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（子計畫四）SARS 病人抗肺部組織及細胞自體抗體之偵測

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嚴重急性呼吸道症候群的免疫致病機轉-（子計畫四）SARS 病人抗肺部組織及細胞自體抗體之偵測

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目錄

中文摘要

關鍵字：嚴重急性呼吸道症候群，自體抗體，內皮細胞，上皮細胞，細胞毒殺

嚴重急性呼吸道症候群 (SARS)是一種由 SARS-associated 冠狀病毒感染而引起肺部嚴重發炎，甚至纖維化的非典型性肺炎。在這個研究中，我們將探討感染此冠狀病毒後身體是否會產生抗血管內皮細胞及肺部上皮細胞之自體抗體。血清來自病人疾病不同時期(phase I, phase II/III)及健康對照組。目標細胞我們選用 1) A549 human pulmonary epithelial cell-line, 2) human umbilical venous endothelial cells (HUVEC), 3) primary human pulmonary endothelial cells (HPEC)。自體抗體的偵測是採用 cell-based ELISA, indirect immunofluorescence staining, 及 flow cytometry 三種方法。結果顯示不管用那一種方法，病人於 phase II/III 的血清中存 IgG anti-A549 cells antibodies, IgG anti-HUVEC antibodies, 及 IgM anti-HPEC antibodies 三種自體抗體。進一步的研究顯示 IgG anti-A549 cells 自體抗體具有 complement-dependent 細胞毒殺作用。本實驗結論：感染 SARS-associated 冠狀病毒後期，病人血清會產生抗內皮細胞及上皮細胞之自體抗體，而這些感染後產生的自體抗體所扮演的角色則須要更多的研究來確定。

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

Abstract

The severe acute respiratory syndrome (SARS), an atypical pneumonia emerged in 21st century, is caused by the invasion of the SARS-associated coronavirus (SARS-CoV) and characterized by severe pulmonary inflammation and fibrosis. In this study, we investigate the possibilities of the development of autoantibodies against human epithelial cells and endothelial cells in patients with SARS at different time periods (the first week: phase I, 1-2 months after the disease onset: phase II/phase III). Antibodies in sera of patients and normal 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 the methods of cell-based ELISA, indirect immunofluorescence staining, and flow cytometry. The results of ELISA 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. Results of the other two tests, indirect immunofluorescence staining and flow cytometry, were consistent with the previous one. Sera from SARS patients at phase II/phase III could mediate complement dependent cytotoxicity against A549 cells. 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 the post-infectious cellular injury and also provide the possibility of SARS-induced immunopathology.

報告內容

Introduction

In early 2003, a new infectious disease-severe acute respiratory syndrome (SARS) swept the world including Taiwan [1-2]. The pathogen was later identified as SARS-associated coronavirus (SARS-CoV) and spread soon among human beings through the close contact of droplets [3-4]. Those who infected by this virus present with persistent high fever, cough, dyspnea, and the disease may eventually progress to respiratory and/or multiple organs failure [1,5]. The 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 [6-7]. Besides, systemic vasculitis has also been 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 [7]. All these pathological changes are now thought to be mediated by direct viral destruction and followed by immune-mediated processes [8].

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 [9-11], 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 [12-17]. Although some of them appear as a result of inflammatory tissue injury, others are proved to 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 the methods of cell-based ELISA, indirect immunofluorescence staining, and flow cytometry, 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 according to the typical clinical presentations with fever, cough, and dyspnea, and positive viral PCR. Informed consents and institutional approval were obtained for this study. Blood was sampled during the first one-week (phase I) and one-two months after the disease onset (phase II or phase III) [8]. Twenty normal healthy adults were enrolled as controls. In the study of indirect immunofluorescence staining, patients with streptococcal necrotizing pneumonia were also recruited as controls. For safety, serum samples of patients derived from the acute stage 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, the 96-well microplate was coated with purified His-N protein at a concentration of 5 μg/ml. Each well was then blocked by PBS containing 0.05% Tween-20 (PBS/Tween 20) (Sigma) and 5%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 [18]. 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°C 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% bovine serum albumin (BSA)/0.05% azide/0.1 M Tris in ddH_2O) for 60 min at 37°C 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°C. 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°C. 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 phosphate buffered saline (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.

Flow cytometry analysis

A549, HUVEC and HPEC cells were detached from the culture plates and suspended at 2×10^5 cells for flow cytometry. Cells were washed once with FACS buffer (2% FCS/0.05% azide/0.5M EDTA in PBS) and incubated with 50 μl sera of SARS patients or healthy controls for one hour at 4℃. After washing with FACS buffer once, cells were incubated with 50 μl of FITC-conjugated antihuman immunoglobulins (1:100 in FACS buffer) for another one hour at 4℃, washed once again, and then fixed by 2% paraformaldehyde in FACS buffer. Isotype matched control mouse IgG was used to eliminate non-specific bindings. The binding activities of antibodies to cells were finally analyzed using FACSort (Becton Dickinson, USA).

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 analysed 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).

AEpCA and 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 statistically different 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 AECA detection by indirect immunofluorescence staining and flow cytometry

In the study of autoantibodies against A549 cells, Fig 3(A) showed that IgG anti-A549 cells 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. In AECA detection, although some binding activities of IgM anti-HPEC and IgG anti-HUVEC antibodies were detected in patients with necrotizing pneumonia, the intensity of immunofluorescence of these antibodies in SRAS patients at phase II/phase III was much higher than pneumonia patients and

healthy controls (Fig 3(B), (C)). By using another method of flow cytometry to re-check AEpCA and AECA, serum was interacted directly with the suspended target cells. The results also showed that the binding activities of IgG antiA549 cells antibodies, IgM anti-HPEC antibodies, and IgG anti-HUVEC antibodies in patients at phase II/phase III were higher than those in healthy controls (Fig 4).

SARS patient sera induce A549 cell lysis

In the present experiment, purified IgG and IgM immunoglobulins could not be available due to the limitations regarding blood sampling from SARS patients. Therefore, in order to investigate if these autoantibodies have the 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 greater A549 cell lysis than sera from healthy controls (cytotoxicity index: 45.43 ± 5.21 vs 29.74 ± 2.86 $p = 0.007$) (Fig 5).

Discussion

SARS is a new emerging infectious disease with global impact. The diagnosis could be confirmed by the positive viral PCR, and patients were also found to have positive serologic results with elevated IgG and IgM antibodies against SARS-CoV nucleocapsid protein at later phases. Although the pathogen has been identified, the real pathogenesis is to be determined. A prospective study from Peiris JSM, et al 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 [8,19]. In addition to pulmonary damage, some autopsies also revealed an impressive finding of systemic vasculitis [7,20]. Taken together with some therapeutic effects of immunoglobulin and steroid to block the disease progression [21-22], all these findings suggested that the later phases of SARS are related to immunopathological damage.

Focusing on the immune-mediated pathogenesis, after SARS-CoV infection, we found in this study that some autoantibodies developed around one-two months

beyond 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 [23-25]. Cell-based ELISA is now the method most widely used. 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 [25-26]. Another method is demonstrated by immunoglobulin binding to suspension of cells in a fluorescence-activated cell sorter (flow cytometry) analysis. This method needs a number of cells in suspension. Besides, the cell surface antigens are at high risk to be contaminated with nuclear and cytoplasmic components through the procedures of detachment of cells, grown in vitro, and enzymatic treatment [25,27]. Each test described above has its own advantages and limitations, in order to confirm the results of our study; we used three methods to re-check the laboratory data and gained consistent results.

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 [9,10,28]. There is no literature concerning about AEpCA detection in those disorders with pulmonary involvement using respiratory tract epithelial cells as target cells. In our study, we use A549 cells, an easily available and commonly used human respiratory epithelial cell-line, as the substrate. Our 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 in the study of the association between autoantibodies development and infectious pulmonary disorders. In contrast to AEpCA, AECA are extensively studied. AECA have been found in a wide range of diseases, especially in systemic autoimmune diseases and primary autoimmune vasculitis [12-17,25]. Vasculopathy or vasculitis would develop after some viral infection including hepatitis C virus (HCV) [29], cytomegalovirus (CMV) [16], and dengue virus [17]. 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 [7,20]. This phenomenon indicates that SARS-CoV like HCV, CMV, or dengue virus has the ability to damage vessels directly or indirectly, and this may be the reason why we could detect IgM anti-HPEC antibodies, and IgG anti-HUVEC antibodies in SARS patients. Another relevant finding in our 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 [30], the virus invades into the epithelial cells, and that can be directly observed by the electro microscope [31]. 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 that have been found increased after SARS-CoV infection [32-33]. 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 that also 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 virus then cross-react with these cells due to the mimic molecules. Autoantibodies against cells like AECA are functionally heterogeneous, most probably depending on their specificity [34]. 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 [17,34-35]. In SARS patients, we found that autoantibodies binding to epithelial cells could activate the complement system and induce 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. Among these autoantibodies, AEpCA have a pathogenic role to damage the epithelial cells, and this reaction provides 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.

References

1. Tsang KW, Ho PL, Ooi GC, Yee WK, Wang T, Chan-Yeung M, Lam WK, Seto WH, Yam LY, Cheung TM, Wong PC, Lam B, Ip MS, Chan J, Yuen KY, Lai KN. A cluster of cases of severe acute respiratory syndrome in Hong Kong. N Engl J Med. 2003;348:1977-85.
2. Twu SJ, Chen TJ, Chen CJ, Olsen SJ, Lee LT, Fisk T, Hsu KH, Chang SC, Chen KT, Chiang IH, Wu YC, Wu JS, Dowell SF. Control measures for severe acute respiratory syndrome (SARS) in Taiwan. Emerg Infect Dis. 2003;9:718-20.
3. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RA, Berger A, Burguiere AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Muller S, Rickerts V, Sturmer M, Vieth S, Klenk HD, Osterhaus AD, Schmitz H, Doerr HW. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med. 2003;348:1967-76.
4. Yeh SH, Wang HY, Tsai CY, Kao CL, Yang JY, Liu HW, Su IJ, Tsai SF, Chen DS, Chen PJ, Chen DS, Lee YT, Teng CM, Yang PC, Ho HN, Chen PJ, Chang MF, Wang JT, Chang SC, Kao CL, Wang WK, Hsiao CH, Hsueh PR. Characterization of severe acute respiratory syndrome coronavirus genomes in Taiwan: Molecular

epidemiology and genome evolution. Proc Natl Acad Sci U S A. 2004 [Epub ahead of print].

5. Fowler RA, Lapinsky SE, Hallett D, Detsky AS, Sibbald WJ, Slutsky AS, Stewart TE; Toronto SARS Critical Care Group. Critically ill patients with severe acute respiratory syndrome. JAMA. 2003;290:367-73.
6. Franks TJ, Chong PY, Chui P, Galvin JR, Lourens RM, Reid AH, Selbs E, McEvoy CP, Hayden CD, Fukuoka J, Taubenberger JK, Travis WD. Lung pathology of severe acute respiratory syndrome (SARS): a study of 8 autopsy cases from Singapore. Hum Pathol. 2003;34:743-8.
7. Ding Y, Wang H, Shen H, Li Z, Geng J, Han H, Cai J, Li X, Kang W, Weng D, Lu Y, Wu D, He L, Yao K. The clinical pathology of severe acute respiratory syndrome (SARS): a report from China. J Pathol. 2003;200:282-9.
8. Peiris JS, Chu CM, Cheng VC, Chan KS, Hung IF, Poon LL, Law KI, Tang BS, Hon TY, Chan CS, Chan KH, Ng JS, Zheng BJ, Ng WL, Lai RW, Guan Y, Yuen KY; HKU/UCH SARS Study Group. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. Lancet. 2003;361:1767-72.

9. Sun A, Hsieh RP, Liu BY, Wang JT, Leu JS, Wu YC, Chiang CP. Strong association of antiepithelial cell antibodies with HLA-DR3 or DR7 phenotype in patients with recurrent oral ulcers. J Formos Med Assoc. 2000r;99:290-4.
10. Snook JA, Lowes JR, Wu KC, Priddle JD, Jewell DP. Serum and tissue autoantibodies to colonic epithelium in ulcerative colitis. Gut. 1991;32:163-6.
11. Ablin RJ. Antiepithelial antibodies in prostatic cancer. Arch Dermatol. 1972;105:759.
12. Carvalho D, Savage CO, Isenberg D, Pearson JD. IgG anti-endothelial cell autoantibodies from patients with systemic lupus erythematosus or systemic vasculitis stimulate the release of two endothelial cell-derived mediators, which enhance adhesion molecule expression and leukocyte adhesion in an autocrine manner. Arthritis Rheum. 1999;42:631-40.
13. Grunebaum E, Blank M, Cohen S, Afek A, Kopolovic J, Meroni PL, Youinou P, Shoenfeld Y. The role of anti-endothelial cell antibodies in Kawasaki disease - in vitro and in vivo studies. Clin Exp Immunol. 2002;130:233-40.
14. Yang YH, Wang SJ, Chuang YH, Lin YT, Chiang BL. The level of IgA antibodies to human umbilical vein endothelial cells can be enhanced by TNF-alpha treatment in children with Henoch-Schonlein purpura. Clin Exp Immunol. 2002;130:352-7.

15. Lee KH, Chung HS, Kim HS, Oh SH, Ha MK, Baik JH, Lee S, Bang D. Human alpha-enolase from endothelial cells as a target antigen of anti-endothelial cell antibody in Behcet's disease. Arthritis Rheum. 2003;48:2025-35.
16. Toyoda M, Petrosian A, Jordan SC. Immunological characterization of anti-endothelial cell antibodies induced by cytomegalovirus infection. Transplantation. 1999;68:1311-8.
17. Lin CF, Lei HY, Shiau AL, Liu CC, Liu HS, Yeh TM, Chen SH, Lin YS. Antibodies from dengue patient sera cross-react with endothelial cells and induce damage. J Med Virol. 2003;69:82-90.
18. Jaffe EA, Nachman RL, Becker CG, Minick RC. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973;52:2745-56.
19. Poon LL, Chan KH, Wong OK, Cheung TK, Ng I, Zheng B, Seto WH, Yuen KY, Guan Y, Peiris JS. Detection of SARS coronavirus in patients with severe acute respiratory syndrome by conventional and real-time quantitative reverse transcription-PCR assays. Clin Chem. 2004;50:67-72.
20. Lang Z, Zhang L, Zhang S, Meng X, Li J, Song C, Sun L, Zhou Y. Pathological study on severe acute respiratory syndrome. Chin Med J (Engl). 2003;116:976-80.

21. Chiang CH, Chen HM, Shih JF, Su WJ, Perng RP. Management of hospital-acquired severe acute respiratory syndrome with different disease spectrum. J Chin Med Assoc. 2003;66:328-38.

22. Ho JC, Ooi GC, Mok TY, Chan JW, Hung I, Lam B, Wong PC, Li PC, Ho PL, Lam WK, Ng CK, Ip MS, Lai KN, Chan-Yeung M, Tsang KW. High-dose pulse versus nonpulse corticosteroid regimens in severe acute respiratory syndrome. Am J Respir Crit Care Med. 2003;168:1449-56.

23. Lindquist KJ, Osterland CK. Human antibodies to vascular endothelium. Clin Exp Immunol. 1971;9:753-60

24. Tan EM, Pearson CM. Rheumatic disease sera reactive with capillaries in the mouse kidney. Arthritis Rheum. 1972;15:23-8

25. Praprotnik S, Blank M, Meroni PL, Rozman B, Eldor A, Shoenfeld Y. Classification of anti-endothelial cell antibodies into antibodies against microvascular and macrovascular endothelial cells: the pathogenic and diagnostic implications. Arthritis Rheum. 2001;44:1484-94.

26. Meroni PL, Khamashta MA, Youinou P, Shoenfeld Y. Mosaic of anti-endothelial antibodies. Review of the first international workshop on anti-endothelial antibodies: clinical and pathological significance. Lupus. 1995;4:95-9.

27. Faulk WP, Rose M, Meroni PL, Del Papa N, Torry RJ, Labarrere CA, Busing K, Crisp SJ, Dunn MJ, Nelson DR. Antibodies to endothelial cells identify myocardial damage and predict development of coronary artery disease in patients with transplanted hearts. Hum Immunol. 1999;60:826-32.

28. Colon JE, Bhol KC, Razzaque MS, Ahmed AR. In vitro organ culture model for mucous membrane pemphigoid. Clin Immunol. 2001;98:229-34.

29. Cacoub P, Ghillani P, Revelen R, Thibault V, Calvez V, Charlotte F, Musset L, Youinou P, Piette JC. Anti-endothelial cell auto-antibodies in hepatitis C virus mixed cryoglobulinemia. J Hepatol. 1999;31:598-603.

30. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature. 2003;426:450-4.

31. Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, Nicholls J, Yee WK, Yan WW, Cheung MT, Cheng VC, Chan KH, Tsang DN, Yung RW, Ng TK, Yuen KY; SARS study group. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet. 2003 Apr 19;361(9366):1319-25.

32. Ng PC, Lam CW, Li AM, Wong CK, Cheng FW, Leung TF, Hon EK, Chan IH, Li CK, Fung KS, Fok TF. Inflammatory cytokine profile in children with severe acute respiratory syndrome. Pediatrics. 2004;113(1 Pt 1):e7-14.

33. Beijing Group of National Research Project for SARS. Dynamic changes in blood cytokine levels as clinical indicators in severe acute respiratory syndrome. Chin Med J (Engl). 2003;116:1283-7.

34. Bordron A, Revelen R, D'Arbonneau F, Dueymes M, Renaudineau Y, Jamin C, Youinou P. Functional heterogeneity of anti-endothelial cell antibodies. Clin Exp Immunol. 2001;124:492-501.

35. Worda M, Sgonc R, Dietrich H, Niederegger H, Sundick RS, Gershwin ME, Wick G. In vivo analysis of the apoptosis-inducing effect of anti-endothelial cell antibodies in systemic sclerosis by the chorionallantoic membrane assay. Arthritis Rheum. 2003;4:2605-14.

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. Cytofluorographic analysis for serum IgG anti-A549 cells antibodies (1^{st} row), IgM anti-HPEC antibodies (2^{nd} row), and IgG anti-HUVEC antibodies (3^{rd} row

in healthy controls and SARS patients at phase II/phase III by using of liquid-phase sandwich assay. Isotype matched control mouse IgG was used to eliminate non-specific bindings.

Figure 5. Complement-dependent cytotoxicity assay. Sera from SARS patients (n = 10) at phase II/phase III with autoantibodies and from healthy controls (n = 10) were used for this assay. 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 (%). (* $P < 0.05$)

附件：

Fig 1

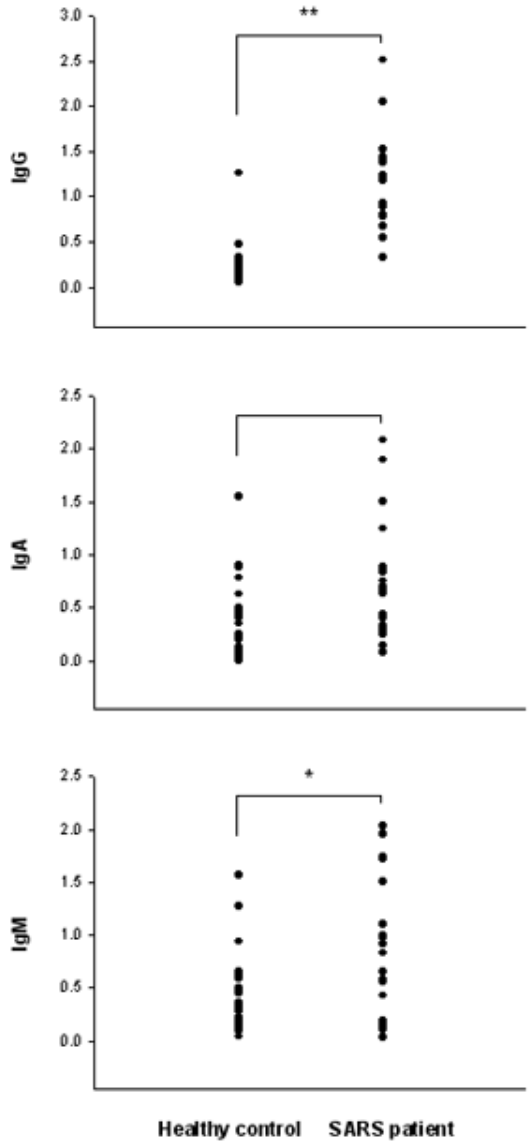

**
IgG
3.0
2.5
2.0
1.5
1.0
0.5
0.0
IgA
2.5
2.0
1.5
1.0
0.5
0.0
*
IgM
2.5
2.0
1.5
1.0
0.5
0.0
Healthy control
SARS patient

Fig 2

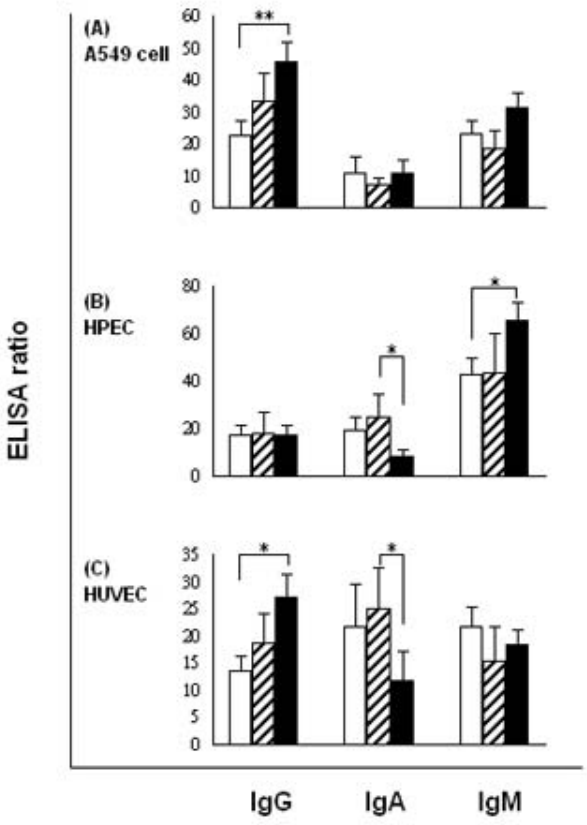


Fig 3

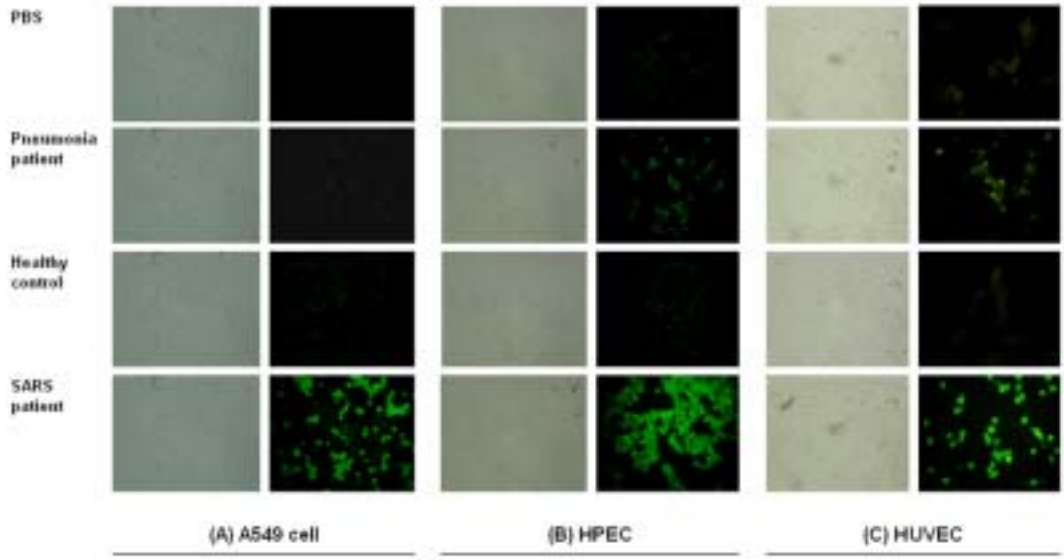

PBS
Pneumonia patient
Healthy control
SARS patient
(A) A549 cell
(B) HPEC
(C) HUVEC

Fig 4

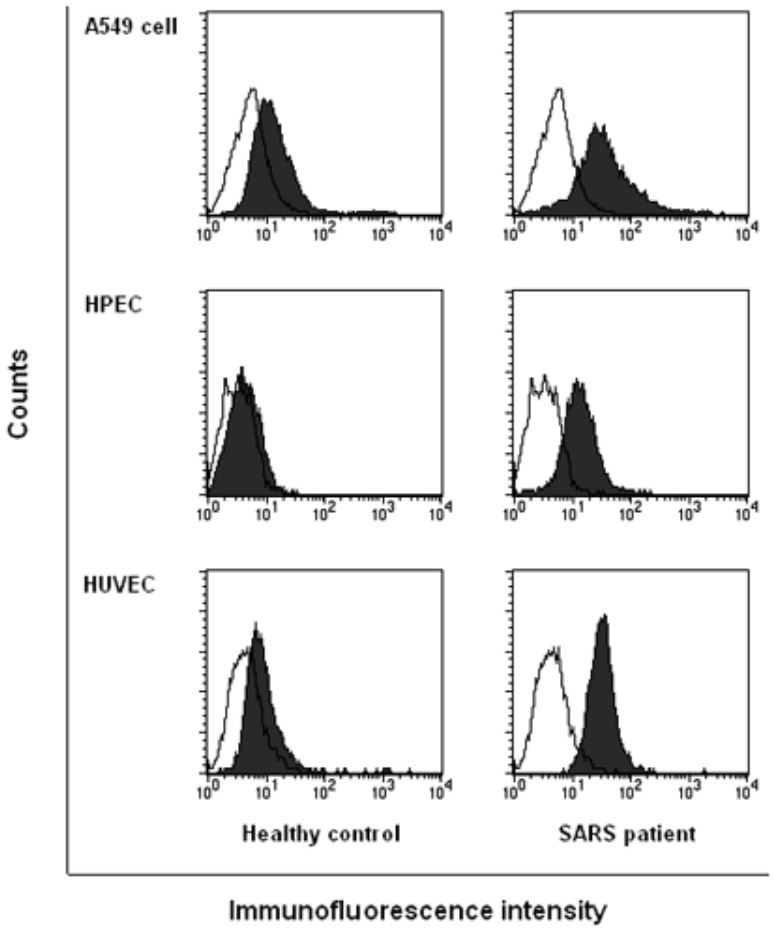
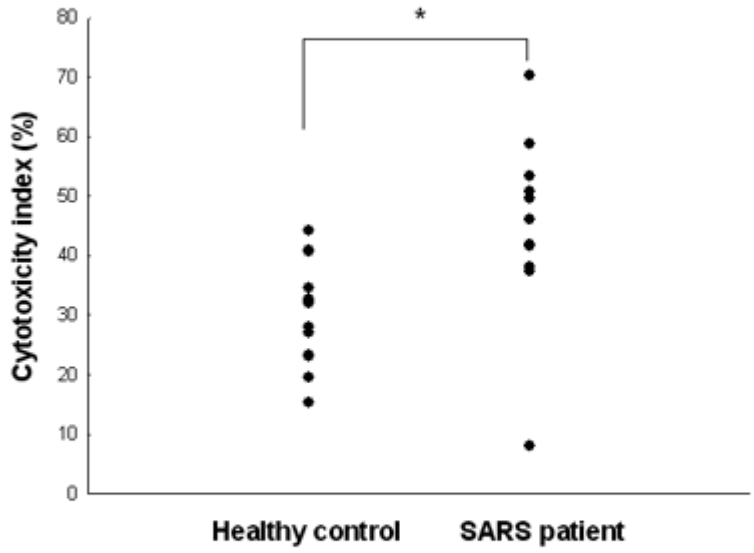

A549 cell
HPEC
HUVEC
Counts
Healthy control
SARS patient
Immunofluorescence intensity

Fig 5


*
80
70
60
50
40
30
20
10
0
Cytotoxicity index (%)
Healthy control
SARS patient