Annexin A2 on lung epithelial cell surface is recognized by severe acute respiratory syndrome-associated coronavirus spike domain 2 antibodies

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ARTICLE INFO

ABSTRACT

Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) infection causes lung failure characterized by atypical pneumonia. We previously showed that antibodies against SARS-CoV spike domain 2 (S2) in the patient sera can cross-react with human lung epithelial cells; however, the autoantigen is not yet identified. In this study, we performed proteomic studies and identified several candidate autoantigens recognized by SARS patient sera in human lung type II epithelial cell A549. Among the candidate proteins, annexin A2, which was identified by mass spectrometry analysis and had the highest score by Mascot data search, was further characterized and investigated for its role as an autoantigen. By confocal microscopic observation, SARS patient sera and anti-S2 antibodies were co-localized on A549 cells and both of them were co-localized with anti-annexin A2 antibodies. Anti-annexin A2 antibodies bound to purified S2 proteins, and anti-S2 bound to immunoprecipitated annexin A2 from A549 cell lysate in a dose-dependent manner. Furthermore, an increased surface expression and raft-structure distribution of annexin A2 was present in A549 cells after stimulation with SARS-induced cytokines interleukin-6 and interferon-γ. Cytokine stimulation increased the binding capability of anti-S2 antibodies to human lung epithelial cells. Together, the upregulated expression of annexin A2 by SARS-associated cytokines and the cross-reactivity of anti-SARS-CoV S2 antibodies to annexin A2 may have implications in SARS disease pathogenesis.

1. Introduction

Infection by severe acute respiratory syndrome-associated coronavirus (SARS-CoV) causes life-threatening atypical pneumonia (Drosten et al., 2003; Ksiazek et al., 2003; Kuiken et al., 2003; Marra et al., 2003; Peiris et al., 2003a; Rota et al., 2003). The pathogenesis of this disease is not fully understood. Pathological studies in SARS patients showed lung lesions with three defined phases including acute inflammation, fibrous proliferation, and the final fibrosis stage. Disease progression is initiated by SARS-CoV acute infection and accelerated by abnormal host immune responses (Gu and Korteweg, 2007; Holmes, 2003; Lai, 2003; Lau and Peiris, 2005; Nicholls et al., 2003a; Peiris et al., 2003b; Perlman and Dandekar, 2005; Perlman and Netland, 2009). SARS-CoV can infect multiple cell types with immune cells and pulmonary epithelial cells representing the main targets (Gu et al., 2005). Furthermore, a cytokine and chemokine storm has been demonstrated and its intensity associated with some clinical manifestations (Cameron et al., 2008; He et al., 2006; Huang et al., 2005; Jiang et al., 2005a; Wong et al., 2004; Zhang et al., 2007). Therefore, it is not sufficient to prevent SARS progression by anti-virus therapy only. Anti-inflammatory agents have also been used for clinical treatment (Fujii et al., 2004; Groneberg et al., 2005; Lai, 2005; So et al., 2003; Tsang and Seto, 2004; Tsang and Zhong, 2003; van Vonderen et al., 2003). SARS vaccines are currently under development and evaluation (Bai et al., 2008; Cheung et al., 2007; Groneberg et al., 2005; Jiang et al.,

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2005b; Lin et al., 2007; Marshall and Enserink, 2004; Martin et al., 2008; Okada et al., 2007; Oxford et al., 2005).

For anti-SARS therapy, the interrelationship must be clarified between viral and host responses in disease pathogenesis. CoV-induced autoimmunity has been characterized previously. Infections of murine CoV such as mouse hepatitis virus induce autoreactive T cells, B cell polyclonal activation, and autoantibody production (Hooks et al., 1993; Kyuwa et al., 1991; Mathieu et al., 2001; Perlman and Dandekar, 2005). Furthermore, our previous studies showed the presence of autoantibodies in SARS patient sera that cross-reacted with the epithelial cell line A549 (Lin et al., 2005). Other groups have also reported the generation of autoantibodies against epithelial and endothelial cells in SARS patients (Lo et al., 2006; Yang et al., 2005). However, the mechanism and implications are still unclear regarding the induction of autoimmunity by SARS-CoV infection. Molecular mimicry-based autoimmunity has been reported in both acute and chronic viral infections (Barzilai et al., 2007; Christen et al., in press; Christen and von Herrath, 2004; Horwitz and Sarvetnick, 1999; Kim et al., 2005; Lin et al., 2006; Regner and Lambert, 2001; Rouse and Deshpande, 2002). The identification of autoantigens is important to verify the molecular basis of autoimmunity.

Antibodies against SARS-CoV spike-protein domain 2 (S2) are, at least in part, responsible for the epithelial cell cross-reactivity of SARS patient sera (Hwa et al., 2008; Lin et al., 2005). In the present study, we performed proteomic approach to identify epithelial cell autoantigens recognized by SARS patient sera and anti-S2 antibodies. Annexin A2 represents a candidate autoantigen. The surface expression of annexin A2 upregulated by SARS-induced cytokines including interleukin-6 (IL-6) and interferon-γ (IFN-γ) was also investigated.

2. Materials and methods

2.1. Patient sera

SARS patient sera were collected by the Center for Disease Control, Department of Health, Taiwan (CDC-Taiwan), from March to June 2003. Diagnosis of SARS was based on the clinical criteria established by the WHO and confirmed by laboratory methods as described previously (Lin et al., 2005). Five SARS patient sera collected from the late stage ($\geq$20 days after fever onset) were included in this study. Patient sera were collected by CDC-Taiwan. The study protocols and procedures were approved by the Institutional Review Board of the National Cheng Kung University Hospital. Sera from healthy individuals were used as controls.

2.2. Cell cultures

Human lung adenocarcinoma cell line A549 was grown in DMEM, and human lung epithelial cell line HL was grown in MEM, both supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamate, and 50 ng/ml gentamycin. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Cell cytosol and membrane preparation

The cytosolic and membrane proteins of A549 cells were extracted using a commercial kit named 2-D Sample Prep for Membrane Proteins (Pierce Biotechnology, Rockford, IL, USA) and performed with TCA/acetone at −20 °C for 15 min, followed by centrifugation at 15,000 × g for 20 min at 4 °C. The pellet was dissolved in rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, IPG buffer pH 3–10 (Amersham Biosciences, London, UK), and a trace amount of bromophenol blue before gel electrophoresis.

2.4. 2-DGE and LC–MS/MS analysis

2-DGE was carried out in a horizontal electrophoresis system (IPGphor; Amersham Biosciences, Buckshire, UK) for the first dimensional IEF. Immobiline DryStrips (18 cm, pH 3–10, nonlinear) were rehydrated in the sample supernatant (in rehydration buffer) in the slot of an 18-cm strip holder (Amersham Biosciences, Buckshire, UK) at 20 °C for 12 h. The first dimensional IEF running conditions were 500 V for 500 V h, 1000 V for 1000 V h, and 8000 V for 32,000 V h. The current limit was set at 50 mA per strip and maintained at 20 °C. Upon completion, the strip was equilibrated first with equilibration buffer (6 M urea, 30% glycerol, 0.2% SDS and 50 mM Tris (pH 8.8), and 1% DTT) for 15 min at room temperature, and then with 2.5% iodoacetamide in equilibration buffer for another 15 min. The equilibrated strip was then applied to the SDS-PAGE for second dimension separation. The SDS-PAGE was carried out on a homogeneous running gel 12% without a stacking gel. Electrophoresis was conducted at 25 V for 1 h, and then at 15 W per gel at 5 °C until the bromophenol blue reached the bottom of the gel. The resulting gel was visualized by silver stain.

The protein spots of interest were excised and subjected to in-gel digestion. The excised gel slice was destained in 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate and then washed twice in 25 mM ammonium bicarbonate with 50% acetonitrile. The protein in the gel slice was reduced in 10 mM DTT for 60 min and alkylated in 55 mM iodoacetamide for 45 min in the dark. The gel slice was then washed twice as described above, air-dried after adding acetonitrile, and subjected to tryptic digestion. The gel slice was suspended and ground into small pieces in digestion buffer containing 1 μg TPCK-treated porcine trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate, and the digestion was carried out at 37 °C for 16 h. Immediately after digestion, the sample was centrifuged, and the supernatant, which contained tryptic peptides, was transferred, mixed with 5% formic acid in 1:1 water/acetonitrile, diluted with an equal volume of water, and submitted to MS analysis.

For protein identification, the tryptic digests were injected into an LCQ Deca XP plus ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with a HPLC system (LC Packings, Amsterdam, Netherlands). Before being introduced into the nano-ESI source of the mass spectrometer, the peptides were fractioned by a reverse-phase capillary C18 column (75 μm i.d. × 15 cm, LC Packings) with a linear 5–60% solvent gradient (buffer A: 95:5 water/acetonitrile in 0.1% formic acid; buffer B: 20:80 water/acetonitrile in 0.1% formic acid) in 50 min at a flow rate of 200 nl/min. The electrospray voltage was 1.2 kV and tandem MS was performed for the peptides eluted from LC (Tyan et al., 2005).

2.5. Sequence database search

The raw data acquired by MS were converted into peak list (DTA) files via Bioworks Browser 3.3 (ThermoFinnigan). The resulting peak lists were used to search against the NCBI nr database (NCBI nr 20090710; 9283978 sequences; 3180197137 residues) using the Mascot MS/MS ion search engine (20090612 updated, http://www.matrixscience.com, Matrix Science Ltd., London, UK). The search parameters were set as follows: trypsin was allowed for proteolytic cleavage; the number of missed cleavage sites was set to 2; fix modifications were not selected; variable modifications included cysteine carboxymidomethylation, asparagines and glutamine deamidation, methionine oxidation, pyroGlu on N-terminal of glutamic acid and glutamine were considered; peptide mass tolerance was set to 1 Da; MS/MS ion mass tolerance was set to 1 Da; peptide charges were set to 2; only human taxonomy was considered; the number of human protein entries in NCBI database

was 226477 and the proteins identified with peptide score >20 (expectation value <0.05) were considered.

2.6. Protein binding assay

SARS S2 proteins were purified from the construct *pMBP-TWsp-S2* in *Escherichia coli* DH5α. The preparation of S2 protein was described in previous study (Lin et al., 2005). For S2 binding assay, an ELISA plate was coated with purified S2 protein in coating buffer (15 mM of sodium carbonate and 35 mM of sodium bicarbonate, pH 9.6) overnight at 4 °C. After blocking with 1% bovine serum albumin (BSA) in coating buffer and repeated washes with 0.05% Tween-20 in phosphate-buffered saline (PBS-T), polyclonal anti-annexin A2 IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Calbiochem, San Diego, CA, USA) in 1/2000 dilution. After washing

with PBS-T, ABTS peroxidase substrate (Sigma–Aldrich, Milwaukee, WI, USA) was added to each well and the absorbance was measured at 405 nm by Emax microplate reader (MDS Analytical Technologies, Sunnyvale, CA, USA). For annexin A2 binding assay, polyclonal anti-annexin A2 IgG in PBS was coated on ELISA plate and annexin A2 in A549 cell lysate was immunoprecipitated by its binding to anti-annexin A2. After washing, polyclonal anti-SARS S2 IgG was added followed by HRP-conjugated anti-mouse IgG (Calbiochem) in 1/5000 dilution. The measurement of absorbance was the same as described above.

2.7. Immunofluorescence staining and confocal microscopy

Monolayers of A549 cells (1×10^5 cells/ml) were cultured on sterile microscope cover glasses in 24-well plate before experiment. Cells were washed with PBS gently and fixed with 1% paraformaldehyde in PBS at room temperature for 10 min. Primary
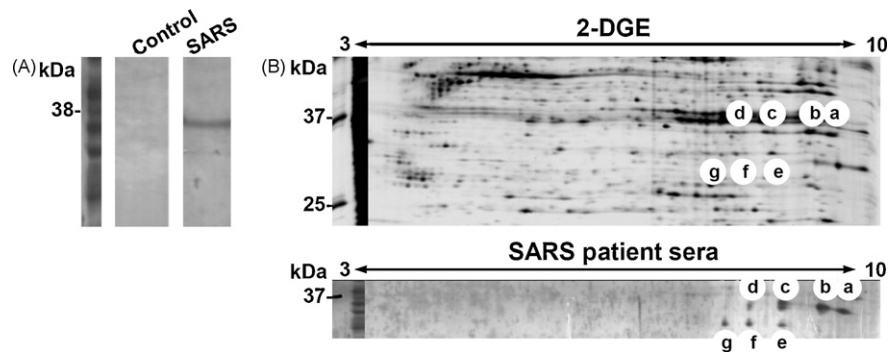

Fig. 1. Epithelial cell self-antigens recognized by SARS patient sera. (A) Human A549 epithelial cell membrane fraction was separated by SDS-PAGE and immunoblotted with SARS patient sera (from a pool of five patients) or normal control sera. (B) Membrane fraction was analyzed by 2-DGE and silver stain (top panel) followed by blotting with SARS patient sera (bottom panel). Seven protein spots (a–g) were detected and protein identification is shown in Table 1.

Table 1
Identification of epithelial cell self-antigens recognized by SARS patient sera.

2-DGE spot designation[a]	Protein name	NCBI accession number[b]	Score[c]	Number of unique peptide[d]	% of sequence coverage[e]	Mr[f] (kDa)	pI[g]
a	Glyceraldehyde-3-phosphate dehydrogenase	31645	314	7	43	36.0	8.26
b	Glyceraldehyde-3-phosphate dehydrogenase	31645	140	4	26	36.0	8.26
c	Annexin A2, isoform 2	4757756	308	8	36	38.6	7.57
	Crystal structure of Akr1b10 complexed with Nadp+ and tolrestat	119388973	94	4	26	36.1	7.79
	Aldo-ketoreductase family 1, member B10	20531983	89	3	26	36.0	7.12
d	Aldo-ketoreductase family 1, member B10	20531983	145	4	29	36.0	7.12
	Crystal structure of Akr1b10 complexed with Nadp+ and tolrestat	119388973	145	4	29	36.1	7.79
	Dihydrodiol dehydrogenase/bile acid-binding protein	1723158	107	3	21	36.7	6.22
	Crystal structure of h3alpha-hydroxysteroid dehydrogenase type 3 mutant Y24a in complex with Nadp+ and Epi–testosterone	150261301	102	2	21	36.4	7.60
	Dihydrodiol dehydrogenase isoform DD1	556516	88	2	23	34.8	8.10

[a] These 2-D gel spot designations are defined as in Fig. 1.
[b] NCBI accession number was given as a part of output from Mascot database search.
[c] Scores calculated from Mascot database search.
[d] Number of unique peptide sequences generated from Mascot database search result for this protein.
[e] Percentage of sequence coverage generated from Mascot database search.
[f] Theoretical molecular weight calculated from protein sequence database.
[g] Theoretical pI calculated from protein sequence database.

antibodies were incubated with cells for 1 h at room temperature. Primary antibodies used were 1/20 diluted SARS patient serum sample, 1 μg of polyclonal anti-S2 IgG, or monoclonal anti-annexin A2 light chain IgG (MP Biomedicals, Aurora, OH, USA). After several washes in PBS, cells were stained with secondary antibodies including 1/100 diluted FITC-conjugated anti-human IgG and TRITC-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at 4 °C. Polyclonal FITC-conjugated anti-annexin A2 IgG (BD Biosciences, San Jose, CA, USA) and TRITC-conjugated anti-caveolin-1 IgG were stained directly with cells for 1 h at room temperature. MitoTracker Red CMXRos (Invitrogen, Eugene, OR, USA) was used for mitochondrial staining. All confocal images were obtained using a Leica TCS SP2 confocal microscope.

2.8. Cell elution

For cytokine stimulation, 1×10^6 cells were added in a 6-cm culture dish containing DMEM with 10% FCS. After IFN-γ or IL-6 treatment, cells were washed with ice-cold HBS for three times. Then cells were eluted with 20 mM EGTA/HBS (Sigma–Aldrich) and mixture of protease inhibitors at 4 °C for 30 min. The eluates were detected using Western blotting.

2.9. Western blotting

Protein concentrations were determined using Bradford assay (Bio-Rad Laboratories, Richmond, CA, USA). Equal amounts of protein of total cell lysates (10 μg) were separated by 12% SDS-PAGE and transferred to PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk in PBS-T, blots were probed with SARS patient sera or polyclonal anti-annexin A2 IgG (Santa Cruz Biotechnology) followed by HRP-conjugated anti-human and anti-rabbit IgG (Jackson ImmunoResearch Laboratories), respectively, in 1/5000 dilution. Blots were developed using Western lightning chemiluminescence reagent (PerkinElmer, Shelton, CT, USA).
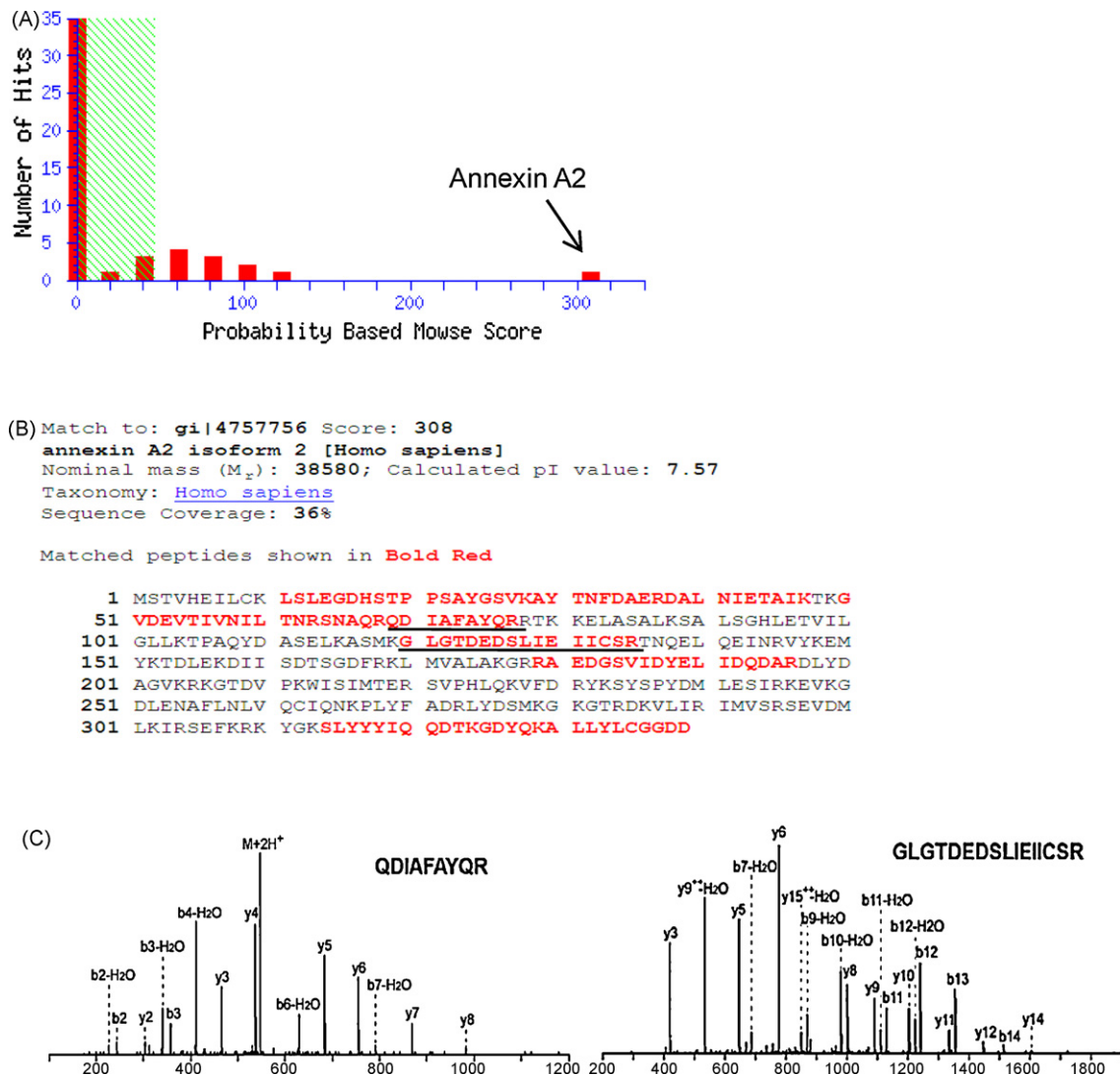

Fig. 2. Mass spectrometry identification of annexin A2. The spot c on the 2D-gel as shown in Fig. 1B was excised and subjected to in-gel digestion, mass spectrometry analysis, and Mascot database search. (A) A summary of the Mascot search result is shown. Annexin A2 scored 308, the highest score among the significant hits (those above the shaded area). (B) Amino acid sequence of annexin A2. The peptide sequences in bold red were those matched in the database search. (C) Two examples, as underlined in (B) of tandem mass spectrometry data leading to positive identification of annexin A2. Using Mascot database search, these two product ion scan spectra were matched to the sequences QDIAFAYQR (left panel) and GLGTDEDSLIEIICSR (right panel), respectively. Most of the peaks were assigned as b and y series ions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.10. Flow cytometry binding assay

Cells were cultured in 6-cm culture dish and treated with 25 ng/ml human cytokines IL-6 or IFN-γ (Cytolab, Rehovot, Israel) at 37 °C in a humidified atmosphere of 5% CO_2. Triplicate samples were suspended by trypsin/EDTA and fixed with 1% formaldehyde in PBS for 10 min at room temperature. Fixed cells were stained with 1 μg of polyclonal anti-S2 IgG for 1 h at 4 °C. After washing with PBS, cells were stained with FITC-conjugated anti-mouse IgG in 1/100 dilution for 1 h at room temperature. Samples were analyzed using FACSCalibur (BD Biosciences).

2.11. Statistics

We used the paired *t*-test for statistical analysis. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Detection of autoantigens recognized by SARS patient sera

We previously showed the presence of autoantibodies in SARS patient sera that reacted with human lung adenocarcinoma A549 cells. We thereby identified the autoantigens in A549 cell membrane extracts recognized by SARS patient sera. Western blotting of the cell membrane fraction showed a major band with a molecular weight approximately 38 kDa recognized by SARS patient sera but not by normal control sera (Fig. 1A). The 38 kDa protein band was also detectable in the A549 cell cytosolic fraction (data not shown). The representative silver stained 2D-gel of total cell extracts from A549 cells was shown (Fig. 1B, top panel). Seven protein spots were detected when immunoblotting with SARS patient sera from 2D-gel transferred membrane (Fig. 1B, bottom panel, a–g). These protein

spots were not detected in the immunoblot which was stained with normal control sera (data not shown). Protein identification from the seven protein spots was established by LC–MS/MS and Mascot search engine described in Section 2. Human epidermal keratins and other proteins below the threshold score were discarded. There were seven candidate proteins identified from the spots a–d and no proteins conforming to the search parameter in spots e–g. The summary of proteins identification including protein name, accession number, number of peptide identified, sequence coverage, mass value, and pI are presented in Table 1. According to the proteomic information, we next investigated whether annexin A2 may act as an autoantigen in the SARS-CoV-infected patients. It is because annexin A2 has been demonstrated as autoantigen in antiphospholipid syndrome (APS) patients and lung cancer patients (Brichory et al., 2001; Cesarman-Maus et al., 2006; Cockrell et al., 2008; Lopez-Pedrera et al., 2008; Salle et al., 2008; Shoenfeld et al., 2008). The spot c from our Mascot search data showed that annexin A2 had the highest score 308 as compared with other proteins (Fig. 2A). The amino acid sequence of annexin A2 (Fig. 2B) and the two examples of identified sequences QDIAFAYQR (Fig. 2C, left panel) and GLGTDEDSLIEIICSR (Fig. 2C, right panel) obtained from tandem MS are shown.

3.2. Annexin A2 is recognized by SARS patient sera and anti-S2 antibody

To further characterize the target protein annexin A2 recognized by SARS patient sera and anti-S2 antibodies, protein–protein co-localization was investigated by confocal microscopic observation. By immunofluorescence staining, the A549 cell-binding sites of SARS patient sera were co-localized with those of anti-S2 (Fig. 3A) and anti-annexin A2 (Fig. 3B). We further demonstrated the co-localization of anti-S2 and anti-annexin A2 in A549 cells (Fig. 3C).
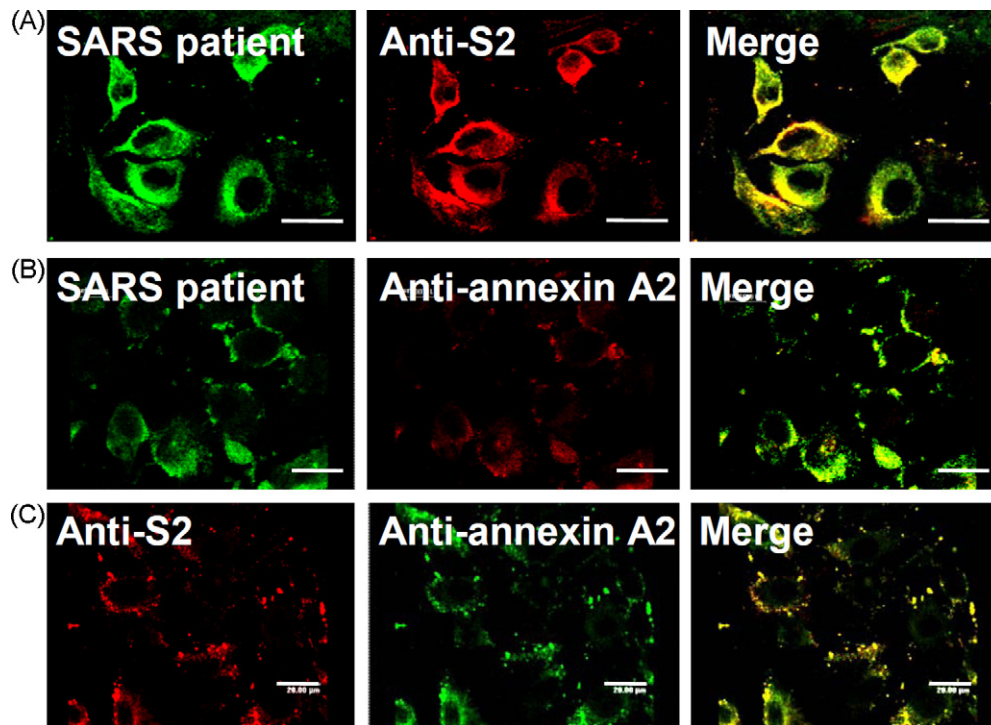

Fig. 3. Co-localization of SARS patient sera and anti-S2 antibodies with annexin A2 expressed on human epithelial cells. Cell binding was detected using immunostaining followed by confocal microscopic observation. (A and B) A549 cells were incubated with a 1:20 dilution of sera from SARS patients, followed by FITC-conjugated anti-human IgG (SARS patient, green). After washing, cells were then stained with anti-S2 (anti-S2, red) or anti-annexin A2 (anti-annexin A2, red) followed by TRITC-conjugated anti-mouse IgG. (C) Cells were incubated with antibodies against S2 followed by TRITC-conjugated anti-mouse IgG (anti-S2, red), and then FITC-conjugated anti-annexin A2 (anti-annexin A2, green). The signals of co-localization are shown (Merge, yellow). Bar: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
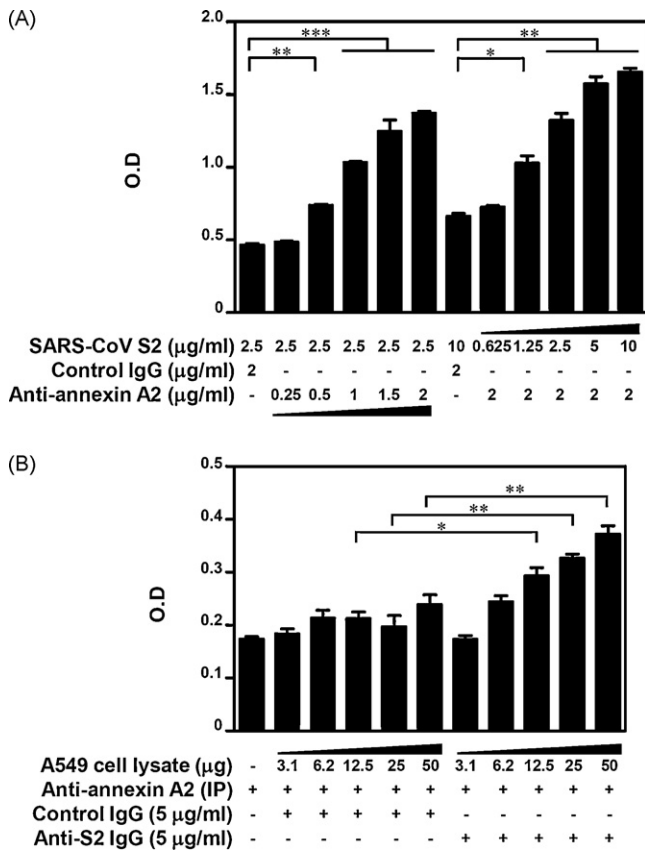
Fig. 4. Anti-annexin A2 antibodies bind to SARS-CoV S2 protein and anti-S2 antibodies bind to immunoprecipitated annexin A2. (A) Recombinant S2 protein was coated on ELISA plate with different doses as indicated, then incubated with control IgG or serial doses of anti-annexin A2 antibodies followed by HRP-conjugated anti-rabbit IgG. Cultures were developed using specific substrate ABTS and detected using an ELISA reader. Experiments were carried out in triplicate, and the averages of the relative OD as mean ± standard deviation (SD) are shown. (B) Annexin A2 was immunoprecipitated from various doses of A549 cell lysate using anti-annexin A2 antibodies. Cultures were then incubated with 5 μg/ml of control IgG or anti-S2 followed by HRP-conjugated anti-mouse IgG. Experiments were carried out in triplicate, and the averages of the relative OD as mean ± SD are shown. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Using ELISA, we next showed that anti-annexin A2 antibodies recognized purified S2 protein in a dose-dependent manner (Fig. 4A). Because purified annexin A2 protein is not available, we immunoprecipitated annexin A2 from A549 cell membrane fraction and a dose-dependent binding ability of anti-S2 to annexin A2 was observed (Fig. 4B).

3.3. IL-6 and IFN-γ upregulate epithelial cell surface expression of annexin A2 and enhance the epithelial cell-binding activity of anti-S2

Immunopathogenesis of SARS-CoV infection mediated by a cytokine storm, including IL-6 and IFN-γ, has been demonstrated (Cameron et al., 2008; He et al., 2006; Huang et al., 2005; Jiang et al., 2005a; Wong et al., 2004; Zhang et al., 2007). Furthermore, upregulated expression of annexin A2 by IL-6 and production of autoantibodies against annexin A2 were observed in lung cancer patients (Brichory et al., 2001). We herein confirmed the induction of surface expression of autoantigen annexin A2 by SARS-induced cytokines IL-6 and IFN-γ in A549 cells. Using Western blotting, both IL-6 and IFN-γ increased the surface expression of annexin A2 in A549 cells (Fig. 5A). By confocal microscopy, we observed that annexin A2 was concentrated to a specific region around the plasma membrane in IL-6- and IFN-γ-treated cells, whereas annexin A2 was evenly distributed in untreated cells (Fig. 5B). By immunostaining using the lipid raft marker caveolin-1, the lipid raft-like structure in A549 cells pretreated with IL-6 and IFN-γ was evident as compared with untreated cells (Fig. 5C). We next analyzed the binding ability of anti-S2 antibody to IL-6- and IFN-γ-treated A549 cells. Results showed that IL-6 and IFN-γ increased the cross-reactivity of anti-S2 to A549 cells (Fig. 6, left panel). Similar results were also obtained in human lung epithelial HL cells (Fig. 6, right panel).

4. Discussion

The failure of human respiratory system resulted from SARS is characterized by lung injury and severe inflammation. Histopathological destruction can be observed both in bronchiolar and alveolar epithelial cells (Gu et al., 2005; Lang et al., 2003; Nicholls et al., 2003b). Previous studies by us (Lin et al., 2005) and others (Lo et al., 2006; Yang et al., 2005) have shown the presence of autoantibodies against human lung epithelial cells in SARS patient sera. In the present study, we further identified candidate autoantigens which can be recognized by patient sera.

Viral autoimmunity has been observed in various viral infections, such as human immunodeficiency virus (HIV), lymphocytic choriomeningitis virus (LCMV), hepatitis C virus (HCV), cytomegalovirus (CMV), and dengue virus (Barzilai et al., 2007; Christen et al., in press; Horwitz and Sarvetnick, 1999; Kim et al., 2005; Lin et al., 2006; Regner and Lambert, 2001; Rouse and Deshpande, 2002). Previous studies have demonstrated that mouse CoV infection can induce autoreactive T and B cell activation and the generation of autoantibodies (Hooks et al., 1993; Kyuwa et al., 1991; Mathieu et al., 2001). Autoimmunity may also be involved in SARS based on previous findings of anti-epithelial cell autoantibodies in SARS patients (Lin et al., 2005; Lo et al., 2006; Yang et al., 2005). Relatively higher titers of autoantibodies appeared at day 20 of disease onset, reached a maximum around day 40, and declined thereafter. Both cytotoxicity and inflammatory activation in epithelial cells were induced by anti-S2 antibodies present in patient sera.

Dysregulation of inflammatory cytokines and chemokines, so-called cytokine and chemokine storm, are involved in the pathogenesis of SARS. Cytokines including IFN-γ, IL-1, IL-6 and TGF-β, chemokines including IL-8, monocyte chemoattractant protein-1 (MCP-1) and IFN-γ-inducible protein-10 (IP-10), as well as inflammatory mediators such as prostaglandin E2 are significantly elevated in SARS patients (Cameron et al., 2008; Cheung et al., 2005; He et al., 2006; Huang et al., 2005; Jiang et al., 2005a; Lee et al., 2004; Wong et al., 2004). Among the candidate proteins we have identified, annexin A2 was considered as an autoantigen in lung cancer patients which was associated with high levels of IL-6 (Brichory et al., 2001). In this study, we treated A549 cells with IL-6 and IFN-γ and found that annexin A2 expression was elevated on the cell surface after cytokine stimulation.

Annexin A2, a Ca^{2+}-dependent binding protein, is found in different cells as a monomer, heterodimer, or heterotetramer. The heterotetramer composed of two annexin A2 monomers and a p11 dimer is the dominant form on the cell plasma membrane. However, annexin A2 tetramer lacks the transmembrane domain. It binds to the plasma membrane via a Ca^{2+}-dependent manner and generally interacts with proteins such as carcinoembryonic antigen cell adhesion molecule-1 and membrane-associated proteins such as β_2 glycoprotein I (Kirshner et al., 2003; Ma et al., 2000; Zhang and McCrae, 2005). A number of studies have shown the diverse roles of extracellular annexin A2, including profibrinolytic coreceptor, tumor progression, and pathogen receptor (Derry et al., 2007;
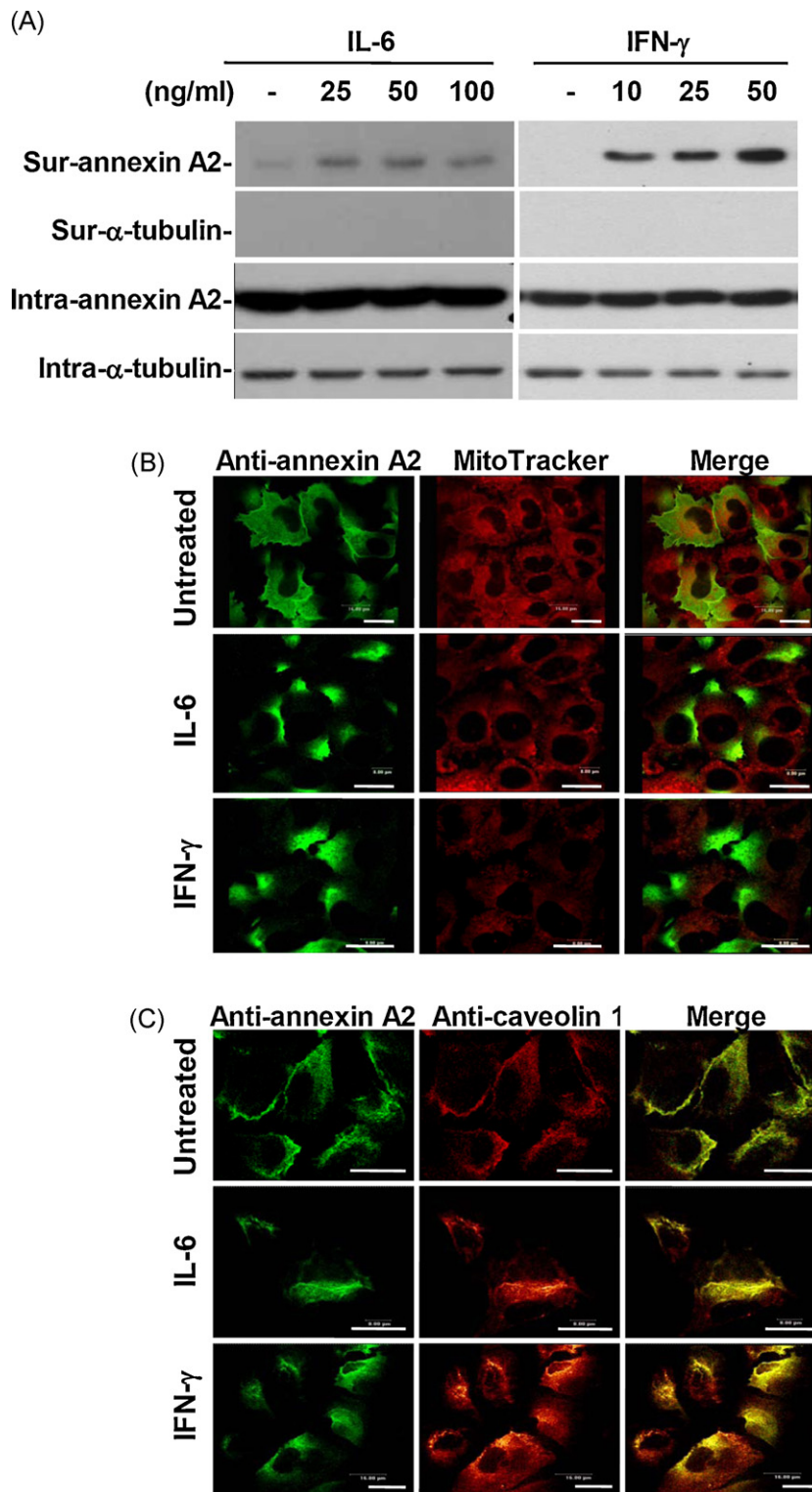
Fig. 5. IL-6 and IFN-γ upregulate epithelial cell surface expression of annexin A2. (A) Surface (Sur) expression of annexin A2 in A549 cells pretreated with various doses of IL-6 or IFN-γ for 48 h was determined by Western blotting. The expression of intracellular annexin A2 is also shown. α-Tubulin was used as the control. (B) The protein expression and distribution of annexin A2 in A549 cells pretreated with 25 ng/ml of IL-6 or IFN-γ for 24 h was determined using confocal microscopic observation. Cells were stained with anti-annexin A2 followed by FITC-conjugated anti-mouse IgG (green). A cytosolic counterstaining was performed by mitochondrial labeling using MitoTracker (red). Bar: 16 μm. (C) The formation of lipid raft-like structure in A549 cells after IL-6 and IFN-γ treatment was demonstrated by caveolin-1 staining. Cells were incubated with FITC-conjugated anti-annexin A2 (green) and TRITC-conjugated anti-caveolin-1 (red). Co-localization of annexin A2 and caveolin-1 is shown (Merge, yellow). Bar: 16 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Kim and Hajjar, 2002; Kirschnek et al., 2005; Ryzhova et al., 2006; Sharma and Sharma, 2007). The involvement of annexin A2 in lipid raft assembly and signaling in response to extracellular stimuli has been reported (Babiychuk and Draeger, 2000; Paradela et al., 2005; Sorice et al., 2007). Anti-annexin A2 autoantibodies have been found in lung cancer patients and anti-phospholipid syndrome patients (Brichory et al., 2001; Cesarman-Maus et al., 2006; Cockrell et al., 2008; Lopez-Pedrera et al., 2008; Salle et al., 2008; Shoenfeld
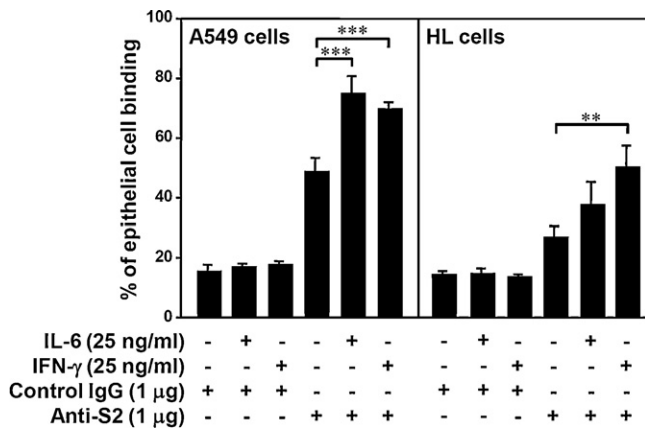
Fig. 6. IL-6 and IFN-γ enhance the epithelial cell-binding ability of anti-S2 antibodies. Human lung epithelial cells A549 and HL were treated with or without 25 ng/ml of IL-6 or IFN-γ for 24 h. Cells were stained with 1 μg of control IgG or anti-S2 followed by FITC-conjugated anti-mouse IgG and analyzed using flow cytometry. Data are presented as mean ± SD of triplicate cultures. **$p < 0.01$; ***$p < 0.001$.

et al., 2008). Clustering of annexin A2-bound β_2 glycoprotein I by anti-annexin A2 autoantibodies triggers endothelial cell activation, which involves an increased cell surface expression of adhesion molecules (Cockrell et al., 2008; Zhang and McCrae, 2005). Our previous study showed that anti-S2 antibody increased immune cell adhesion to epithelial cells (Lin et al., 2005). A role of annexin A2 in mediating phagocytosis of apoptotic cells by macrophages has been demonstrated (Fan et al., 2004). Interestingly, our unpublished data showed that annexin A2-mediated apoptotic cell recognition and phagocytosis by macrophages was inhibited by anti-S2 pretreatment. The effects of anti-S2 antibodies cross-reactive with annexin A2 need to be further elucidated.

Recent reports have shown that the S protein may provide protective effects against SARS-CoV infection (Bai et al., 2008; Chou et al., 2005; Du et al., 2008; He et al., 2004; Lokugamage et al., 2008; Zeng et al., 2004). However, S2 shows sequence homology with self-antigens and the potential pathogenic role of the cross-reactivity of anti-S2 remains a concern. However, S2 shows sequence homology with self-antigens and the potential pathogenic role of the cross-reactivity of anti-S2 remains a concern. We previously showed that two synthetic spike-protein peptides, located at the S2 domain, were bound by SARS patient sera (Lin et al., 2005). The local alignment by JEMBOSS-Water analysis showed that the sequence similarity between annexin A2 and two regions on S2 (residues 927–937 and residues 940–951) are 50 and 66.7%, respectively. Our results strongly suggest that SARS-CoV-induced autoimmunity raises an alert not only for effective therapy but also for the development of a safe vaccine. Also, the SARS-associated cytokine storm may upregulate the expression of autoantigens. In addition to annexin A2, the roles of other candidate autoantigens found in this study identified by proteomic approach remain to be further investigated.

Conflict of interest

The authors have no financial conflict of interest.

Acknowledgments

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