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（子計畫二）嚴重急性呼吸道症候群 SARS 與肺部發炎反應

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Title: Inflammatory Response in SARS

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Abstract

Severe acute respiratory syndromes (SARS) stimulate cells of the immune system to produce proinflammatory cytokines and chemokines which mediated acute lung inflammation. To investigate the immunopathogenesis of SARS, we infected a number of human cell lines with SARS-CoV. By immunoflourescent staining with sera from SARS patients, we identified A549 and THP-1 cell lines that are susceptible to the virus. SARS-CoV-infected A549 epithelial cell expressed adhesion molecules, P-selectin and VCAM-1. In addition, total RNA was extracted from the cells to assay for the expression of chemokines. Results of Multi-Probe RNase protection assay demonstrated that the THP-1 cell line expressed CXCL5, CXCL10, CCL2, CCL3, CCL4, and CXCL8 after SARS-CoV infection, while A549 the epithelial cell line expressed only CCL2. Comparing DC-SIGN transfected cells to their parental cell line; we demonstrated that expression of DC-SIGN did not change the kinetics of chemokines induced by SARS-CoV.

Chemotaxis assay showed that exposure of peripheral leukocytes to mixture of recombinant chemokines CXCL5, CXCL10, CCL2, CCL3, CCL4, and CXCL8, migration of neutrophils and monocytes out-competed lymphocytes. These data may explain why neutrophils and monocytes were dominant cell types in pulmonary inflammatory response in patients with SARS.

Introduction

Severe acute respiratory syndrome (SARS), a newly identified illness caused by human coronavirus SARS-CoV, has claimed 812 death worldwide (1). SARS in adult is characterized by an acute onset of fever with subsequent progression to pneumonia.(2, 3), absence of upper respiratory symptoms, new pulmonary infiltration on the chest radiography, the presence of lymphopenia, thrombocytopenia and high levels of proinflammatory cytokines, chemokines(4) and C-reactive protein (CRP) levels (5) in the sera. The clinical picture is highly suggestive of an abnormal pathological reaction to pulmonary viral infection, characterized by a cascade of immunological events leading to pulmonary inflammation and respiratory failure (6). The disease is fatal in about 10% of cases and resembles adult acute respiratory distress syndrome (ARD) (7). At the onset of fever, the majority of patients demonstrate air-space consolidation and death may result from progressive respiratory

failure due to alveolar damage. It is believed that there is a direct correlation between the high levels of chemokines and cytokines in serum-"cytokine storm", triggered by host immune response to SARS-CoV attributes to overwhelming pulmonary infiltration, diffuse alveolar damage and fibrosis(3). However, the type of cells that produce cytokines and chemokines still remains to be determined.

Histopathological evaluation of biopsies of lung tissue from autopsy patients with SARS showed diffuse alveolar damage with abundant infiltrates of foamy macrophages, polymorphonuclear phagocytes (8) and multinucleated synctial cells (9). By *in situ* hybridization, the viral nucleic acids in the lungs were shown to be associated with type II pneumocytes (8)as well as macrophages (10). In addition, a rapid decrease of peripheral T cell subsets is a unique characteristic in patients with SARS during acute infection (11). In recovering patients, a rapid and dramatic restoration of peripheral T cell subsets is seen (11). These results indicate that immune cell trafficking is a hallmark of SARS.

The purpose of this study was to investigate cellular response to SARS-CoV. The clinical, laboratory and histopathological findings strongly indicated that although the intestinal mucosa is involved in SARS, the most severe pathology is the lungs. We employed human lung epithelial carcinoma cell line (A549) and DC-SIGN-transfected monocytic cells (DC-SIGN-THP-1) to investigate chemokine and cytokine response to SARS-CoV. The results show that SARS-CoV induces CCL2 expression and the upregulation of P-selectin and VCAM-1 by lung epithelial cells. In addition, SARS-CoV also induces DC-SIGN-transfected THP-1 monoytic cells to express CCL5, CXCL10, CCL2, CCL3, CCL4 and CXCL8. Based on the results of our study, we propose the inflammatory response in the lungs of patients that follows SARS-CoV infection is as follows: (i) SARS-CoV infects pulmonary epithelial cells. (ii) The infected epithelial cells express high-affinity adhesion molecules VCAM and CCL2, a chemoattractant for monocytes to attract monocytes. (iii) The recruited monocytes in turn are induced to produce chemokines that attract polymorphonuclear phagocytes, T cells and more monocytes into the lungs, which results in overwhelming pulmonary infiltrates and subsequent tissue damage and eventual respiratory distress.

Material and methods

A549 Cell

A549 type II-like lung epithelial cells were purchased from the American Type Culture Collection, Rockville, MD. A549 cells are a cell line derived from a patient with alveolar cell carcinoma of the lung. These cells retain features of type II alveolar epithelial cells, including cytoplasmic multilamellar inclusion bodies and the synthesis of surfactant. A549 cells were grown as monolayers in tissue flasks or dishes incubated in 100% humidity and 5% CO 2 at 37 ^{o} C. Ham's F12K medium supplemented with fetal calf serum (FCS) (10%) was used as growth media. The cells from the monolayers were harvested with trypsin (0.25%) and ethylenediaminetetraacetic acid (EDTA) (0.1%) in phosphate-buffered saline (PBS), centrifuged at low speed (250 x *g,* 5 min), and resuspended in fresh medium before growing them in T75. Confluent monolayers of culture were used for experiments.

THP-1 cells and THP-1-DC-SIGN

THP-1 cells (American Type Culture Collection) at a concentration of 5x 10^5/ml were differentiated with 20 nM 12-*O* tetradecanoylphorbol 13-acetate (PMA) in Dulbecco's modified Eagle medium (DMEM) (BioWhittaker) with 10% fetal calf serum (FCS) (Life Technologies). Human AM were prepared as previously described (4, 52). Briefly, healthy volunteers underwent bronchoscopy with bronchoalveolar lavage with 100 ml of saline. Fluid was filtered to remove debris. Cells were spun at 800 x *g* for 5 min and resuspended in DMEM plus 10% fetal calf serum (FCS) at 5 x 10^5/ml.

Infection of cells with SARS-CoV

Monocytic THP-1 and epithelial cell line A549 were infected with SARS-CoV in the P3 facility in the National Taiwan University College of Medicine. Permission to perform experiments was obtained from the P3 Biosafety Committee. Cell lines were infected with SARS-CoV TW1 at 100 TCID and cultured at 37^{o}C. The cells were harvested at different time points after infection. Uninfected cells were left in culture and used as controls.

Immunoflorescence assay

Cells harvested from cultures were resuspended in 40 ml of PBS containing 2% heat-inactivated fetal bovine serum. The cell suspension was spotted onto multiwell slides. The slides were allowed to air dry before being fixed in cold acetone. Slides were then stored at -70^{o}C until staining. Fc fragments were applied to block FcR on theTHP-1. SARS virus-infected cells were detected by staining with SARS patient convalescent serum followed by FITC-conjugated rabbit anti-human Ig antibody.

FITC-conjugated anti-VCAM and anti-P-seletcin antibodies were used to detect the

expression of VCAM and P-selectin.

RNA preparation and semiquantitaive RT-PCR

Total RNA was extracted from cell lines using TRIzol (Life Technologies, Rockville, MD) according to the manufacturer's instruction. The cDNA was generated from 1 μg of total RNA with oligo(dT) primer using Superscript First-Strand Synthesis system for RT-PCR (Life Technologies), according to the protocol, and filled up to 80μg. A total of 4μl of cDNA solution was used for RT-PCR in a total volume of 40 μl containing 0.5 μM of sense and antisense primers. Quantitation of the expression of transcriptsof interest and of housekeeping transcripts was performed by a competitive RT-PCR technique using internal cDNA. The amount of transcripts of interest was related to the amount of housekeeping mRNA. Primer sequences, annealing temperatures, the number of cycles carried out, the size of the target amplification products, and the size of the internal standard amplification products are given:

P-selectin:5'-CCAGTGCTTATTGTCAGC-3',
5'-CACATTGCAGGCTGGAAT-3' ,35 cycles, 610 bp,
VCAM-1:5'-CCCTTGACCGGCTGGAGATT-3',
5'-CTGGGGGCAACATTGACATAAAGTG-3', 30 cycles, 241 bp
B-actin:5'-CCAGAGCAAGAGAGGCATCC-3'
5'-CTGTGGTGGTGAAGCTGAAG-3', 40 cycles, 436bp

Ribonuelease protection assay (RPA)

Total RNA from cells was isolated using TRIzol. The expression of various chemokines and cytokine were analyzed by multiprobe ribonuclease protection assay. Multiprobe templates were purchased from BD PharMigen, and the assay was performed according to the manufacturer's instructions. Probes for chemokine include lymphotactin, CCL5, CXCL10, MIP-1β, MIP-1α, CCL2, CXCL8, I-309; probes for cytokine include IL-12p35, IL-12p40, IL-10, IL-1α, IL-1β, IL-1 Ra, IL-6, IL-18, and IFN-γ. Briefly, the radiolabelled RNA probes were generated from the multiprobe templates in the presence of nucleotide pool (G, A, C, U) with [32P] UTP using T7 RNA polymerase. The probes were hybridized overnight with 10-20 μg total RNA, and digested with RNase mixtures followed by proteinase K digestion. RNase resistant double RNAs are extracted with phenol and precipitated with ammonium acetate. The RNA pellets were solubilized and resolved on 5% sequencing gel. Gels were dried and subjected to autoradiograph and phosphoimage analysis. The expression of each cytokine and chemokine is normalized against the housekeeping genes.

Isolation of PBL

PBMC were isolated from whole blood from all subject groups by sedimentation on Ficoll-Paque Plus, followed by centrifugation on a discontinuous lymphocyte layer undisturbed at the interface gradient. After separation, the cells were counted and resuspended at $1X10^6$ cells/ml in suspension buffer [RPMI-1640 medium supplemented with 0.5% (w/v) BSA]. To remove residual RBC, subject cells to hypotonic lysis by resupending each neutrophil/RBC pellet in 20 ml cold 0.2% NaCl for exactly 30 sec. At the end of this period, restore isotonicity by adding 20 ml ice-cold 1.6% NaCl. Centrifuge 6 min at 1000 rpm (250 X g), 5^oC. Discard supernatant. Repeat wash step until the cell pellets appear relatively free of RBC. Resuspend cells in ice-cold PBS/glucose and combine tubes. Adjust cell concentration in ice-cold PBS/glucose as desired and keep suspension in an ice bath until ready for chemotaxis assay.

Chemotaxis assay

Chemotaxis was assayed in 24-well plate (Nunc) having Transwell inserts of 5-μm pore size. Briefly, PBMC were washed twice and resuspended in RPMI 1640 medium containing 2% albumin and then loading onto inserts at 10^6 cells per 100μl for each well; 600μl RPMI 1640 medium containing the THP-1DC-SIGN culture supernatant at the indicated concentrations was placed in the button compartment. After difference time point of incubation at 37^oC with 5% CO_2, cells were scraped from the upper chamber and wash with phosphate-buffered saline (PBS) (100μl) to remove nonmigrated cells. This was followed by addition of PBS containing 2 mM EDTA. The filters were removed, and collect cells. Each sample was tested in triplicate. The cell was mounted on a glass slide, and the PMN that had migrated were counted under a light microscope. A minimum of 300 total cells were counted. The extent of migration was expressed as the migration rate, which was calculated as the number of cells per high power field migrated cells/total number of cells counted. Each stimulus was applied in duplicate.

Results

Pulmonary epithelial cells are targets of SARS-CoV. Epithelial cells of different origin NL-20, NCI-H292, and A549 were infected with SARS-CoV at 100 TCID to test for their permissiveness to infection. At different time points after infection, cells were fixed and stained by convalescent sera from SARS patients. Results show that A549, a pulmonary epithelial cell line, but not NL-20 or NCI-H292 stained positive by SARS patient convalescent sera. It was noted that SARS-CoV infection

did not cause cytopathic effect in A549 cells, respectively, at day 7 after infection.

Pulmonary epithelial A549 cells express adhesion molecules after SARS-CoV infection. We then examined whether SARS-CoV induces changes in infected epithelial cells. We showed that SARS-CoV infected A549 cells expressed VCAM-1 and P-selectin. VCAM-1 and P-selectin mRNA expressions were upregulated at 24 h, respectively, after infection.

Pulmonary epithelial A549 cells express CCL2 after SARS-CoV infection. In addition to the expression of adhesion molecules, we went on to determine whether infected-A549 cells express chemokines. Results of RPA show that the expression of CCL2 and CXCL8 but not other chemokines were upregulated in A549 cells at day 1 after SARS-CoV infection. However, it returned to the level comparable to that in control uninfected cells at day 2 after infection, indicating CCL2 and CXCL8 expression was transient.

DC-SIGN transfected THP-1are targets for SARS-CoV. Since CCL2 chemoattracts monocytes, we reasoned that monocytic cells might be the second population of cells that might be affected by SARS-CoV. It has been reported that SARS-CoV S protein binds to DC-SIGN; we employed DC-SIGN transfected THP-1 to test their susceptibility to SARS-CoV. Our data shows that THP-1 was permissive for SARS-CoV infection, but the infectivity was much higher in DC-SIGN-transfected THP-1 cells. The results were confirmed by mouse anti-SARS-CoV antibody.

DC-SIGN-transfected THP-1 cells express multiple chemokines and inflammatory cytokines after SARS-CoV infection. RNA extracted from infected THP-1 cells was subject to RPA to determine chemokine expressions. Our data show that SARS-CoV infection induced the expression of multiple chemokines, including CCL5, CXCL10, CCL4, CCL3, CCL2, and CXCL8 but not CCL1 at as early as day 1 after infection. Multiple chemokine expression peaked at day 1 and sustained until day 3. Interestingly, these chemokines include those that are chemoattractansts for T cells (CCL5, CXCL10), monocytes (CCL2, CCL3, and CCL4) and neutrophils (CXCL8). Moreover, SARS-CoV infection induced monocytic cells to express IL-1β, IL-1Ra and IL-6 but not IL-12, IL-10 or IFN-γ. The peak of expression was at day 1 of infection. While the expression of IL-1Ra and IL-6 returned to a level comparable to the control, IL-1β expression remained upregulated up to day 3 of infection.

Neutrophils and monocytes out-compete other cells in chemotaxis. To model the recruitment of leukocytes to tissues where multiple chemokines were present, we exposed peripheral blood leukocytes to a mixture of chemokines in a transwell chamber. In 15 min after exposure to a mixture of CCL5, CXCL10, CCL2, CCL3, CCL4, and CXCL8, neutrophils and monocytes in the peripheral blood leukocytes migrated across the well. By 30 min after exposure, neutrophil and monocyte migration in wells with mixed chemokines were significantly higher than that in wells without chemokine. The cell number continued to increase up till 60 min after exposure. However, the number of lymphocytes migrated across was not different in wells with or without chemokines. These data indicated that neutrophils and monocytes out-compete lymphocytes in response to a mixture of chemoattractants for neutrophils, monocytes and lymphocytes. These results implied that neutrophils and monocytes are the first wave of cells that migrate to the lungs of SARS patients even in the face of multiple chemokines.

Conclusion

The results of this study showed that in lungs of patients with SARS, CCL2 and CXCL8 are major chemokines produced by infected epithelial cells. Monocytic cells that chemoattracted by CCL2 can be infected by SARS-CoV. The infected monocytes in turn produce chemokines to attract other cells. By chemotaxis assay, we deomonstrated that in the presence of mixed chemokines that were produced SARS-CoV-infected monocytic cells, neutrophils and monocytes outcompete other cells. These data may explain the reason for the presence of large number of neutrophils and monocytes in pulmonary inflammatory response in SARS patients.

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