

SARS Coronavirus Induces Apoptosis in Vero E6 Cells

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Severe acute respiratory syndrome (SARS) is an emerging infectious disease. Its etiological agent has been convincingly identified as a new member of family Coronaviridae (SARS-CoV). It causes serious damage to the respiratory system yet the mechanism is not clear. Infection-induced apoptosis or necrosis is suspected but no direct evidence for this yet exists. To date, Vero E6 cells are the only cell line that could be used to replicate the virus with obvious CPE (cytopathic effect) in vitro. It is known for some viruses (including members of family Coronaviridae) that CPE can be caused either by virus-induced apoptosis (active death) or cell necrosis (passive death). In this study, we examined the apoptosis in the SARS-CoV infected Vero E6 cells. Indeed, the results do show that the CPE was induced by apoptosis rather than necrosis, shown by typical DNA fragmentation, through the existence of apoptotic bodies and swollen mitochondria. This observation has some implications for the SARS-CoV pathogenicity: SARS-CoV does induce apoptosis in cell cultures and might have the same effect in vivo, responsible for the severe damage of the respiratory system. **J. Med. Virol. 73:323–331, 2004.** © 2004 Wiley-Liss, Inc.

KEY WORDS: SARS virus; apoptosis; Vero E6 cells; pathogenesis; pathogenicity

INTRODUCTION

Severe acute respiratory syndrome (SARS) is a newly identified emerging infectious disease and its causative agent has been convincingly identified as a new member of family Coronaviridae (SARS-CoV) within a record time [Drosten et al., 2003a,b; Ksiazek et al., 2003; Rota et al., 2003]. It has also been shown that its genomic sequence is unrelated to any known coronaviruses isolated either from humans or animals though a closely-related virus has been isolated recently from some wild animals, e.g., civet cat [Guan et al., 2003;

Marra et al., 2003; Rota et al., 2003]. The disease has been reproduced in monkeys, therefore the Koch's postulates was fulfilled by proxy [Fouchier et al., 2003]. Currently, the virus biology, pathogenesis, pathogenicity and preventive measures, including vaccine development, are under detailed scrutiny. It is as yet unclear why such an acute virus infection becomes so severe and why the respiratory system is heavily damaged, causing patients a fatal consequence.

Virus-induced apoptosis is a well documented phenomenon in many viruses, especially RNA viruses, including the members of family Coronaviridae [Taki-zawa et al., 1993; Hinshaw et al., 1994; Esolen et al., 1995; Mori et al., 1995; Shen and Shen, 1995; Haagmans et al., 1996; Hardwick, 1997; O'Brien, 1998; Hofmann et al., 1999; Kang et al., 1999; Eleouet et al., 2000; Aleman et al., 2001; Liu et al., 2001; Brydon et al., 2003; Clarke and Tyler, 2003; Vuorinen et al., 2003]. This was first observed from work on an adenovirus in 1981 [Pilder et al., 1984]. Apoptosis is an active and physiological type of cell death, which is different fundamentally from necrosis (passive accidental death) [White, 1996; Peter et al., 1997]. It is characterized morphologically by nuclear fragmentation and cellular breakdown into apoptotic bodies [Kerr et al., 1972] and biochemically by chromosomal DNA fragmentation into oligonucleosomes [Wyllie, 1998]. It can be induced either directly by the virus itself to assist virus dissemination,

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or by triggering inadvertently cellular sensors that initiate cell death [O'Brien, 1998; Koyama et al., 2000]. No matter what mechanism is involved, virus-induced apoptosis is recognised as a normal response in the virus-infected cells, but in general could be detrimental to the host, e.g., the influenza virus damages epithelial cells in the human and animal respiratory tracts through apoptosis [Hinshaw et al., 1994; Julkunen et al., 2001; Ito et al., 2002; Brydon et al., 2003; Lowy, 2003], leading to the respiratory system failure. Apoptosis is a common event in lytic viral infection [Shen and Shen, 1995; O'Brien, 1998]. As SARS-CoV is observed as an acute lytic infection [Drosten et al., 2003a; Fouchier et al., 2003; Rota et al., 2003; Zhao et al., 2003], apoptosis-induced cell damage might be responsible for the severe clinical manifestations.

Recently, two reports from Chinese literatures on SARS patients showed that lung epithelial cells had undergone apoptosis [Lang et al., 2003; Zhang et al., 2003]. This implies that SARS-CoV could be a pro-apoptotic agent. Therefore, we examined the apoptotic effect of SARS-CoV in the *in vitro* cell line, Vero E6 cells, which is the only CPE-induced cell line for SARS-CoV available to date. The results show that SARS-CoV induced apoptosis in Vero E6 cells is evident due to the observed DNA fragmentation, existence of apoptotic body and swollen mitochondria. This might explain why SARS-CoV induces respiratory epithelial cell damage similar to that observed in influenza infection through apoptosis. The mechanism under which the apoptosis occurs and its effect on the SARS patients, could lead to a greater future understanding of its pathology.

MATERIALS AND METHODS

Cells and Viruses

The Vero E6 cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). SARS-CoV isolation was undertaken from a blood specimen of a fatal SARS case in Hubei province belonging to the original case cluster from Beijing, China as described by Ksiazek et al. [2003]. The virus isolate was verified by electron-microscopy (EM), indirect fluorescence antibody test and RT-PCR as described previously [Drosten et al., 2003a; Ksiazek et al., 2003]. This virus isolate was named after WHU strain and its genomic sequence was deposited in GenBank with Accession No. AY394850.

For virus propagation, SARS-CoV-WHU was grown in Vero E6 cells and was harvested 3–5 days post-infection (p.i.). Virus induced cytopathic effect (CPE) was observed under reverse-phase light microscope. Virus titres were determined by TCID₅₀ test with Vero E6 cells (TCID₅₀ was virus titres causing 50% of CPE on Vero E6 cell monolayer). To infect cells, cell monolayer was washed with phosphate-buffered saline (PBS) and then was incubated with viruses in MEM–2.5% fetal calf serum (FCS) at a multiplicity of infection (MOI) of 0.1 for 1–2 hr at 37°C. The monolayer was washed and incubated with MEM with 2.5% FCS at 37°C in an incubator with 5% CO₂. Vero E6 cells were maintained

in MEM containing 10% FCS. Virus-infected cells were grown in MEM containing 2.5% FCS.

Purification of Cellular Genomic DNA and Electrophoresis

Cellular genomic DNAs were purified as described previously [Zhang et al., 2002]. Briefly the confluent Vero E6 cells in T25 (25 cm²) flasks were infected with SARS-CoV-WHU at an MOI of 0.1. At 0, 12, 24, 48 and 72 hr p.i., cells were washed twice with PBS without Mg²⁺ or Ca²⁺ and then were incubated in a 0.5 ml solution containing 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 10 mM NaCl, 1% (w/v) SDS and 1 mg/ml proteinase K at 60°C for 2 hr. Cell lysates were harvested into Eppendorf-tubes and extracted twice with phenol:chloroform (1:1 v/v), followed by precipitation with ethanol overnight at –20°C. The precipitates were washed with 70% ethanol. Pellets were air dried and re-dissolved in 100 µl of Tris-EDTA and 10 µl of 1 mg/ml RNase at 37°C for 30 min. Electrophoresis was performed on 2% agarose gels with DNA-size markers.

Hoechst 33258 Staining and Indirect Fluorescence Staining of the Infected Cells

Confluent Vero E6 cells in 96-well plates were infected with SARS-CoV-WHU at an MOI of 0.1 as described above. At 0, 12, 24, 48 and 72 hr p.i., cells were fixed without disruption in 2% paraformaldehyde in PBS (pH 7.4) for 10 min, permeabilised with 0.1% Triton X-100 in PBS for 3 min, and washed with PBS containing 1% bovine serum albumin. Two-colour immunofluorescence was used to detect cell infection and cell nuclei simultaneously with different excitation light. Cell infection was localised with serum from a convalescent patient (in dilution of 1:100) and followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Southern Biotechnology, Inc., Birmingham, Ala.). Nuclei were stained with Hoechst 33258 as described previously [Zhang et al., 2002; Zhou et al., 2002]. A Nikon Eclipse TE300 microscope equipped with a digital camera was used for image capture and collection. Images of FITC fluorescence were collected with a B filter group. Images of Hoechst 33258 stained nuclei in the same area as FITC images were collected with UV filter group and CPE images were taken under phase contrast light.

Propidium Iodide (PI) Staining and DNA Content Assay

For PI staining, confluent Vero E6 cells in T25 (25 cm²) flasks were infected with SARS-CoV-WHU at an MOI of 0.1. At 0, 12, 24, 48 and 72 hr p.i., cells were trypsinised and collected. The harvested cells were then centrifuged at 250g for 10 min at 4°C and were washed with PBS between each step. The cells were fixed with 75% ethanol at –20°C overnight, then were centrifuged and washed with PBS twice. For detecting DNA content, cells were incubated with 500 µl of a 50 µg/ml con-

centration of PI (Sigma-Aldrich, St. Louis, MO) and 0.1% of RNase A for 30 min at 25°C. The stained cells were analysed by using FACscan (Becton Dickinson, San Jose, CA). The percentage of apoptotic cells was determined using the CellQuest program (Becton Dickinson).

Electron Microscopy (EM)

Vero E6 cells grown in T25 (25 cm²) flasks were infected with SARS-CoV-WHU at an MOI of 0.1. At 48 hr p.i., cells were scraped off and collected. The harvested cells were fixed with 2.5% glutaraldehyde, rinsed, and post-fixed in 1.5% osmium tetroxide. Then the cells were dehydrated in a graded ethanol series and embedded in spur resin. Ultra-thin sections were cut and stained with lead citrate and were then viewed under a Hitachi H800 transmission electron microscope.

RESULTS

DNA Fragmentation of SARS-CoV Infected Vero E6 Cells

A number of viruses had been shown to cause cell death by induction of apoptosis, including the members of the family Coronaviridae [e.g., Shen and Shen, 1995; Haagmans et al., 1996; Hardwick, 1997; Teodoro and Branton, 1997; O'Brien, 1998; Sur et al., 1998; Eleouet et al., 2000; Liu et al., 2001]. SARS-CoV, which causes severe acute respiratory syndrome, may infect human lung tissue causing diffuse alveolar damage, abundant foamy macrophage, and multinucleated syncytial cells in the infected lung tissues [Lang et al., 2003; O'Donnell et al., 2003; Zhang et al., 2003; and Website: <http://www.afip.org/Departments/Pulmonary/SARS/pathogen1b.html>]. To test whether the cell damage and death are due to induction of apoptosis, the presence of fragmented chromosome DNA in SARS-CoV-infected cells were examined. Vero E6 is the only cell line to support the SARS-CoV replication with obvious CPE. In this study, Vero E6 cells were mock infected or were infected with SARS-CoV-WHU, and were subsequently harvested at different time point p.i. Genomic DNA was purified as described in "Materials and Methods" and was resolved in 2% agarose gels (Fig. 1). DNA fragmentation was seen in Vero E6 cells from 24 hr p.i. Increased amount of fragmented DNA was detected with longer infection times, suggesting that SARS-CoV induced apoptosis in Vero E6 cells. The fragments formed a typical apoptotic ladder in the gel, with most of the DNA accumulated in the lower bands at 48 and 72 hr p.i. No fragmentation phenomenon was detected in uninfected cells (Fig. 1).

Virus Multiplication and Apoptosis

To confirm further that Vero E6 CPE and death were due to induction of apoptosis in SARS-CoV-infected cells and to quantify the apoptosis, indirect immunofluorescence staining and Hoechst 33258 staining were carried out for infection and nuclei morphologic observation

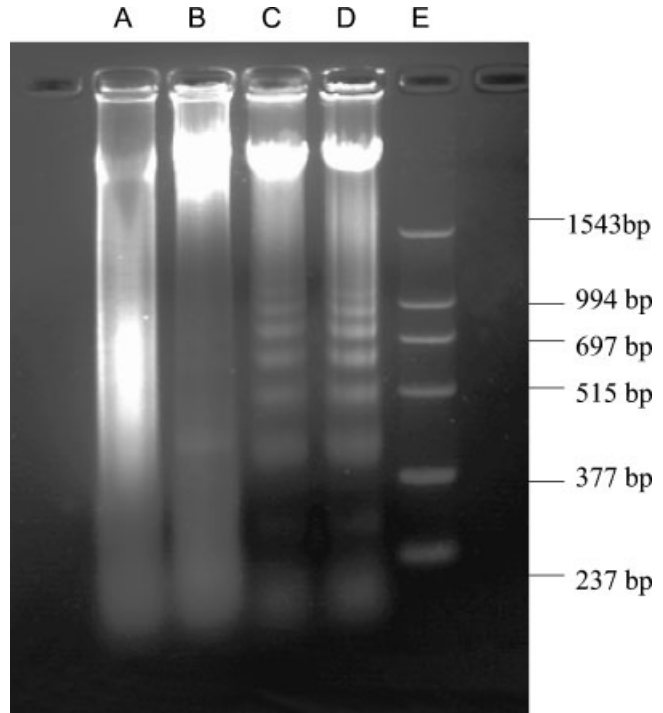


Fig. 1. DNA fragmentation ("DNA ladder") observed in SARS-CoV infected Vero E6 cells at different times post infection (p.i.). Increased amount of fragmented DNA was detected with the increase of infection time. A: Uninfected cells; (B) 24 hr p.i.; (C) 48 hr p.i.; (D) 72 hr p.i.; (E) DNA markers as indicated in bp (basepairs).

respectively. The characteristic CPE of SARS-CoV infection in vitro for Vero E6 is cellular round-up and shrinking without production of syncytia (Fig. 2, column A). Infection (Fig. 2, column B) could be detected as early as 12 hr after infection, but no apparent CPE and apoptosis was observed (condensed nuclei) (Fig. 2, column C). Most of the cells were infected as shown with indirect immunofluorescence staining 24 hr after infection but only a few cells (about 5%) developed CPE and nuclei with condensed chromatin (Fig. 2, 3rd row). More cells (about 16%) showed CPE and nuclei with condensed chromatin 48 hr p.i. (Fig. 2, 4th row). Most of the infected cells underwent CPE and shrinkage 72 hr p.i., in which almost all cells with CPE contained nuclei with condensed chromatin, or fragmentation into apoptotic bodies (Fig. 2, 5th row). Detection of cells developing nuclei with condensed chromatin, or fragmentation into apoptotic bodies increased with longer infection times (Fig. 3). This result illustrates that apoptotic cells can also be infected with the SARS-CoV.

Further Evidence of Apoptosis Rather Than Necrosis

The ability of SARS-CoV to induce apoptosis in Vero E6 was further confirmed by PI staining and analysed using a flow cytometer. Apoptotic cells show a decreased DNA content below the G₀/G₁ level. Sub-G₁ is one of the reliable biochemical markers of apoptosis and this

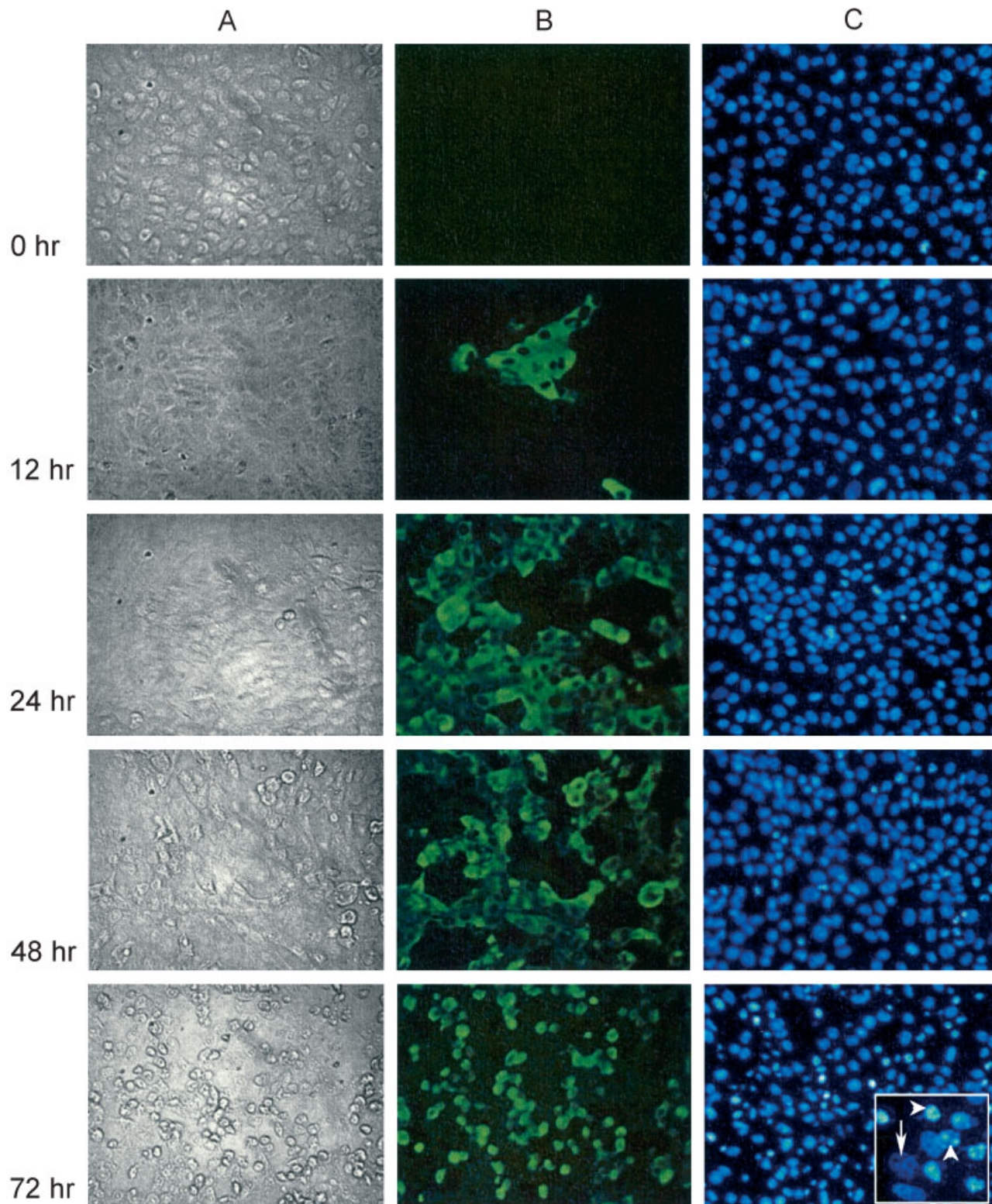


Fig. 2. SARS CoV infected Vero E6 cells observed simultaneously for CPE, infection and nuclei morphology at 0, 12, 24, 48, 72 hr p.i. **Column A:** SARS CoV induced CPE in Vero E6 cells; **column B:** Infected Vero cells were shown by indirect fluorescence (IF) antibody staining using the sera from a convalescent patient; **column C:** SARS

CoV induced apoptosis in Vero E6 cells by observation of nuclei with condensed chromatin. Many more cells showed nuclei with condensed chromatin, or fragmentation into apoptotic bodies at 72 hr p.i. Inset picture at 72 hr showed the nuclei with clear condensed chromatin at higher magnification.

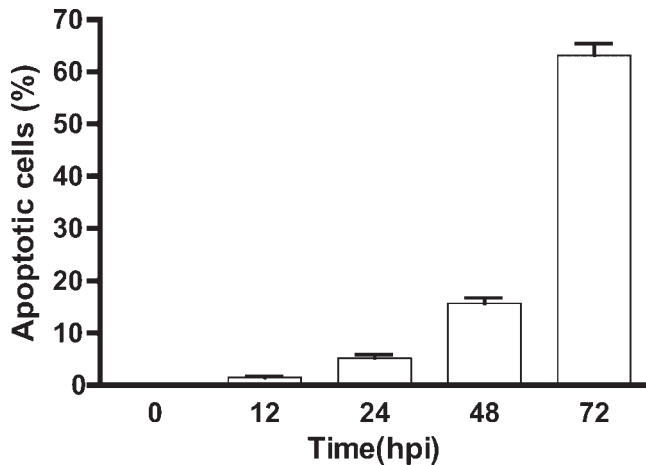


Fig. 3. Percentage of SARS CoV induced apoptosis in Vero E6 cells by observation of nuclei with condensed chromosome at different time points p.i. Data are summaries of the results from analysing the cells at time the points indicated. Samples were triplicates. Error bars are standard errors of means.

technique also discriminates between apoptosis and necrosis [Zhou et al., 2002]. Figure 4 illustrates changes in DNA content distribution in SARS-CoV infected Vero E6 cells at different time point p.i. The histograms of DNA content frequency distribution clearly indicates that the SARS-CoV infection results in the appearance of cells with a fractional DNA content, which was defined as “sub- G_1 ” peak, 24 hr p.i. Furthermore, as the infection progressed, the percentage of cells in sub- G_1 was increased accordingly. This is typical characteristic of apoptosis rather than necrosis.

EM Ultra-Structural Evidence of SARS-CoV Induced Apoptosis

EM experiments were carried out as described in the “Materials and Methods” section. In the Vero E6 cell section, coronavirus particles could be seen, which confirmed SARS-CoV-WHU cell infection (Fig. 5A). Typical swollen mitochondria, with an irregular widened space between the cristae could also be seen easily in the infected cells (Fig. 5A). A puffed nuclear membrane was also observed (Fig. 5A,B). SARS-CoV infected cells show distinctive condensation and margination of chromatin in the nuclei (Fig. 5C), which is another hallmark of apoptotic pathways, whilst uninfected cells appear healthy (Fig. 5D). All these ultra-structural changes indicate that apoptosis occurs in the infected cells.

DISCUSSION

In this study, apoptosis was observed in SARS-CoV infected Vero E6 cells as confirmed by morphological and biochemical analyses. Taking into account the recent reports on the apoptosis pattern of SARS-CoV infected cells in vivo [Lang et al., 2003; Zhang et al., 2003], it is clear that SARS-CoV is a pro-apoptotic agent. Apoptosis was observed in patient’s lung epithelial cells [Lang

et al., 2003; Zhang et al., 2003] and other types of cells, including monocytes. This might explain the severe damage of the respiratory system in SARS patients as reported for the influenza virus, which induces lung epithelial cell apoptosis responsible for severe damage of the respiratory system in influenza infection [Hinshaw et al., 1994; Mori et al., 1995; Julkunen et al., 2001; Ito et al., 2002; Brydon et al., 2003].

Virus-induced apoptosis is a common phenomenon in viral infection, especially RNA virus infections, including some coronaviruses (e.g., swine transmissible gastroenteritis virus, avian infectious bronchitis virus and feline infectious peritonitis virus) [Esolen et al., 1995; Shen and Shenk, 1995; Haagmans et al., 1996; Hardwick, 1997; O’Brien, 1998; Everett and McFadden, 1999; Hofmann et al., 1999; Kang et al., 1999; Eleouet et al., 2000; Koyama et al., 2000; Liu et al., 2001; Schultz-Cherry et al., 2001; Cuconati and White, 2002; Clarke and Tyler, 2003; Koyama et al., 2003; Markotic et al., 2003; Maruoka et al., 2003; Schlitt et al., 2003; Shafee and AbuBakar, 2003]. As observed in our study, SARS-CoV replicates while cells undergoing apoptosis. This is consistent with some other previous observations, e.g., some animal viruses can produce considerable amounts of progeny virus in ongoing apoptotic cells [O’Brien, 1998; Koyama et al., 2000]. SARS-CoV was also observed in macrophages or T cells and infection of these cells were suspected [Lang et al., 2003; O’Donnell et al., 2003; Zhang et al., 2003]. The apoptotic cells are subjected to phagocytosis by macrophages, therefore it is possible for the SARS virus seen in macrophages to be the consequence of phagocytosis of the apoptotic infected cells by macrophages. This needs to be addressed further in the future.

Apoptosis is a “double-edged” sword for virus infection: it is used by the host to remove infected cells by committing suicide, thereof clearing the virus, and for the virus to assist itself dissemination by the release of viral particles, thereof facilitating its survival in vivo [Shen and Shenk, 1995; Krakauer and Payne, 1997; O’Brien, 1998; Everett and McFadden, 1999; Koyama et al., 2000; Aubert and Blaho, 2003]. Therefore, apoptosis can have two opposite roles on the pathogenicity of viral infection, enhancing or suppressing the infection. In the case of SARS, as observed in human patients [Lang et al., 2003; Zhang et al., 2003], the apoptosis occurs massively, thus SARS-CoV induced apoptosis would certainly have a deleterious pathogenic role in the patients, leading to severe tissue damage. This might be one of the explanations of the serious nature of SARS infection as observed in clinics. In most cases of animal virus-induced apoptosis, enhancing infection and detrimental effects are common [Shen and Shenk, 1995; Hardwick, 1997; O’Brien, 1998; Everett and McFadden, 1999; Koyama et al., 2000]. Though killing off the infected cells might be a mechanism for the body to limit the spread of the virus, it is still detrimental if the lung cells are destroyed.

Future studies need to be designed to elucidate the mechanism of SARS-CoV induced apoptosis, e.g., the

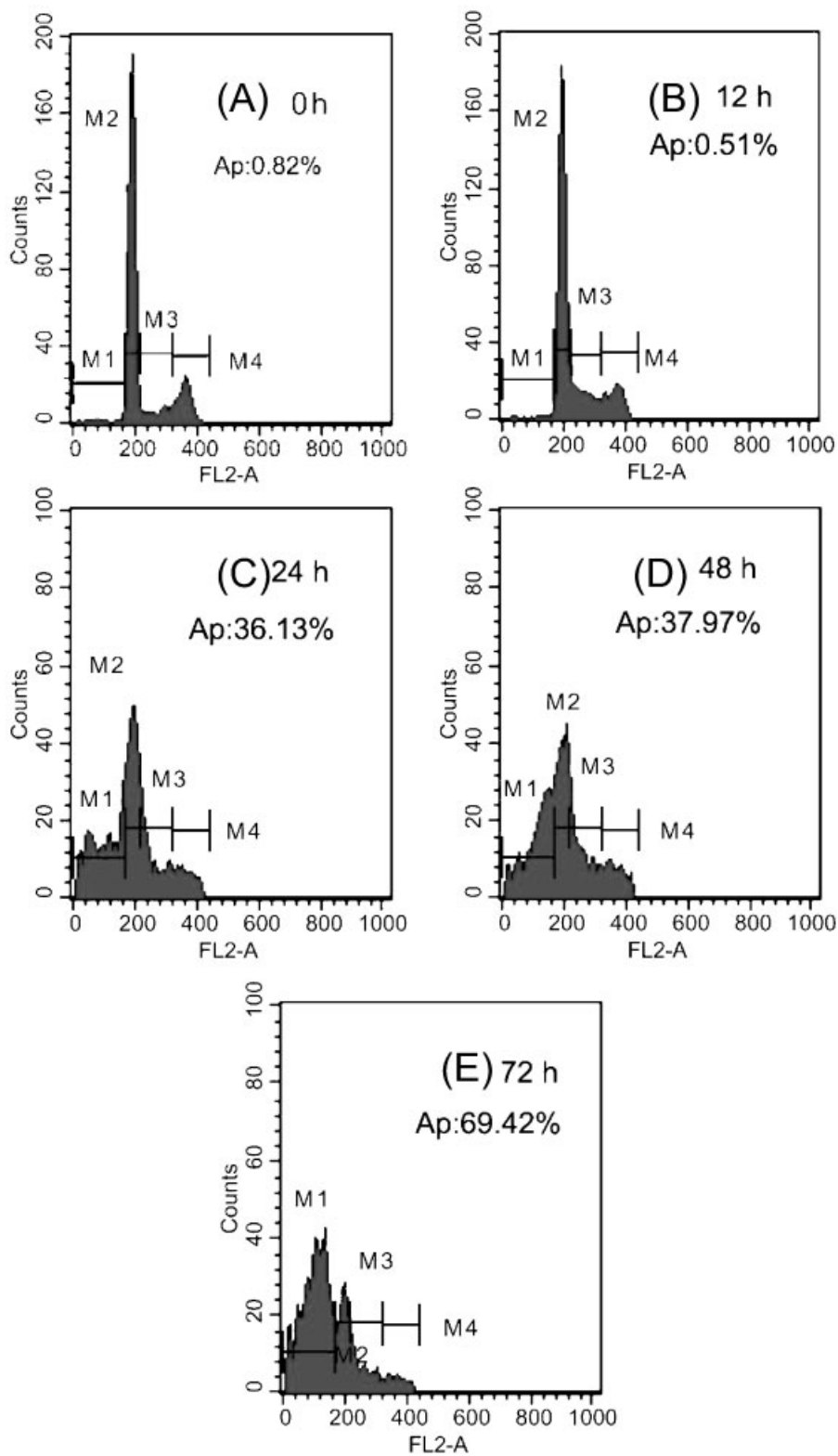


Fig. 4. DNA content analysis on FACScan. **A:** 0 hr; **(B)** 12 hr; **(C)** 24 hr; **(D)** 48 hr; **(E)** 72 hr of p.i. M1, sub-G₁ phase cells (apoptosis); M2, G₁ phase cells; M3, S phase cells; M4, G₂/M phase cells. Sub-G₁ peak appears evidently from 48 hr p.i.

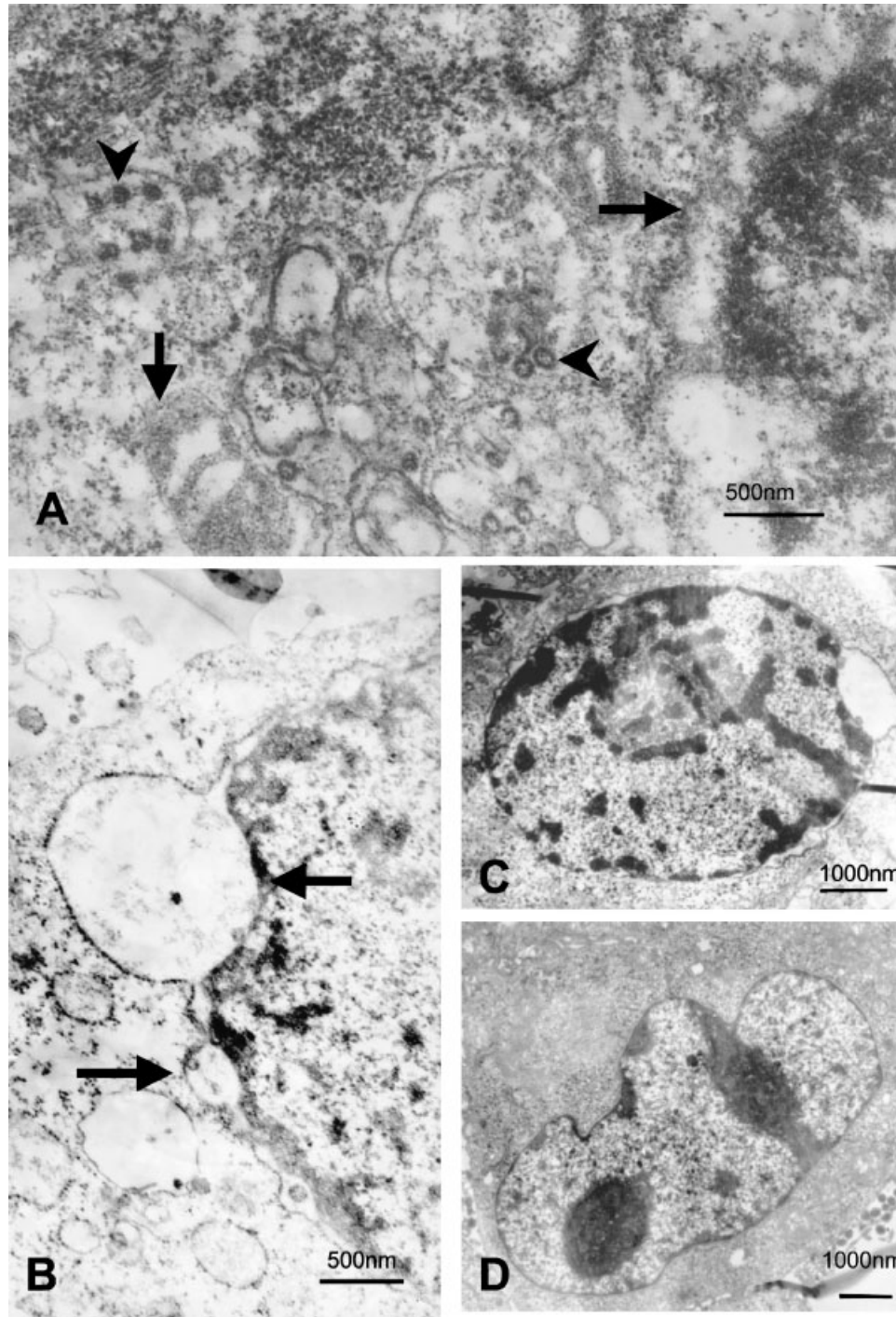


Fig. 5. Electron microscopy (EM) of SARS CoV infected Vero E6 cells. **A:** SARS-CoV-WHU infected Vero E6 cells showed swollen mitochondria (vertical arrow) and nuclear membrane puff (horizontal arrow). Black arrowhead indicates virus particles (approximately 90–110 nm in size). Bar, 500 nm. **B:** Evident nuclear membrane puff was observed

in SARS CoV infected Vero E6 cell. Bar, 500 nm. **C:** Margination and condensation of chromatin can be seen in SARS CoV infected Vero E6 cell nuclei. Bar, 1,000 nm. **D:** Normal nuclei was observed in uninfected Vero E6 cells. Bar, 1,000 nm.

pro-apoptotic components of the virus genes and the apoptosis pathway. It has been shown that in other coronaviruses, e.g., avian infectious bronchitis virus [Liu et al., 2001] and swine transmissible gastroenteritis virus [Eleouet et al., 2000], that apoptosis is induced by a caspase-dependent mechanism. Similar mechan-

ism could exist for SARS-CoV-induced apoptosis and should be considered for future work. It is also essential to understand the relationships between apoptosis in vivo and the severe outcome of SARS-CoV infection in the human population in order for the rational design of clinical medical treatment strategy.

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