which produced 20 times more progeny virus than M1 at 1 day postinfection, induced a high degree of apoptotic cell death before the virus replication cycle was completed. Accordingly, the production of progeny virus was strongly inhibited thereafter. In the lungs of mice infected with MVC11, virus antigens and signals of DNA fragmentation detected by the in situ terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling technique colocalized in bronchial epithelial cells, clearly demonstrating that infection by MVC11 triggered apoptosis in vivo as well as in vitro. These results suggest the possibility that induction of apoptosis by MVC11 plays an important role in attenuation of mouse pathogenicity by restricting progeny virus production in the lung. The C protein was shown to have the capacity to induce apoptosis, and the increased level of the C protein in MVC11-infected cells was considered to account partly, if not entirely, for the induction of apoptosis.

Sendai virus (SeV), a member of the paramyxoviruses, is also called murine parainfluenza virus type 1 and often causes outbreaks of lethal pneumonia in mouse colonies. Experimental infection in mice has been studied as a model for respiratory viral infection. Although there are SeV strains differing remarkably in pathogenicity to mice, the determinants of their pneumovirulence are largely unknown. In our previous work, we demonstrated that the pathogenicity of SeV closely correlates with virus replication in the mouse lung (35). We then showed that susceptibility of the fusion (F) envelope glycoprotein to trypsin and to the activating proteases in the mouse respiratory tract determines the efficiency of virus replication, and therefore the virulence, by supporting multiple-cycle replication through cleavage and activation of the F protein (16). Kato et al. (20) reported that an SeV mutant lacking the V protein replicated less efficiently in the lungs of mice and was strongly attenuated. However, the mechanism by which the lack of the V protein leads to the decreased production of progeny virus is not known.

When infected with a virus, the host attempts to suppress virus replication in infected cells and viral spread to neighboring cells by means of host defense mechanisms such as induction of immune responses, interferon production, and suicidal cell death (apoptosis). By turning on the switch for apoptosis before the virus has completed the replication cycle, the host cells prevent the virus from producing progeny virions that infect neighboring cells. This idea is supported by recent findings that many viruses, such as poxviruses (2, 13), adenovirus (30, 31), Epstein-Barr virus (9–11), hepatitis C virus (7, 32),...
and baculovirus (3), contain genes whose function appears to interfere with the apoptotic process, presumably to allow cell survival and continued virus replication (36).

Recently, we reported the characterization of an avirulent mutant of SeV, the Ohita-MVC11 (MVC11) strain, which was derived from a highly virulent field strain, the Ohita-M1 (M1) strain, and possessed two amino acid mutations, one in the C protein and the other in the L protein (17). MVC11 exhibited strongly suppressed virus replication in mouse lungs and had almost entirely lost pathogenicity to mice. In this work, we studied replication of M1 and MVC11 in cultured mouse pulmonary epithelial cells, as well as in LLC-MK2 cells, in order to elucidate the mechanism of restricted replication of MVC11 in mouse lungs. We found that whereas M1 maintained to produce progeny virus for a long period of time without killing the host cells, MVC11 induced apoptotic cell death that interfered with the following progeny virus production. We propose a hypothesis that the increased capacity of MVC11 to induce apoptosis may play an important role in attenuation of virulence through restricting virus spread in mouse lungs.

**MATERIALS AND METHODS**

**Viruses, cells, and antibodies.** The M1 strain of SeV, a fresh isolate from an outbreak of SeV in experimental animal facilities, and the MVC11 strain, a laboratory-adapted mutant of M1 obtained through passaging in rhesus monkey (LLC-MK2) cells, were described elsewhere (17). Infective virus titers were determined as described previously and expressed as PFU or cell-infecting units (CIU) (19). CIU were essentially equivalent to PFU. LLC-MK2 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 8% fetal bovine serum (FBS). Polyclonal anti-SeV antibodies were made by immunizing rabbits with purified SeV strain Fushimi. Polyclonal anti-C guinea pig serum (29) was a kind gift from K. Iwasaki (Tokyo Metropolitan Institute of Medical Science).

**Primary culture of mouse pulmonary epithelial cells.** Tracheotomy was performed on 6-week-old male ICR/CRJ (CD-1) mice under ether anesthesia, and 1 ml of protease type X (2 mg/ml; Sigma) was infused into the lung with a syringe. After incubation for 10 min at room temperature, the lung was taken and minced in phosphate-buffered saline (PBS). Blocks of the tissues were removed by filtration through four layers of sterilized gauze, and single cells were collected by...
centrifugation, suspended in Dulbecco’s MEM supplemented with 10% FBS, and cultivated at 38°C.

**Determination of apoptotic cell death.** Occurrence of apoptosis was determined by the following two methods.

For detection of chromatin condensation of the nuclei, cells grown on coverslips were fixed with ethanol for 20 min at room temperature and stained with 10 μg of Hoechst 33342 per ml in PBS for 10 min at room temperature. For detection of DNA fragmentation, cells (5 × 10^5) were lysed in 0.5% Triton X-100–10 mM Tris-HCl (pH 7.5)–10 mM EDTA, and nuclei were removed by centrifugation at 10,000 × g for 5 min at 4°C. The supernatants were treated with 50 μg of proteinase K per ml and 10 μg of RNase A per ml for 1 h at 37°C, and DNAs were extracted with phenol-chloroform and precipitated with ethanol. The pellets were dissolved in Tris-EDTA (pH 7.5) and separated by electrophoresis in 1% agarose gels.

**Western blot analysis.** Cells were lysed by treatment with 0.5% Triton X-100 in 10 mM Tris-HCl (pH 7.5) for 15 min on ice, and nuclei were removed by centrifugation at 10,000 × g for 5 min at 4°C. Supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8 or 16% acrylamide for detection of the C protein) under reducing conditions, and the proteins were electrotransferred onto nitrocellulose membranes. After blocking with 3% skim milk in PBS, the membranes were incubated with anti-SeV rabbit antiserum and subsequently with peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG). To detect the C protein, the membranes were treated with anti-C guinea pig antiserum and then with peroxidase-conjugated goat anti-guinea pig IgG. Virus-specific proteins were visualized with an ECL chemiluminescence kit (Amersham) according to the manufacturer’s instructions.

**In situ terminal end-labeling.** The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) technique was used to label DNA strand breaks in apoptotic cells. Paraformaldehyde-fixed lung sections were deparaffinized with absolute and 95, 75, and 50% ethanol solutions and then washed with PBS. After endogenous peroxidase was inactivated in 3% hydrogen peroxide, slide preparations were treated with 50 μg of proteinase K per ml for 30 min at room temperature. Subsequent steps were performed with a kit (Apop Tag; Oncor), according to the manufacturer’s instructions, to end label the fragmented DNA with digoxigenin-11-dUTP and peroxidase-conjugated antidigoxigenin antibody. Color development was achieved with diaminobenzidine as the substrate.

**Double labeling of tissue sections for detection of SeV antigens and TUNEL signals.** The TUNEL reaction was completed as far as the wash steps after the labeling reaction. The sections were then incubated sequentially with anti-SeV rabbit antiserum, biotin-conjugated goat anti-rabbit IgG, and alkaline phosphatase-conjugated streptavidin. Finally, color reactions were performed with diaminobenzidine to detect the peroxidase-labeled DNA fragmentation and then with 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium to detect alkaline phosphatase-labeled SeV antigens.

**Expression of the C protein.** Sau3AI-BglII fragments (nucleotides [nt] 107 to 812) of cDNAs of the P genes of M1 and MVC11, both containing the start codon for the C protein (nt 114) but lacking those for the P (nt 104) and the C9 (nt 81) proteins, were inserted into the unique BamHI site of the pSG5 mammalian expression vector (8). EcoRI linker-ligated NP cDNA (nt 26 to 1674) derived from M1 was inserted into the EcoRI site of pSG5. The resulting plasmids, pSG-CM, pSG-CMVC, and pSG-NP, which express the C protein of M1 (C170F), that of MVC11 (C170S), and the NP protein, respectively, under the control of the simian virus 40 early promoter, were introduced into cells by calcium phosphate-mediated transfection or by using Lipofectin (GIBCO BRL). We also used pSV2-C, which expresses the C protein of SeV strain Z (kindly supplied by H. Taira, Faculty of Agriculture, Iwate University).

FIG. 4. Chromatin condensation in primary cultures of mouse pulmonary epithelial cells infected with SeV. Primary cultures of mouse pulmonary epithelial cells were infected with either M1 (a to d) or MVC11 (e to h). At 2 or 6 days p.i., cells were fixed with ethanol and subjected to immunofluorescence staining with anti-SeV rabbit antiserum (αSeV) as the first antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG as the second antibody for detection of virus antigens (a, c, e, and g), followed by staining with Hoechst 33342 for detection of chromatin condensation (b, d, f, and h). The arrows in panel d show the nuclei of dividing cells infected with M1, and the arrowheads in panels f and h depict chromatin condensation in the nuclei.
RESULTS

Detection of apoptosis in SeV-infected LLC-MK₂ cells. We previously showed that progeny virus production of MVC11 in LLC-MK₂ cells within 24 h postinfection (p.i.) was higher than that of M1 through increased mRNA synthesis (17). We also reported that, although it was higher than that of M1 within 1 day p.i., replication of MVC11 in mouse lungs was strongly restricted at 2 days, p.i. and thereafter (17). In the present study, therefore, we first examined M1 and MVC11 virus replication in LLC-MK₂ cells for a longer period of time. The culture media of M1- and MVC11-infected LLC-MK₂ cells were replaced every 6 h (0 to 48 h p.i.) or every 12 h (48 to 96 h p.i.), and the virus titers were assayed. M1-infected LLC-MK₂ cells continued to release high titers of progeny virus until 96 h p.i., with approximately 90% of infected cells being alive (Fig. 1a). All the living cells were confirmed to be infected with M1 by immunofluorescence analysis with anti-SeV antiserum (data not shown). Since the cells were infected at a multiplicity of infection (MOI) of 10 and cultivation was performed in the absence of trypsin, multiple-cycle replication would not take place. Thus, a high degree of virus replication was maintained in M1-infected LLC-MK₂ cells throughout the observation period without killing the host cells. On the other hand, MVC11-infected LLC-MK₂ cells underwent injury and died. Accordingly, MVC11 progeny virus production diminished rapidly after 24 h p.i. (Fig. 1b).

Since SeV was reported to induce apoptosis (38), we examined whether the strong cytopathic effect of MVC11 was caused by apoptosis. Staining of nuclei with Hoechst 33342 clearly demonstrated condensed chromatin in MVC11-infected, but not M1-infected, LLC-MK₂ cells (Fig. 2a). Also, strong DNA fragmentation was detected at 36 h p.i. and later in MVC11-infected cells, whereas slight DNA fragmentation was observed in M1-infected cells only at 48 h p.i. (Fig. 2b).

Restricted production of progeny virus as a result of apoptosis in MVC11-infected mouse pulmonary epithelial cells. MVC11 has almost completely lost lethality against mice, with its replication being extremely suppressed in the mouse lungs (17). To get information on the mechanism(s) of suppression of progeny virus production, we investigated whether MVC11 could trigger apoptosis to interrupt virus production in mouse pulmonary epithelial cells, the target cells of SeV in vivo, as observed with LLC-MK₂ cells. In a primary culture of mouse pulmonary epithelial cells, M1 virus growth took place more slowly than in LLC-MK₂ cells and reached a maximum titer at 3 days p.i., which was maintained at least until 11 days p.i. (Fig. 3a). On the other hand, virus titers in MVC11-infected cells reached a maximum level at 1 day p.i., and diminished rapidly.
thereafter. Figure 3b shows synthesis of SeV-specific proteins in the primary culture of epithelial cells. In accordance with the time course of virus production (Fig. 3a), synthesis of viral proteins in M1-infected mouse pulmonary epithelial cells took place relatively slowly, reaching a maximum level at 3 days p.i., which was maintained throughout the observation period. On the other hand, protein synthesis in MVC11-infected cells occurred more rapidly to a maximum level at 1 day p.i. and was strongly suppressed thereafter.

SeV-infected mouse pulmonary epithelial cells were examined for apoptosis by staining the nuclei with Hoechst 33342. The nuclei of M1-infected cells appeared to be intact at both 2 and 6 days p.i. (Fig. 4b and d). Some of the M1-infected cells were even found to proliferate (Fig. 4d), suggesting that M1 caused persistent infection without killing the host cells. On the other hand, the nuclei of MVC11-infected cells demonstrated condensed chromatin at both 2 and 6 days p.i. (Fig. 4f and h), with large numbers of cells having detached from the bottom of the plastic dish on day 6. These results indicate that MVC11 could induce strong apoptosis of the infected cells, which interrupted the following synthesis of viral proteins and progeny virus production. On the other hand, M1 possessed a very limited capacity, if any, to trigger apoptosis, which allowed M1 to replicate for a prolonged period of time.

**Induction of apoptosis in the lungs of mice infected with MVC11.** To examine whether MVC11 could induce apoptotic cell death in mouse lungs in vivo, lung sections obtained from M1- and MVC11-infected mice were stained for TUNEL signals. TUNEL signals were detected only slightly in the lungs of mice infected with M1 at 2 days p.i. (Fig. 5a). Although M1 infection spread over the lung, even to the alveoli, and produced high titers of virus on day 6 (17), only a few TUNEL signal-positive cells were detected (Fig. 5b). On the other hand, nuclei of bronchial epithelial cells in MVC11-infected mice were strongly stained by TUNEL at 2 days p.i. (Fig. 5c). On day 6, the number of cells with TUNEL-positive nuclei decreased and the intensity of the signals diminished (Fig. 5d), which was associated with both the elimination of MVC11 antigen-positive cells and the sharp decline of virus titers in the lung (17).

Lung sections prepared from the mice described above were then dually stained for SeV antigens and DNA fragmentation to confirm that the cells dying by apoptosis were infected with MVC11. Although bronchial epithelial cells of mice inoculated with M1 were stained dark purple, which verified the infection by SeV, brown TUNEL signals were not detected in those cells (Fig. 6a). In MVC11-infected mice, bronchial epithelial cells with DNA fragmentation (brown) were shown to be positive for SeV antigens (purple) (Fig. 6b). The lungs of control mice inoculated with PBS alone demonstrated neither SeV antigens nor TUNEL signals (Fig. 6c). These results clearly demonstrate that MVC11 triggered apoptosis as a result of virus infection, while M1 did not.

**Induction of apoptosis by the C protein.** MVC11 possesses two amino acid mutations; one is in the C protein at position 170 (Phe→Ser), and the other is in the L protein at position 2050 (Glu→Ala) (17). We tested possible involvement of the C protein in the induction of apoptosis. As demonstrated in Fig. 7, COS-7 cells transiently expressing the C protein exhibited condensation of chromatin. There was no apparent difference in apoptosis-inducing capacity among the C proteins of strains M1 (C<sup>170F</sup>) (Fig. 7b), MVC11 (C<sup>170S</sup>) (Fig. 7d), and Z (Fig. 7f). It was unlikely that the apoptosis was induced simply by overexpression of a protein, since strong expression of the NP protein did not induce apoptosis in the cells (Fig. 7h). Induction of apoptosis by C<sup>170F</sup> and C<sup>170S</sup>, but not by the NP protein, was confirmed also in HeLa and L929 cells (data not shown).

Since the C proteins of M1 and MVC11 induced apoptosis equally, we then examined the synthesis of the C protein in M1- and MVC11-infected cells. In M1-infected LLC-MK<sub>2</sub> cells, the C protein accumulated gradually until 96 h p.i. (Fig. 8a). In MVC11-infected LLC-MK<sub>2</sub> cells, on the other hand, synthesis of the C protein took place more rapidly and to a larger extent than with M1 until 24 h p.i. but decreased rapidly thereafter. In mouse pulmonary epithelial cells, the C protein of M1 was not detected throughout the course of infection, prob-
ably due to the limited number of cells, whereas that of MVC11 was detected at 1 day p.i. and diminished thereafter (Fig. 8b).

**DISCUSSION**

Apoptosis is a built-in cell suicide program required for normal embryonic development, tissue homeostasis, and several immunological processes. Infection of cultured cells with a wide variety of viruses, including herpesviruses (14, 18, 21, 22, 25), paroviruses (27), retroviruses (5, 23, 26, 28, 33), paramyxoviruses (4), myxoviruses (6, 12, 34), alphaviruses (24, 39), and picornaviruses (37), results in activation of the apoptosis pathway. It has been shown that virus-induced activation of programmed cell death in certain cell populations, such as neurons and immune cells, may be directly associated with viral pathogenicity (1). In such cases, virulent strains cause apoptosis more strongly than avirulent strains. On the other hand, data have accumulated that host cells trigger apoptosis when infected with viruses, which interferes with virus production, offering an important host defense mechanism to combat virus infection (36). Suppression of virus production by apoptosis was reported with some viruses, such as poliovirus (37) and vaccinia virus (13).

In this study, we demonstrated that MVC11, an avirulent mutant of SeV derived from the virulent wild-type isolate M1, induced apoptosis in mouse pulmonary epithelial cells within 2 days p.i. (Fig. 4f). As shown in the one-step growth experiment (Fig. 3a), replication of SeV appears to take place relatively slowly in primary cultures of mouse epithelial cells, and probably in vivo as well, reaching a maximum titer at 3 days p.i. Apoptosis triggered by MVC11 therefore could have caused cell death before the virus replication cycle was completed and, as a result, the following synthesis of virus proteins and pro-
duction of progeny virus were strongly suppressed. Considering that about 10^4 mouse pulmonary epithelial cells were used for the virus growth experiment of Fig. 3a, a single MVC11-infected cell produced approximately 100 virus particles on the first day of infection and much less thereafter. On the other hand, an M1-infected cell released 1,000 virus particles every day throughout the cultivation period without undergoing apoptosis. Therefore, it is clear that apoptosis induced by MVC11 inhibited virus production in the culture. The same concept could be applied to the in vivo experiments, where apoptosis interfered with the replication and spread of MVC11 in mouse lungs. Thus, it is likely that induction of apoptosis by MVC11 plays an important role in attenuation of the mouse pathogenicity of the virus.

There is increasing evidence that many viruses encode proteins that interact with the cellular pathways regulating programmed death (36). However, the molecular mechanism by which SeV infection activates the death pathway is unknown. Tropéa et al. (38) reported that alpha interferon had no effect on SeV-induced apoptosis. We demonstrated in the present study that the C protein of SeV induces apoptosis when transiently expressed in COS-7 cells (Fig. 7) and in HeLa and L929 cells (data not shown). To our knowledge, this is the first study that pinpointed an SeV protein as an apoptosis-inducing protein.

Despite the marked difference between M1 and MVC11 in the capacity to induce apoptosis through viral infection, the C protein of M1 (C^{1708}) induced apoptosis to practically the same extent as the C protein of MVC11 (C^{1708}) in transient-expression experiments (Fig. 7). A possible explanation for this discrepancy is that apoptosis is triggered by the increased level of C protein expression in MVC11-infected cells; the amounts of the C protein in MVC11-infected cells in the early stage of infection (12 to 24 h p.i. in LLC-MK2 cells and 1 day p.i. in mouse pulmonary epithelial cells) were significantly larger than those in M1-infected cells throughout the infection (Fig. 8). Another possibility that should also be taken into consideration is that another SeV protein(s) is involved in the induction of apoptosis. Further analysis to elucidate the mechanism of SeV-induced apoptosis is in progress.

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