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Comparative host gene transcription by microarray analysis early after infection of the Huh7 cell line by SARS coronavirus and human coronavirus 229E

Key Message

During the early stages of infection, SARS-CoV produces more severe perturbation of host cell gene expression in a human epithelial cell line of liver origin than the HCoV-229E.

Introduction

SARS-CoV is the aetiological agent of SARS, which is associated with a high mortality and morbidity. Such an unfavourable clinical effect is different from that of other known human coronaviruses, including the group 1 coronavirus 229E and NL63 and the group 2 coronavirus OC43.

SARS-CoV is an enveloped positive-sense single-stranded RNA virus that can grow in embryonic monkey cell lines including the Vero E6 and foetal rhesus monkey kidney (FRhk-4) cells. It can be sub-cultured onto other Vero cells and colonic carcinoma cell lines such as Caco-2 or LoVo. Unlike other human coronaviruses, SARS-CoV proliferates rapidly and causes obvious cytopathic effects in Vero E6 within 48 h of inoculation. There are no other human cell lines known to be susceptible to infection to both SARS-CoV and other human coronaviruses. It has been reported that a human hepatoma cell line (Huh7) can be infected by the pseudotyped lentiviral particles carrying the Spike protein of the SARS-CoV and the wild type replicative SARS-CoV.[1-3]

Aims and objectives

To report the susceptibility of the cell line Huh7 to infection by both the SARS-CoV and HCoV-229E and perform a comparative gene transcriptional profile at an early stage of such infection by these two viruses to elucidate differences in pathogenesis.

Methods

Cell lines and virus

Huh7 cells (courtesy of Prof David Ho, Aaron Diamond AIDS Research Center) were used throughout this study. The cells were incubated at 37ºC in Minimal Essential Medium (MEM) supplemented with 10% foetal calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin. Our prototype virus (SARS-CoV, HKU-39849) was isolated from the lung-tissue biopsy of the brother-in-law of the index SARS patient who travelled to Hong Kong from Guangzhou and started a superspreading event leading to the pandemic.[4] The HCoV-229E strain (American Type Culture Collection Number: VR-740) was used in this study. The SARS-CoV and HCoV-229E used in our experiments had undergone 3 passages in FRhk-4 cells and MRC-5 cells, respectively, and were stored at -70ºC. Viral titres were determined as the median tissue culture infective dose ($TCID_{50}$) per mL in confluent Huh7 cells in 96-well microtitre plates. The plates were used to standardise the viral inoculum and measure the relative susceptibility of the Huh7 cell line to these two viruses. The relative susceptibilities of Vero 1008, Vero 76, Vero, and Huh7 cell lines to SARS-CoV and HCoV-229E were also tested by $TCID_{50}$. One hundred $TCID_{50}$ was confirmed by plaque assays to be equivalent to 85 plaque-forming units. All work with infectious viruses was performed inside a type II Biosafety Cabinet, in a Biosafety Containment level III facility, and the

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personnel wore powered air-purifying respirators.

Monitoring of virus-induced cytopathic effect, antigen detection, semi-quantitative and quantitative PCR

Huh7 cells and culture supernatants infected with either SARS-CoV or HCoV-229E at a multiplicity of infection of 100 $TCID_{50}$ per cell were collected at 2, 4, 12 and 24 h post-infection. A washing step was performed 1 h post inoculation. The percentages of cells developing cytopathic effects (CPE) were counted by inverted light microscopy at 24 and 48 h. The rate of viral replication was measured semi-quantitatively by RT-qPCR on the culture filtrate. The amount of coronavirus antigen expression in infected cells was measured by indirect immunofluorescence tests, using convalescent serum of patients suffering from SARS-CoV and HCoV-229E infection.[5,6] RT-PCR for SARS-CoV and HCoV-229E was performed directly on culture filtrate according to our previous protocol.[5]

Microarray analysis

Human genome-wide gene expression was examined with the Affymetrix GeneChip system HG-U133A microarray, which is composed of more than 22 000 oligonucleotide probe sets interrogating approximately 18 400 unique transcripts, including 14 500 well-characterised human genes. Quality control, GeneChip hybridisation, data acquisition and analysis were performed at the Genome Research Centre, The University of Hong Kong, according to the standard protocols available from Affymetrix. Data analysis was performed using the Microarray Suite Expression Analysis software (Version 5.1; Affymetrix). For comparison across different arrays, the data for each array were normalised by a global scaling strategy, using a scaling target intensity of 500.

Gene expression analysis by semi-quantitative PCR, quantitative RT-PCR and immunoassays

Genes with significant transcriptional changes known to be associated with biological significance were selected for further analysis by semi-quantitative PCR, RT-qPCR and immunoassays. RT-qPCR was performed according to our previous protocol.[7] The extracted RNA was pre-treated with DNase. Primers that specifically amplified the nine genes related to apoptosis, inflammation and coagulation were designed. The housekeeping gene porphobilinogen deaminase (PBGD) was used to standardise the initial RNA content of a sample. Experiments were performed in duplicate and the results for individual samples were expressed as mean expression level of a specific gene/PBGD relative to the reference cDNA. The relative expression of each infected sample versus the uninfected controls were then calculated and expressed as fold changes. Three sets of immunoassays (human IL8, PAI1 and TFPI2) were performed according to our previous protocols and the manufacturers' instruction.[8,9]

Statistical analysis

The fold changes in the target gene expression and the differences in the concentration of protein expression between SARS-CoV and HCoV-229E at different post-inoculation time points were compared by Student's *t* test. A P value of <0.05 was considered significant. A statistical package (SPSS 10.0) was used for all analyses.

Results

Susceptibility of Huh7 cell line to SARS CoV and HCoV-229E

Using a multiplicity of infection of 100, CPE was visible in Huh7 cells at 24 h and progressed to about 50% cell death at 48 h in both viruses. Both viruses produced a comparable $TCID_{50}$ of around 10^7 per mL in the culture supernatant of Huh7 cells at 48 h. In terms of viral load, one log increase of viral genome copy was noted at 12 h post-infection in both viruses, which was followed by a peak at 24 h. For both viruses, antigen expression could be observed by indirect immunofluorescence in over 50% of the cells at 24 h post-infection.

Effects on gene expression of host cells by microarray

Based on the gene expression analysis, 224 genes were significantly altered within 4 h post-infection. Only 21 genes were disturbed by HCoV-229E per se, whereas 164 genes were altered by SARS-CoV infection only, and the remaining 39 by both coronaviruses. Out of the 164 genes with altered expression in SARS-CoV, 38 were up-regulated and only one was down-regulated at both 2 and 4 h post inoculation. At 2 h post inoculation, 43 were up-regulated and 16 down-regulated. At 4 h post inoculation, 49 were up-regulated and 17 down regulated. In contrast, for HCoV-229E infection, only one gene was up-regulated and no genes were down regulated at both 2 and 4 h post inoculation. At 2 h post inoculation, no genes were up-regulated and only two genes were down-regulated. At 4 h post-inoculation, 14 genes were up-regulated and four were down-regulated. When multiple transcripts of the same gene were eliminated and analysed, genes related to apoptosis (n=23), inflammatory or immune response (n=34) and coagulation (n=5) were identified in addition to the expected genes of stress response, metabolism and other unknown genes. Of the 23 apoptotic genes affected, 13 were pro-apoptotic and 11 were up-regulated in SARS-CoV infection compared to only three in HCoV-229E infection. As for inflammation and immune response, 32 genes were up-regulated in SARS-CoV compared to only three in HCoV-229E. These included NFKB1A, NFKB2, IL8, TGFβ2, chemokines CXCL1, 2, 3, 5, 6 and 10, ICAM1, and TNFα induced proteins. Surprisingly, genes of the pro-coagulation pathway were also affected by SARS-CoV infection with up-regulation of PLSCR1 (phospholipid scramblase 1), EGR1 (early growth response 1 gene), PAI1/SERPINE1 (plasminogen activator inhibitor 1) and THBS1 (thrombospondin 1). In terms of stress response, seven genes were up-regulated in SARS-CoV infection compared to only one in HCoV-229E infection. Overall

there were far more changes in gene expression related to cell cycle, transcription, metabolism, and miscellaneous and unknown functions in SARS-CoV infection. When the Pathway Assist software (Ariadne Genomics Inc.) was used for linking altered genes in cellular pathways for SARS-CoV, there was clear clustering of altered genes related to apoptosis, inflammation and coagulation.

Confirmation of cellular gene and protein expression by semi-quantitative PCR, RT-qPCR and immunoassay

A similar trend to up-regulation of gene expression with SARS-CoV showed a 1.4-10.8 fold increase, compared to HCoV-229E infection for coagulation (TFPI2, PAI1 and THBS1), inflammation (IL8 and NFKB2), transcription (JUNB) and apoptotic (PHLDA1, CARD10 and BAX). Enzyme immunoassay showed SARS-CoV induced higher concentrations of PAI1 and IL8 compared to HCoV-229E at 2, 4 12 and 24 h post-inoculation. Both SARS-CoV and HCoV-229E induced similar TFPI2 expression 4, 12 and 24 h post-inoculation, but at 2 h post-inoculation SARS-CoV induced a lower concentration of this protein.

Discussion

SARS-CoV causes respiratory failure in over 60% of those infected and has a mortality rate of around 15%.[4,10] Apart from pneumonia, occasionally SARS also manifests clinically as pulmonary vasculitis and thrombosis in the lungs among those who died.[11,12] Much has been studied including the virology, genomics, diagnostics, clinical features and progression in relation to viral load, treatment, infection control and immunisation.

No pneumocyte cell line has yet been found to support lytic or non-lytic infection by SARS-CoV. In this study, Huh7 cells were found to be susceptible to SARS-CoV.[13] HCoV-229E produced lytic infection within 48 h post-infection. A high multiplicity of infection of 100 $TCID_{50}$ per cell was used to ensure reproducibility of the gene expression study. Since the expression of a large number of genes was expected to change significantly when virus-induced cytopathology followed a rapidly lytic viral infection, we studied the difference in gene and protein expression profiles at a relatively early stage of infection (ie 2 and 4 h post-infection). This time frame is biologically relevant as proliferation of the Golgi complex and related vesicles and swelling of trans-Golgi sacs were observed in infected cells within the 1st hour of infection. Extracellular virus particles were present in 5% and 30% of the cell populations at 5 and 6 h post-infection, respectively.[14] This also facilitated the analysis as a lower number of altered genes were involved.

Comparative transcriptomic analysis indicated that far more genes (n=136) were up-regulated by SARS-CoV than HCoV-229E. Contrary to the reported findings of increased anti-apoptotic/inflammatory gene expression and decreased pro-apoptotic/inflammatory gene expression in the enterocyte cell lines,[15] far more pro-apoptotic and pro-inflammatory genes were expressed in Huh7 cells infected by SARS-CoV but not HCoV-229E. For instance, expression of BCL2 was induced by SARS-CoV in enterocytes, yet we observed up-regulation of its antagonists, including BAX and BCL2L11, in Huh7 cells. Moreover, much higher expression of other pro-apoptotic proteins, including CASP7, CARD10, PMAIP1, and GADD45B were also induced by SARS-CoV in contrast to HCoV-229E. Furthermore, there was marked perturbation of genes involved in cell cycle regulation, including induction of the CDKN2B gene, which can mediate growth arrest at the G1-phase.

The induction of pro-inflammatory cytokines by SARS-CoV was even more prominent compared to HCoV-229E. The induction of IL8 may be of pathogenic importance as its concentration was positively correlated with disease severity in pulmonary infection with RSV. Thus, the observed significantly higher level of IL8 induced by SARS-CoV in Huh7 cells, compared to HCoV-229E, may recapitulate the host response to these viruses by pneumocytes. The induction of various chemokines of the CXC or CCL families may mediate the chemotaxis of lymphocytes and neutrophils.

These alterations in gene expression are in keeping with the histological changes of SARS hepatitis in which cellular apoptosis, marked accumulation of cells in mitosis with ballooning degeneration of hepatocytes, and moderate lymphocytic infiltration were found in biopsied liver tissues.

The up-regulation of genes involved in pro-coagulation and platelet activation is interesting. TFPI2 inhibits thrombin generation by binding and inactivation of the TF: FVIIa (tissue factor: factor VIIa) complex. Up-regulation of the gene probably represents an inhibitory response to restrain the activation of the coagulation pathway during acute inflammation. In contrast, TFPI2 also inhibits both free and matrix/cell-associated plasmin, thus favouring fibrin deposition and may have a positive role in matrix turnover. Up-regulation of the gene of PAI1 accompanied by a dramatic increase in protein level results in an anti-fibrinolytic response. This may favour fibrin deposition during the acute inflammatory phase of the disease. It is important to note that the mouse hepatitis virus can activate the immune coagulation system by fgl2 gene encoding a prothrombinase. This enzyme can induce macrophage pro-coagulation activity resulting in fibrin deposition on the endothelium of intrahepatic veins and hepatic sinusoids. The result could be confluent hepatocellular necrosis. The low number of liver biopsies performed in these patients may account for the lack of reports on these large-scale changes related to vascular damage. However, systemic vasculitis including oedema, localised fibrinoid necrosis and infiltration by monocytes, lymphocytes, and plasma cells into vessel walls of various tissues has been reported.[12] Thrombosis was found in small veins. Marked up-regulation of a pro-apoptotic gene, PHLDA1, was observed in SARS-

CoV infection of Huh7. Over-expression of this gene in vascular endothelial cells leads to decreased cell adhesion and induces detachment-mediated apoptosis.[16] If similarly induced in vascular endothelial cells infected by SARS-CoV, this gene may contribute to the vascular damage induced SARS-CoV infection.[16]

Conclusions

SARS-CoV produces more severe disturbance of host cell gene expression in a human epithelial cell line of liver origin than the HCoV-229E during the early stage of infection. There are marked alterations in gene expression related to apoptosis, inflammation and pro-coagulation. These findings are consistent with the histological changes of SARS, especially in the liver and blood vessels.

Besides antivirals against SARS-CoV, other modalities of treatment such as anti-apoptotic agents, immunomodulators against inflammation and modifiers of coagulation should be considered in future research on the treatment of SARS. It is important to note that many patients continued to deteriorate 2 to 3 weeks after the onset of SARS, despite a decreasing viral load.

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