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（子計畫二）SARS 病毒感染與 T 細胞趨化性遷移

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In vitro Study of SARS Virus Infection and Cell Migration

Yu-Ting Yen and Betty Wu-Hsieh

Gradute Instite of Immunology, National Taiwan University College of Medicine

Abstract

To investigate the immunoapthogenesis of SARS, we infected a number of human cell lines with SARS-CoV. By immunoflourescent staining with sera from SARS patients, we identified one monocytic cell line that is susceptible to the virus. Total RNA was extracted from the cells to assay for the expression of chemokines. Results of Multi-Probe RNase protection assay demonstrated that the monocytic cell line expressed a panel of chemokines after SARS-CoV infection, while the epithelial cell line expressed only one. In addition, SARS-CoV-infected epithelial cell expressed adhesion molecules. Western blot analysis showed that the monocytic as well as the epithelial cells express ACE-2, a putative SARS-CoV receptor. 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.

Based on our data, we concluded that both human epithelial cells and monocytic cells are hosts for SARS-CoV. The possible scenario of severe acute respiratory distress that occurs in patients infected by SARS-CoV is as follows :(1) SARS-CoV infects pulmonary epithelial cells; (2) The infected epithelial cells express adhesion molecules and produce chemokine to attract monocytes; (3) The recruited monocytes in turn are infected by SARS-CoV to produce a panel of chemokines; (4) These chemokines are important in recruiting T cells, neutrophils and more monocytes into the lungs, which result in tissue damage and eventual respiratory distress.

Introduction

The disease of severe acute respiratory syndrome (SARS) has killed more than 750 people worldwide and infected more than 8300 since it first appeared in Guangdong Province late 2002. Absence of upper respiratory symptoms, new pulmonary infiltration on the chest radiography, the presence of lymphopenia and leucopenia, and high levels of proinflammatiry cytokines in the sera, are some outstanding features that suggest the SARS pathology is immune-mediated. However, it is not clear what induces the strong inflammatory response in patients infected with the SARS virus, a noval coronavirus, distantly related to the known coronaviruses.

Methods

Infection of cells with SARS-CoV

Monocytic cell line P and epithelial cell line A 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 Biosafty Committee. Cell lines were infected with SARS-CoV TW1 at 100 $TCID_{50}$ 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 μl 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 the cell line P. 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.

· **Western blotting**

Total protein was extracted in Laemmli Sample Buffer (Bio-Rad) and applied to SDS gel electrophoresis. The electrophoretically separated proteins were blotted onto nitrocellulose membrane and hybridized by purified anti-ACE2 antibody.

· **Ribonuelease protection assay (RPA).**

Total RNA from cells was extracted with TRIzol. The expression of various chemokines and cytokine were analyzed by multiprobe ribonuclease protection assay. Multiprobe templates was purchased from BD PharMigen, and the assay was performed according to the manufacturer's instructions. Probes for chemokines include lymphotactin, RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, IL-8, I-309. Briefly, the radiolabelled RNA probes were generated from the multiprobe templates in the presence of nucleotide pool (G, A, C, U) with [^{32}P] 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-stranded RNAs were extracted with phenol and precipitated with ammonium acetate. The RNA pellets were solubilized and resolved on 5% sequencing gel. Gels were dried and subject to autoradiograph and phosphoimage analysis. The expression of each cytokine and chemokine was normalized against the housekeeping genes.

Results

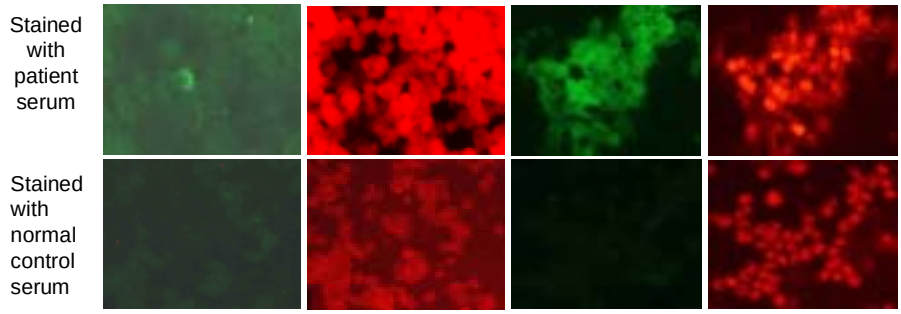
Susceptibility of cell line A and cell line P cells to SARS-CoV infection

Several established cell lines of human epithelial cells and monocytic leukemia and macrophage cell lines were investigated for their permissiveness to SARS-CoV

infection. Cell lines were infected with 100 $TCID_{50}$ of SARS-CoV. At different time points after infection, cells were fixed and stained with SARS patient serum. Before staining, Fc block was used to block non-specific binding of human immunogobulin to FcR on macrophages of monocytes. None of them showed cytopathic effects during the 7 days of incubation. Real time PCR and indirect immunofluorescence staining revealed the presence of SARS-CoV in cell line A and cell line P cells, whereas all other cell lines were virus-free after 7 days of incubation. Indirect immunofluorescence staining also indicated the presence of viral antigens in the cytoplasm of infected cell line A and P cell. SARS-CoV antigens were appeared as fluorescence green, whereas the counter stain appeared red. While no morphological changes of infected cell line A and P cells could be observed with light microscopy at magnification of 400x.

Figure 1

Susceptibility of cell line P and DC-SIGN transfected cell line P to SARS-CoV infection



Expression of adhesion molecules by alveolar epithelial cells

The expression of VCAM-1 by epithelial cells was reported for bronchial and renal epithelium(Garner et al., 1994; Oertli et al., 1998), whereas contradictory results were reported for alveolar epithelial cells(Cunningham and Kirby, 1995; Cunningham et al., 1994). Because adhesion of neutrophils and monocytes to epithelial cells occurs via adhesion molecules, we then decided to study the expression of VCAM-1 and P-selectin on the cell surface of SARS-CoV-induced cell line A cells using RT-PCR and immunofluorescence. The results showed VCAM-1 expression was upregulated at as early as 24 hours after infection, and the expression increased thereafter. The results showed P-selectin expression was upregulated at as early as 6 hours after

infection, and the expression increased thereafter.

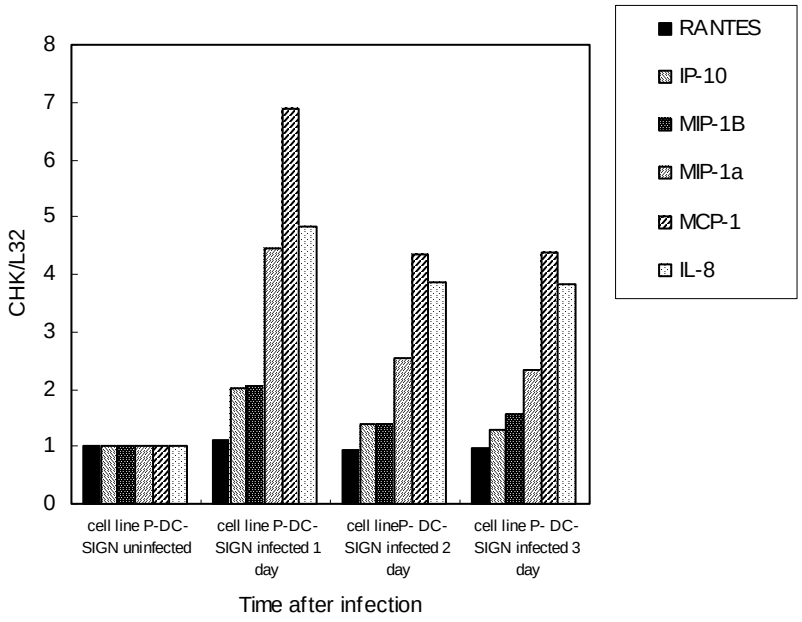
SARS-CoV induce MCP-1 production by cell line A

MCP-1 production by cell line A at 1 day after SARS-CoV infected by RPA assay.

SARS-CoV induce chemokines production by cell line P transfected DC-SIGN

SARS-CoV infection of monocyte, induce cytokines and chemokines mRNA expression. As shown in Figure 2 mRNA expression of RANTES, IP-10, MIP-1β, MIP-1α, MCP-1, and IL-8 were optimally up-regulated in the infected cell at day 1. After this time point, mRNA levels of these cytokines and chemokines returned to basal level. These data indicate that SARS-CoV infection of monocytic cells induces chemokines that chemoattract T cells, NK cells, and monocyte.

Figure 2



Expression of ACE-2 in cell line A and cell line P and other Non-permissive cell lines

In view of the current evidence of ACE-2 as the cellular receptor for SARS-CoV, we investigated the relationship between permissiveness to SARS-CoV infection and ACE-2 expression in these lung epithelial cell and monocytic cell lines. Our data show that ACE-2 was expressed on cell line P.

Conclusion

1. **SARS-CoV infects epithelial cells of pulmonary origin.**

2. **The infected epithelial cells express adhesion molecules and express chemokine that attract monocytes.**
3. **Monocytes are infected by SARS-CoV and express a panel of chemokine that are important in recruiting T cells, neutrophils and monocytes.**