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M and N proteins of SARS coronavirus induce apoptosis in HPF cells

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Abstract

Background: SARS-associated coronavirus (SARS-CoV) induced cell apoptosis and its structural proteins may play a role in this process. *Objectives:* To determine whether the structural proteins M and N of SARS-CoV induce apoptosis. *Study design:* We investigated human pulmonary fibroblast (HPF) cells, were transfected with plasmids containing the M or N gene, by TdT-mediated dUTP nick end labeling (TUNEL), Hoechst 33342 staining for nuclei, and observation of morphology. *Results:* We found that in the absence of serum about 16.34% of cells transfected by pcDNA3.1-M and 21.72% of N-transfected cells showed typical apoptotic characteristics, significantly different from mock-transfected cells (only 6.23%, $p < 0.01$). Furthermore, the cells that were co-transfected with M and N proteins showed more obvious phenomena of cell death (about 36.03%). There was a statistical significance between M-transfected cells and co-transfected cells ($p < 0.01$), and a remarkable difference between N-transfected cells and co-transfected cells ($p < 0.01$). *Conclusions:* The results show that M and N proteins of SARS-CoV can induce apoptosis of HPF cells. Co-transfection of M and N enhances the induction of apoptosis by M or N alone, which also suggests that the structural proteins of SARS-CoV may play an important role not only in the process of invasion but also in the pathogenetic process in cells.

Abbreviations: HPF, human pulmonary fibroblast; SARS, severe acute respiratory syndrome; SARS-CoV, SARS-associated coronavirus; TUNEL, TdT-mediated dUTP nick end labeling

Introduction

The pathogen of severe acute respiratory syndrome (SARS) is SARS-associated coronavirus (SARS-CoV), a novel coronavirus. Sequence analysis of a limited region of the replicase (*rep*) gene and phylogenetic analyses of the sequence of SARS-CoV suggested that it was distinct from all other coronaviruses. However, the genomic organization of SARS-CoV is typical of coronaviruses, and the genome encodes proteins of similar structures to those of other coronaviruses, including S, E, M, and N proteins. All of them play an important role during host cell entry and virion morphogenesis and release (Rota et al., 2003).

N protein can bind to a defined packaging signal on viral RNA, leading to the formation of the nucleocapsid. It also contains two nuclear localization signals. It is possible that the SARS virus nucleocapsid protein has a novel nuclear

function, which could play a role in pathogenesis (Marra et al., 2003). M protein is localized at specialized intracellular membrane structures. Interactions between the M and E proteins and nucleocapsid result in budding through the membrane. By interaction with S protein, the latter is incorporated into the viral envelope, which is an important step of the assembly of virus particles. Therefore, all of these proteins may correlate with the pathogenesis of SARS-CoV and study of them could help reveal the pathogenic mechanism of SARS-CoV and indicate effective means of SARS therapy.

We have studied the infectious effect of SARS-CoV virus on juvenile and adult Brandt's vole (*Microtus brandtii*) by the nasal cavity spraying method. SARS virus causes death in adults, who exhibit hemorrhage from the mouth, nasal cavity, and intestine, hemorrhagic interstitial pneumonia, and a clot in liver, spleen, and kidney. Surviving adults showed local hemorrhagic spots in lung and emphysema, but the other organs showed no pathological abnormality (Gao et al., 2005). These interesting results suggest some lethal mechanism by which SARS-CoV destroys natural cells, which should be further investigated by experiments *in vitro* and *in vivo*.

Some recent reports of research on the proteins of SARS-CoV refer to the cell apoptosis induced by proteins of SARS-CoV. Apoptosis is a kind of programmed cell death that does not cause any inflammatory reaction *in vivo*. Generally, virus can promote or inhibit the apoptosis of host cells. During SARS, apoptosis is a common phenomenon. Apoptotic cells increased significantly in the spleen, lung, and lymph nodes of SARS patients as compared with normal tissues. Yan et al. (2004) reported that SARS-CoV infection induced the apoptosis of Vero E6. Jin et al. (2004) reported that the apoptosis of T lymphocytes mediated by Fas/FasL might play an important role in the cytoimmunological pathogenesis and pathophysiology of SARS. Surjit et al. (2004) showed that SARS-CoV N is capable of inducing apoptosis of COS-1 monkey kidney cells in the absence of growth factors by regulating the p38 MAPK pathway and inducing actin reorganization in cells devoid of growth factors. Tan et al. (2004) demonstrated that the overexpression of 7a protein induces apoptosis in many cell lines derived from different organs, including lung, kidney, and liver. Chow et al. (2005) demonstrated that the adenovirus-mediated overexpression of SARS-CoV spike (S) protein and its C-terminal domain (S2) induces apoptosis in Vero E6 cells in a time- and dose-dependent manner. Yuan et al. (2005) demonstrated that overexpression of 3b-EGFP fusion protein could induce cell cycle arrest at G_0/G_1 phase in Vero, 293, and COS-7 cells and could induce apoptosis in COS-7 cells. Yang et al. (2005) reported that SARS-CoV E protein induced apoptosis in transfected Jurkat T-cells, and the apoptosis rate was amplified in the absence of growth factors. The apoptosis could be inhibited by overexpressed antiapoptotic protein Bcl-xL. Law et al. (2005) showed that the 3a protein of SARS-CoV could induce apoptosis in Vero E6 cells.

Acute lung injury is the key feature of SARS and the pathological changes in lungs are one of the major causes of death in SARS patients. However, no reports of studies on the relation between SARS-CoV proteins and human lung cells have been retrieved. Based on the previous reports, we chose tom use human pulmonary fibroblast (HPF) cells derived from humans to investigate whether N and M proteins of SARS-CoV could induce apoptosis *in vitro*.

Materials and methods

Plasmids

The pcDNA3.1-M and -N plasmids containing full-length SARS-CoV M and N cDNA were kindly provided by Professor Yang Huan-Ming (Genomics Institute, Chinese Academy of Science). The Genbank accession number is AY278487 (SARS coronavirus BJ02).

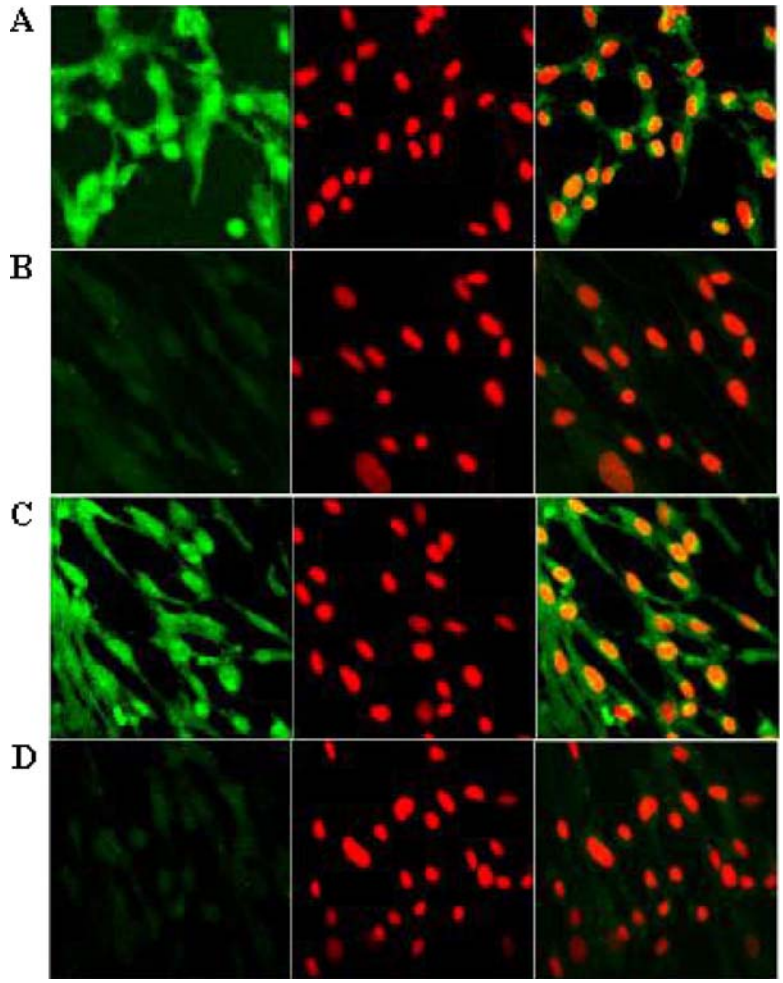

Figure 1. Analysis of expression of pcDNA3.1-M and -N in HPF cells. HPF cells that were transfected with pcDNA3.1-N or -M or with mock plasmid reacted with antisera and secondary antibody conjugated with FITC, as described in Materials and Methods. These cells were counterstained with propidium iodide for visualization of nuclei of all cells and analyzed for expression by confocal microscopy 24 h post transfection by lipofectamine 2000. (A) HPF cells transfected with pcDNA3.1-M, reacted with the antisera from pcDNA3.1-M-immunized mice. (B) HPF cells transfected with pcDNA3.1, reacted with the antisera from pcDNA3.1-M-immunized mice. (C) HPF cells transfected with pcDNA3.1-N, reacted with the antisera from pcDNA3.1-N-immunized mice. (D) HPF cells transfected with pcDNA3.1, reacted with the antisera from pcDNA3.1-N-immunized mice. The red fluorescence represents the nuclei of cells and the green fluorescence represents expression. The figure comprises data of three or four independent experiments.

Cells

HPF cells were provided by the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, and the initial population doubling level was 8. Cells were grown in DMEM medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 U/ml streptomycin, in a humidified atmosphere of 5% CO_2 at 37°C.

Reagents

Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). Hoechst 33342 was

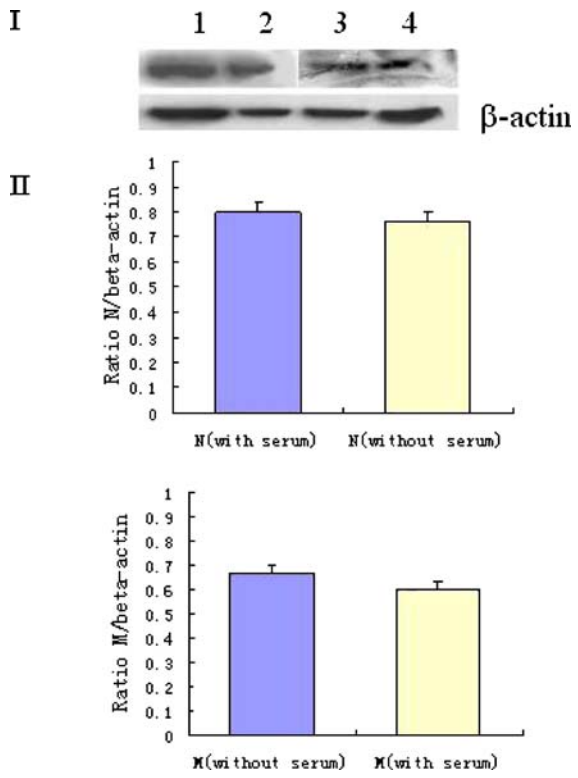

Figure 2. (I) Western blotting analysis of M and N proteins. Lane 1, total proteins of N-transfected cells in the absence of serum; lane 2, total proteins of N-transfected cells in the presence of serum; lane 3, total proteins of M-transfected cells in the absence of serum; lane 4, total proteins of M-transfected cells in the presence of serum. Lanes 1 and 2 were probed with N-immunized serum and lanes 3 and 4 were probed with M-immunized serum. Beta-actin was also detected. (II) Semiquantitative evaluation. Ratio of M/beta-actin or N/beta-actin is shown as relative content of protein (mean ± SD; protein with serum versus protein without serum).

obtained from Sigma (St Louis, MO, USA). *In Situ* Cell Death Detection Kit, Fluorescein was from Roche Biochemical (Mannheim, Germany).

Transfection method

One day before transfection, plate 2×10^5 cells in 500 μl growth medium without antibiotics in 6-well plates so that cells will be 90–95% confluent at the time of transfection. For each transfection sample, dilute 4 μg DNA in 50 μl DMEM. Mix gently and then dilute 10 μl lipofectamine 2000 in 50 μl DMEM without serum. Incubate for 3 min at room temperature. Combine the diluted DNA with diluted lipofectamine 2000, mix gently, and incubate for 20 min at room temperature. Add the 100 μl of complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth. Incubate cells in a humidified atmosphere of 5% CO_2 at 37°C.

Expression of pcDNA3.1-M and -N in cultured HPF cells in vitro

The pcDNA3.1-M, pcDNA3.1-N and pcDNA3.1 were respectively transfected into the HPF cells, with lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfected cells and the culture medium were collected 24 h post transfection. A portion of the transfected cells were fixed in 4% paraformaldehyde–phosphate-buffered saline (PBS) for 1 h at room temperature, washed 3 times in PBS, and then permeabilized (0.1% Triton X-100 in 0.1% sodium citrate) for 5 min. After washing with PBS, they were blocked with 3% bovine serum albumin (BSA)–PBS at room temperature for 30 min. The cells were directly incubated with the mock or pcDNA3.1-M or pcDNA3.1-N vector immunized sera (diluted 1:50 with PBS, pH 7.4) at 37°C for 2 h. These immunized sera were obtained from mice 10 weeks after they had been injected with the plasmids. Cells were incubated with the second antibody (goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC); diluted 1:100 in PBS) at room temperature for 1 h and then washed with PBS. The cells were counterstained with propidium iodide (Sigma) for visualizing nuclei and analyzed for expression using a Leica confocal microscope.

Western blotting

Total protein of HPF cells was extracted according to the protocol recommended for the TRIzol reagent provided by Invitrogen. Proteins

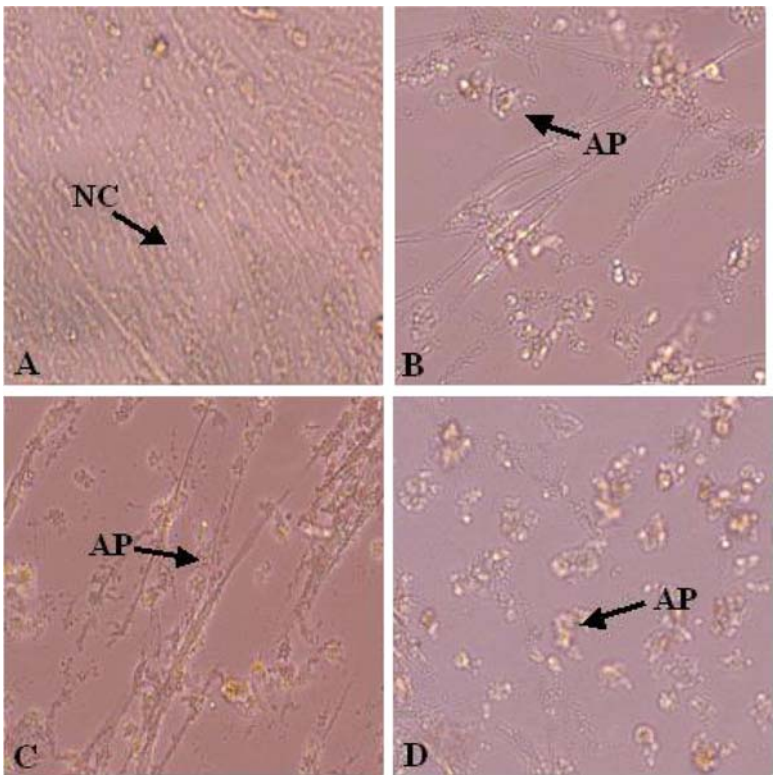

Figure 3. Morphological changes of HPF cells transfected by pcDNA3.1-M and-N. Twenty-four hours post transfection, cells were maintained for a further 24 h in the absence of serum. (A), (B), (C), and (D) show, respectively, the light-microscopic results with mock-, M- or N-transfected cells, and co-transfected cells. NC, normal cells; AP, apoptotic cells (×400).

were separated by 15% SDS-PAGE. Western blots were probed with immunized mouse antisera at a 1:100 dilution for 2 h at 37°C. Antibody binding was detected with horseradish peroxidase-conjugated goat antibody to mouse IgG (Santa Cruz, Heidelberg, Germany) at a 1:10 000 dilution for 2 h at 37°C and ECL development (Pierce, Rockford, IL, USA).

Nuclear condensation

Cells were harvested and re-suspended in 2% paraformaldehyde in PBS for 15 min. The nuclear dye Hoechst 33342 (1 mg/ml in PBS) was then added and cells were incubated at room temperature for 15 min. Apoptotic nuclei were evaluated using a fluorescence microscope with a ×40 objective (Jiang et al., 2003).

TUNEL assay

Cell death was detected using a TUNEL assay kit according to the manufacturer's instructions. The slides with transfected cells were air-dried for 15 min. Method: Fix samples with a freshly prepared fixation solution (4% paraformaldehyde in PBS, pH 7.4) for 1 h at room temperature. Rinse slides 3 times with PBS and then incubate in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate, freshly prepared) for 2 min on ice (2–8°C). At the same time, we prepare the TUNEL reaction mixture. Remove 100 µl Label

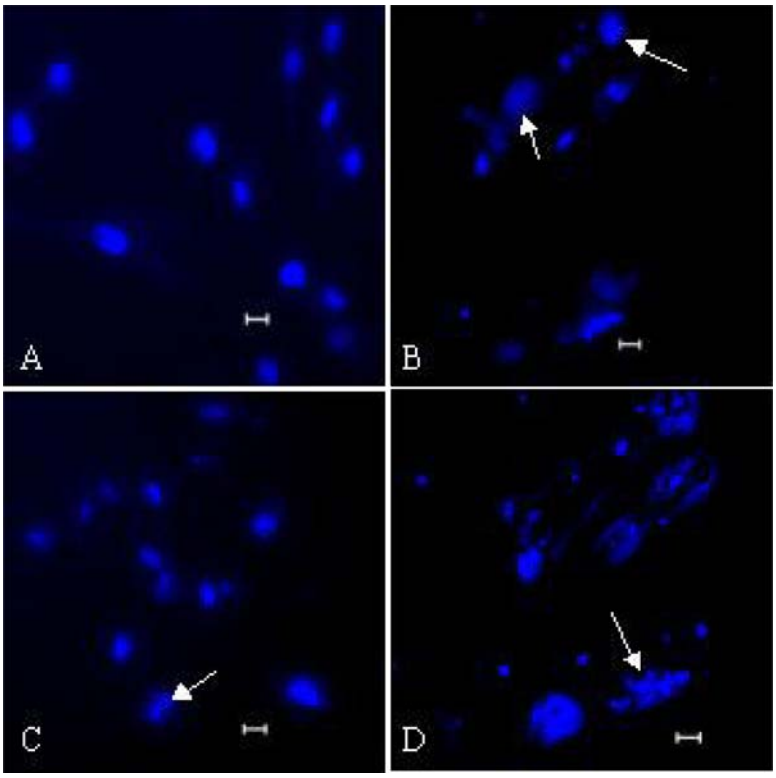

Figure 4. (a) Results of Hoechst 33342 staining. Twenty-four hours post transfection, cells were maintained for a further 24 h in the absence of serum and then were stained with Hoechst 33342. (A), (B), (C), and (D) show, respectively, the fluorescence microscopy results with control, M- or N-transfected cells, and co-transfected cells. White arrows indicate typical morphological features of apoptotic cells (DNA condensation and fragmentation, and nuclear shrinkage). The scale bar corresponds to 20 μm. (b) Ratio of Hoechst staining-positive cells; 500 cells were counted. Bar 1 shows normal cells; bar 2 shows mock-transfected cells; bars 3, 4, and 5 show, respectively, M-, N-, and co-transfected cells in the absence of serum. * $p < 0.05$; ** $p < 0.01$.

Solution (nucleotide mixture in reaction buffer) for two negative controls and add the total volume (50 μl) of Enzyme solution to the remaining 450 μl Label Solution to obtain 500 μl TUNEL reaction mixture. Mix well to equilibrate components. Rinse slides twice with PBS. Dry the area around the samples. Add 50 μl TUNEL reaction mixture to sample (for the negative control, add 50 μl Label solution) and cover with parafilm to ensure a homogeneous spread of TUNEL reaction mixture across the cell monolayer and to avoid evaporative loss. Incubate slides in a humidified atmosphere for 60 min at 37°C in the dark. Rinse slides 3 times with PBS. Samples can directly be analyzed by fluorescence microscopy using an excitation wavelength of 488 nm and detection wavelength of 520 nm (green).

Statistical analysis

All values are reported as the mean ± SD of at least three independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA). When significant treatment effects were indicated, the Student–Newman–Keuls multirange test was employed among the groups using SPSS software. A value of $p < 0.05$ was considered statistically significant.

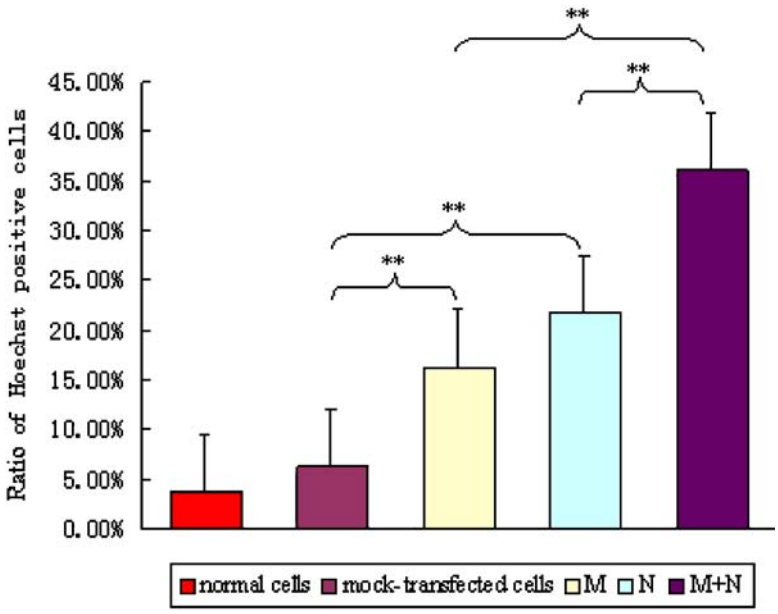

Figure 4. (*Continue*)

Results

Expression of vectors in cultured HPF cells in vitro

To identify pcDNA3.1-M and -N expressed *in vitro*, we transfected them into HPF cells; pcDNA3.1 vector was used as the negative control. The transfected HPF cells were reacted with antisera from the corresponding plasmid-immunized mice and subsequently reacted with the goat anti-mouse IgG conjugated with FITC as the secondary antibody. Green fluorescence was observed in cytoplasm of N-transfected HPF cells, and was also found in cytoplasm and nucleus in M-transfected HPF cells (Figure 1). This suggests that pcDNA3.1-M and-N can be expressed in HPF cells.

Western blotting was also performed to detect the expression level of M and N proteins in the absence and presence of serum. M- and N-immunized sera were respectively used as the primary antibodies. Beta-actin protein was also detected as an internal control. The result was analyzed for semiquantitative evaluation by Quantity One software (Bio-Rad, Hercules, CA, USA). For the expression level of M or N protein, there was no statistical difference between the absence and presence of serum (Figure 2), demonstrating that the difference we observed was not due to protein expression levels.

M and N proteins induce apoptosis of HPF

Twenty-four hours post transfection, cells were maintained for a further 24 h either in the absence or in the presence of serum. Mock-transfected cells were transfected with the respective empty vectors. Results of Hoechst 33342 staining and observation under the light microscope showed that cells transfected with plasmids containing M or N genes in the absence of serum had obvious morphological and biochemical changes that differed from normal cells. Nuclear condensation, apoptotic bodies, and fragments could be seen (Figures 3 and 4). Hoechst-positive cells were

scored from a total of 500 cells viewed from three different parts of the culture and the bar graph (Figure 4b) represents the mean proportion of them. Hoechst-positive cells constituted approximately 20–30% of the total cell count. In the absence of serum, about 16.34% of cells transfected with pcDNA3.1-M and 21.72% of N-transfected cells showed typical apoptotic characteristics, which showed statistical significance when compared with mock-transfected cells (only 6.23%; $p < 0.01$).

We also performed TUNEL assay to detect cell death. Apoptosis was detected in cells transfected with plasmids containing M or N genes in the absence of serum (Figure 5). The statistical method was the same as that for Hoechst staining and similar results were found (data not shown).

Two kinds of plasmids (pcDNA3.1-M and pcDNA3.1-N) were co-transfected into HPF with a ratio of 1:1 (the total amount of DNA was constant). Twenty-four hours post transfection, the same assays were performed to detect apoptosis. The co-transfected cells had remarkably higher ratio ($p < 0.01$) of cell death than cells transfected with M or N alone (see Figure 3D, Figure 4a (D), and Figure 5E).

The effect of serum on the apoptosis of HPF cells

In presence of serum, few transfected cells were dead, even though they were co-transfected with both plasmids. This protective effect is shown in Figure 6 as a typical example.

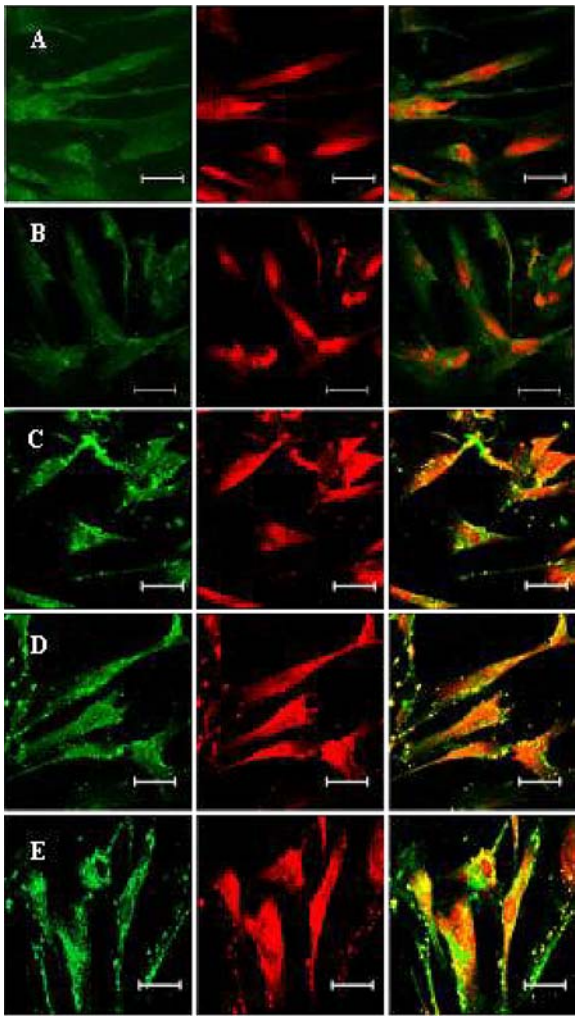

Figure 5. Results of TUNEL assay. Twenty-four hours post transfection, cells were maintained for a further 24 h in the absence of serum and then TUNEL assay was performed. (A), (B), (C), (D), and (E) show, respectively, the fluorescence microscopy results with control, mock-, M- or N-transfected cells, and co-transfected cells. The scale bar represents 20 μm.

Discussion

The fact that proteins of coronaviruses can induce apoptosis of host cells has been known for a long time. Although overall sequence conservation is low, the predicted M and N proteins of SARS-CoV contain conserved motifs that are found in other coronaviruses. Therefore, SARS-CoV may share the similar pathogenic mechanism with other coronaviruses.

Our study suggests that SARS-CoV M and N proteins can induce apoptosis of HPF cells. To our knowledge, this is the first report showing that SARS proteins can induce apoptosis in primary human cells. This conclusion confirms our previous findings. Adult Brandt's voles that were infected with SARS-CoV showed hemorrhagic interstitial pneumonia, a clot in liver, spleen, and

kidney, and local hemorrhage spot in lung (Gao et al., 2005). Cell apoptosis was one of the major reasons for these changes. To verify this, we chose two major structural proteins (M and N) for study. Plasmids containing them were transfected into HPF cells and were shown to be expressed by immunofluorescence staining and Hoechst staining, TUNEL assays were performed to detect cell death 48 h post transfection. The results demonstrated that M and N proteins can induce apoptosis of HPF to some degree. Furthermore, the apoptosis was observed only in the absence of serum and few cells were dead in the presence of serum, as in the control. This finding corresponds to previous reports (Surjit et al., 2004). It may be that some survival pathway is activated by growth factors and other components in the serum, and inhibits apoptotic pathways (Surjit et al., 2004).

The induction of apoptosis by SARS-CoV has been detected in some cell lines, such as Vero E6 and COS-1, and in lung, liver, and other organs. However, some cell lines are insensitive to the proteins. Surjit et al. (2004) did not observe significant cell death in human hepatoma (Huh7) cells. Recently, Tan et al. (2004) demonstrated that the protein 7a, which was specifically expressed in SARS-CoV, could induce apoptosis in many cell lines. These reports illustrate a diversity of mechanisms by which SARS-CoV induces apoptosis.

We co-transfected two kinds of plasmids into HPF cells and saw a significantly higher ratio of cell death. This may be concerned with the complex formation of SARS proteins in cells and suggests that SARS proteins may work in a complex form. However, the effect of co-transfection is not simply the sum of the effects of transfection with the individual plasmids. The co-transfected cells were almost all dead 48 h post transfection in the absence of serum, and only a few cells were still adhesive, while most of the cells transfected with only one plasmid were still adhesive in the same condition. Therefore, the com-

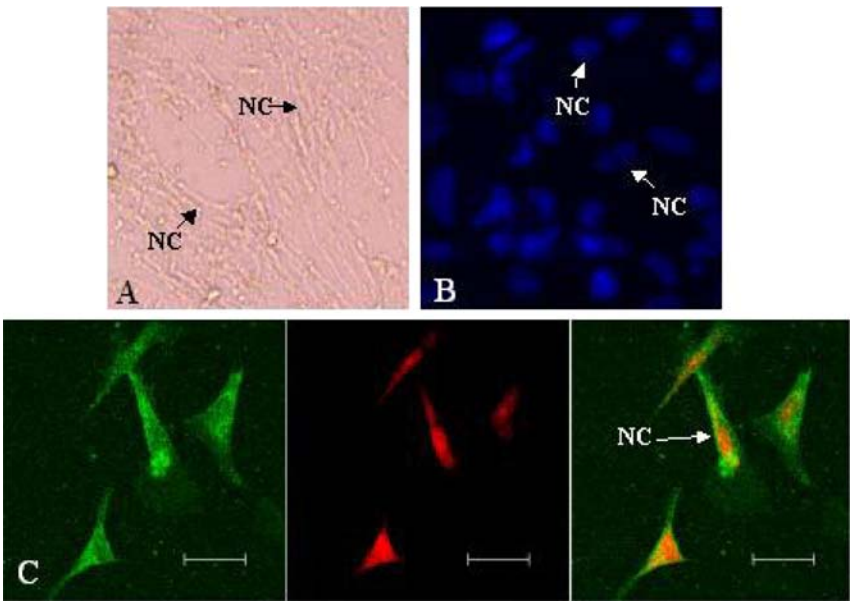

Figure 6. The effect of serum on the apoptosis of HPF cells. (A), (B), and (C) show, respectively, the results of Hoechst 33342 staining, TUNEL, and observation of morphology of cells co-transfected with pcDNA3.1-M and -N in presence of serum. NC, normal cells; ×400.

plex may be the natural and toxic form of SARS proteins in cells. He et al. (2004) reported that interaction of the N and M proteins could take place *in vivo* and identified that a stretch of amino acids (AA residues numbers 168–208) in the N protein may be critical for such protein–protein interactions. It could be a new target for SARS therapy by destroying the complex or making it as a new specific antigen. We injected a mixture of pVAX1-M and pVAX1-N into Balb/c mice, which elicit humoral and cellular responses. (Shu-Qun Shi et al., in press). The result showed that membrane protein could augment the N-specific immune responses. N-transfected cells show more notable differences than M-transfected cells. This seems to suggest that N protein is the core of the apoptosis inducers and that M proteins might enhance the effect of N protein by formation of protein complexes or by other means.

We investigated the induction of apoptosis by SARS proteins *in vitro* and found that M and N proteins could induce apoptosis of HPF cells and that co-transfection of M and N clearly enhances this effect. M and N proteins, especially complexes of these structural proteins, may play an important role in the pathogenetic process in these cells. However, the molecular mechanism and the effects of the proteins *in vivo* have not been identified. In which survival and apoptosis pathways do SARS proteins participate? Why is the induction of apoptosis of SARS proteins detected *in vivo* in serum-rich conditions? These questions will be addressed in subsequent work.

Acknowledgments

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References

Chow KY, Yeung YS, Hon CC, Zeng F, Law KM, Leung FC. Adenovirus-mediated expression of the C-terminal domain of SARS-CoV spike protein is sufficient to induce apoptosis in Vero E6 cells. FEBS Lett. 2005;579(30):6699–704.

Gao Hong, Peng Jingpian, Deng Wei, et al. Infection of SARS-CoV on juvenile and adult Brandt's vole *Microtus brandtii*. Chinese Science Bulletin. 2005;50(12):1199–204.

He R, Leeson A, Ballantine M, et al. Characterization of protein– protein interactions between the nucleocapsid protein and membrane protein of the SARS coronavirus. Virus Res. 2004;105(2):121–5.

Jiang Z, Zhang Y, Chen XQ, et al. Apoptosis and activation of Erk1/2 and Akt in astrocytes postischemia. Neurochem Res. 2003;28(6):831–7.

Jin Rong-Hua, Zhang Yong-Hong, Ren Yi, et al. Apoptosis related proteins expression of T lymphocyte in SARS convalescent patients. Chinese J Immunol. 2004;20(2):97–9.

Law PT, Wong CH, Au TC, et al. The 3a protein of severe acute respiratory syndrome-associated coronavirus induces apoptosis in Vero E6 cells. J Gen Virol. 2005;86(Pt 7):1921–30.

Marra MA, Jones SJ, Astell CR, et al. The genome sequence of the SARS-associated coronavirus. Science. 2003;300(5624):1399–404.

Rota PA, Oberste MS, Monroe SS, et al. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. Science. 2003;300(5624):1394–9.

Shi SQ, Peng JP, Li YC, et al. The expression of membrane protein augments the specific responses induced by SARS-CoV nucleocapsid DNA immunization. Mol Immunol. 2006;43(11):1791–8.

Surjit M, Liu B, Jameel S, Chow VT, Lal SK. The SARS coronavirus nucleocapsid protein induces actin reorganization and apoptosis in COS-1 cells in the absence of growth factors. Biochem J. 2004;383(Pt 1):13–8.

Tan YJ, Fielding BC, Goh PY, et al. Overexpression of 7a, a protein specifically encoded by the severe acute respiratory syndrome coronavirus, induces apoptosis via a caspase-dependent pathway. J Virol. 2004;78(24):14043–7.

Yan H, Xiao G, Zhang J, et al. SARS coronavirus induces apoptosis in Vero E6 cells. J Med Virol. 2004;73(3):323–31.

Yang Y, Xiong Z, Zhang S, et al. Bcl-xL inhibits T-cell apoptosis induced by expression of SARS coronavirus E protein in the absence of growth factors. Biochem J. 2005;392(Pt 1):135–43.

Yuan X, Shan Y, Zhao Z, Chen J, Cong Y. G0/G1 arrest and apoptosis induced by SARS-CoV 3b protein in transfected cells. Virol J. 2005;2:66.

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