Severe acute respiratory syndrome coronavirus 3C-like protease-induced apoptosis

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Abstract
The pathogenesis of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is an important issue for the treatment and prevention of severe acute respiratory syndrome. Recently, SARS-CoV has been demonstrated to induce cell apoptosis in Vero-E6 cells. The possible role of SARS-CoV 3C-like protease (3CLpro) in virus-induced apoptosis is characterized in this study. Growth arrest and apoptosis via caspase-3 and caspase-9 activities were demonstrated in SARS-CoV 3CLpro-expressing human promonocyte cells. The fluorescence intensity of dihydrorhodamine 123 staining indicated that cellular reactive oxygen species were markedly increased in SARS-CoV 3CLpro-expressing cells. Moreover, in vivo signalling pathway assay indicated that 3CLpro increased the activation of the nuclear factor-kappa B-dependent reporter, but inhibited activator protein-1-dependent transcription. This finding is likely to be responsible for virus-induced apoptotic signalling.

Introduction
Severe acute respiratory syndrome (SARS), with high fever, malaise, headache, dry cough and generalized, interstitial infiltrates in the lung, is caused by a novel virus, SARS-associated coronavirus (SARS-CoV) (Lee et al., 2003; Tsang et al., 2003; Hsueh et al., 2004). SARS-CoV is rapidly transmitted through aerosols, and caused 8447 reported cases with 811 deaths worldwide during a short period from February to June 2003 (Peiris et al., 2003; Poutanen et al., 2003). Around 20% of SARS cases developed an acute respiratory distress syndrome requiring mechanical ventilation. Pathological examination of the lung indicated that bronchial epithelial denudation, loss of cilia, multinucleated syncytial cells and squamous metaplasia were found in lung tissues of SARS patients (Lang et al., 2003; Nicholls et al., 2003). Abnormal laboratory findings in SARS patients included lymphopenia, leucopenia, thrombocytopenia and an increase in aminotransferase, lactate dehydrogenase, creatine kinase, interleukin-6 (IL-6) and IL-8 in serum (Wang et al., 2004; Huang et al., 2005).

SARS-CoV particles contain a single positive-stranded RNA genome that is approximately 30 kb in length and has a 5' cap structure and 3' polyA tract (Enjuanes et al., 2000; Holmes, 2001; Lai & Holmes, 2001). The SARS-CoV genome encodes for replicase, spike, envelope, membrane and nucleocapsid. The replicase gene encodes two large overlapping polypeptides (replicase 1a and 1ab, c. 450 and c. 750 kDa, respectively), including 3C-like protease (3CLpro), RNA-dependent RNA polymerase and RNA helicase for viral replication and transcription (Ziebuhr et al., 2000). SARS-CoV replicates in Vero-E6 cells with cytopathic effects (Ng et al., 2003; Yan et al., 2004), and also induces AKT signalling-mediated cell apoptosis (Mizutani et al., 2004). Recently, the SARS-CoV nucleocapsid (SARS-CoV N) protein has been demonstrated to induce actin reorganization and apoptosis in COS-1 monkey kidney cells by down-regulating extracellular signal-regulated kinase (ERK) and up-regulating c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways (Surjit et al., 2004). As the 3C protease of the picornaviruses poliovirus, enterovirus 71 and rhinovirus induces cell apoptosis (Li et al., 2002; Calandria et al., 2004; Funkhouser et al., 2004), the possible role of SARS-CoV 3CLpro in virus-induced apoptosis is of interest.

In this study, we investigated the effects of SARS-CoV 3CLpro on cellular functions. The cell growth curve, annexin V staining, caspase-3 and caspase-9 activities, oxidative stress and in vivo signalling pathways were measured to determine the effects of SARS-CoV 3CLpro-induced apoptosis.
Therefore, this study demonstrated the significance of SARS-CoV 3CLpro-induced apoptosis.

Materials and methods

Cell culture and transfection

To examine the effect of SARS-CoV 3CLpro in cells, the 3CLpro gene was amplified using PCR and then cloned into the pcDNA3.1 vector, as described previously (Lin et al., 2004b). The resulting plasmid pcDNA3.1-SARS-CoV 3CLpro (pSARS-CoV 3CLpro) (4.5 μg), plus indicator vector pEGFP-N1 (0.5 μg), was transfected into HL-CZ cells, a human promonocyte cell line (Liu et al., 1991), using the GenePorter reagent. According to the manufacturer’s instructions (Gene Therapy Systems, San Diego, CA), the transfected cells were maintained in 2 mL of RPMI 1640 medium containing 20% fetal bovine serum (FBS) after 5 h of incubation with a mixture of the plasmid DNA and GenePorter reagent. For the selection of the transfected cell clones, cells were incubated with RPMI 1640 medium containing 10% FBS and 800 μg mL⁻¹ of G418. Enhanced green fluorescent protein (EGFP) expression in transfected cells with pSARS-CoV 3CLpro plus pEGFP-N1 was observed using immunofluorescence microscopy.

Immunofluorescence staining and Western blotting of 3CLpro-expressing cells

The stably transfected cells were washed once in phosphate-buffered saline (PBS), and then fixed in ice – 10% methanol for 2 min. The cells were subsequently incubated with anti-His tag antibody for 1 h, followed by rhodamine-conjugated antimouse immunoglobulin G (IgG) antibody for 1 h. After washing three times in PBS, photographs of the cells were taken using immunofluorescence microscopy. For western blotting, the cell lysates were dissolved in 2× sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer without 2-mercaptoethanol, and boiled for 10 min. Proteins were resolved on 12% SDS-PAGE gels and transferred to nitrocellulose paper. The resultant blots were blocked with 5% skimmed milk, and then incubated with the appropriately diluted anti-His tag monoclonal antibody (Serotec, Oxford, UK) for 3 h. The blots were then washed with 1× Tris-buffered saline Tween 20 (TBST) three times and overlaid with a 1 : 5000 dilution of goat antimouse IgG antibodies conjugated with alkaline phosphatase (PerkinElmer Life Sciences Inc., Boston, MA). Following 1 h of incubation at room temperature, the blots were developed with tetrazolium salt/5-bromo-4-chloro-3-idolyphosphate (TNBT/BCIP) (Gibco, Invitrogen, Merelbeke, Belgium).

Annexin V staining for apoptosis detection

After washing with cold PBS, transfected cells were resuspended in annexin-binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl2, pH 7.4). Each 100 μL of cell suspension was incubated with 5 μL of antiannexin V antibody (Biocompare, Abcom, Cambridge, UK) at room temperature for 15 min. The apoptotic cells were further stained using a horseradish peroxidase/diaminobenzidine (HRP/ DAB) system of the SuperPicTure™ Polymer Detection Kit (Zymed Laboratories Inc., San Francisco, CA). The percentage of apoptotic cells with a permanent intense brown deposit was counted at ×200 magnification using bright field microscopy.

Fluorimetric assay of caspase profiling

Transfected cells were harvested to measure the activity of caspase-2, -3, -8 and -9 using BD ApoAlert Caspase Fluorescent Assay Kits (BD Biosciences, Franklin Lakes, NJ); 2 × 10⁵ cells per well were lysed in 50 μL of lysis buffer and centrifuged at 12 000 g for 5 min at 4 °C. The supernatant was transferred into the well of the caspase profiling assay plate and incubated at 37 °C for 2 h. Finally, the caspase profiling assay plate was analysed in a fluorescent plate reader with excitation at 380 nm and emission at 460 nm.

Detection of reactive oxygen species

Reactive oxygen species (ROS) of the 3CLpro-expressing cells were quantified by staining with dihydrodihorodamine 123 (DHR, Molecular Probes, Invitrogen). After incubation with 20 μM DHR in RPMI 1640 medium for 15 min, the cells were washed three times with PBS, and the fluorescence intensity of the cells was measured with excitation at 492 nm and emission at 553 nm.

Transient transfections of cis-reporter plasmids for signalling pathway assay

The cis-reporter plasmids pISRE-Luc, pGAS-Luc, p53-Luc, pAP1-Luc and pNF-κB-Luc were purchased from the Stratagene Company (La Jolla, CA). The SARS-CoV 3CLpro-expressing and mock cells were transfected with a cis-reporter plasmid using the GenePorter reagent. After 18 h of incubation, the luciferase activity in the indicated cells was measured using the dual Luciferase Reporter Assay System (Promega, Madison, WI) and the Luminometer TROPIX TR-717 (Applied Biosystems, Foster City, CA).

Results

Expression of SARS-CoV 3CLpro in HL-CZ promonocyte cells

To examine the effect of SARS-CoV 3CLpro on cell function, co-transfection of human promonocyte HL-CZ cells with
the recombinant plasmid pSARS-CoV 3CL\textsuperscript{pro} and a green fluorescent protein (GFP) reporter was carried out (Fig. 1). Immunofluorescent staining of transfected cells with anti-His tag antibody revealed that the 3CL\textsuperscript{pro} His-tag fusion protein localized predominantly in the cytoplasm and nucleus (Fig. 1d), in contrast with mock cells expressing the His-tag only (Fig. 1b). Western blotting analysis of cell lysates with anti-His tag antibody showed that a major 68 kDa band for the dimer and a minor 34 kDa band for the monomer were observed in cells transfected with pSARS-CoV 3CL\textsuperscript{pro} (Fig. 2, lane 3), but not in mock cells (Fig. 2, lane 2). This result demonstrates the functional expression of SARS-CoV 3CL\textsuperscript{pro} in HL-CZ cells.

**Cellular effect of SARS-CoV 3CL\textsuperscript{pro} in promonocyte cells**

To further analyse the cellular effect of SARS-CoV 3CL\textsuperscript{pro}, the growth curves of 3CL\textsuperscript{pro}-expressing cells and mock cells were obtained by direct counting assay (Fig. 3a). The cell growth curve of 3CL\textsuperscript{pro}-expressing cells increased more slowly than that of mock cells. This result demonstrates a significant inhibitory effect of SARS-CoV 3CL\textsuperscript{pro} on the growth of human promonocyte cells. In addition, annexin V staining indicated a significant difference in the percentage of apoptotic cells between 3CL\textsuperscript{pro}-expressing cells (20%) and mock cells (3%) (Figs 3b and c). SARS-CoV 3CL\textsuperscript{pro}-induced apoptosis was also measured with a fluorimetric caspase profiling assay (Figs 4a and b). Caspase-3 activity in 3CL\textsuperscript{pro}-expressing cells was 3.04-fold greater than that in the presence of the caspase-3 inhibitor and 2.1-fold greater than that in mock cells (Fig. 4a). Caspase-9 activity in 3CL\textsuperscript{pro}-expressing cells was about 1.3-fold greater than that in mock cells and in the presence of the caspase-9 inhibitor (Fig. 4b). The activities of caspase-2 and caspase-8 were also determined, but no significant difference was found between 3CL\textsuperscript{pro}-expressing cells and mock cells (data not shown).

**Increase in ROS and activation of nuclear factor-kappa B signalling pathway by SARS-CoV 3CL\textsuperscript{pro}**

To test the possible mechanism of 3CL\textsuperscript{pro}-induced apoptosis, ROS production and the \textit{in vivo} signal transduction pathway in SARS-CoV 3CL\textsuperscript{pro}-expressing cells were investigated (Figs 5 and 6). DHR staining was carried out for ROS detection. A significant increase in ROS was measured in 3CL\textsuperscript{pro}-expressing cells (2.3-fold higher) relative to pcDNA3.1-expressing cells (Fig. 5). To measure signal...
transductions, transient transfection of 3CL\textsuperscript{pro}-expressing cells and mock cells with a cis-reporter plasmid was subsequently performed to detect the in vivo signal transduction pathway (Fig. 6). The luciferase activity of cis-reporter plasmids indicated that SARS-CoV 3CL\textsuperscript{pro} activated the expression of a nuclear factor-kappa B (NF-κB)-dependent reporter gene in HL-CZ cells (2.15-fold increase), but inhibited activator protein-1 (AP1)-dependent transcription (0.45-fold decrease) (Fig. 6). In addition, no significant effect on interferon-stimulated response element (ISRE)- and interferon-gamma-activated site (GAS)-dependent transcriptions was induced by SARS-CoV 3CL\textsuperscript{pro}.

**Discussion**

A mixture of the active dimer form and the inactive monomer form of SARS-CoV 3CL\textsuperscript{pro} was generated in human promonocyte HL-CZ cells (Fig. 2), consistent with bacterial expression systems (Fan et al., 2004; Lin et al., 2004b). Interestingly, the dimer was the major 3CL\textsuperscript{pro} in HL-CZ cells, different from that in Escherichia coli-synthesized HL-CZ cells, where it was the monomer (Fan et al., 2004; Lin et al., 2004b). SARS-CoV 3CL\textsuperscript{pro} influenced cell growth and induced apoptosis (Figs 3 and 4). Activation of caspase-3 and caspase-9, but not caspase-2 and caspase-8,
was correlated with SARS-CoV 3CL\textsuperscript{pro} -induced apoptosis in HL-CZ cells (Fig. 4). A similar caspase activity profile has been reported in poliovirus 3C-induced apoptosis via caspase-3 activation (Calandria et al., 2004).

SARS-CoV 3CL\textsuperscript{pro} caused a significant increase in ROS production in HL-CZ cells (Fig. 5) which, in turn, were involved in 3CL\textsuperscript{pro} -induced apoptosis. Recently, ROS-mediated apoptosis has also been reported in viral infections, such as Japanese encephalitis virus (Lin et al., 2004a) and influenza virus (Uchide et al., 2002). In vivo signalling pathway assay indicated that 3CL\textsuperscript{pro} increased the activation of the NF-κB-dependent reporter, but inhibited AP1-dependent transcription (Fig. 6). This finding indicates that NF-κB, but not c-Jun, is involved in 3CL\textsuperscript{pro} -induced apoptosis of human promonocyte cells. The transcription factor NF-κB is produced in response to oxidative stress, being widely proposed to be involved in the mediation or prevention of apoptosis, and ROS serve as second messengers in the induction of the transcription factor NF-κB (Bonizzi et al., 1999). The activation of an NF-κB-dependent reporter gene induced by SARS-CoV 3CL\textsuperscript{pro} (Fig. 6) was correlated with an increase in ROS production in HL-CZ cells. Consensus NF-κB sites exist in the promoters of apoptosis-related genes and proinflammatory genes (Chen et al., 2001), such as transforming growth factor-β (TGF-β), tumour necrosis factor-α (TNF-α), IL-1α, IL-6 and IL-8. Interestingly, TGF-β, IL-6 and IL-8 are greatly increased in the acute phase sera of SARS patients (Huang et al., 2005). Therefore, the activation of the NF-κB signal transduction pathway induced by SARS-CoV 3CL\textsuperscript{pro} could be useful for elucidating the pathogenesis of SARS.

In conclusion, the cellular effects of SARS-CoV 3CL\textsuperscript{pro} in human promonocyte cells, such as growth arrest and apoptosis via caspase-3 and caspase-9 activities, have been well characterized in this study. ROS production and NF-κB signalling are likely to be associated with SARS-CoV 3CL\textsuperscript{pro} -induced pathology.

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