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Identification of human cell line model of persistent SARS coronavirus infection and studies of the response to cytokines and chemokines

Key Messages

1. The human intestinal cell line LOVO, human lung epithelial cell lines NCI-H1650 and NCI-H1563, and normal nasopharyngeal epithelial cell lines NP69 and NP460 were permissive to SARS coronavirus (SARS-CoV) infection.
2. These human cell lines could be useful for future in vitro investigations of SARS.
3. The cytokine profiles induced following SARS-CoV infection varied among different cell lines, suggesting that the cellular response to SARS-CoV infection was tissue specific and may also correlate with the degree of permissiveness.
4. The results provide important insights into possible pathogenic mechanisms and potential novel therapeutic targets such as CXCL10/IP-10 in SARS.

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Introduction

Severe acute respiratory syndrome (SARS) is typified by its high infectivity, morbidity and mortality caused by a new SARS coronavirus (SARS-CoV).[1] The pathogenesis of SARS remains unclear, and therefore the current choices for specific, effective treatments remains limited.

Clinically, SARS gave rise to a predominantly lower respiratory tract illness. Diarrhoea and other organ dysfunction were also common. Based on our virological, ultrastructural, and in situ hybridisation (ISH) studies in human tissues, we identified persistent SARS-CoV infection in human lung pneumocytes and intestinal enterocytes, but not in other organs or cell types,[2] indicating both lung and intestinal epithelial cells in the pathogenesis of SARS. For practical reasons, animal studies were not feasible in most laboratories and had limitations and may not be relevant to the human situation. Identification of relevant human cell line models of SARS-CoV infection greatly facilitates further laboratory investigations into the virology of SARS-CoV, pathogenesis and various therapeutic approaches. We identified persistent SARS-CoV infection in human lung pneumocytes and intestinal enterocytes, and a human intestine cell line that can maintain persistent SARS-CoV infection. It was not clear whether other cell types, in particular in the human upper respiratory tract, could be infected by SARS-CoV. We investigated the susceptibility of human lung, intestinal, and nasopharyngeal epithelial cell lines to SARS-CoV infection, in order to identify in vitro experimental models for SARS studies.

Altered inflammatory or immune responses have been implicated in the pathogenesis of SARS and in its various manifestations. Thus, the cellular expression profiles of cytokines/chemokines induced by SARS-CoV were investigated in the selected permissive cell line models. These findings may enhance our understanding of the pathogenic mechanisms of this new human infectious disease and provide insights into the identification of potential therapeutic targets. We aimed to (1) identify human lung and intestinal epithelial cell lines that can maintain persistent SARS-CoV infection, and (2) study the expression profiles of cytokines and chemokines in cell lines infected with SARS-CoV.

Methods

This study was conducted from January 2005 to January 2006.

The study samples were human cell lines. The tested cell lines included: (1) lung epithelial cells (including lines derived from normal epithelial cells and lung adenocarcinoma): HBE4-E6/E7, NCI-H23, NCI-H522, NCI-H1563, NCI-H1650, NCI-H526, NCI-H520, NCI-H292; (2) intestine cell lines (including lines derived from intestinal adenocarcinoma): LS-180, SW-480, SW-620, HT-29, DLD-1, LOVO, HCT-116; (3) nasopharyngeal epithelial cell lines (including immortalised normal nasopharyngeal epithelial cell lines and nasopharyngeal carcinoma cell lines): C666-1, HONE1, HK1, NP69, NP460. NP69 and NP460

were kindly provided by Dr SW Tsao, Department of Anatomy, University of Hong Kong.

Viral preparation

The CUHK-W1 strain of SARS-CoV (GenBank accession no AY278554) was grown in Vero cells and the third passage at a concentration of $5x10^6$ 50% tissue culture infective dosed ($TCID_{50}$)/mL was kept at -70°C for experiments.

Inoculation with SARS-CoV

Cell lines were maintained in recommended media and incubated in a 37ºC, humidified, 5% CO_2 incubator. Cells at 60 to 70% confluence in 25-cm^2 flasks were inoculated with 300 μL of virus suspension to provide a multiplicity of infection (MOI) of 10. Inoculated cells were maintained at 37ºC for 7 days. A mock infection was performed in parallel for each cell line. The cells were examined daily for cytopathic effects (CPE). Cells were harvested after 7 days of incubation for virus detection. All laboratory investigations related to viral culture were performed in a class-III viral laboratory.

Indirect immunofluorescence assay

Intracellular viral antigens were detected by standard indirect immunofluorescence staining, using hybridoma fusion mouse-anti-SARS antibody as described previously.[3]

In situ hybridisation

In situ hybridisation was developed for the detection of SARS-CoV as described previously.[2]

Electron microscopy

Cell pellets harvested after 7 days of SARS-CoV incubation were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and processed for examination by transmission electron microscopy.

Quantitative real-time detection of SARS-CoV by reverse transcriptase–polymerase chain reaction

Cells at 60 to 70% confluence were inoculated with SARS-CoV at 10, 1 and 0.1 MOI, respectively. The concentration of virus in cell culture supernatant was monitored by quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) as described previously.[3]

Expression profile of cytokines and chemokines in permissive cell lines

The expression profile of the cytokines, chemokines and their receptors was investigated in selected cell lines confirmed to be SARS-CoV permissive. Cells were harvested at different time points (0, 6 and 24 hours after SARS-CoV inoculation) and total RNA was prepared using Qiagen RNeasy RNA isolation kit (QIAGEN). cDNA synthesis was carried out using Superscript III reverse transcriptase (Invitrogen). For quantitative real-time RT-PCR analysis, either the Taqman PCR Master Mix or the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used. The reaction was performed using specific primers for CXCL13/BCA-1, CCL2/MCP-1, CCR2, CXCL14/BRAK, CXCR5, IFN-γ, IL-10, IL-12α, IL-12b, IL-18, IL-α, IL-1b, IL-6, CXCL8/IL-8, IL-8Rα, IL-8Rβ, CXCL10/IP-10, CXCL9/MIG, CXCL12/SDF-1, TGF-β1, TGF-β2, TNFα, CCL5/RANTES, CCL20/MIP-3α, CXCR4 and run on an ABI 7900 Sequence Detection System. All reactions were performed in triplicate. Relative levels of expression were normalised, using GAPDH as an internal reference control, and calculated using the 2[-ΔΔC(T)] method for comparing the mRNA amount of each sample to that of time zero.

Results

Susceptibility of human intestinal, lung and nasopharyngeal epithelial cell lines to SARS-CoV infection

The susceptibility of the tested cell lines is summarised in Table 1. Of the seven human intestinal cell lines tested (LS-180, SW-480, SW-620, HT-29, DLD-1, LOVO, and HCT-116), only LOVO showed permissiveness to SARS-CoV infection. None of the infected intestinal cell lines showed CPE during the 7 days of incubation. Indirect immunofluorescence staining indicated the presence of viral antigens in the cytoplasm of 75% of infected LOVO cells. In situ hybridisation also revealed the presence of SARS-CoV RNA in LOVO cells, whereas all other cell lines were virus-free after incubation (Fig). Under electron microscopy, the infected LOVO cells contained cytoplasmic vesicles, consistent with dilated endoplasmic reticulum and Golgi apparatus, which were packed with numerous spherical viral cores at different stages of maturation; mature virus particles sizes ranged from 80 to 120 nm. In situ hybridisation, indirect immunofluorescence, and ultrastructural studies confirmed the permissiveness of LOVO cells to SARS-CoV.

Out of eight cell lines from human lung tissue (HBE4-

Table 1. Susceptibility of cell lines to SARS-CoV inoculation

Cell line	Organ	Cytopathic effects (day 7)	% of infected cells
Vero	Kidney	++	70
NCI-H1563	Lung	None	50
NCI-H1650	Lung	None	80
NCI-H23	Lung	None	5
NCI-H292	Lung	None	5
NCI-H520	Lung	None	5
NCI-H522	Lung	None	5
NCI-H526	Lung	None	5
HBE4-E6/E7	Lung	None	5
C666-1	Nasopharynx	None	5
Hone-1	Nasopharynx	None	5
NP69	Nasopharynx	+++	60
NP460	Nasopharynx	++	5
HK-1	Nasopharynx	None	0
SW620	Colon	None	5
SW480	Colon	None	15
LOVO	Colon	None	75
HCT-116	Colon	None	0
DLD-1	Colon	None	0
LS180	Colon	None	5
HT-29	Colon	None	0

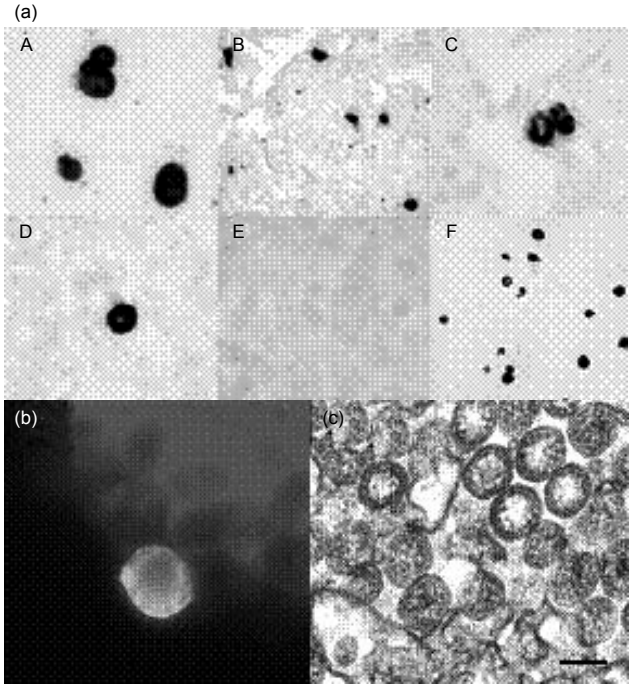

Fig. (a) In situ hybridisation analysis of cells inoculated with SARS-CoV. Presence of SARS-CoV RNA is shown by dark spots. A: NCI-H1650, B: LOVO, C: NP69, D: NP460, E: negative control, F: Vero (positive control). (b) Indirect immunofluorescence staining: SARS-CoV infected cells (LOVO) indicating presence of SARS-CoV antigens is shown as a light spot. (c) Viral particles in dilated vesicles are seen in ultra structural examination (LOVO cell). The cross bar represents 100 nm

E6/E7, NCI-H23, NCI-H522, NCI-H1563, NCI-H1650, NCI-H526, NCI-H520, NCI-H292), none showed CPE after 7 days of SARS-CoV infection at a MOI of 10. Indirect immunofluorescence staining at day 7 indicated the presence of viral antigens in over 80% of SARS-CoV–infected NCI-H1650 cells and 50% of NCI-H1563 cells. Viral RNA was also detected by ISH in infected NCI-H1650 cells (Fig). The other six cell lines were negative for SARS-CoV by immunofluorescence and ISH.

Inoculation of SARS-CoV at a MOI of 10 produced CPE in two immortalised normal nasopharyngeal epithelial cell lines (NP69 and NP460) at day 7. Whereas no CPE was observed in all three nasopharyngeal carcinoma cell lines: C666-1, HK-1 and HONE-1. After 7 days of inoculation, indirect immunofluorescence staining was performed to confirmed viral replication in NP69 and NP460 cells. Greater than 60% of NP69 and around 5% NP460 cells showed positive immunofluorescence for SARS-CoV antigen. In situ hybridisation demonstrated that SARS-CoV infected NP69 and NP460 cells contained detectable viral RNA (Fig).

Expression profiles of selected cytokines, chemokines, and their receptors in SARS-CoV infected by LOVO, NCI-H1650, NP69, and NP460 cells

Four cell lines demonstrated permissive infection of SARS-CoV, namely LOVO, NCI-H1650, NP69 and NP460 cells. They were selected for expression profiling of selected cytokines, chemokines, and their receptors. The selected significant findings were summarised in Table 2.

α-Chemokine CXCL10/IP-10 and CXCL9/MIG are potent chemoattractants for activating T and NK cells. Up-regulation of IP-10 and MIG mRNA expression was detected in all four cell lines. The up-regulation was detected at 6 hours after SARS-CoV infection, and remained at a high level at 24 hours. Notably, in cell lines more susceptible to SARS-CoV infection, higher expression of CXCL10/IP-10 and CXCL9/MIG was detected. The expression level of CXCL10/IP-10 and CXCL9/MIG at 24-hour post-inoculation correlated with the proportion of infected cells determined by indirect immunofluorescence assay (CXCL10/IP-10, P=0.02, CXCL9/MIG, P=0.001). The ubiquitous up-regulation of IP-10 and MIG after SARS-CoV inoculation independent of cell types suggested that it might be a general response to viral infection in susceptible cells.

In NCI-H1650 cells, the CXCL8/IL-8 and CCL20/MIP-3α level were 70-fold and 112-fold higher, respectively, at 6-hour post-inoculation, and remained at a higher level (13-fold and 9-fold, respectively) at 24-hour post-inoculation. A transient surge of CXCL8/IL-8 and CCL20/MIP-3α was also observed 6 hours after SARS-CoV inoculation

Table 2. Cytokines/chemokines gene expression levels in SARS-CoV–infected human epithelial cells by quantitative reverse transcriptase–polymerase chain reaction

	NCI-H1650		LOVO		NP460		NP69	
	6 hours	24 hours	6 hours	24 hours	6 hours	24 hours	6 hours	24 hours
CXCL10/IP-10	82.28 (6.26)	156.74 (11.42)	2.77 (0.78)	7.07 (4.67)	4.69 (2.21)	5.37 (2.69)	2.29 (0.71)	1.88 (0.30)
CXCL9/MIG	2.85 (0.29)	9.11 (0.39)	4.03 (1.37)	3.47 (0.84)	11.31 (3.51)	2.31 (0.32)	2.08 (1.70)	2.06 (0.46)
CXCL8/IL-8	70.2 (2.24)	13.49 (0.45)	1.36 (0.52)	1.6 (0.32)	3.44 (0.99)	0.24 (0.16)	4.37 (3.13)	0.97 (0.67)
CCL20/MIP-3α	112.61 (6.28)	9.21 (0.18)	0.3 (0.04)	2.21 (0.49)	3.11 (0.11)	0.64 (0.05)	2.58 (0.32)	0.66 (0.03)
IL-6	14.29 (0.65)	5.65 (0.61)	0.73 (0.031)	14.07 (15.09)	0.6 (0.05)	0.16 (0.05)	0.56 (0.09)	0.66 (0.06)
TNF-α	30.89 (5.02)	13.69 (3.86)	2.39 (0.47)	23.2 (17.50)	0.03 (0.01)	0.16 (0.06)	0.12 (0.04)	0.05 (0.01)
IL-18	385.67 (260.51)	2088.44 (1302.49)	38 (14.03)	0.69 (0.24)	0.01 (0.01)	0.67 (0.57)	3.18 (1.89)	4.79 (1.81)
IFN-γ	0.81 (1.06)	11.51 (6.26)	1.92 (0.77)	29.94 (37.69)	0.01 (0.01)	0.19 (0.04)	0.03 (0.01)	0.01 (0.01)
IL-10	0.5 (0.07)	4.79 (0.45)	0.64 (0.17)	7.75 (10.32)	0.02 (0.004)	0.14 (0.02)	0.11 (0.01)	0.04 (0.005)
IL-12β	0.73 (0.11)	2.4 (0.76)	0.74 (0.19)	16.84 (12.19)	0.05 (0.01)	0.18 (0.02)	0.11 (0.01)	0.13 (0.03)

in NP69 and NP460 cells, and then decreased by 24-hour post-inoculation. In LOVO cells, infection with SRAS-CoV slightly induced the expression of CXCL8/IL-8 and CCL20/MIP-3α at 24-hour post-inoculation, while no significant effect above background level was detected in these genes at the 6-hour time point. The expression level of CXCL8/IL-8 and CCL20/MIP-3α in NCI-H1650, NP69, NP460 and LOVO also correlated with the proportion of infected cells, indicated by immunofluorescence of viral antigen (P<0001). Significant up-regulation of CXCL8/IL8 receptors IL8Rα and IL8Rβ was observed at 24-hour post-inoculation in NCI-H1650 and LOVO cells, but not in NP69 and NP460.

In NCI-H1650 and LOVO cells, proinflammatory cytokines IL-6, TNF-α, IL-18 were strongly induced by SARS-CoV at 6-hour post-inoculation. The early induction of IL-6, TNF-α, and IL-18 was seen at 6-hour post-inoculation in NCI-H1650 cells and sustained a high expression level till 24-hour post-inoculation. Induction of TNF-α was detected at 6 hours and continued to increase in LOVO cells, reaching a much higher level (23-fold) at 24 hours. On the contrary, expression of IL-18 peaked at 6 hours (38-fold) and decreased to background levels at 24 hours in LOVO cells. Marked elevation of IFN-γ and to a lesser extends IL-10, and IL-12-β expression in these cell lines was seen at 24-hour post-inoculation.

Discussion

Confirming our earlier observations, intestinal cell line LOVO can maintain a permissive infection of SARS-CoV. Four more cell lines were also found to be permissive to SARS-CoV, including human lung adenocarcinoma cell line NCI-H1650, NCI-H1563, immortalised normal nasopharyngeal epithelial cell lines NP69 and NP460. In American green monkey kidney cells Vero, typical CPE was observed several hours post-inoculation. However CPE was not observed in LOVO, NCI-H1650 and NCI-H1563 cells after 7 days inoculation of SARS-CoV. In contrast, CPE was observed in SARS-CoV–inoculated nasopharyngeal epithelial cells NP69 and NP460. The basis of the differences in cellular response is unclear. Although SARS-CoV was detected in the lungs and intestine specimens from SARS patients, severe cellular damage was characteristically observed in the lungs, while no morphological change was detected in the intestine. The discrepancies in clinical manifestations and in vitro experiments highlight the importance of host cell responses in SARS infection. The mechanisms underlying SARS-CoV–mediated pathogenesis remain largely unexplained. Our findings of persistent infection in human intestinal, lung, and nasopharyngeal epithelial cells provide useful in vitro models for SARS-CoV–related studies.

In NCI-H1650 and LOVO cells, SARS infection significantly induced the expression of α-chemokines CXCL10/IP-10 and CXCL9/MIG, cytokines IL-6, IL-10, TNF-α, IL-18, IFN-γ, IL-10, and IL-12-β. Infection with SARS-CoV did not induce the expression of α-chemokines CXCL13/BCA-1, CXCL14/BRAK, receptor CXCR5, cytokines TGF-β1, TGF-β2, IL-1α, IL-1β and IL-12α in NCI-H1650 and LOVO cells. Nonetheless, cell type–dependent and/or time-dependent differences between NCI-H1650 and LOVO cells were noted. CCL20/MIP-3α and CXCL8/IL-8 markedly elevated in NCI-H1650 but not in LOVO.

NP69 and NP460 cells were less inducible in comparison to NCI-H1650 and LOVO. Induction of chemokines and inhibition of proinflammatory and anti-viral cytokines has been reported in different cell types after in vitro exposure to SARS-CoV. CCL2/MCP-1 and CXCL8/IL-8 were induced in lung adenocarcinoma cell line A549. SARS-CoV strongly induced IP-10 and IL-18 but suppressed anti-viral system such as IFN-α, IFN-β CCL5/RANTES, and IL-6 in colon carcinoma cell line Caco-2. CXCL10/IP-10 and CCL2/MCP-1 but not IFN-β was induced in macrophage. In monocytes-derived human dendritic cells, SARS-CoV induced CCL3/MIP1α, CCL5/RANTES, CXCL10/IP-10 and CCL2/MCP-1. Unlike the usual response of dendritic cells to viral infection, anti-viral cytokines such as IFN-α, IFN-β, IFN-γ and IL-12β, proinflammatory cytokines TNF-α and IL-6 were not activated. Significant up-regulation of chemokines in our cell line models was in concordance with previous reports. Proinflammatory and antiviral cytokines IFN-γ, TNF-α and IL-6 were also markedly up-regulated in our highly permissive models LOVO and NCI-H1650. These two cell lines are the only in vitro models in which successful induction of proinflammatory cytokines could be demonstrated. The cellular mechanisms underlying the differential proinflammatory cytokine induction remain poorly understood. The difference in cell type and degree of permissiveness to SARS-CoV infection might play a role.

After SARS-CoV infection, the circulating concentrations of most of the cytokines showed only transient and short-lived activation in patients. In contrast, circulating CXCL9/MIG, CXCL10/IP-10 and CCL2/MCP-1 were markedly increased in SARS patients. We have previously demonstrated that high levels CXCL10/IP-10 mRNA were present in autopsy lung specimens from SARS victims and that serum level of CXCL10/IP was an independent indicator of poor prognosis.[4] In the lungs of SARS patients, CXCL10/IP-10 was expressed in the pneumocytes, CD3+ T lymphocytes and monocytes/macrophages and was a potent chemoattractant for activated T cells, natural killer cells, leading to immune and inflammatory reactions. In our lung cell line model NCI-H1650, marked elevation of CXCL10/IP-10 (82-fold) was detected at 6-hour post-inoculation and reached 156-fold after 24 hours, whereas up-regulation of IFN-γ was not detected until 24-hour post-inoculation. The initial induction of CXCL10/IP-10 occurred without the presence of IFN-γ, suggesting that SARS-CoV induced expression of CXCL10/IP-10 ensues in an IFN-γ-independent

manner. These in vivo data were consistent to our in vitro observations. These chemokines appear to be important elements in the pathogenesis of SARS. CXCL10/IP-10 has been used as a therapeutic target in a viral model of multiple sclerosis and also in autoimmune and inflammatory renal disease.[5] Thus, our findings provide information for rational therapeutic approaches, such as targeting CXCL10/IP-10 for anti-SARS treatment.

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