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（子計畫四）SARS 病人抗上皮細胞及內皮細胞自體抗體致病角色之探討

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Autoantibodies against Human Epithelial Cells and Endothelial Cells after Severe Acute Respiratory Syndrome (SARS)-Associated Coronavirus Infection

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Abstract

The severe acute respiratory syndrome (SARS) is caused by infection with the SARS-associated coronavirus (SARS-CoV) and characterized by severe pulmonary inflammation and fibrosis. In this study, the development of autoantibodies against human epithelial cells and endothelial cells in patients with SARS at different time periods (the first week: phase I, one month after the disease onset: phase II/phase III) were investigated. Antibodies in sera of patients and healthy controls against 1) A549 human pulmonary epithelial cell-line, 2) human umbilical venous endothelial cells (HUVEC), 3) primary human pulmonary endothelial cells (HPEC) were detected by cell-based ELISA and indirect immunofluorescence staining. The results revealed that serum levels of IgG anti-A549 cells antibodies, IgG anti-HUVEC antibodies, and IgM anti-HPEC antibodies were significantly higher in SARS patients at phase II/phase III than those in healthy controls. Sera from SARS patients at phase II/phase III could mediate complement dependent cytotoxicity against some A549 cells and HPEC. It is concluded that some autoantibodies against human epithelial cells and endothelial cells would be developed after SARS-CoV infection and this phenomenon may indicate post-infectious cellular injury and also induce SARS-induced immunopathology.

Key words: SARS, autoantibodies, epithelial cell, endothelial cell, cytotoxicity

Introduction

In early 2003, a new infectious disease, the severe acute respiratory syndrome (SARS) swept the world including Taiwan [Tsang et al., 2003; Twu et al., 2003]. The pathogen was later identified as SARS-associated coronavirus (SARS-CoV) and spread through close contact of droplets [Drosten et al., 2003; Yeh et al., 2004]. Those who were infected by this virus presented with persistent high fever, cough, dyspnea, and the disease may eventually progress to respiratory and/or multiple organs failure [Fowler et al., 2003; Tsang et al., 2003]. Autopsies of patients died from SARS have revealed extensive pulmonary consolidation, localized hemorrhage and necrosis, proliferation and desquamation of alveolar epithelial cells, monocytes, lymphocytes and plasma cells infiltration in alveoli, and hyaline membrane formation [Ding et al., 2003; Franks et al., 2003]. Systemic vasculitis was also found and characterized by edema, thrombosis, localized fibrinoid necrosis, and infiltration of monocytes, lymphocytes, and plasma cells into vessel walls in many organs including heart, lung, liver, kidney, and the stroma of striated muscles [Ding et al., 2003]. All these pathological changes are now thought to be mediated by direct viral destruction and followed by immune-mediated processes [Peiris et al., 2003a].

Epithelial cells and endothelial cells, according to the pathological findings, may be the two major target cells that are damaged in the inflammatory process of SARS.

Autoantibodies against epithelial cells have been detected in recurrent oral ulcer, ulcerative colitis and prostate cancer [Ablin., 1972; Snook et al., 1991; Sun et al., 2000], and anti-endothelial cell antibodies (AECA) have also been found in many disorders such as systemic lupus eyrthematosus (SLE), Kawasaki disease, Henoch-Schönlein purpura (HSP), Behcet's disease, and some post-infectious immune-mediated diseases [Carvalho et al., 1999; Grunebaum et al., 2002; Lee et al., 2003; Lin et al., 2003; Toyoda et al., 1999; Yang et al., 2002]. Although some of these conditions appear as a result of inflammatory tissue injury, others have a pathogenic potential to induce further damage. The aims of this study was to investigate the development of autoantiboies against human epithelial cells and endothelial cells after the SARS-coronavirus infection by using cell-based ELISA and indirect immunofluorescence staining, and to identify the pathogenic role of these antibodies.

Materials and Methods

Patients and controls

Twenty-two previously healthy Chinese adults suffering from SARS in early 2003 were included in this study. The diagnosis was confirmed by the typical clinical presentations with fever, cough, and dyspnea, and positive viral PCR. Informed consent and institutional approval were obtained for this study. Blood was sampled during the first one-week (phase I) and one month after the disease onset (phase II or phase III) [Peiris et al., 2003a]. Twenty healthy adults were enrolled as controls. In the study by indirect immunofluorescence staining, patients with streptococcal necrotizing pneumonia were also recruited as controls. For safety, serum samples derived from patients were inactivated at 56℃ for 30 min before testing.

Antibodies against SARS-CoV nucleocapsid protein

To detect the presence of anti-SARS-CoV nucleocapsid (N) antibodies in SARS patients, a 96-well microplate was coated with purified His-N protein at a concentration of 5 μg/ml. Each well was then blocked by phosphate buffered saline (PBS) containing 0.05％Tween-20 (PBS/Tween 20) (Sigma) and 5％bovine serum albumin (BSA) at 37℃ for 2 hours. Diluted serum samples from SARS patients at phase II/phase III and healthy controls (1: 100 or 1: 400 with 1％BSA) were added to

the wells at room temperature for 2 hours and then removed. Following the washing procedure, peroxidase-conjugated mouse anti-human IgG, IgA and IgM (1: 5000 in 1 %BSA) was added to each well and incubated at room temperature for 1 hour. The plates were washed by PBS/Tween 20 before adding teramethylbezidine (TMB) (KPL, USA) substrate and the reactions were stopped by the addition of 2 N H_2SO_4. The optical density (OD) of each well was read at a wavelength of 450nm minus 540 nm by an ELISA reader. The serum levels of antibodies between patients and controls were expressed as OD values.

Cells culture

A549 cells, a human pulmonary epithelial cell-line, were cultured with DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamin, 150 mM HEPES, and 100 μg/ml penicillin/streptomycin. Primary human pulmonary endothelial cells (HPEC) were cultured with EGM-2 MV (SingleQuots, USA) supplemented with EBM (Cambrex Bio Science Walkersville, Inc. USA). Human umbilical venous endothelial cells (HUVEC) were obtained from human umbilical vein by collagenase (GIBCO BRL Life Technologies) digestion as described previously [Jaffe et al., 1973]. The separated cells were seeded in 75 ml flasks precoated with 1% gelatin solution and grown in medium 199 (GIBCO BRL, Life

Technologies) supplemented with 15% heat inactivated FCS, heparin sulfate, L-glutamine, endothelial cell growth factor (BM) (final concentration, 20 μg/mL), and 100 μg/ml penicillin/streptomycin. All cultures were incubated at 37℃ in 5% CO_2, and the cells were used between the 2nd and the 6th passage.

Cell-based ELISA

A549, HUVEC, and HPEC were prepared to detect autoantibodies in sera of SARS patients. Cells were seeded on gelatin-coated 96-well microtitre plates (Nunc™, Denmark) at a concentration of 1×10^5 cells/well. When the cellular growth became confluent 3-4 days later, cells were fixed with 0.2% glutaraldehyde in PBS for 10 min at room temperature and incubated with blocking buffer (1% BSA/0.05% azide/0.1 M Tris in ddH_2O) for 60 min at 37℃ to prevent non-specific binding. After washing with PBS/Tween 20, the serum samples, diluted in blocking buffer at 1:200 for IgG/IgM detection; 1:25 for IgA detection, were incubated for 2 h at 37℃. The sera were then removed and the plates were washed, 100 μl of peroxidase-conjugated rabbit antihuman IgG, IgM and IgA immunoglobulins were added to each well for a further 2 h at 37℃. After washing, TMB solution was added for 15 min, and stop solution (1M hydrochloric acid) for 5 min. The optical density of each well was read at 450 nm by an ELISA reader. Initial screening of SLE and HSP patients by

immunofluorescence staining and ELISA had identified the patients with high antibody binding activity to three cell types, and who were adopted as the positive control (SLE serum for IgG and IgM detection; HSP serum for IgA detection). A normal control serum with relative low binding activity was used as the negative control. The results were expressed as ELISA ratio (ER) = 100×(S-A)/(B-A), where S is absorbance of sample, A is absorbance of negative control and B is positive control.

Indirect immunofluorescence staining

A549, HPEC and HUVEC were prepared on 12-well Teflon-printed slides, fixed in 4% paraformaldehyde overnight at 4℃, and incubated with blocking buffer (5% fetal calf serum in(PBS) for 30 min at 37℃. Cells on slides were then incubated with sera of SARS patients, patients with necrotizing pneumonia, and healthy controls for 1 h at 37℃. The slides were washed three times by PBS and FITC-conjugated antihuman immunoglobulins (CHEMICON, Australia), diluted in blocking buffer at 1:100, were added to each well for a further 1 h at 37℃. The interactions of cells and PBS only (without adding any serum) were as negative controls to establish backgrounds of various immunofluorescence staining. The specimens were then washed three times, mounted in glycerol and examined using a fluorescence microscope.

Complement dependent cytotoxicity assay

Cells were seeded in 48-well culture plates at 1×10^4 cells/well overnight for cell lysis assay. The culture medium was replaced by test medium (RPMI-1640 supplemented with 2 mM L-glutamine but without phenol red) before the addition of patient sera. Patient and normal control sera were preheated at 56℃ for 30 min to inactivate complement, diluted (1:25 dilution) and incubated with Low-Tox-M rabbit complement (1:20 dilution; Celardane Laboratories Ltd., Hornby, Ontario, Canada) at 37℃ for 60 min before being added to the cells. After incubation for 48 hours, the levels of lactate dehydrogenase activity in the culture supernatant were determined using a Cytotoxicity Detection Kit (Boehringer Mannheim GmbH, Germany). The absorbance of the sample was measured at 490 nm and the reference wavelength was 620 nm. To determine the cytotoxicity index, the absorbance values are substituted in the following equation: cytotoxicity index (%) = (sample value-low control)/(high control-low control) × 100%. Low control is the absorbance from the supernatant of the cells cultured with test medium, and high control is the absorbance from the supernatant of the cells cultured with 1% Triton X-100 in test medium.

Statistical analysis

The values of OD, ELISA ratio, and cytotoxicity index were expressed as mean ±

SEM. Each two-group comparison was conducted using the Mann-Whitney U test. A two-tailed p value of less than 0.05 was considered statistically significant.

Results

Anti-N protein antibodies in SARS patients

Serum levels of antibodies against SARS-CoV nucleocapsid protein in SARS patients at phase II/phase III and those in healthy controls were examined and compared. The results showed that IgG and IgM anti-N protein antibodies elevated significantly in SARS patients (IgG: 1.16 ± 0.10 vs 0.23 ± 0.04, $p < 0.001$, IgM: 0.84 ± 0.13 vs 0.46 ± 0.07, $p = 0.03$), and there was no statistical difference of IgA isotype between SARS patients and healthy controls (0.69 ± 0.12 vs 0.42 ± 0.07, $p = 0.08$) (Fig 1).

Anti-epithelial cell antibodies (AEpCA) and anti-endothelial cell antibodies (AECA) detection by cell-based ELISA

Figure 2 summarized the ELISA ratios of serum antibodies (IgG, IgA, IgM) against A549 cells, HPEC, and HUVEC in healthy controls and SARS patients at different time periods, phase I and phase II/phase III. During the first week (phase I), patients presented with high fever, general malaise, myalgia and cough. The levels of these all antibodies in this period were not different between patients and healthy controls. When the disease progressed, patients received combined therapy of ribavirin, intravenous immunoglobulin, and steroid. Although serum samples in this period were collected during or after treatment, the serum levels of IgG anti-A549 cells antibodies,

IgM anti-HPEC antibodies, and IgG anti-HUVEC antibodies were significantly increased when compared with healthy controls (IgG anti-A549 cells: 45.44 ± 6.26 vs 22.63 ± 4.57, p = 0.009; IgM anti-HPEC antibodies: 65.45 ± 7.38 vs 42.94 ± 6.67, p = 0.036; IgG anti-HUVEC antibodies: 27.12 ± 4.28 vs 13.47 ± 2.92, p = 0.025). The levels of IgA anti-HPEC antibodies and IgA anti-HUVEC antibodies in patients, no matter at phase I or phase II/phase III, were not different statistically from healthy controls; however, these two antibodies were decreased significantly after the acute phase (comparisons between different phases, IgA anti-HPEC antibodies: 25.04 ± 9.17 vs 7.94 ± 3.26, p = 0.018; IgA anti-HUVEC antibodies 25.13 ± 7.52 vs 11.75 ± 5.51, p = 0.018) (Fig 2(B), (C)).

Anti-epithelial cell antibodies (AEpCA) and anti-endothelial cell antibodies (AECA) detection by indirect immunofluorescence staining

In the study of autoantibodies against A549 cells, Fig 3(A), (B), and (C) showed that IgG anti-A549 cells, IgM anti-HPEC, and IgG anti-HUVEC antibodies existed in SARS patients during phase II/phase III, but not in healthy controls and patients with necrotizing pneumonia that also had severe pulmonary inflammation and damage.

SARS patient sera induce A549 cell and HPEC lysis

In the present experiments, purified IgG and IgM immunoglobulins were not available due to the limitations regarding blood sampling from SARS patients. Therefore, in order to investigate if these autoantibodies have pathogenic effects, sera from SARS patients with high-level autoantibodies were used for the cytotoxic assay. The results showed that in the presence of complement, sera from patients at phase II/phase III induced more A549 cells and HPEC lysis than sera from healthy controls (cytotoxicity index %: A549 cells, 30.06 ± 3.54 vs 11.77 ± 2.65, p = 0.002; HPEC, 30.52 ± 4.67 vs 11.35 ± 3.19, p = 0.005) (Fig 4 (A), (B)). For HUVEC, cytotoxicity indexes between patients and healthy control were not different significantly (17.8 ± 6.31 vs 10.55 ± 2.6, p = 0.06) (Fig 4 (C)).

Discussion

SARS is a new emerging infectious disease with global impact. The diagnosis is confirmed by the positive viral PCR, and patients were also found to have elevated IgG and IgM antibodies against SARS-CoV nucleocapsid protein at later phases. Although the pathogen has been identified, the underlying pathogenesis is yet to be determined. A prospective study by Peiris et al (2003a) concluded that the clinical progression of SARS had a tri-phasic pattern according to the clinical presentations and pathological changes. Phase I (the first week), characterized by fever, myalgia and other systemic symptoms was supposed to be the effect of viral rapid replication and cytolysis. As the disease progressed into phase II and phase III, the rates of viral shedding from nasopharynx, stood, and urine decreased gradually, however, severe clinical worsening often occurred at this time [Peiris et al., 2003a; Poon et al., 2004]. In addition to pulmonary damage, some autopsies also revealed systemic vasculitis [Ding et al., 2003; Lang et al., 2003]. Taken together with some therapeutic effects of immunoglobulin and steroids to block disease progression [Chiang et al., 2003; Ho et al., 2003], these findings suggest that the later phases of SARS are related to immunopathological damage.

Focusing on the immune-mediated pathogenesis after SARS-CoV infection, It was found in this study that some autoantibodies developed around one month after the

onset of the disease; including IgG anti-A549 cell antibodies, IgM anti-HPEC antibodies, and IgG anti-HUVEC antibodies. There are many well-established methods to detect antibodies against whole cells. The use of fluorescein-conjugated antisera to human immunoglobulins (indirect immunofluorescence staining) is the standard method with high specificity; however, this method is limited by low sensitivity [Lindquist, Osterland., 1971; Praprotnik et al., 2001; Tan, Pearson., 1972]. Cell-based ELISA is now the method used most widely. In this assay, whole cells from different sources are used as the substrate and fixed by glutaraldehyde treatment. The procedure of cell fixation may lead to false-positive results, probably because that autoantibodies reacting to intracellular antigens are also detected as well, therefore, the specificity of this method is limited [Meroni et al., 1995; Praprotnik et al., 2001]. Each test described above has its own advantages and limitations. In order to confirm the results of our study; we used these two methods to obtain and confirm the laboratory data.

Previous studies of AEpCA were limited to some certain epithelial cells from different tissue such as mucosal epithelial cells in pemphigus and recurrent oral ulcer, and intestinal epithelial cells in ulcerative colitis [Colon et al., 2001; Snook et al., 1991; Sun et al., 2000]. There is no literature concerning AEpCA detection in those disorders with pulmonary involvement using respiratory tract epithelial cells as target

cells. A549 cells, an easily available and commonly used human respiratory epithelial cell-line, were used as the substrate in this study. The results showed the development of IgG anti-A549 cell antibodies in SARS patients but not in patients with streptococcal necrotizing pneumonia, and this is the first report of the association between autoantibodies development and infectious pulmonary disorders. In contrast to AEpCA, anti-endothelial cell antibodies (AECA) are extensively studied. AECA have been found in a wide range of diseases, especially in systemic autoimmune diseases and primary autoimmune vasculitis [Carvalho et al., 1999; Grunebaum et al., 2002; Lee et al., 2003; Lin et al., 2003; Praprotnik et al., 2001; Toyoda et al., 1999; Yang et al., 2002]. Vasculopathy or vasculitis may develop after some viral infections including hepatitis C virus (HCV) [Cacoub et al., 1999], cytomegalovirus (CMV) [Toyoda et al., 1999], and dengue virus [Lin et al., 2003]. In these conditions, AECA could also be detectable. SARS primarily affects lung, but vasculopathy/vasculitis of other organs can also be found as the disease progresses [Ding et al., 2003; Lang et al., 2003]. This phenomenon indicates that SARS-CoV like HCV, CMV, or dengue virus may have the ability to damage vessels directly or indirectly, and this may be the reason why those IgM anti-HPEC antibodies and IgG anti-HUVEC antibodies could be detected in SARS patients. Another relevant finding in this study revealed that IgA anti-HPEC and IgA anti-HUVEC antibodies, although were not statistically increased

at phase I, decreased significantly when the disease progressed to phase II/phase III. This phenomenon may be explained by the invasion of SARS-CoV that activates mucosa-associated immune system, a specialized system for IgA globulins production, and induces the formation of IgA AECA that decline gradually when the viral load is decreased.

The mechanisms of these autoantibodies development in SARS patients were speculated: after the contact of SARS-CoV, possibly through the epithelial cell surface receptor recently identified as angiotensin-converting enzyme 2 [Li et al., 2003], the virus invades into the epithelial cells, and that can be directly observed by the electro microscope [Peiris et al., 2003b]. During the first week (phase I), SARS-CoV replicates rapidly and induces cytolysis. At the same time, macrophages accumulate around local inflammatory site; and these activated macrophages and other cells may release tumor necrosis factor α, interleukin-1, and other proinflammatory cytokines are increased after SARS-CoV infection [Beijing Group of National Research Project for SARS., 2003; Ng et al., 2004]. The damaged cells and the stimulation by proinflammatory cytokines may reveal some cryptic autoantigens. Macrophages infiltrated around the lesion may play another role as the antigen presenting cells, initiate the process of adaptive immunity, and lead to the formation of autoantibodies. However, patients with necrotizing pneumonia characterized by severe pulmonary

cells damage and increased proinflammatory cytokines did not have the same phenomenon. Another possibility may be that SARS-CoV shares some specific antigenic determinants with epithelial cells and endothelial cells individually. The antibodies primarily against the virus then cross-react with these cells due to molecular mimicry. Autoantibodies against cells like anti-endothelial cell antibodies (AECA) are functionally heterogeneous, most probably depending on their specificity [Bordron et al., 2001]. They may only be epiphenomenon of pulmonary epithelial and vascular injury, but they could also have pathogenic roles to cause further cellular damage by apoptosis, complement or antibody-dependent cytotoxic pathway [Bordron et al., 2001; Lin et al., 2003; Worda et al., 2003]. In SARS patients, it was found that those autoantibodies binding to epithelial cells and endothelial cells could activate the complement system and induce some of these cells lysis.

In summary, although more studies should be designed and performed to identify the disease-specific autoantigens, the presence of AEpCA and AECA after SARS-CoV infection may represent the severe pulmonary injury and vascular damage in these SARS patients. These autoantibodies also seem to have the potential to damage some epithelial cells and endothelial cells, and these reactions provide another immunological clue for a better understanding of the pathogenesis of SARS. Because of the possible pathogenic potential of these autoantibodies, it is suggested to

exclude the possibility of cross-reactions to these primary cells in the development of SARS vaccine.

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Legends

Figure 1. The comparisons of serum IgG, IgA, and IgM antibodies against SRAS-CoV nucleocapsid protein between SARS patients at phase II/phase III and healthy controls. The relative serum levels of these immunoglobulins were expressed as optical density (OD) values. (* $P < 0.05$, ** $P < 0.01$)

Figure 2. Quantitative analysis of serum levels of IgG, IgA, and IgM autoantibodies against (A) A549 cells, (B) human pulmonary endothelial cells (HPEC), and (C) human umbilical venous endothelial cells (HUVEC) in healthy controls (□) and SARS patients at phase I (hatched bars) and phase II/phase III (■). The levels of antibodies (ELISA ratios) are expressed as mean ± SEM. (* $P < 0.05$, ** $P < 0.01$)

Figure 3. Immunofluorescence analysis for the binding activities of (A) IgG antibodies against A549 cells, (B) IgM antibodies against HPEC, and (C) IgG antibodies against HUVEC in patients with streptococcal necrotizing pneumonia (2^{nd} row), healthy controls (3^{rd} row), and SARS patients at phase II/phase III (4^{th} row). PBS was used in this test as a control to eliminate non-specific bindings (1^{st} row).

Figure 4. Complement-dependent cytotoxicity assay. Sera from SARS patients (n = 9)

at phase II/phase III with autoantibodies and from healthy controls (n = 9) were used against (A) A549 cells, (B) HPEC, and (C) HUVEC. Low-Tox-M rabbit complement (1:20 dilution) was incubated with pre-inactived sera, and the cytotoxic effects were evaluated by the supernatant levels of lactate dehydrogenase. Data were calculated and expressed as cytotoxicity index (%). (* P = 0.001, ** P = 0.005)

Figure 1

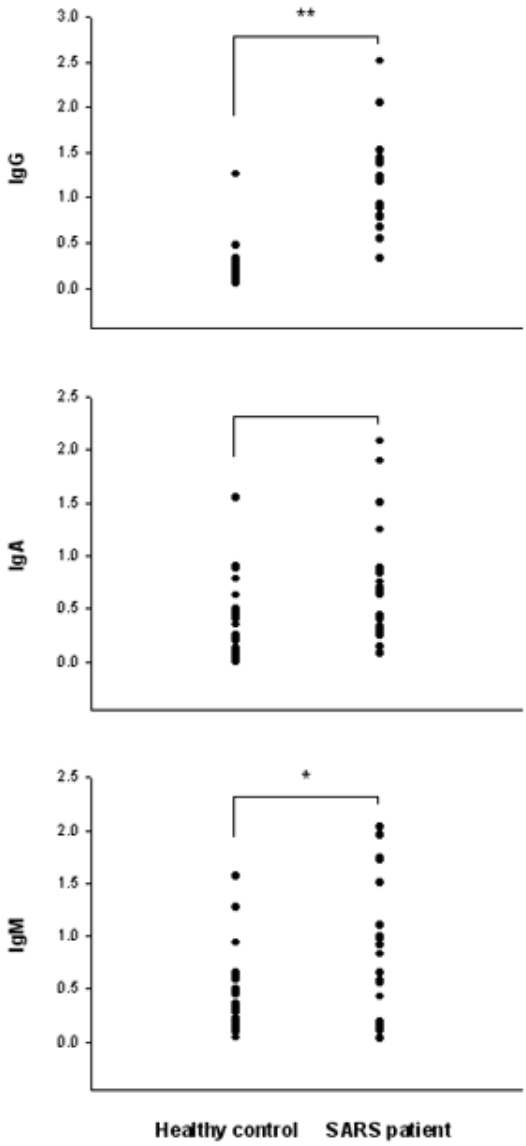

**
IgG
3.0
2.5
2.0
1.5
1.0
0.5
0.0
IgA
2.5
2.0
1.5
1.0
0.5
0.0
*
IgM
2.5
2.0
1.5
1.0
0.5
0.0
Healthy control
SARS patient

Figure 2

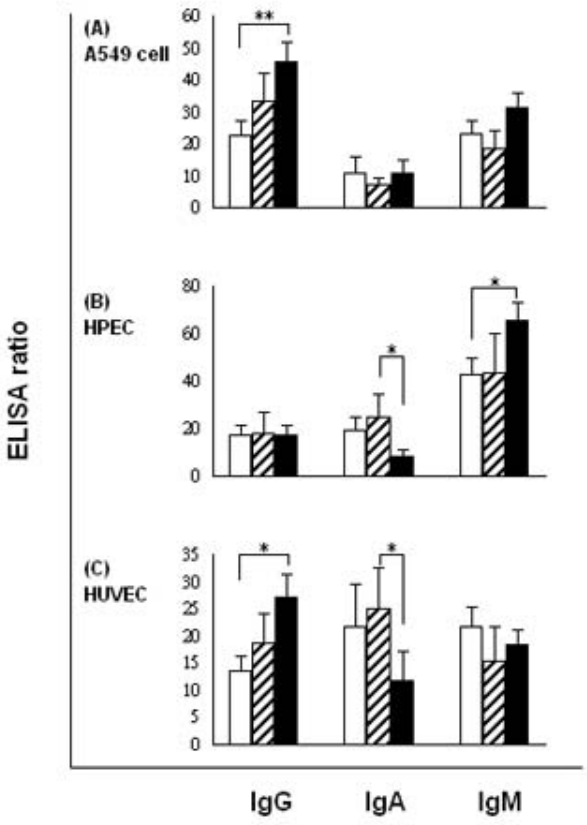


Figure 3

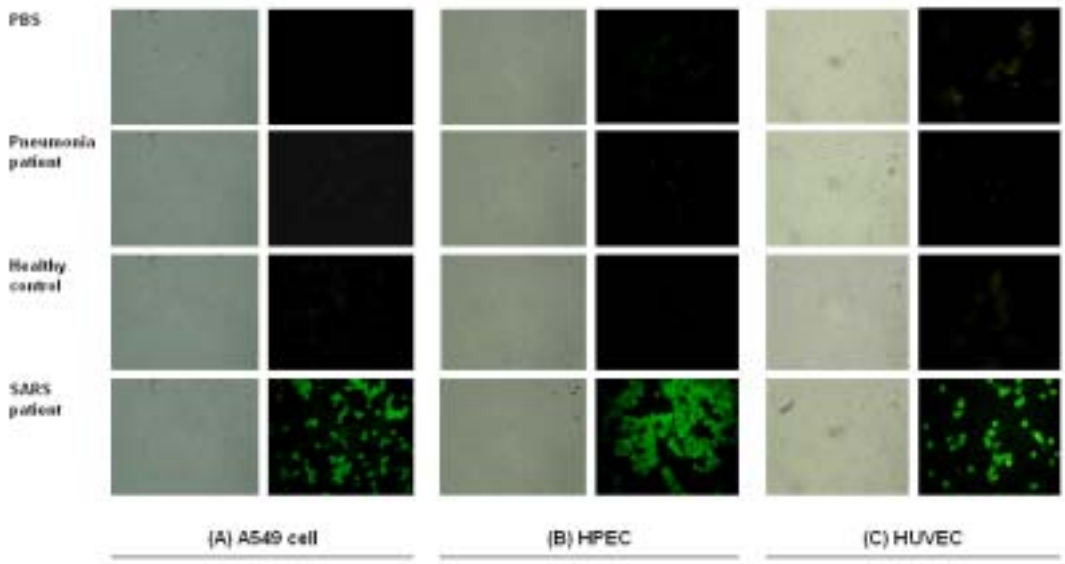
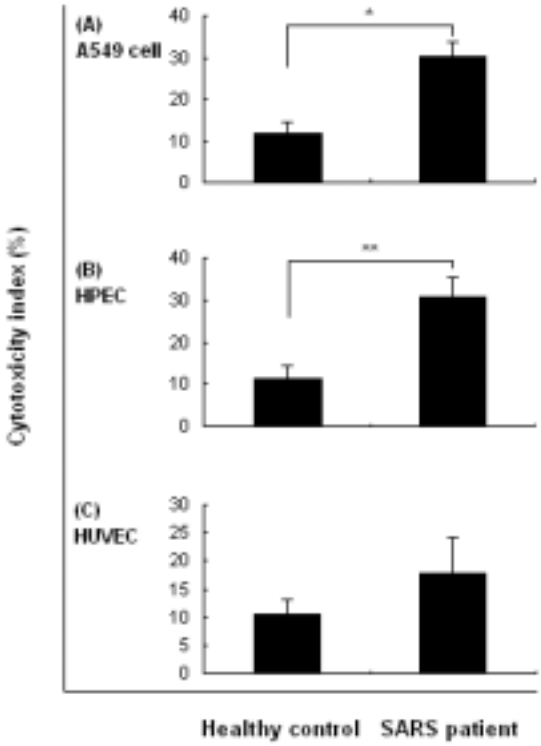

PBS
Pneumonia patient
Healthy control
SARS patient
(A) A549 cell
(B) HPEC
(C) HUVEC

Figure 4


(A)
A549 cell
(B)
HPEC
(C)
HUVEC
Cytotoxicity index (%)
Healthy control
SARS patient
*
**