Role for Nonstructural Protein 1 of Severe Acute Respiratory Syndrome Coronavirus in Chemokine Dysregulation[▽]

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Severe acute respiratory syndrome (SARS) is an emerging infectious disease caused by a novel coronavirus. Since its associated morbidity and mortality have been postulated to be due to immune dysregulation, we investigated which of the viral proteins is responsible for chemokine overexpression. To delineate the viral and cellular factor interactions, the role of four SARS coronavirus proteins, including nonstructural protein 1 (nsp-1), nsp-5, envelope, and membrane, were examined in terms of cytokine induction. Our results showed that the SARS coronavirus nsp-1 plays an important role in CCL5, CXCL10, and CCL3 expression in human lung epithelial cells via the activation of NF- κ B.

The severe acute respiratory syndrome coronavirus (SARS-CoV) is a newly emerging pathogen which caused the worldwide outbreak in 2003 (21), resulting in more than 8,000 cases of SARS and 774 deaths in 30 countries (WHO website, http://www.who.int/csr/sars/country/table2003_09_23/en). SARS patients mainly present with a severe pneumonia with extensive lung injury (19). Additionally, SARS-CoV has been detected in extrapulmonary organs, including the gastrointestinal tract, lymph nodes, spleen, liver, heart, and kidney (7).

The pathogenesis of SARS may be caused by rapid viral replication and the hyperactivated host immune response. A murine model of SARS-CoV infection showed the induction of chemokines, including CCL2, CCL3, CCL5, CXCL9, and CXCL10, and their respective cognate receptors in the lung (10). In addition, SARS-CoV induces the expression of CXCL10 and CCL2 in primary human blood macrophages as

well as the transcription of CXCL10, CCL2, CCL3, and CCL5 in monocyte-derived dendritic cells (5, 13). Furthermore, elevated levels of chemokines and cytokines, including CXCL10, CCL2, interleukin 8 (IL-8), IL-6, IL-1 β , and gamma interferon, were found in the sera of SARS patients (11). Taken together, the massive production of chemokines, induced by SARS-CoV, seems to play pathological roles in the patients.

The SARS-CoV genome encodes 4 structural proteins, including spike (S), membrane (M), nucleocapsid (N), and envelope (E), 8 accessory proteins with undefined functions, and 16 nonstructural proteins (nsp) that are responsible for virus replication (24). Chang et al. reported that a functional fragment of SARS-CoV S protein is capable of inducing IL-8 expression, as mediated by mitogen-activated protein kinase and activator protein-1 (AP-1) signaling pathways, in lung epithelial cells (4). Additionally, pseudoparticles formed from the

TABLE 1. Coding regions and primers used for cloning expression plasmids

Gene encoding:	GenBank accession no.	Coding region (nt ^a)	Forward and reverse PCR primers
SARS-CoV nsp1	gi:30023963	265–804	5'-ATGGAGAGCCTTGTTCTTGGTG-3' 5'-TTATTGGAAGGTAACACCAGAGCAT-3'
SARS-CoV nsp5	gi:30023963	9985–10902	5'-AGTGGTTTTAGGAAAATGGCA-3' 5'-TTATTGGAAGGTAACACCAGAGCAT-3'
SARS-CoV E	gi:30023963	26117–26347	5'-ATGTACTCATTCTGTTTCGGAAGAA-3' 5'-TTAGACCAGAAGATCAGGAAGTC-3'
SARS-CoV M	gi:30023963	26398–27063	5'-ATGGCAGACAACGGTACTATTACC-3' 5'-TTACTGTACTAGCAAAGCAATA-3'
H-CoV 229E nsp1	gi:12175745	293–625	5'-ATGGCCTGCAACCGTGTGAC-3' 5'-TTAACCACCACCTCTCTTACCGA-3'
H-CoV OC43 nsp1	gi:22347588	211–948	5'-ATGTCGAAGATCAACAAATACG-3' 5'-TTAACCACGATACCCCTTAAT-3'
MHV nsp1	gi:9629812	210–950	5'-ATGGCAAAGATGGGCAAAATACG-3' 5'-TTAACCAGCATAGCCCTTAAGAAG-3'

^a nt, nucleotide.

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FIG. 1. Expression of SARS-CoV proteins in human lung epithelial cells. Myc-tagged expression plasmids containing SARS-CoV (SARS) genes encoding nonstructural protein 1 (nsp1), nsp5, envelope (E), and membrane (M) were transfected into A549 cells. Cells expressing HCoV-229E nsp1 (229E nsp1), HCoV-OC43 nsp1 (OC43 nsp1), MHV nsp1, and parental plasmid (Mock) were used as controls. Total RNA was extracted at 20 h after transfection. (A) The transcriptional levels of the viral genes were examined using semiquantitative RT-PCR. The full-length transcripts encoding the viral proteins were amplified by the corresponding cloning primers. The level of GAPDH was used as an internal control. (B) Total cell lysates were harvested at 20 h after transfection. Western blot analysis using an antibody against the myc epitopes showed the expression of the indicated viral proteins.

coexpression of the M and E viral proteins of a group 1 coronavirus, the transmissible gastroenteritis virus, can induce interferon production in porcine blood mononuclear cells (1). However, the identification of the SARS-CoV factors in chemokine induction remains to be investigated.

To delineate the mechanism of chemokine induction in SARS-CoV infection, we cloned two structural proteins, M and E, as well as two nonstructural proteins, nsp1 and nsp5, of SARS-CoV (strain 39849). The nsp1 and nsp5 proteins are predicted to be mature replicase proteins and are derived from the enzymatic cleavage of the polyprotein 1a (22). The nucleotide sequences of these four viral genes are conserved among 14 isolates of SARS-CoV (23). They were obtained from cDNA of virus-infected Vero cells and cloned into an expression plasmid tagged with four myc epitopes, pcDNA3_Myc. To construct the pcDNA3_Myc fusion expression plasmid, we first released the DNA sequence encoding four Myc epitopes from the pCS2+MT plasmid (gift from J. W. Yam, The University

of Hong Kong) by use of EcoRI and BamHI restriction endonucleases. The four-myc DNA fragment was purified by use of a QiaxII kit (QIAGEN) and then subcloned into the EcoRI and BamHI sites of the pcDNA3.0 expression vector (Invitrogen) to generate the pcDNA3_Myc expression plasmid. The cDNA sequences containing the viral genes were amplified from total RNA of SARS-CoV-infected cells by reverse transcription-PCR (RT-PCR). The encoding regions of viral genes and the primers used for cloning of expression plasmids are shown in Table 1. The PCR products of SARS-CoV genes were purified and cloned into the pDrive vector by use of a QIAGEN PCR cloning kit (QIAGEN) according to the supplier's protocol and were then subcloned into the pcDNA3_Myc expression plasmid. The sequences of the viral genes, which are in frame to that of the myc sequence, were confirmed by DNA sequencing.

Each plasmid was transiently transfected into a lung epithelial cell line, A549, by using Lipofectamine 2000 transfection

FIG. 2. SARS-CoV nonstructural protein 1-induced chemokine expression in human lung and liver epithelial cells. At 20 h after transfection, the relative mRNA levels of CCL5 (A), CXCL10 (B), CCL3 (C), and CCL2 (D) in the viral protein-expressing A549 cells and CCL5 (H) and CXCL10 (I) in SARS-CoV nsp1-expressing HepG2 cells were assayed by TaqMan gene expression assays. Levels of CCL5 (E), CXCL10 (F), and CCL3 (G) in the culture supernatants from A549 cells at 24 h after transfection were assayed by ELISA. *, $P < 0.05$; #, $P < 0.01$.

reagent (Invitrogen). At 20 h after transfection, the transcriptional level of the viral genes was measured by semiquantitative RT-PCR. The full lengths of the transcripts encoding the viral proteins were amplified by the corresponding cloning

primers (Fig. 1A, upper panel). The level of GAPDH was used as an internal control (Fig. 1A, lower panel). All of the viral genes were detected at the level of transcription, and the sizes of the transcripts were consistent with the calculated values.

Also, the whole-cell lysates were prepared by using total lysis buffer containing a protease inhibitor cocktail. The levels of myc-tagged protein expression were demonstrated by Western analysis (Fig. 1B). The observed molecular masses for the expressed fusion proteins were as follows: for SARS-CoV nsp1, 35 kDa; for SARS-CoV nsp5, 45.3 kDa; for SARS-CoV E, 31 kDa; and for SARS-CoV M, 38 kDa. The M protein from SARS-CoV appeared as two bands in Western analysis. This may due to N glycosylation of the protein as demonstrated in a previous report (18). Moreover, Liao et al. reported that posttranslational modification of the E protein contributes to the differences in the observed and calculated molecular masses (16).

The levels of mRNA encoding CCL5, CXCL10, CCL3, and CCL2 in each of the transfectants at 20 h posttransfection were assayed by using TaqMan gene expression assays (Applied Biosystems) (14). Surprisingly, the SARS-CoV-encoded E and M proteins, as well as nsp5, did not show any significant induction effects on the indicated chemokines. In contrast, the SARS-CoV nsp1 protein was a potent inducer of CCL5, CXCL10, and CCL3 expression ($P < 0.05$), with increases in the range of 25- to 200-fold from what was seen for mock-transfected cells (Fig. 2A to C). However, no significant increases in CCL2 expression were induced by any of the four SARS-CoV genes (Fig. 2D). We also examined the protein levels of CCL5, CXCL10, and CCL3 in the culture supernatants of the SARS-CoV nsp1-overexpressing cells by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) at 24 h posttransfection. Consistent with the real-time RT-PCR results, the production of CCL5, CXCL10, and CCL3 was significantly increased by 10-fold in the SARS-CoV nsp1-expressing cells from what was seen for the mock-transfected cells by ELISA ($P < 0.05$ or $P < 0.01$; Fig. 2E to G).

To examine the specificity of the SARS-CoV gene in chemokine dysregulation, we cloned the corresponding nsp1 genes of the human CoV 229E (HCoV-229E) and OC43 (HCoV-OC43) and mouse hepatitis virus (MHV), the coronavirus prototype. The nsp1 PCR products amplified from cDNA of HCoV-229E-, HCoV-OC43-, and MHV-infected cells were cloned into pCR2.1 vector by use of a TOPO-TA cloning kit (Invitrogen) and were then subcloned into the pcDNA3_Myc expression plasmid. The viral proteins were also expressed in A549 cells (Fig. 1B). The observed molecular masses for the expressed proteins were as follows: for HCoV-229E nsp1, 26.5 kDa; for HCoV-OC43 nsp1, 46.7 kDa; and for MHV nsp1, 46 kDa. As shown, the nsp1 proteins from HCoV-229E, HCoV-OC43, and MHV did not significantly induce chemokine expression, compared to what was seen for mock-transfected cells (Fig. 2A to G). Furthermore, the SARS-CoV nsp1 induced the mRNA levels of CCL5 and CXCL10 in HepG2 cells by greater than 80- and 200-fold, respectively (Fig. 2H and I). These findings suggested that the chemokine-inductive property of the SARS-CoV nsp1 was not cell type specific. Moreover, we did not observe cytotoxicity in cells transfected by either the SARS-CoV nsp1 or parental plasmids at 24 h posttransfection (data not shown).

To further investigate the mechanism of chemokine induction, we measured the nuclear translocation of the transcription factor NF- κ B in the SARS-CoV nsp1-expressing A549 cells by using Western analysis. At 14 h posttransfection, the

FIG. 3. SARS-CoV nsp1-induced nuclear translocation of NF- κ B in A549 cells. (A) At 14 h after transfection, NF- κ B levels in the nuclear portions of the A549 cells expressing SARS-CoV (SARS) nsp1, nsp5, E, and M were assayed by Western blot analysis (lanes 2 to 5). Cells expressing HCoV-229E nsp1 (229E nsp1) and mock-transfected cells were used as controls (lanes 6 and 1). (B) The kinetics of NF- κ B activation by SARS-CoV nsp1 was also examined by Western blotting. Nuclear proteins of the SARS-CoV nsp1-expressing and mock-transfected cells were harvested at 10, 14, and 20 h after transfection. Lamin B was used as a loading control for nuclear protein. The densities of the protein bands were determined by using Bio-Rad Quantity One imaging software. The NF- κ B intensity value was normalized to the corresponding lamin B value. The values in parenthesis are the relative normalized intensities compared to those of mock-transfected cells (panel A) or 0-hour normalized intensity (panel B).

nuclear proteins were analyzed by Western analysis as described previously (14, 15), using antibodies against the p65 subunit of NF- κ B (Santa Cruz). Our results showed that a strong NF- κ B nuclear signal was detected in the SARS-CoV nsp1-expressing cells but not in the cells expressing the HCoV-229E nsp1 or other SARS-CoV genes, including those encoding nsp5, E, or M (Fig. 3A). Further kinetics studies showed that the SARS-CoV nsp1-induced NF- κ B activation started at 10 h and peaked at 14 h posttransfection (Fig. 3B).

Activation of the I κ B kinase- β (IKK)/NF- κ B signaling pathway has been shown to play important roles in chemokine induction (17). To investigate the role of NF- κ B in nsp1-induced chemokine dysregulation, we first performed time course experiments to demonstrate that the onset of CCL5, CXCL10, and CCL3 induction was at 14 h posttransfection by real-time RT-PCR assays (data not shown). In parallel experiments, we treated the transfected cells with or without sodium salicylate (NaSAL), a well-described IKK- β inhibitor (25), at

FIG. 4. SARS-CoV nsp1-induced chemokine expression in A549 cells is mediated through the NF- κ B pathway. (A) The involvement of NF- κ B in the SARS-CoV (SARS) nsp1-induced chemokine expression was investigated by using an inhibitor of I κ B kinase- β , NaSAL. At 12 h after transfection, SARS-CoV nsp1-expressing cells were treated with sodium salicylate at the indicated concentrations. After treatment for 7 h, NF- κ B levels in the nuclear portion of the cells were assayed by Western blotting. Lamin B was used as a loading control. The densities of the protein bands were determined by using Bio-Rad Quantity One imaging software. The NF- κ B intensity value was normalized to the corresponding lamin B value. The values in parenthesis are the relative normalized intensities compared to the normalized values in the absence of sodium salicylate. (B to D) The relative mRNA levels of CCL5 (B), CXCL10 (C), and CCL3 (D) were examined by TaqMan gene expression assays at 19 h posttransfection. (E) The quantity of CCL5 in the culture supernatant after treating the mock-transfected or SARS-CoV nsp1-expressing cells with sodium salicylate for 10 h was assayed by ELISA. *, $P < 0.05$; #, $P < 0.01$.

12 h after transfection. This was followed by NF- κ B assays using Western analysis as well as chemokine measurements by real-time RT-PCR at 19 h posttransfection. Our results showed that the nuclear translocation of NF- κ B was suppressed by NaSAL at 10 or 20 mM (Fig. 4A). There were concomitant decreases in the induction of CCL5, CXCL10, and CCL3 transcription in the SARS-CoV nsp1-expressing cells with increasing concentrations of NaSAL. At 20 mM NaSAL, the levels of induction for the respective chemokines were reduced by 44%, 75%, and 59%, respectively, at 19 h after transfection (Fig. 4B to D). Also, the chemokines secreted in the supernatant were also reduced by suppressing the NF- κ B activation. After treating the SARS-CoV nsp1-expressing cells with NaSAL at 20 mM for 10 h (i.e., 22 h posttransfection), the production of CCL5 decreased by 52% as determined by ELISA (Fig. 4E). Thus, our results clearly showed that chemokine induction by SARS-CoV

nsp1 is mediated through the NF- κ B signaling pathway in human lung epithelial cells.

Unlike the other three human coronaviruses, i.e., HCoV-229E, HCoV-OC43, and HCoV-NL63, which are associated with mild upper respiratory tract diseases, SARS-CoV causes life-threatening atypical pneumonia with severe lung damage (19) as well as the dysfunction of multiple organ systems. Elevated serum levels of chemokines found in the patients suggested that SARS pathogenesis may be due to immune dysregulation and rapid viral replication. Chemokines are a family of small proteins that play a functional role in the transition from innate to adaptive immunity (9), and their dysregulated expression could cause destructive inflammation (8).

Expression of CCL5 and CCL3 has been shown to be associated with acute viral infection (9). Here, we showed that expression of the SARS-CoV nsp1, but not the nsp5, E, or M,

induced the secretion of chemokines including CCL5, CXCL10, and CCL3 in human lung epithelial cells. By contrast, S protein from the SARS-CoV was shown to induce IL-8 through the AP-1 pathway. However, it did not activate the NF- κ B pathway (4). Furthermore, our results showed that the nsp1 protein from two other human coronaviruses, HCoV-229E and HCoV-OC43, were weak inducers of the chemokines, with effects 10-fold less than that of the nsp1 of SARS-CoV (Fig. 2E to G). The results indicate that the SARS-CoV nsp1 has a unique property of chemokine induction in human lung cells. It has recently been shown that the SARS-CoV nsp1, while not fully characterized, is one of the mature replicase proteins that are distributed in the cytoplasm and the perinuclear region (22). Moreover, a significant amount of SARS-CoV nsp1 protein was detected in Vero cells with the CoV infection by use of Western blot analysis (22). Like other coronaviruses, the mature replicase proteins of SARS-CoV have been postulated to mediate the formation of the replication complex, the transcription of subgenomic RNA, and the replication of the viral genome (3, 22). In DNA viruses such as the Epstein-Barr virus, the leader protein EBV-LP has been shown to enhance EBNA-1-mediated transactivation of latent membrane protein-1, leading to Epstein-Barr virus-induced B-cell transformation (20). Moreover, EBV-LP induces the expression of thymus- and activation-regulated chemokines in B cells (12).

By blocking the IKK/NF- κ B pathway, we demonstrated that the SARS-CoV nsp1-induced dysregulation of chemokines is mediated by NF- κ B activation (Fig. 4B to E). Our results were consistent with a previous report that showed the activation of NF- κ B in SARS-CoV-infected colon carcinoma cells (6). The incomplete suppression of the chemokine induction by sodium salicylate suggests that other transcription factors and pathways may also be involved. These may include interferon regulatory factors, CCAAT enhancer binding protein, and AP-1 (17). We have investigated the involvement of interferon regulatory factor 3 and CCAAT enhancer binding protein in the chemokine induction in SARS-CoV nsp1-expressing cells by use of Western analysis. However, no significant activation of these transcription factors was detected (data not shown). Other critical signaling pathways for the recognition of pathogens, such as Toll-like receptors, may also contribute to the induction of chemokines in SARS-CoV infection.

It has been postulated that in acute virus infection, both the pathogen and the host response contribute to the disease pathogenesis (9). For instance, recent reports showed beneficial effects from combined treatment with an antiviral drug and anti-CCL3 antibody, resulting in mortality being reduced from 100% to 30% in pneumovirus-infected mice (2). Therefore, a better understanding of the mechanism of induction of specific proinflammatory chemokines and the nature of pathogen-host interactions may steer the direction of therapeutics development for emerging infectious diseases, including SARS.

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