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Key Messages

1. The adenovirus-mediated overexpression of SARS coronavirus (SARS-CoV) spike protein (S) and its C-terminal domain (S2) induce apoptosis in Vero E6 cells.
2. Such apoptosis in Vero E6 cells is time- and dose-dependent.
3. The adenovirus-mediated over-expression of SARS-CoV N-terminal domain (S1) and other structural proteins, including E, M and N protein do not induce apoptosis.

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RFCID project number: 02040222

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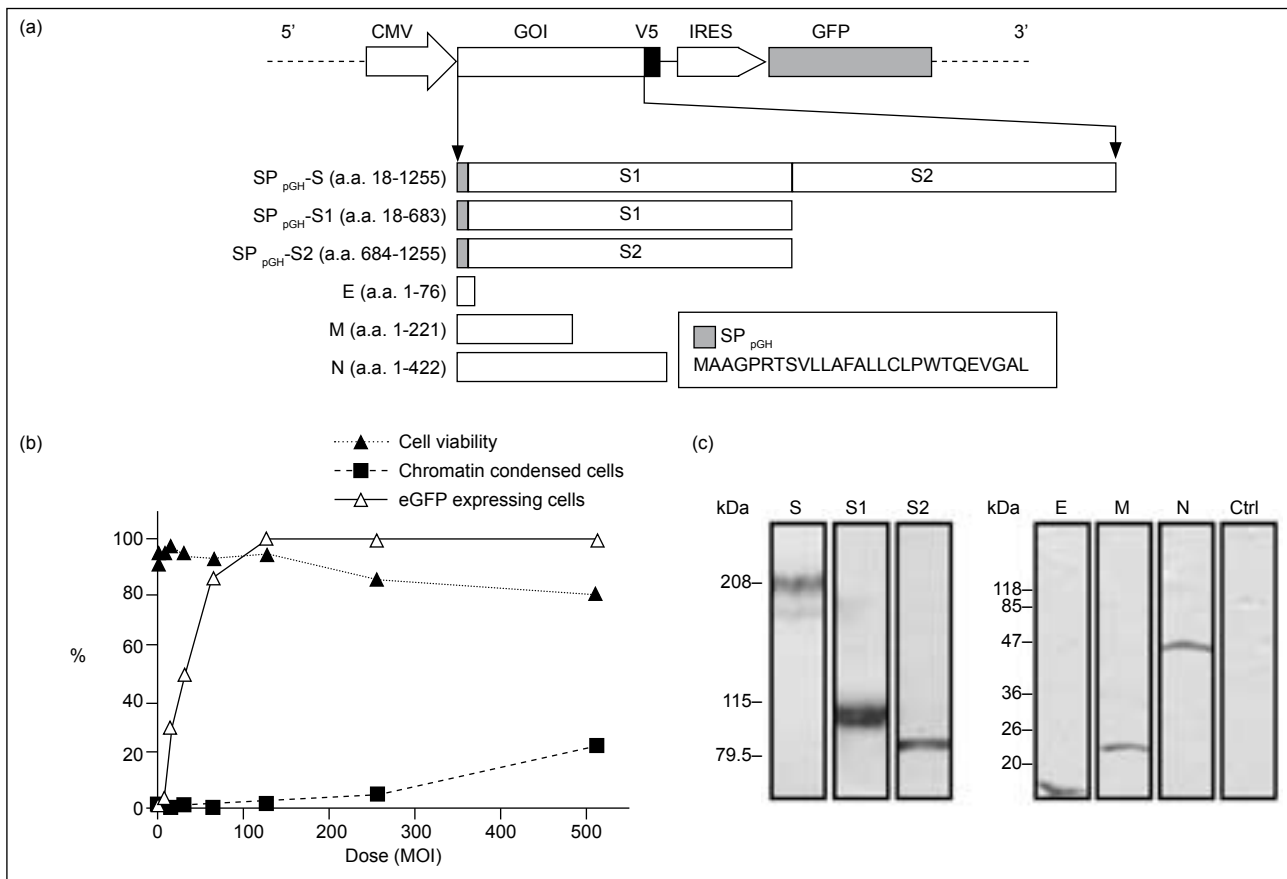Fax: (852) 2857 4672
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SARS coronavirus and apoptosis

Introduction

SARS coronavirus (SARS-CoV) was identified as the causative agent of SARS early in 2003.[1] Fever, dyspnoea, lymphopaenia, neutropaenia, and lower respiratory tract infection were commonly encountered in infected individuals. Comparative genomic analysis revealed that SARS-CoV is a novel member of the viral family coronaviradae, with an RNA genome of 29.7 kbp.[1,2] At least five viral structural proteins (VSPs), namely the spike (S), envelope (E), membrane (M) and nucleocapsid (N) protein, together with the newly identified ORF3a, were encoded from the viral genome. Among these proteins, expression of S, M and N are necessary and sufficient for pseudovirus assembly mimicking those found in SARS-CoV infected cells. Accumulated evidence has demonstrated that survival of the viruses depends on successful modulation of apoptosis, initiated either by the hosts or the viruses themselves. Several studies have associated apoptosis with the pathogenesis of coronaviruses,[3] including suggestions that overexpression of certain coronaviral proteins could induce apoptosis in vitro. For SARS-CoV, certain features, such as depletion of hepatocytes and T lymphocytes, ie, lymphopaenia, were appeared related to apoptosis.[4] It was also demonstrated that in-vitro replication of SARS-CoV induces apoptosis. Recently, the ORF3a and the accessory protein 7a, but not the N, M and E protein of SARS-CoV, were shown to induce apoptosis in Vero E6 cells. In contrast, the E and N protein of SARS-CoV appeared to induce apoptosis in Jurkat T and COS-1 cells, respectively, under serum depletion conditions. It was also reported that baculovirus-mediated expression of the N-terminal (S1) but not the C-terminal (S2) domain of the S protein of SARS-CoV triggers the cell survival–related AP-1 signalling pathway in lung cells. Nevertheless, the possible role(s) of the SARS-CoV VSPs in virus-induced apoptosis is largely unknown. In this study, we set out to demonstrate a possible role of SARS-CoV S protein in virus-induced apoptosis using recombinant adenovirus (rAd)–mediated expression system. The apoptotic properties of S, S1 and S2 protein, as well as other VSPs, including E, M and N protein, were investigated in Vero E6 cells.

Methods

This study was conducted from January 2005 to December 2006.

Cell culture

HEK293-derived AD-293 cells (Stratagene) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL), supplemented with 10% heat-inactivated foetal bovine serum (FBS; Gibco-BRL) containing a 1% concentration of the antibiotic-antimycotic 100X (Bibco-BRL) at 37°C and 5% CO_2. Vero E6 cells were maintained in Eagle's Minimal Essential Medium (EMEM; Gibco-BRL) supplemented with 10% FBS and 1% antibiotics-antimycotic.

Generation of recombinant adenoviral virus

Cloning of the SARS-CoV VSPs from viral cDNA, including S, S1 and S2, as well as three other structural genes—E, M, and N gene (Fig 1a), was described elsewhere.[2,5] The cloned cDNA fragments were tagged at the carboxy-terminal with a V5 epitope. The signal peptide of pig growth hormone was placed upstream of the coding sequences of S (18–1255), S1 (18–683) and also S2 (684–1255). This was to ensure comparable post-translational modifications for all spike protein fragments used in the study. The transgenes were then subcloned into a modified bicistronic shuttle vector designated pShuttle-CMV-GOI-IRES-eGFP, which was derived from the pShuttle vector of the AdEasy XL Adenoviral Vector

Fig 1. Construction of the recombinant adenovirus–viral structural proteins (rAd-VSPs) and the evaluation of rAd-mediated transduction and expression

(a) A schematic diagram showing the organisation of the bicistronic expression cassette of the modified shuttle vector (pShuttle-CMV-GOI-IRES-eGFP) [upper panel] used for rAd construction and the cloned coding regions of SARS-CoV VSPs, including S, S1, S2, E, M and N (lower panel). The amino acids were numbered according to the corresponding proteins of SARS-CoV strain HK-39. The amino acid sequence of signal peptide of pig growth hormone (SPpGH) is shown in the key and the detailed sequence information of the IRES-eGFP fragment is available at http://www.addgene.org/pgvec1?f=c&identifier=1736&cmd=findpl

(b) Assessment of the optimal multiplicity of infections (MOI) for maximal transduction efficiency. The percentage of enhanced green fluorescent protein (eGFP) expressing cells was accessed by flow cytometer with at least 1 · 105 cells were counted for each sample. Each data point of the three assays were determined in triplicate and represents the average of three independent experiments ± standard error mean (S.E.M)

(c) Expression of SARS-CoV VSPs in Vero E6 cells. The expressed proteins were detected by using anti-V5 antibody and the sizes of the molecular marker were shown on the left of each blot

System (Stratagene) and the plasmid pBMN-I-GFP (the generous gift of Dr GP Nolan, Stanford University School of Medicine, US). The bicistronic expression cassette contains the gene of interest (GOI) and the enhanced green fluorescent protein (eGFP), which are driven by a human cytomegalovirus immediate–early gene promoter (CMV) and an internal ribosomal entry site (IRES), respectively (Fig 1a). The recombinant adenovirus containing the VSPs (rAd-VSPs) was then generated by incorporating the expression cassette into the pAdEasy-1 vector (Stratagene) according to the manufacturer's instructions. A control adenovirus (rAd-Ctrl) with no transgene was also constructed. The rAds were propagated in AD-293 cells and CsCl-purified as described.[6]

Immunoblotting

To assess the expression of SARS-CoV VSPs, Vero E6 cells were transduced with the corresponding rAds at

multiplicity of infections (MOI) of 100 for over-expression. Cells were harvested 84 hours (h) post-transduction (p.t.) and cell lysate was denatured and subjected to SDS–PAGE (S, S1 and S2 in 5% PAGE; other VSPs in 10% PAGE). To detect the expressed VSPs, Western blotting was carried out as described[5] using AP-conjugated anti-V5 antibody (Invitrogen).

Cell viability assay

Viability of cells transduced at indicated MOIs was accessed by trypan blue exclusion assay. Cells were harvested and stained with 0.025% trypan blue dye (Invitrogen) for 10 min, and the percentage of dead cells (blue) was counted using a haemocytometer.

Nuclear morphology

To detect chromatin condensation, cells transduced at indicated MOIs, were collected by low-speed centrifugation

and stained with Hoechst 33342 (Molecular Probes) phosphate buffered saline (PBS) solution (1:1000 v/v) at 37°C for 5 min. At least 200 cells from three random fields were counted under fluorescence microscope.

DNA laddering assay

Cellular DNA fragmentation into characteristic ladders in apoptotic cells was assayed as described[7] with modifications. Briefly, cells were transduced with indicated rAds at MOI of 100. Both floating and adherent cells were collected at indicated time points p.t. and were subjected to low-speed centrifugation. Cell pellets were then washed once in ice-cold PBS and subsequently resuspended in 80 mL of the same solution. Three hundred microlitres of lysis buffer [10 mM Tris–HCl (pH 7.6), 10 mM EDTA, and 0.6% SDS] were added to the cell suspension, prior to the addition of 0.1 mL of 5M NaCl. Lysates were then incubated at 4°C overnight. Cell debris was pelleted by centrifugation and the supernatants were treated with 10 mL of 20 mg/mL proteinase K (Gibco-BRL) at 37°C for 1 h. Low-molecular-weight DNA was concentrated by ethanol precipitation overnight at 20°C after phenol:chloroform extraction and subsequently analysed by 2% agarose gel electrophoresis.

Flow cytometry analysis of early apoptosis by 7-AAD and Annexin V staining

The asymmetry of the plasma membrane of rAd-S and-S2 transduced cells at 84 h p.t. was monitored by dual staining with Annexin V-PE and 7-aminoactinomycin D (7-AAD), which is a phosphatidylserine (PS)-binding protein and an impermeable DNA-labelling dye, respectively (Annexin V-PE apoptosis detection Kit I, BD Pharmingen BioSciences). Data were acquired by Coulter Epics Elite Flow Cytometer and were analysed with the WinMDI v2.81 software package (Scripps Research Institute). Early apoptotic cells were recognised as PS-externalised (Annexin V-PE labeled) with an intact cell membrane that resists 7-AAD staining (lower-right quadrant), which allows the exclusion of necrotic cells that are indistinguishable from the late apoptotic cells (upper-right quadrant). At least 10^5 cells were counted for each data point.

Statistical analysis

Student's *t* tests were used to compare the significance of differences between specified groups, with $P < 0.05$ or < 0.01 be defined as statistically significant.

Results

Adenovirus-mediated expression of SARS-CoV viral structural proteins

To determine the rAd dosage needed for maximum transduction efficiency with minimal cytopathic effects, Vero E6 cells were transduced with rAd-Ctrl at different MOIs and were examined at 84 h p.t. (Fig 1b). At a MOI of 100, about 95% of cells were expressing eGFP, while no substantial apoptotic effect (ie <5% of non-viable and chromatin condensed cells) was observed. Therefore, a

MOI of 100 was chosen as the upper dose limit of the rAd transductions in this study. The successful and comparable transductions of all rAd-VSPs were ensured in which at least 95% of cells showed the expression of eGFP and V5 epitope as detected by flow cytometer (data not shown), while the expression of SARS-CoV VSPs was further confirmed by Western blots (Fig 1c). The well-resolved double-band pattern was observed for S and S1 at around 200 and 110 kDa, respectively, which mirrored previous reports that these two proteins are heavily glycosylated. Among the bands of S protein doublets, the one with lower molecular weight is at about 180 kDa, which is expected to be the glycosylated protein found in the endoplasmic reticulum, and the one with the higher molecular weight, which is about 200 kDa, and is expected to represent its more complexly glycosylated form found in Golgi bodies.

Transduction by recombinant adenovirus–viral structural proteins induces apoptosis in Vero E6 cells

We next compared the apoptotic effects induced by rAd-VSPs transductions in terms of cell morphology, cell viability, chromatin condensation and cellular DNA fragmentation at 12 h intervals p.t. At 84 h p.t., cytopathic effects with abnormal cell morphology (ie shrinkage and detachment) [Fig 2a] and chromatin condensation (Fig 2b) were observed in a substantial proportion of cells transduced by rAd-S and-S2, but neither in mock nor other rAds-transduced cells. As shown in Figure 2c, cells transduced by rAd-S and rAd-S2 collected at 84 h p.t. showed significantly stronger apoptotic effects in terms of both cell viability and chromatin condensation. Moreover, cellular DNA fragmentation into characteristic ladders was only observed in rAd-S and -S2 transduced cells (Fig 2d), in which increments of about 200 bp became weakly observable at 36 h p.t. Although random shearing of DNA was also observed in parallel, the intensity of the ladder was substantially increased at 84 h p.t. These observations indicate that both S and S2 proteins are able to induce apoptosis in Vero E6 cells while the other VSPs do not.

Transduction of rAd-S2 showed a stronger apoptotic effect than that of rAd-S

To further confirm the observed apoptotic effect of the S proteins, Vero E6 cells were transduced with rAd-Ctrl, -S, -S1 and -S2 at different MOIs and the percentage of apoptotic cells at 84 h p.t. were evaluated by chromatin condensation and PS-externalisation using fluorescent microscopy and flow cytometry, respectively. As shown in Figure 3a, the percentage of chromatin condensed cells induced by either rAd-S or -S2 transduction at all indicated MOIs was significantly higher than that of the others in a dosage-dependent manner. Moreover, at MOIs of 50 and 100, the percentage of chromatin condensed cells induced by rAd-S2 transduction was significantly higher than those induced by rAd-S transduction. A similar phenomenon was observed when the cell membrane asymmetry of cells were examined (Fig 3b); the percentage of early apoptotic cells in
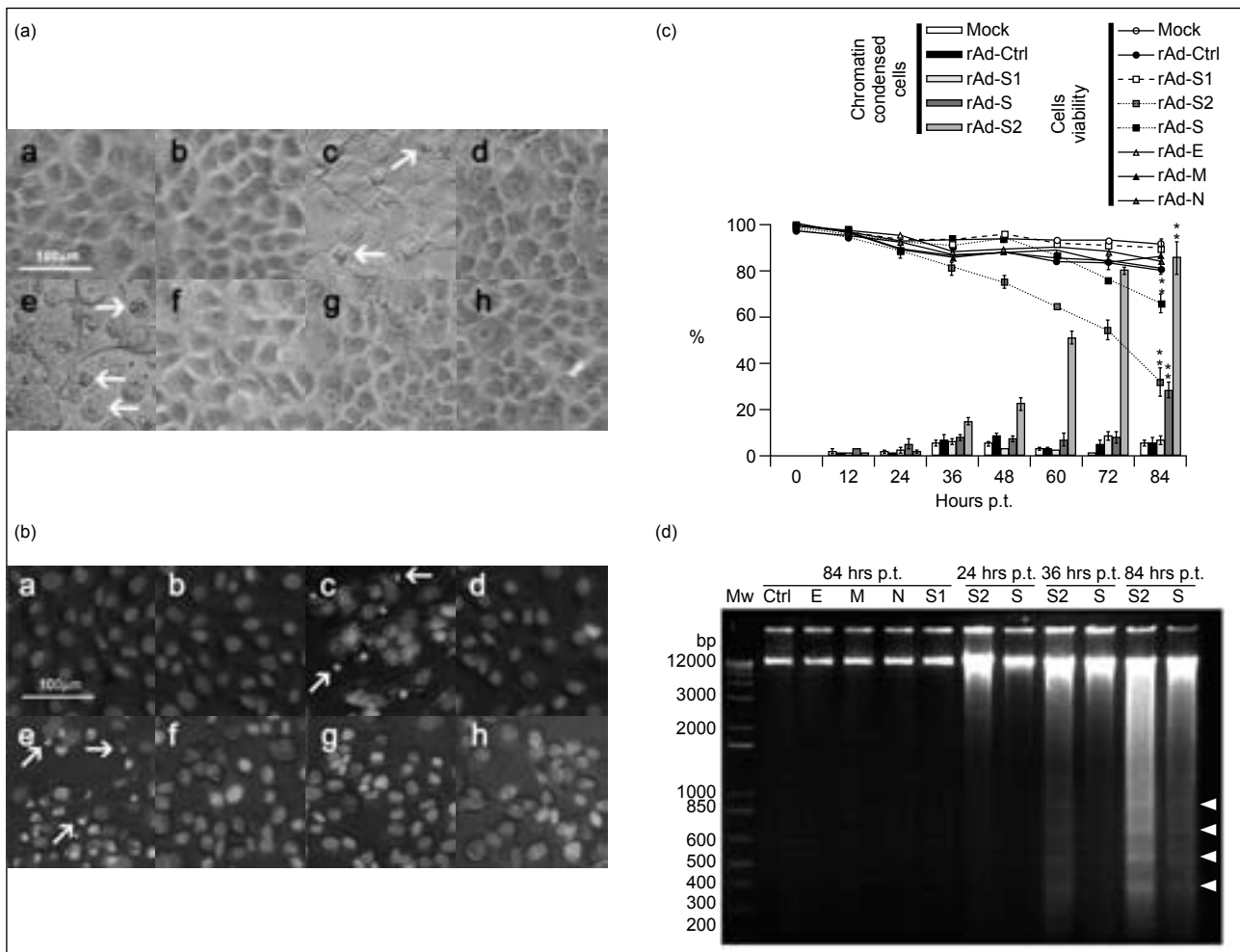
Fig 2. Transduction of Vero E6 cells by rAd-S and rAd-S2 induced cell death and apoptosis
(a) Photomicrographs showing the cell morphology of Vero E6 cells at 84 h after transduction with the indicated rAds (a–h, representing Mock, rAd-Ctrl, -S, -S1, -S2, -E, -M and -N, respectively) at a MOI of 100. Cells undergoing cytoplasmic shrinkage are indicated by arrows
(b) Phase contrast/Hoechst 33342 fluorescence merged photographs of the Vero E6 cells transduced as in (a). Cells undergoing chromatin condensation are indicated by arrows
(c) The apoptotic effects induced by the transduction of all rAds at a MOI of 100 were compared quantitatively in terms of cell viability (lines) and percentage of chromatin condensed cells (bars) using trypan blue exclusion assay and Hoechst 33342 staining, respectively, as in Fig 1b; ** denotes $P<0.01$
(d) Cellular DNA fragmentation analysis. Characteristic DNA ladders with approximately 200 bp increments are indicated by arrowheads. Results shown in (a), (b) and (d) are representatives of three independent experiments

rAd-S and -S2 transduction was at least 2 fold higher than that of the controls at MOIs of 50 and 100. In summary, the above data strongly suggest that rAd-mediated over-expression of S and S2 protein induces apoptosis in Vero E6 cells, and that rAd-S2 induced substantially stronger apoptosis than rAd-S under the condition tested.

Discussion

Infection of SARS-CoV in Vero E6 cells induces extensive apoptosis through a caspase-3 and p38 MAPK–dependent pathway. Using an rAd-mediated expression system, we assessed the apoptotic effect of the major structural proteins of SARS-CoV, including S, S1, S2, E, M and N protein. Typical features of apoptosis such as cell rounding,

shrinkage, nuclear condensation, DNA fragmentation and PE-externalisation were only observed in cells transduced with rAd carrying S and S2, but not S1, nor any other structural proteins studied. These data suggest that the over-expression of SARS-CoV S and S2 could induce apoptosis. The present findings seem to be unique within the coronavirus family. In MHV and IBV, over-expression of S protein did not induce observable apoptosis in vitro. On the other hand, the observed in-vitro apoptotic effects of the VSPs of other coronaviruses, such as the E protein of MHV and the N protein of TGEV, were not observed when we overexpressed the SARS-CoV homologues in Vero E6 cells. Interestingly, the E and N protein of SARS-CoV has been reported to induce apoptosis in Jurkat T and COS-1 cells, respectively, under serum depletion conditions. This was not
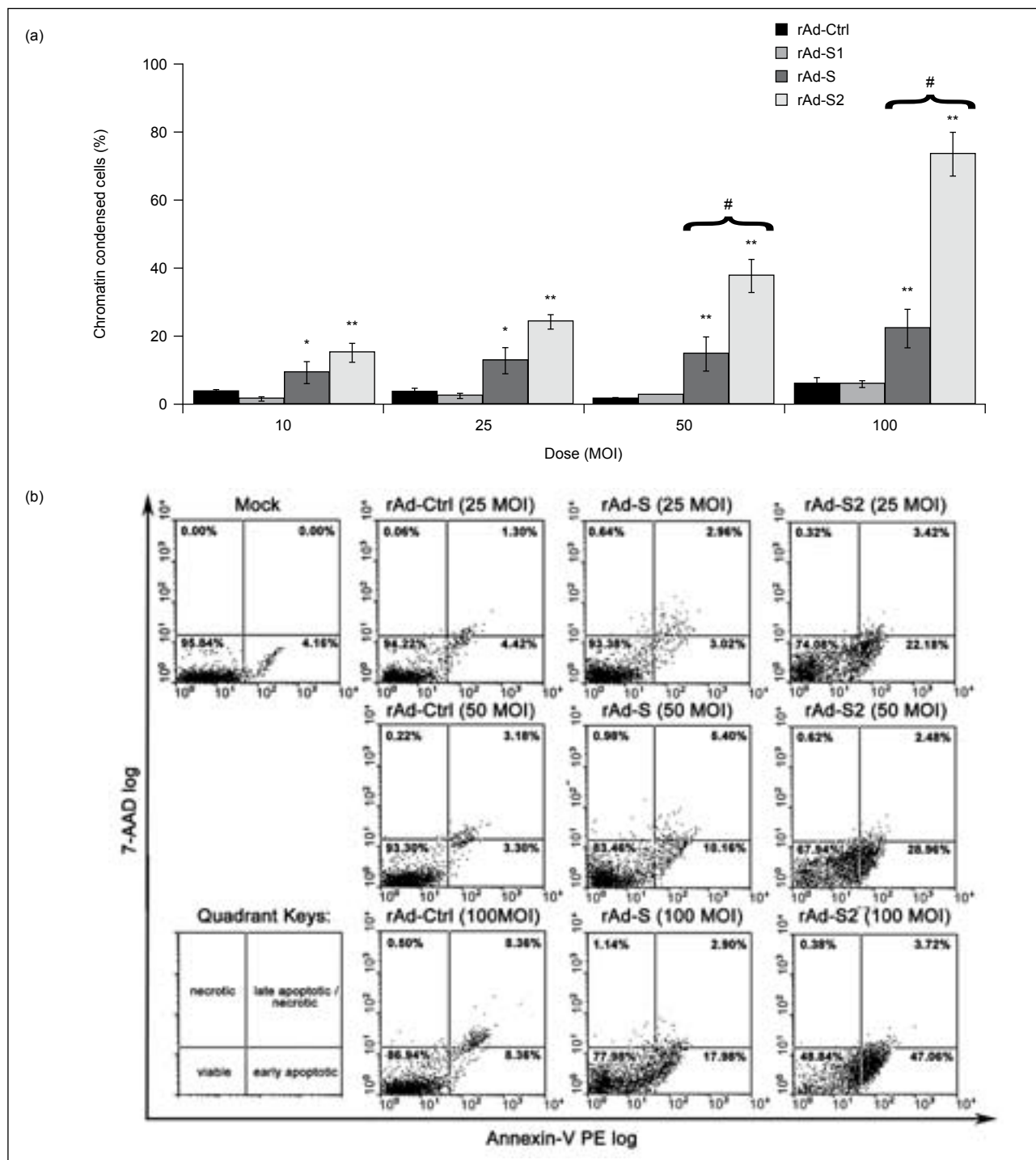
Fig 3. Transduction of rAd-S2 showed a stronger apoptotic effect than that of rAd-S in Vero E6 cells
(a) Dosage-dependence of the apoptotic effect induced by rAd-S and -S2 in terms of chromatin condensation. Cells were transduced by rAds at indicated MOIs. The percentage of apoptotic cells was determined by Hoechst 33342 staining at 84 h p.t as in Fig 1b; * denotes P<0.05, ** P<0.01, and # P<0.01
(b) Early apoptosis of Vero E6 cells transduced by rAd-S and -S2. The asymmetry of the plasma membrane of rAd-S and -S2 transduced cells at 84 h p.t. was monitored by double staining with the Annexin V and 7-AAD detected via flow cytometry. Quadrant keys are showed at the lower-left corner. The percentage of cells in each quadrant is showed at the top of each dot-plot. Results shown in (b) are representative of three independent experiments

observed under the conditions prevailing in the current and a previous study.[8] Recently, over-expression of two newly identified viral proteins of SARS-CoV, ORF3a and 7a, were shown to induce apoptosis in Vero E6 cells as well, which

is associated with caspase-8 and -3 activity, respectively. Since the expression level of these viral proteins in SARS-CoV infected cells has not been clearly demonstrated, their pro-apoptotic properties may not be the only contributing

factor in SARS-CoV induced apoptosis. In contrast, the S protein is one of the major viral proteins in SARS-CoV–infected cells apart from N protein. In SARS-CoV–infected Vero E6 cells, cleavage of the S protein into fragments was suggested in previous studies, which includes a form that resembles the S2 protein in this study; inhibition of such protein processing completely abrogated the virus-induced cytopathic effects in vitro, suggesting potential roles for S and S2 in SARS-CoV–induced apoptosis. Although activation of the mitochondrial apoptotic pathway, caspase cascade, and the p38 MAPK–dependent pathway are reported in several in-vitro models of SARS-CoV–induced apoptosis, the viral component(s) responsible for these observations remain unclear. Ren et al[9] demonstrated that the addition of inactivated SARS-CoV viral particles to Vero E6 cells is unable to induce apoptosis, implying that expression of viral genes is indispensable for the viral-induced apoptosis in vitro. Regarding these findings, the pro-apoptotic properties of S and S2 in this report and the comparative study of apoptotic pathways initiated by expression of individual viral genes and viral infection could provide clues as to the molecular components responsible for the SARS-CoV–induced apoptosis. The demonstrated roles of SARS-CoV S protein in viral entry and elicitation of neutralising immune responses make it an attractive target for antiviral therapies.[10] Investigations into the molecular basis of S protein induced apoptosis ongoing in our laboratory, together with findings from this study may provide important insights. Such insights could aid the rational design of anti-viral therapies and an understanding of the molecular pathogenesis of SARS-CoV infection.

Acknowledgement

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