Adenovirus-mediated expression of the C-terminal domain of SARS-CoV spike protein is sufficient to induce apoptosis in Vero E6 cells

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Abstract The pro-apoptotic properties of severe acute respiratory syndrome coronavirus (SARS-CoV) structural proteins were studied in vitro. By monitoring apoptosis indicators including chromatin condensation, cellular DNA fragmentation and cell membrane asymmetry, we demonstrated that the adenovirus-mediated over-expression of SARS-CoV spike (S) protein and its C-terminal domain (S2) induce apoptosis in Vero E6 cells in a time- and dosage-dependent manner, whereas the expression of its N-terminal domain (S1) and other structural proteins, including envelope (E), membrane (M) and nucleocapsid (N) protein do not. These findings suggest a possible role of S and S2 protein in SARS-CoV induced apoptosis and the molecular pathogenesis of SARS.

Keywords: Apoptosis; Virus–cell interaction; Severe acute respiratory syndrome coronavirus; Spike protein

1. Introduction

Severe acute respiratory syndrome-coronavirus (SARS-CoV) was identified as the causative agent of SARS early in 2003 [1]. Fever, dyspnea [1], lymphopenia, neutropenia [2,3] and lower tract respiratory infection [1] were commonly found 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 [4–6]. 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 [7,33], were encoded from the genome [9,10]. Among these proteins, expression of S, M and N are necessary and sufficient for pseudovirus assembly mimicking those found in SARS-CoV infected cells [11,12].

Accumulated evidences have demonstrated that survival of viruses depends on the successful modulation of apoptosis initiated either by the hosts or the viruses themselves [13–16]. Several studies have associated apoptosis with the pathogenesis of coronaviruses [17–21]. Previous reports suggested that over-expression of certain coronaviral proteins could induce apoptosis in vitro [22,23]. For SARS-CoV, clinical symptoms, such as depletion of hepatocytes and T lymphocytes, i.e., lymphopenia, were suggested to be related to apoptosis [24–26]. It was also demonstrated that in vitro replication of SARS-CoV induces apoptosis [27–31]. Recently, the ORF3a and the accessory protein 7a, but not the N, M and E protein of SARS-CoV, was demonstrated to induce apoptosis in Vero E6 cells [8,32]. In contrast, it is reported that the E and N protein of SARS-CoV induces apoptosis in Jurkat T and COS-1 cells, respectively, under serum depletion conditions [34,35]. 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 signaling pathway in lung cells [36]. Nevertheless, the possible role(s) of the SARS-CoV VSPs in the virus-induced apoptosis is largely unknown. In this study, we demonstrated 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.

2. Materials and methods

2.1. Cell culture

HEK293-derived AD-293 cells (Stratagene) were maintained in Dulbecco's modified eagle medium (DMEM; Gibco-BRL), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL) and 1% antibiotics–antimycotic (Gibco-BRL) at 37 °C and 5% CO_2. Vero E6 cells were maintained in minimum essential medium with eagle's salts (EMEM; Gibco-BRL) supplemented with 10% FBS and 1% antibiotics–antimycotic.

2.2. 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 [6,37]. The cloned cDNA fragments were tagged at the carboxy-terminal with a V5 epitope. The signal peptide of pig growth hormone (SP_{pGH}) [38] was placed upstream of the coding sequences of S (18–1255), S1 (18–683) and also S2 (684–1255), so as to ensure a comparable post-translational modifications for all the spike protein fragments used in the study. The transgenes were then subcloned into a modified bicistronic shuttle vector designated as pShuttle-CMV-GOI-IRES-eGFP, which is derived from the pShuttle vector of the AdEasy™ XL Adenoviral Vector System (Stratagene) and the plasmid pBMN-I-GFP (Dr. G.P. Nolan, Stanford University School of Medicine). The bicistronic expression cassette contains the gene of interest (GOI) and the enhanced green fluorescent protein (eGFP), which are driven by a CMV promotor and an internal

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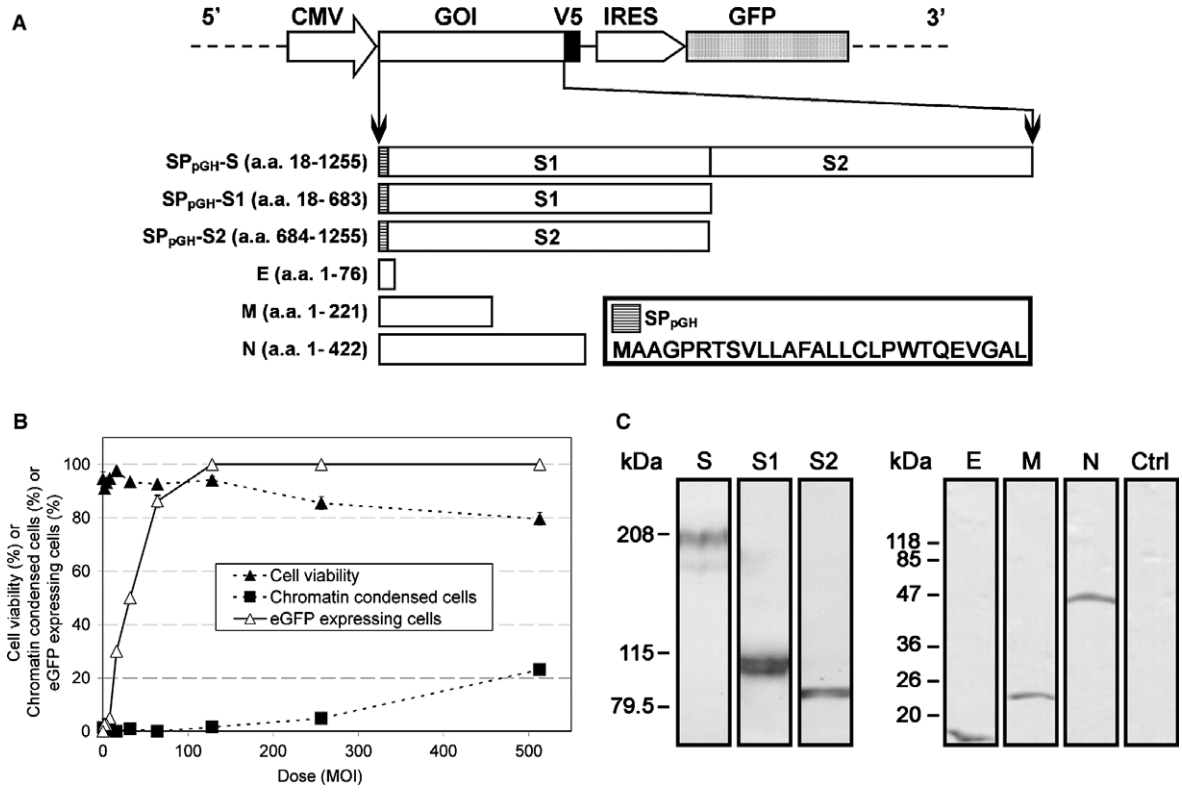

Fig. 1. Construction of the rAd-VSPs and the evaluation of rAd-mediated transduction and expression. (A) A schematic diagram showing the organization of the bicistronic expression cassette of the modified shuttle vector (pShuttle-CMV-GOI-IRES-eGFP) (upper panel) used for rAd construction and the cloned 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 SP_{pGH} 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 MOI for maximal transduction efficiency. The percentage of eGFP expressing cells was accessed by flow cytometer with at least 1×10^5 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.

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 manufacturer's instructions (Stratagene). A control adenovirus (rAd-Ctrl) with no transgene was also constructed. The rAds were propagated in AD-293 cells and CsCl-purified as described [39].

2.3. Immunoblotting

To access the expression of SARS-CoV VSPs, Vero E6 cells were transduced with the corresponding rAds at multiplicity of infections (MOI) of 100. 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 [37] using AP-conjugated anti-V5 antibody (Invitrogen).

2.4. Cell viability assay

Viability of cells transduced at indicated MOI 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 haemocytometer.

2.5. Nuclear morphology

To detect chromatin condensation, cells transduced at indicated MOI 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 of view were counted under fluorescence microscope.

2.6. DNA laddering assay

Cellular DNA fragmentation into characteristic ladders in apoptotic cells was assayed as described [40] 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 were subsequently resuspended in 80 μl of the same solution. Three hundred microliters 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 100 μl of 5 M NaCl. Lysates were then incubated at 4 °C overnight. Cell debris was pelleted by centrifugation and the supernatants were treated with 10 μl 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.

2.7. 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 analyzed with the WinMDI v2.81 software package (the Scripps Research Institute). Early apoptotic cells were recognized as PS-externalized (Annexin V-PE labeled) with intact cell membrane that resists 7-AAD staining (lower-right quadrant), which allows the exclu-