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Apoptosis Induced by the SARS-Associated Coronavirus in Vero Cells Is Replication-Dependent and Involves Caspase

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ABSTRACT

The pathogenesis of the severe acute respiratory syndrome (SARS), a newly emerging life-threatening disease in humans, remains unknown. It is believed that the modulation of apoptosis is relevant to diseases that are caused by various viruses. To examine potential apoptotic mechanisms related to SARS, we investigated features of apoptosis induced by the SARS-associated coronavirus (SARS-CoV) in host cells. The results indicated that the SARS-CoV-induced apoptosis in Vero cells in a virus replication-dependent manner. Additionally, the downregulation of Bcl-2, the activation of casapse 3, as well as the upregulation of Bax were detected, suggesting the involvement of the caspase family and the activation of the mitochondrial signaling pathway. Although there is a positive correlation between apoptosis and virus replication, the latter is not significantly blocked by treatment with the caspase inhibitor z-DEVD-FMK. These preliminary data provide important information on both the pathogenesis and potential antiviral targets of SARS-CoV.

INTRODUCTION

Apoptosis, or programmed cell death, is a morphological and biochemically defined form of cell death that occurs in many diseases including virus infections (Kerr *et al*., 1972; Razvi and Welsh, 1995). It is believed that viruses belonging to different families, including coronaviruses, such as infectious bronchitis virus (IBV; Liu *et al*., 2001), transmitted gastroenteritis virus (TGEV; Eleouet *et al*., 1998), and murine hepatitis virus (MHV; Lang *et al*., 2003), encode gene products to maximize their replication by modulating the process of apoptosis during their life cycle (Granville *et al*., 1998; O'Brien, 1998; Roulston *et al*., 1999; Thomson *et al*., 2001; Boya *et al*., 2001; Liu *et al*., 2001). Induction or inhibition of apoptosis in infected cells has been implicated as one of the most important aspects of viral pathogenesis (Hasnain *et al*., 2003; DeBiasi *et al*., 2004; Liu and Zhang, 2005). Studies of such events have provided a crucial understanding of viral diseases (e.g., AIDS), and have led to the development of novel antiviral strategies (Rudin and Thompson, 1997; Selliah and Finkel, 2001; Stanley, 2002; Shao *et al*., 2004).

Cell death caused by apoptosis is characterized by chromatin condensation, DNA fragmentation to nucleosome-sized pieces, membrane blebbing, cell shrinkage, and compartmentalization of the dead cells into membrane-enclosed vesicles or apoptotic bodies (Arends and Wellie, 1991). Several different cellular apoptosis signaling pathways are known at present (Khosravi-Far and Esposti 2004; Thibodeau *et al*., 2004), and in most instances the caspases are deemed as central players. Caspases are a family of cysteine-dependent aspartate-directed proteases that exist in cells as inactive zymogens and are triggered to cleave a set of proteins at specific sites containing aspartic acid to cause cell death. Initiator caspases (e.g., caspase 8 and caspase 9) are activated to trigger the full caspase cascade, and caspase 3 is activated as an effector to induce apoptosis. Bax, Bcl-2, and other proteins belonging to the Bcl-2 family are also important factors in regulating apoptosis *in vivo* (Adams and Cory 1998).

SARS-associated coronavirus (SARS-CoV), which was recognized to be the etiological agent of severe acute respiratory syndrome (SARS) in humans, is a newly identified member of the *Coronaviridae* family. SARS-CoV is enveloped, and shares similar composition and structure with other coronaviruses. It possesses a genome of approximate 29.7 kb in length and is organized with the open reading frames (ORF) encoding replicase, spike (S), small envelope (E), membrane (M), and nucleo-

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capsid (N) in the sequence of 5′ to 3′ terminus. Genes encoding several putative nonstructural proteins are located between the ORFs of S and E, M and N, and downstream of N (Rota *et al*., 2003). Previous studies have shown that SARS-CoV can be cultured in several cell lines with or without cytopathic effects (CPE; Chan *et al*., 2004; Cinatl *et al*., 2004). It was recently reported that SARS-CoV induces apoptosis on Vero E6 cells, but the relevant mechanisms have not yet been fully elucidated (Yan *et al*., 2004).

After the SARS outbreak in 2003, and with the epidemic being effectively controlled so far, the mechanism(s) by which the virus causes the disease remains unknown. To explore this, we investigated SARS-CoV-associated apoptosis. We found that caspase-relevant apoptosis with Bax upregulation was induced in a virus replication-dependent manner in SARS-CoV-infected Vero cells and the inhibition of caspases had a slight effect on virus replication.

MATERIALS AND METHODS

Cell culture and virus infection

The SARS-CoV used in this study was isolated from a pharyngeal swab of a confirmed SARS patient. African green monkey kidney Vero cells from the American Type Culture Collection (ATCC, Rockville, MD), cat. # CCL-81) were maintained in minimal essential medium (MEM; Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. For virus infection, SARS-CoVs were absorbed with Vero cells at a specific multiplicity of infection (m.o.i.) in serum-free MEM for 1 h at 37°C. The inocula were then aspired and replaced with MEM containing 2% FBS and antibiotics.

Inactivation of SARS-CoV

SARS-CoV was inactivated by ultraviolet (UV) irradiation as previously described (Duan *et al*., 2003). The inactivation of the virus was confirmed by three continuous passages on Vero cells.

Virus titration

Virus titer was determined as previously described (Cunningham, 1973). Supernatants harvested from SARS-CoV-infected cells were serially diluted and added to Vero cells grown in 96-well plates in triplicate. After 72 h of infection, the CPEs were observed and median tissue culture infection dose ($TCID_{50}$) values were calculated.

DNA fragmentation assay

Cells undergoing apoptosis were determined by DNA fragmentation as described previously (Zhou *et al*., 2000). Briefly, Vero cells mock infected or infected with SARS-CoV at an m.o.i. of 5 were collected together with the floating cells in the supernatant at different time points postinfection (p.i.). Cells were lysed in a buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% Triton X-100, and were digested with RNase A (0.1 mg/ml) at 37°C for 1 h. The chromosomal DNA was pelleted by centrifuging at 12,000 rpm for 30 min. The collected supernatant fluids were digested with Proteinase K (1 mg/ml) at 50°C for 2 h in the presence of 1% sodium dodecyl sulfate (SDS), extracted with phenol and chloroform, precipitated in cold ethanol at −80°C overnight, then examined by 2.0% agarose gel electrophoresis containing 0.5 mg of ethidium bromide per ml.

Hoechst staining assay

Vero cells were grown on slides (Cel-line/Erie Scientific Co., Portsmouth, NH) and infected with SARS-CoV at an m.o.i. of 5. After fixation in methanol: acetic acid (v/v, 3:1) for 5 min at 4, 8, 12, 24, and 48 h p.i., the cells were stained with Hoechst 33342 (Sigma, St. Louis, MO, B2261) and visualized under a fluorescence microscope with UV filters (DMLS, Leica, Deerfield, IL).

Western blotting

Total soluble proteins from SARS-CoV-infected Vero cells were extracted and quantitated as previously reported (Wang *et al*., 2004). Cellular proteins of equal amount were separated by 15% SDS-PAGE gel and transferred onto nitrocellulose membranes. After blocking the membranes with phosphate-buffered saline (PBS) containing 5% skim milk overnight at 4°C, the blots were incubated with rabbit anti human caspase 3, Bax, or Bcl-2 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or anti-SARS-CoV nucleocapsid protein antibody (Imgexe IMG-543; Imgenex Co., San Diego, CA). Proteins were detected by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP; Pierce, Rockford, IL) or chemiluminescence (Amersham Pharmacia, Arlington Heights, IL). The blots were stripped and reprobed with antiactin antibodies (Sigma) for loading controls following the manufacturer's instructions.

Caspase inhibition assay

The virus-containing inoculums were aspirated and maintained in MEM supplemented with 2% FBS (Invitrogen). The caspase inhibitor z-DEVD-FMK (Merk, Chicago, IL) was diluted in DMSO following the instructions provided by the manufacturer and added to the cell culture medium at a concentration of 40 μM. Cells treated with an equal concentration of DMSO were used as the controls. Cells were collected and detected by Western blotting at indicated times (see above) and supernatants were harvested for viral titration.

RESULTS

SARS-CoV induces apoptosis in cultured Vero cells

To investigate the effects of SARS-CoV infection on Vero cells, several techniques were used to analyze the features of apoptosis. As shown in Figure 1, virus-infected cells have obvious morphological changes. SARS-CoV efficiently infected Vero cells and resulted in typical CPE, i.e., cellular swelling, bead clustering, membrane blebbing, cell bodies rounding up and shrinking of cell bodies, detachment of infected cells from the cultured dishes, and also cell lysis and death. No such changes occurred in mock-infected cells. When the cells were

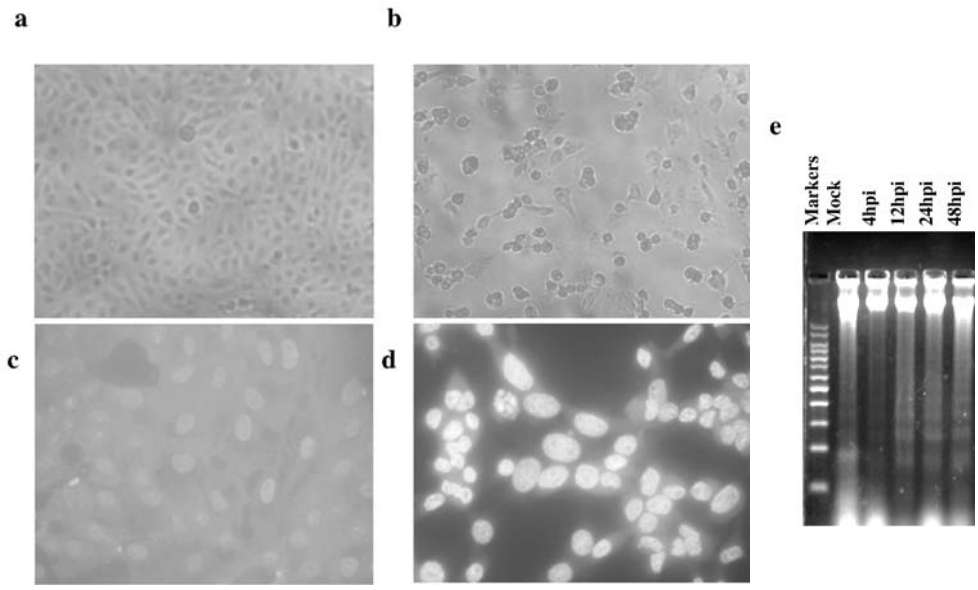

FIG. 1. Morphological and biochemical changes in SARS-CoV-infected Vero cells. (**a, b**) CPE observed under by light microscopy. Vero cells were grown in chamber slides and were either mock- (**a**) or SARS-CoV-infected (m.o.i. = 2.) (**b**) at 24 h p.i. (**c, d**) Fluorescent micrographs of the cells with CPE. Treated cells were fixed and stained with Hoechst 33342 to view the chromatin condensation. (**e**) Time course of internucleosomal DNA cleavage in Vero cells infected with SARS-CoV. Low-molecular-weight DNA fragments were isolated at the indicated times p.i. from SARS-CoV- or mock-infected Vero cells and separated by 2% agarose gel electrophoresis. DNA ladders (1 kb) (Takara) were used as markers.

examined with Hoechst 33342, a membrane permeable DNA dye, chromatin condensation in the nuclei of viral infected cells was apparent when visualized under a fluorescent microscope.

DNA fragmentation in SARS-CoV-infected Vero cells was also examined. The cells mock infected or infected with SARS-CoV at an m.o.i. of 5 were collected along with the floating cells at 4, 12, 24, and 48 h p.i. Low-molecular-weight apoptotic DNA fragments were extracted and separated by 2% agarose gel electrophoresis. As shown in Figure 1e, typical DNA ladders were detectable from 12 h p.i. (lanes 4, 5, and 6). No obvious DNA fragmentation was observed in mock-infected and SARS-CoV-infected cells harvested at 4 h p.i. (lanes 2 and 3). The above results confirmed that apoptosis was induced by SARS-CoV during the course of infection.

Apoptosis induced by SARS-CoV in Vero cells requires virus replication

The relationship between virus life cycle and apoptosis in cultured cells was studied by analyzing chromatin condensation and viral protein expression. Vero cells cultured on slides were infected with live or killed SARS-CoV of equivalent doses and stained with Hoechst 33342 at different time points p.i. as described in Materials and Methods. We found that the nuclei became fragmented at 24 h p.i. in the cells exposed to live SARS-CoV. In contrast, the signs of apoptosis were consistently negative in cells treated with killed viruses. To evaluate the degree of apoptosis induced by SARS-CoV at different time points p.i. 600 to 800 cells exhibiting nuclei with chromatin condensation on each slide were counted to calculate the positive rate of apoptotic cells (Fig. 2d). The results showed that the percentage of apoptotic cells increased as the time p.i. increased. At 48 h p.i., apoptosis appeared in more than 90% of the infected cells. These results suggest that only live virus is capable of inducing apoptosis in Vero cells.

To further confirm the relationship between virus replication and apoptosis, we analyzed the expression of SARS-CoV nucleocapsid protein. Cellular proteins were extracted from cells infected with live or UV-inactivated SARS-CoV and analyzed by Western blotting with an antibody against NP. The results showed that the expression of NP was detectable at 8 h after the infection of live SARS-CoV and increased expression with the increased time p.i., whereas the NP expression was undetectable in the UV-inactivated SARS-CoV group. Additionally, the virus titer in cell culture supernatant was monitored at different time points p.i. to show the link between virus replication and apoptosis. From Figure 2d, we can see that there is a correlation between SARS-CoV titer and the percentage of apoptotic cells. Taken together, we conclude that apoptosis triggered by SARS-CoV in Vero cells is replication dependent, with a possible association between apoptosis and viral replication.

Caspase family plays a role in SARS-CoV-induced apoptosis

SARS-CoV is a newly identified pathogen, and little is known about how the virus results in death of infected cells. Since the caspase family has been recognized as the main mediators of apoptotic responses in cells, we examined the expression of several apoptosis-associated proteins to explore the pathway involved in SARS-CoV-induced apoptosis in Vero

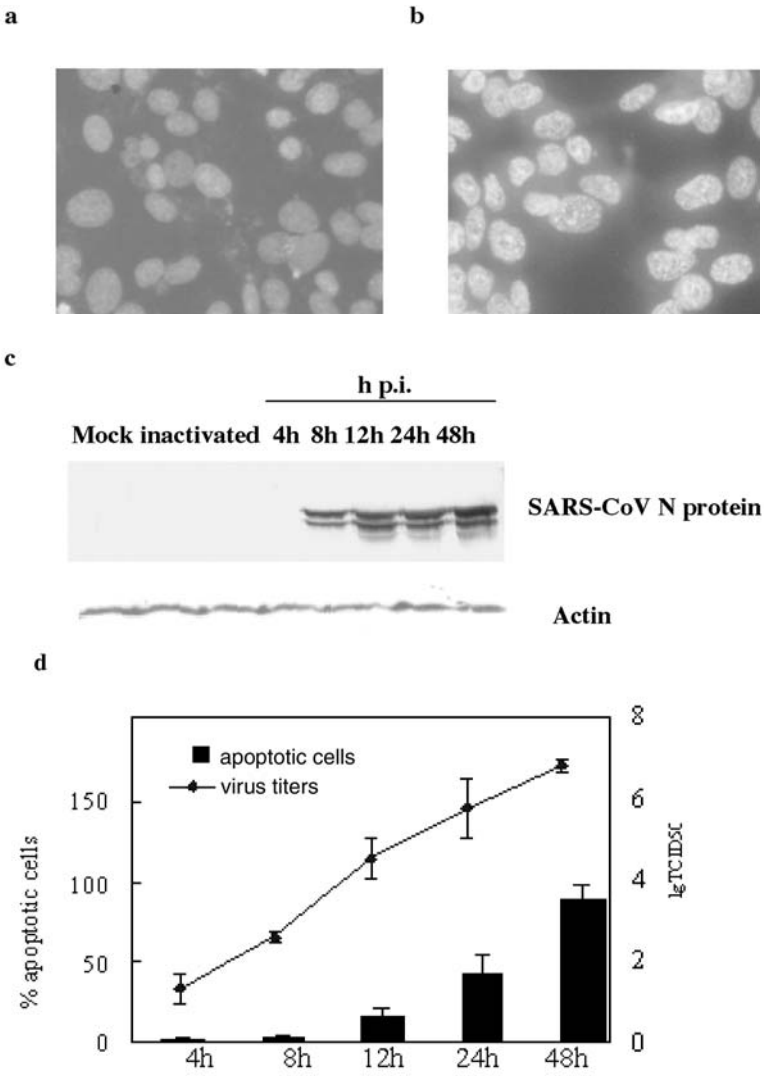

FIG. 2. Apoptosis induced by SARS-CoV requires virus replication. (**a,b**) Nuclei condensation visualized in Vero cells. Vero cells were infected with UV-inactivated (**a**) or live SARS-CoV (**b**) at an m.o.i. of 5. After 24 h of infection, cells were fixed with methanol: acetic acid (v/v 3:1) and stained with Hoechst 33342. (**c**) Expression of nucleocapsid protein after SARS-CoV infection. Cells cultured in 25 cm^2 flasks were infected with live or killed virus as described above, and were collected at different time point p.i. Analysis of increased expression of N protein by Western blotting was carried out with the rabbit anti-SARS-CoV nuocleocapsid antibody. Actin was used as a loading control. (**d**) Correlation between virus titer and percentage of apoptotic cell. The percentage of apoptotic cells is expressed in a column diagram and the virus titer ($TCID_{50}$) is shown in a logarithmic scale (linear chart). Vero cells were infected with SARS-CoV at an m.o.i. of 5. Cells were fixed for Hoechst staining and supernatants were harvested for $TCID_{50}$ measurement at indicated time p.i. There are significant differences in the percentage of apoptotic cells between 12 h and 24 h p.i., as well as 24 h and 48 h p.i. (Student *t*-test, $P < 0.01$).

cells. Proteins of equal amount were collected from mock- or SARS-CoV-infected Vero cells between 4 h and 48 h p.i., and were analyzed by Western blotting. As shown in Figure 3a, the cleavage of procaspase 3 could only be detected up to 24 h p.i., which implies that caspase 3 was activated after viral infection. To test if the apoptotic changes were caspase-dependent, the general caspase inhibitor z-DEVD-FMK was added to the culture media of infected cells. In the presence of z-DEVD-FMK (40 μM), chromatin condensation shown by Hoechst 33342 staining was significantly reduced. In addition, there was no cleaved caspase-3 detected at 24 h and 48 h p.i. by Western blotting. Vero cells incubated with DMSO, the vehicle to dissolve z-DEVD-FMK, showed no detectable apoptosis (data not shown).

We also analyzed the expression of the pro-apoptotic factor Bax and antiapoptotic factor Bcl-2. We determined that after viral infection the expression of Bax protein increased as the expression of Bcl-2 protein decreased (Fig. 4). The shift in protein expression of infected cells was obvious between 8 h and 24 h p.i. Since Bax protein is upregulated, this suggests that the mitochondrial signaling pathway is involved in the apoptotic process triggered by SARS-CoV infection.

Inhibition of caspases slightly reduces SARS-CoV yield

It has been previously reported that the replication of influenza virus was dramatically suppressed when the activity of caspase 3 was inhibited (Boya *et al*., 2001). Based on this hypothesis, the effects of caspase 3 inhibition on SARS-CoV propagation were evaluated by the addition of z-DEVD-FMK to the virus-infected cells. The results showed that the titer of SARS-CoV from z-DEVD-FM-treated cells increased as the postinfection time interval increased (Fig. 3b). Although the titer in the treated group was lower than that in the untreated group, there was no significant difference between the virus titer of the two groups at a given time point p.i. (Student *t*-test, $P > 0.05$). These results indicate that despite the fact that apoptosis is associated with viral replication, the inhibition of capsase 3 does not affect the yield of SARS-CoV.

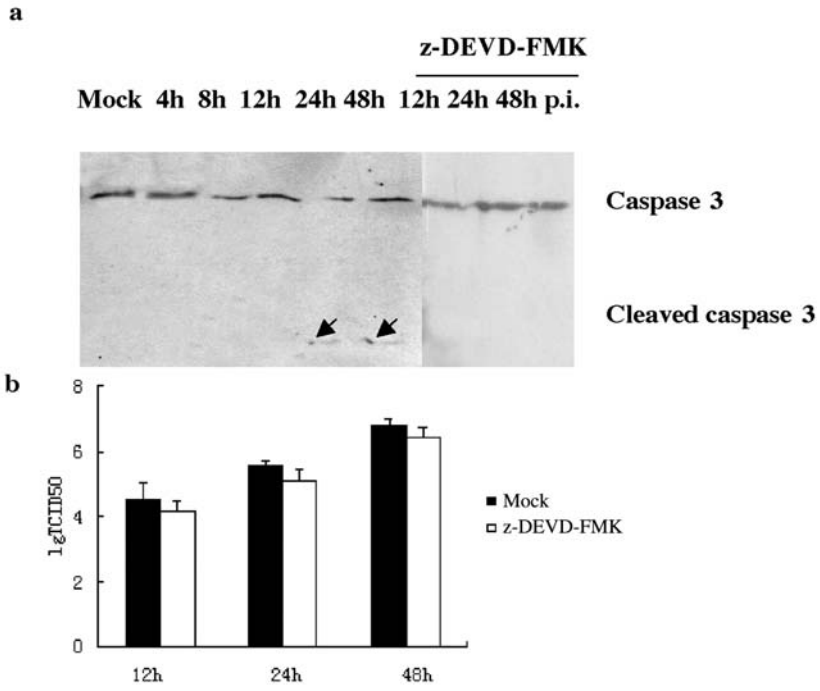

FIG. 3. Activation of Caspase 3 during apoptosis and effects of Caspase 3-inhibition on SARS-CoV yield. Vero cells were infected with SARS-CoV and treated with or without z-DEVD-FMK. At the indicated time point p.i., supernatants and cells were harvested separately. (**a**) Analysis of cell lysates for caspase 3 activity by Western blotting. Without treatment of z-DEVD-FMK, the cleaved caspase 3 was detectable beginning at 24 h p.i. with the cleaved caspase 3 being undetectable in the presence of z-DEVD-FMK. (**b**) Viruses from supernatant samples were titrated on Vero cells in triplicate. The titer of the virus is shown as $TCID_{50}$.

DISCUSSION

The mechanism by which SARS-CoV induces severe dysfunction of the respiratory system is unclear. Direct viral killing of sensitive cells and indirect immunopathological mechanisms which destroy of the cells are presumed to be involved in the process of the disease. The presence of apoptosis in patient's lung epithelial cells led us to hypothesize that apoptosis may play a key role in SARS pathogenesis (Lang *et al*., 2003; Zhang *et al*., 2003). In this report, we show that typical morphological and biochemical features of apoptosis, such as chromatin condensation and DNA fragmentation, were both detected in SARS-CoV-infected Vero cells. These results are similar with those reported in Vero E6 cells by others (Yan *et al*., 2004).

It is not clearly understood how and when infected cells die from SARS-CoV infection. The roles of viral replication during apoptosis in infected cells are different among RNA viruses. Some RNA viruses, such as MHV, infectious bursal disease virus, influenza virus, or enterovirus 71, induce replication-dependent apoptosis (An *et al*., 1999; Fernandez-Ariasa *et al*., 1997; Schultz-Cherry *et al*., 2001; Kuo *et al*., 2002). Whereas other RNA viruses, such as Sindbis virus or Reovirus, induce apoptosis without the requirement for viral replication (Jan and Griffin, 1999; Connolly and Dermody, 2002). To clarify whether viral replication is required for apoptosis induced by SARS-CoV, we compared the apoptosis features of UV-inactivated- or live SARS-CoV-infected Vero cells. The results showed that inactivated viruses lose the ability to induce apoptosis in cultured Vero cells, although they do possess the ability to bind to cells (Kang *et al*., 1999). Additional results showed that there is a positive correlation between the number of cells undergoing apoptosis and viral titer or viral protein expression after infection. From these data, we conclude that for SARS-CoV to induce apoptosis it requires viral replication.

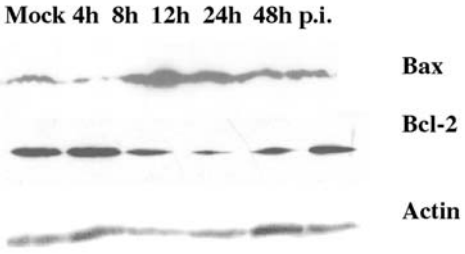

FIG. 4. Expression of Bax and Bcl-2 in SARS-CoV-infected cells. Cell proteins were collected, after being infected with SARS-COV, at indicated time points and examined by Western blotting using anti-Bax or Bcl-2 antibodies (Santa Cruz). Actin was used as a loading control.

It has been proposed that apoptosis may represent a byproduct of viral replication (Liu *et al*., 2001). In the late stage of virus infection, apoptosis has the ability to facilitate viral releasing and spreading, while in the early stage, apoptosis may abort virus infection and evoke a host defense (Savill *et al*., 1993; Liu *et al*., 2001). We detected an increased number of apoptotic cells after 24 h of SARS-CoV infection. The increase in both NP expression and viral titers with the increase in infection time led us to believe that apoptosis might contribute to SARS-CoV replication. Among the cellular apoptosis-related proteins, it has been shown that caspase 3 plays an important role in cellular decision processes in addition to apoptosis (Fernando *et al*., 2002). It participates in the maturation of viral proteins and morphogenesis in both red sea bream iridovirus

(RSIV) and feline calicivirus (FCV; Al-molawi *et al*., 2003; Imajoh *et al*., 2004). To further elucidate the mechanisms by which apoptosis augments viral replication, the replication of SARS-CoV was also assayed by comparing virus titers in the presence or absence of the caspase 3 inhibitor, z-DEVD-FMK. However, the treatment did not decrease virus titers as reported in other viruses, such as influenza virus (Wurzer *et al*., 2003). This implies that caspase 3 may not play a central role in viral spread. The mechanism by which apoptosis promotes the replication and maturation of SARS-CoV needs to be further investigated.

The process of apoptosis is complex. It involves many factors in addition to the caspase family. The Bcl-2 family, which comprises pro-apoptotic and antiapoptotic members, represents a critical intracellular checkpoint in the apoptotic pathway. Among these proteins, Bax is a pro-apoptotic protein containing BH1, BH2, and BH3 domains. Bcl-2 is an antiapoptotic factor and has four conserved BH domains (Cross *et al*., 1999). By binding with other BH3–only proteins, Bax increases the permeability of the intermembrane of mitochondria and causes the release of cytochrome *c*. This release is a major event during apoptosis. Once released into the cytosol, cytochrome *c* forms a molecular complex with Apaf-1 and then activates a caspase cascade leading to many apoptotic events. In contrast to the downregulation of Bcl-2, we found that the expression of Bax increased after the infection of SARS-CoV. These observations indicate that the mitochondrial signaling pathway might be involved, but it needs to be confirmed by additional experiments in future.

Which components of SARS-CoV are responsible for apoptosis in cultured cells are unclear. During the preparation of this manuscript it was reported that only overexpressing 7a of SARS-CoV induces apoptosis among the viral proteins S, N, E, M, 7a, and 3a (Tan *et al*., 2004). This conclusion is different from previous observations for other coronaviruses. For example, in the case of MHV, structural protein E was proven to cause apoptosis (An *et al*., 1999). Since the results reported by Tan and his colleagues were obtained from overexpression by plasmid transfection, their conclusion needs to be validated by further efforts.

In summary, our results suggest that there is a correlation between apoptosis and viral replication in a cell culture system. Since apoptosis potentially facilitates the spread of virus and minimizes the inflammatory reaction evoked by virus-infected host cells, we propose that SARS-CoV-induced apoptosis might contribute to virus infection *in vivo* and be involved in viral pathogenesis. These studies will hopefully lead to more effective therapies for this significant disease by elucidating the mechanisms of SARS-CoV-induced apoptosis.

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