The Severe Acute Respiratory Syndrome Coronavirus 3a Protein Up-Regulates Expression of Fibrinogen in Lung Epithelial Cells

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Here we analyzed the gene expression profile of cells that stably express the severe acute respiratory syndrome coronavirus (SARS-CoV) 3a protein to determine its effects on host functions. A lung epithelial cell-line, A549, was chosen for this study because the lung is the primary organ infected by SARS-CoV and fatalities resulted mainly from pulmonary complications. Our results showed that the expression of 3a up-regulates the mRNA levels of all three subunits, Aα, Bβ, and γ, of fibrinogen. Consequently, the intracellular levels as well as the secretion of fibrinogen were increased. We also observed increased fibrinogen levels in SARS-CoV-infected Vero E6 cells.

A novel coronavirus was identified as the etiological agent for the recent severe acute respiratory syndrome (SARS) epidemic (5). Besides the replicase 1a/1b gene and the major structural proteins, the SARS coronavirus (CoV) genome contains open reading frames with no homologues in other coronaviruses (16, 21, 30). One of these is the 3a protein, which has been detected in SARS-CoV-infected cells and virions (12, 24, 29, 36, 37).

To understand the role of 3a during SARS-CoV infection, A549, a lung cell line with properties of type II epithelium (15), was transfected with plasmid pXJ40neo-3a as previously described (28). The 3a gene was obtained from isolate SIN2774 (29) and cloned into the pXJ40neo vector (38). Cells stably expressing 3a were obtained after antibiotic selection as previously described (27) and the expression of 3a in two independent clones (U1 and U2) was analyzed by Western blot analysis (Fig. 1A) using a specific antibody (29). Control cells were stably transfected with an empty vector.

An oligonucleotide microarray analysis was performed to determine changes in the mRNA levels of host proteins. Total RNA was extracted from these cells using the RNeasy kit (QIAGEN) and hybridized to the HGU133A array, which contains ≈22,000 human transcripts, according to standard protocols available from Affymetrix. The results showed that all three subunits, Aα, Bβ, and γ, of fibrinogen (Table 1) were strongly up-regulated in the 3a-expressing clones. Compared to control cells, the mRNA levels of these genes increased by 26- to 294-fold. The increases in the mRNA levels of the fibrinogen genes were verified independently by reverse transcription-PCR (Fig. 1B) as previously described (26).

The only other fibrinogen-related gene that showed an increase in the mRNA level in the 3a-expressing clones was Fgl-1 (Table 1, Fig. 1B), but the degree of up-regulation was less.

Fgl-1 belongs to the fibrinogen superfamily and contains domains homologous to fibrinogen Bβ and γ proteins (35). All other genes that were up-regulated by at least eightfold are shown in Table 1. Twenty-four transcripts, representing 0.11% of the total transcripts analyzed, were up-regulated in the 3a-expressing cells suggesting that 3a did not cause massive changes to the host gene profile. Interestingly, the mRNA level of CSPG2, which is involved in the extracellular matrix assembly (32), was also specifically up-regulated, with four different transcripts giving similar results (Table 1). The significance of the changes in these genes will need further evaluation.

The fibrinogen subunits are assembled to form the circulating 340-kDa fibrinogen complex, which consists of two units of each of the subunits linked by disulfide bonds (1, 9, 20). Under reducing conditions, the complex dissociates into the three subunits with expected molecular weights of 66,000 (Aα), 52,000 (Bβ), and 46,000 (γ). To determine the intracellular levels of fibrinogen, cells were harvested and lysed in Laemmli's sodium dodecyl sulfate buffer (containing 200 mM dithiothreitol), then heated at 100°C, and subjected to Western blot analysis using monoclonal antibodies (Accurate Chemical and Scientific Corporation) against the Aα, Bβ, and γ fibrinogen subunits. Human plasma and serum (Sigma) were used to test the specificity of the antibodies.

Monoclonal antibody against the Aα subunit detected a major protein of ≈75 kDa in the human plasma and two proteins of ≈75 kDa and ≈70 kDa in Huh7 cells (Japan Health Sciences Foundation), a liver cell line that constitutively expresses fibrinogen (Fig. 2A, panel b, lane 3). Consistent with the increase in the mRNA, the Aα protein levels in the 3a-expressing clones were significantly higher than in the vector control (Fig. 2A, panel b, lanes 4 to 6). In contrast to Huh7 cells, only the 75-kDa protein was detected in clones U1 and U2 (Fig. 2A, panel b, lanes 3, 5, and 6). It is unclear why two forms of Aα were detected in Huh7 cells, but differences in the processing of fibrinogen in exhepatic and hepatic cells have been reported (22).

As shown in Fig. 2A, the Bβ subunit in the plasma and Huh7

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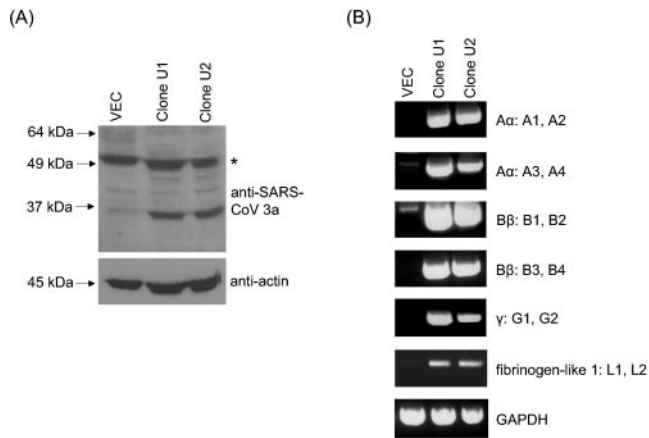
(A)

(B)

TABLE 1. Increase in the mRNA levels of fibrinogen and fibrinogen-related genes, as well as other cellular genes, in 3a-expressing stable A549 cell lines, clones U1 and U2, as determined by oligonucleotide microarray analysis

Accession no. (gene symbol)	Probe set ID[a]	Description	Fold increase[b]	
			U1	U2
NM_000508.2 (FGA)	205649_s_at	Fibrinogen, Aα	294	181
	205650_s_at	Fibrinogen, Aα	60	26
NM_005141.1 (FGB)	204988_at	Fibrinogen, Bβ	181	74
	216238_s_at	Fibrinogen, Bβ	79	32
NM_000509.3 (FGG)	219612_s_at	Fibrinogen, γ	158	60
NM_004467.1 (FGL1)	205305_at	Fibrinogen-like 1	15	10
NM_004385.1 (CSPG2)	204620_s_at	Chondroitin sulfate proteoglycan 2 (versican)	17	16
	221731_x_at	Chondroitin sulfate proteoglycan 2 (versican)	24	21
	211571_s_at	Chondroitin sulfate proteoglycan 2 (versican)	21	21
	215646_s_at	Chondroitin sulfate proteoglycan 2 (versican)	21	20
NM_001680.2 (FXYD2)	205674_x_at	FXYD domain containing ion transport regulator 2	26	32
	207434_s_at	FXYD domain containing ion transport regulator 2	17	26
NM_002937.1 (RNASE4)	205158_at	Ribonuclease, RNase A family 4	14	13
	213397_x_at	Ribonuclease, RNase A family 4	21	18
NM_003982.1 (SLC7A7)	204588_s_at	Solute carrier family 7 (cationic amino acid transporter, y+ system)	24	30
AB002155.1 (UPK1B)	210065_s_at	Uroplakin 1B	18	21
BC001386.1 (TM4SF4)	209937_at	Transmembrane 4 superfamily, member 4	11	26
NM_024795.1 (FLJ22800)	220639_at	Hypothetical protein FLJ22800	26	26
U80736.1 (TNRC9)	215108_x_at	Trinucleotide repeat containing 9	74	119
NM_014059.1 (RGC32)	218723_s_at	Response gene to complement 32	28	42
NM_005980.1 (S100P)	204351_at	S100 calcium binding protein P	15	11
AF088867.1 (AGR2)	209173_at	Anterior gradient 2 homolog (Xenopus laevis)	23	23
NM_004915.2 (ABCG1)	204567_s_at	ATP-binding cassette, subfamily G (WHITE), member 1	18	15
NM_000187.1 (HGD)	205221_at	Homogentisate 1,2-dioxygenase (homogentisate oxidase)	12	10

FIG. 1. Stable expression of SARS-CoV 3a in A549, a lung epithelial cell line, and effects on the mRNA levels of fibrinogen Aα, Bβ, and γ subunits and fibrinogen-related proteins. (A) Western analysis showing the expression of 3a in the stable cell lines clones U1 and U2 but not in the vector control (VEC) cells (top panel). An unknown cellular protein cross-reacting with the anti-3a mouse polyclonal is marked with an asterisk. Equal amounts of cells were used in each lane as verified by the level of endogenous actin (bottom panel). (B) Reverse transcription-PCR results showing the higher mRNA levels of fibrinogen subunits Aα, Bβ, and γ in clones U1 and U2 compared to the vector control. The mRNA level of fibrinogen-like 1, a member of the fibrinogen superfamily, was also increased in the presence of 3a. The primers used (5′ to 3′) were A1: TCACTGAATCTAACCATAGCT GACC (sense) and A2: AAGGCAAGACCACCAGGATTAAAGA (antisense) for probe set ID205649_s_at; A3: TTCGACACTGCCTC AACTGGAAAAA (sense) and A4: GGGCGAGATTTAGCATGGC CTCTCT (antisense) for probe set ID205650_s_at; B1: GTCATGCA GCCAATCCAAACGGCAG (sense) and B2: CGACAAGGATAAA AGACCCCTCTTC (antisense) for probe set ID204988_at; B3: GGT CATCGACCCCTTGACAAGAAGA (sense) and B4: GCATGGGG TGCGACAATATTCCATT (antisense) for probe set ID216238_s_at; G1: CTTCGCTGGTGGGGATGCTGGAGAT (sense) and G2: CAT AACCATTAGGAGTAGATGCTTT (antisense) for probe set ID219612_s_at; and L1: CAGCTGGAGATTCCCTTGCGGGGAA (sense) and L2: ATTAAGTAACAAAGGCAAGTGAGAA (antisense) for probe set ID205305_at. The level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used to verify that equal amounts of RNA were used in each reaction, and the primers used were GAPDHfor: CTGAGAACGGGAAGCTTGTCATCA (sense) and GAPDHrev: CGTCTAGCTCAGGGATGACCTTG (antisense).

[a] Probe set ID refers to the identification given to each transcript on the Affymetrix array, and details on each transcript are available at https://www.affymetrix.com.

[b] Only genes that showed at least an eightfold increase compared to a vector control clone in both clones were selected. A comparison was also made between two independent vector control clones to ensure that none of these up-regulated genes showed significant variance in basal mRNA levels.

cells migrated at ≈64 kDa (panel c, lane 3). As for the γ subunit, the protein in the plasma migrated at ≈60 kDa but the protein in Huh7 cells migrated at only ≈45 kDa, suggesting that the intracellular γ polypeptide underwent some form of posttranslational modifications before secretion into the extracellular matrix (panel a, lane 3). Consistently, the levels of both Bβ and γ proteins in the 3a-expressing clones were also higher than that in the vector control (Fig. 2A, panels c and a, lanes 4 to 6).

To determine the secretion of fibrinogen, 10⁶ cells were resuspended in 1.5 ml of Opti-MEM (Invitrogen). After 24 h, the amounts of fibrinogen and interleukin-6 present in the culture supernatants were determined using the ZYMUTEST Fibrinogen enzyme-linked immunosorbent assay (ELISA) (Hyphen BioMed) and the human interleukin-6 Quantikine ELISA (R&D systems), respectively. Concurrently, the cell numbers were counted using a hemacytometer and used to compute the amount of fibrinogen secreted per cell. All exper-
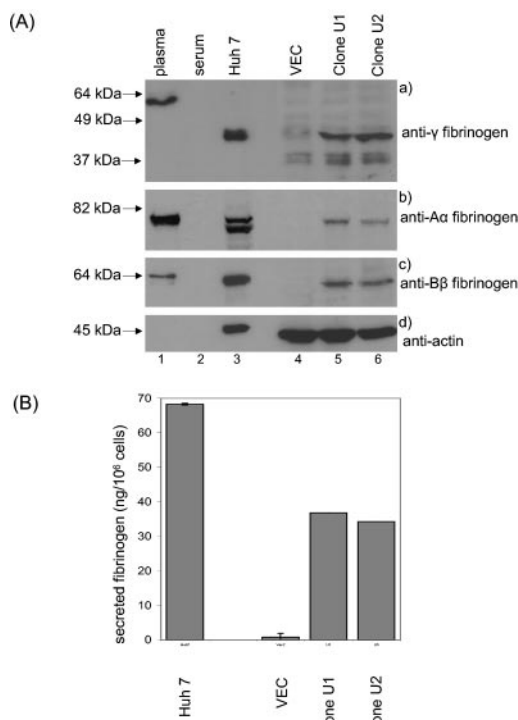
(A)

(B)

FIG. 2. Effects of SARS-CoV 3a protein on the intracellular level of fibrinogen subunits and the secretion of fibrinogen complex into culture supernatant. (A) Western blot analysis with specific monoclonal antibodies was used to detect the Aα, Bβ, and γ subunits of fibrinogen in human plasma (lane 1) and Huh7 cells (lane 3), a human liver cell line that constitutively expresses fibrinogen. Lane 2 was loaded with a similar amount of human serum as a negative control and for all three antibodies did not cross-react with proteins present in human serum. The intracellular levels of the Aα, Bβ, and γ subunits of fibrinogen were higher in the 3a-expressing clones (U1 and U2) compared to the vector control cells (VEC) (panels a to c). Equal loadings of cell lysates was verified by the level of endogenous actin (panel d). (B) The amounts of fibrinogen secreted into the cell culture supernatants, as determined by an ELISA, were higher for the 3a-expressing stable clones U1 and U2 compared to the vector control cells (VEC) (columns 2 to 4). The amount of fibrinogen secreted by Huh7 cells was also determined (column 1). This experiment was repeated three times, and similar results were obtained each time. A representative set of data (mean ± standard deviation) is presented.

iments were performed in duplicate, and quantifications were performed according to the manufacturer's protocol. As shown in Fig. 2B, high levels of fibrinogen ($\approx$30 ng per 10^6 cells) were secreted from the 3a-expressing clones.

Previously, interleukin-6 was found to synergize with dexamethasone to increase the fibrinogen levels (6, 25). However, no significant increase in interleukin-6 was detected in culture supernatant from the 3a-expressing clones (data not shown), suggesting that this process may be independent of proinflammatory cytokines. No change in the mRNA of cytokine-related genes was observed in the microarray analysis, although we cannot rule out that there may be changes at the protein level.

As A549 cells do not support SARS-CoV replication (8), the intracellular level of fibrinogen γ (and secretion of fibrinogen) in SARS-CoV-infected Vero E6 cells was determined. Infection was carried out as previously described (24). The expression of fibrinogen γ was higher in infected cells than mock-
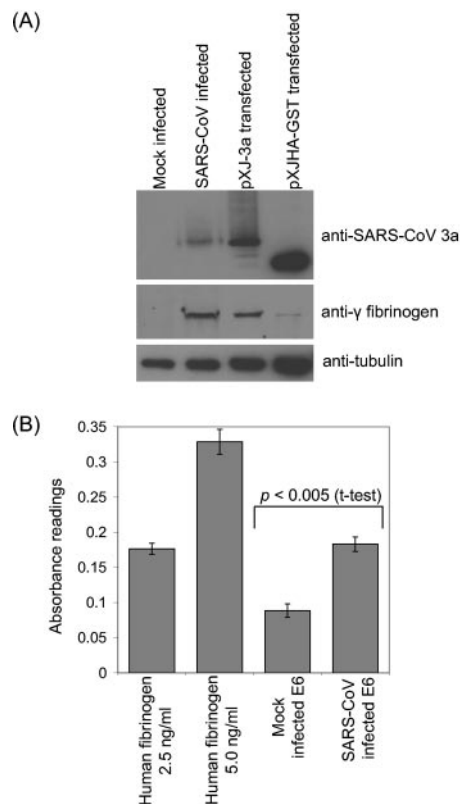
(A)

(B)

FIG. 3. Changes in the intracellular level of fibrinogen γ subunit and secretion of fibrinogen complex into culture supernatant of Vero E6 cells infected with SARS-CoV. (A) Mock- and SARS-CoV-infected Vero E6 cells (lanes 1 and 2, respectively) were subjected to Western blot analysis with the anti-3a polyclonal to determine the expression level of 3a (first panel). Vero E6 cells transiently expressing 3a or a control protein, hemagglutinin (HA)-glutathione S-transferase (GST), was similarly analyzed (lanes 3 and 4, first panel). Since the anti-3a antibody was obtained using a GST fusion protein, it recognizes both the 3a and GST proteins. Monoclonal antibody against fibrinogen γ subunit was used to determine the intracellular level of this protein (second panel). The level of endogenous tubulin was used to compare the amount of total cell lysates used (third panel). (B) The amount of fibrinogen secreted by mock- and SARS-CoV-infected cells into the cell culture supernatants was determined using an ELISA for measuring human fibrinogen. Vero E6 cells were infected at a multiplicity of infection of 0.1 for 2 h and then replaced with the unsupplemented medium. At approximately 14 h postinfection, the cells showed approximately 50% cytopathic effect, and the culture supernatants were collected. Determination of the exact concentration of fibrinogen secreted by Vero E6 cells was not possible as no standard for fibrinogen from African green monkeys was available. Two independent infection experiments were performed, and for each sample, two readings were obtained, and the data were subjected to paired two-sample Student t tests for means (Excel, Microsoft) to determine if the difference in fibrinogen secreted from mock- and SARS-CoV-infected cells was significant ($n = 4$, two-tailed, $P = 0.003$). Data represent mean ± standard deviation. The absorbance readings for human fibrinogen standards are shown for comparison.

infected cells (Fig. 3A). Similarly, Vero E6 transiently transfected with a 3a cDNA construct also showed a higher level of fibrinogen γ than mock-transfected cells (Fig. 3A). The monoclonal antibodies against Aα and Bβ could not recognize these proteins in Vero E6 (derived from African green monkey), probably due to species differences (data not shown). The

level of fibrinogen in the culture supernatant from infected Vero E6 cells was also significantly higher than that from mock-infected cells (Fig. 3B, $P < 0.005$), but we could not determine the concentration, as the assay was designed for measuring human fibrinogen and no standard from the African green monkeys was available.

Coincidently, up-regulation of fibrinogen mRNA was also observed in peripheral blood mononuclear cells infected by SARS-CoV in vitro (17). During an acute-phase response to infection, injury, or neoplasia, the production of fibrinogen by the liver increases to restore homeostasis (1, 3, 4, 9). Increased production of fibrinogen at exhepatic tissues, as in the lung (13, 23), also helps in this process. However, the excessive production of fibrinogen and formation of fibrin at the site of injury may enhance cytokines production or imbalance procoagulant and/or fibrinolytic activities (11, 31).

Postmortem examinations of SARS victims revealed extensive lung damage that is typical of acute respiratory distress syndrome (2, 7, 10, 18). In addition, most SARS patients have thrombocytopenia, elevated D-dimers, and prolonged activated partial thromboplastin time, which suggest dysregulation of the coagulation and fibrin polymerization pathways (14, 19, 33, 34). Taken together with the fibrinogen up-regulation in both infected peripheral blood mononuclear cells and Vero E6 cells (reference 17 and data therein), it seems that increased fibrinogen expression and fibrin degradation products could play an important role in SARS pathogenesis. Our data also demonstrated that expression of 3a alone can up-regulate the expression of fibrinogen, suggesting that 3a may contribute to SARS pathogenesis.

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