Role of epithelial-endothelial cell interaction in the pathogenesis of SARS-CoV-2 infection

Kenrie PY Hui, Man-Chun Cheung, Ka Ling Lai, Ka-Chun Ng, John CW Ho, Malik Peiris, John M Nicholls, Michael CW Chan

Supplementary materials

Methods

Viruses

A SARS-CoV-2 (BetaCoV/Hong Kong/VM20001061/2020, SCoV2) virus was isolated from a COVID-19 patient in Hong Kong in 2020 [1]. For comparison, we used SARS-CoV (strain HK39849/2003, SCoV) isolated from a hospitalized SARS patient in Hong Kong in 2003, MERS-CoV (Human EMC prototype MERS-CoV, MCoV) (strain EMC, provided by Prof R Fouchier, Erasmus University Medical Center, Rotterdam); a highly pathogenic avian influenza (HPAI) H5N1 virus (A/Hong Kong/483/1997, H5N1) from a fatal human infection and a 2009 pandemic influenza virus (A/Hong Kong/415742/2009, H1N1pdm) isolated from a patient in Hong Kong. SARS-CoV-2, SARS-CoV and MERS-CoV viruses were passaged in Vero-E6 cells and virus stock was aliquoted and titrated in Vero-E6 cells. Influenza viruses were passaged and titrated in Madin-Darby canine kidney (MDCK) cells. The experiments were carried out in a Bio-safety level 3 (BSL-3) facility at the School of Public Health, LKS Faculty of Medicine, The University of Hong Kong.

Isolation and maintenance of endothelial cells

Human primary pulmonary microvascular endothelial cells (HPMVECs) were isolated with modifications as previously described [2, 3]. In brief, human lung tissues were digested with collagenase and dispase for 1 h in 37°C with agitation. The mixture was filtered with 40µm cell strainers and subjected to Ficoll-paque PLUS (Sigma) gradient density centrifugation at 400 g for 20 min [2]. Endothelial cells were isolated from the interphase followed by positive selection with anti-CD31-tagged dynabeads (Thermo Fisher) according to the manufacturer instructions. The isolated endothelial cells were maintained in complete endothelial cell growth medium-2 (cEGM-2) (Lonza) with 1% P/S and 5% FBS [3]. Cells were seeded on transwell inserts with 0.4µm pore size (Costar) at a density of 150,000. Medium was changed every 48 h. The cells were subjected to infection or treatment after 5 days.
**Virus infection**

Cells in transwell inserts were washed with PBS and infected with viruses at a multiplicity of infection (MOI) of 0.1 for viral replication kinetics at the apical side or basolateral side. Virus titers were measured in culture supernatants collected from both apical and basal chambers at indicated time points. Infection was done at MOI 2 for the analysis of cytokine production and real-time PCR analysis. Mock-infected cells served as negative controls. Viral titers in supernatant were determined by median tissue culture infectious dose (TCID$_{50}$) assay. Cell lysates were collected at 24 and 96 hpi for mRNA expression analysis using real time-PCR. Wd-Calu-3 cells after infection with SARS-CoV-2 for 96 hours were stained with cleaved-caspase 3.

**Conditioned medium treatment and drug treatment**

Well differentiated Calu-3 cells were infected with SARS-CoV-2 at a MOI of 2 for 96 h. Culture supernatants from basolateral side were collected and filtered with a 100kDa-membrane (Millipore) to remove virus particles. Mock-infected culture supernatants served as control medium. HPMVECs were either treated with conditioned medium from mock (CM-MK), conditioned medium from SARS-CoV-2 infected cells (CM-SARS2), or direct infected with SARS-CoV-2 virus. HPMVECs were treated with cytomix—a mixture composed of IL-1β, TNF-α and IFN-γ at 50ng/ml, impaired epithelial integrity [4]. Mock infection served as negative control. Cell lysates were collected after 24 h treatment with the conditioned media or direct infection for mRNA expression analysis.

After 24h treatment with conditioned medium, HPMVECs were infected with SARS-CoV-2. Viral titers in the culture supernatants were monitored by using TCID$_{50}$ assay. Cell lysates were collected after 24h infection for mRNA expression analysis. 10 µM of imatinib was added to HPMVECs 1 h before and all the way during the treatment with conditioned medium. Cell lysates were collected after 24 h treatment with the conditioned media.

**Cytometric Bead Array (CBA)**

Protein expression of cytokine and chemokine in the supernatants collected from in-vitro cultures was determined by multiplexed bead-based immunoassays—cytometric bead array (BD Bioscience, USA), according to the manufacturer’s protocol. The tailor-made kit combined beads able to measure the levels of human IL-6, IP-10, MCP-1, RANTES and TNF.
In brief, 50 μl of cell culture supernatant and a 10-point standard curve (ranging from 0 to 2500 pg/mL) was used for the measurement of each cytokine and chemokine. The samples were analyzed using a BD LSR Fortessa Analyzer (BD Bioscience). Standard curves for the cytokines and chemokines were built and the concentrations with respective to fluorescence intensity were calculated using FlowJo (Version: 7.6.1).

**Immunofluorescence staining of caspase-3**

To indicate the apoptotic cells, fixed cells were subjected to immunofluorescence staining as follows. Cells were permeabilized, blocked and stained with anti-cleaved-caspase-3 (cell signaling), followed with the secondary goat anti-rabbit-AF488 antibody (Invitrogen). The nuclei were counterstained with DAPI. The stained membranes were mounted with fluorescent mounting medium (Dako) and imaged using Nikon Eclipse Ti-S microscope. The caspase-3 positive cells were determined by using the cell scoring application of MetaMorph software (ver 7.7.0.0)

**Quantitative RT-PCR**

The gene expression profiles of viral gene, cytokines, chemokines and adhesion molecules were quantified and normalized with β-actin as previously described [1].

**MTT assay**

*In vitro* cytotoxicity of the tested drug was evaluated using thiazolyl blue tetrazolium bromide (MTT)-based cell viability assay. HPMVECs were treated with indicated concentrations of imatinib for 24 h. Treated cells were incubated with growth medium with 0.5 mg/mL MTT for 3 h, and formazan crystal products were dissolved with DMSO. Cell viability was calculated by the colorimetric absorbance at 595 nm using the BMG FLUOstar OPTIMA plate reader (BMG Labtech).

**Statistical analysis**

Statistical analysis was done using GraphPad Prism software version 9. Viral titers were compared using two-way ANOVA with Tukey’s multiple comparisons test. Area-under-curve (AUC), mRNA expression and protein concentrations of cytokine and chemokine were compared by one-way ANOVA with Tukey’s multiple comparisons test. Differences were considered significant at $p < 0.05$. 
Supplementary Table 1. Information of tissue donors for the isolation of endothelial cells

| Donor | Tissues used | Gender | Age | Diagnoses       |
|-------|--------------|--------|-----|-----------------|
| 1     | Lung         | M      | 44  | Lung cancer     |
| 2     | Lung         | F      | 75  | Lung cancer     |
| 3     | Lung         | F      | 63  | Lung cancer     |
| 4     | Lung         | M      | 63  | Lung cancer     |
| 5     | Lung         | M      | 75  | Lung cancer     |
**Supplementary Figure 1. Viral replication kinetics and cytokine profile of coronaviruses and influenza A viruses in monolayer Calu-3 cells.** Calu-3 cells were infected with SARS-CoV-2 (SCoV2), SARS-CoV (SCoV), MERS-CoV (MCoV), pandemic influenza A (H1N1pdm) and influenza A H5N1 (H5N1) viruses at MOI 0.1. (a) Viral load in culture supernatants were determined at indicated time points by TCID\(_{50}\). Graphs show the mean virus titer pooled from three independent experiments ± standard deviation of mean. The horizontal dotted line denotes the limit of detection in TCID\(_{50}\) assay. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\), compared to SCoV2; #, \(P < 0.05\); ##, \(P < 0.01\); ###, \(P < 0.001\), compared to SCoV; +, \(P < 0.05\); ++, \(P < 0.01\); ++++, \(P < 0.001\), compared to MCoV. (b) Graphs show the area under the curve (24-72h) with datapoints from three independent experiments ± standard deviation of mean. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\). (c) Calu-3 cells were infected with SARS-CoV-2 (SCoV2), SARS-CoV (SCoV), MERS-CoV (MCoV), pandemic influenza A (H1N1pdm) and influenza A H5N1 (H5N1) viruses at MOI 2. At 96 h post-infection, concentrations of cytokines and chemokines in the culture supernatants were measured by cytometric bead assay. Graphs show the cytokine and chemokine production by coronaviruses and influenza A viruses infection in Calu-3 cells. Results are the calculated mean from three independent experiments ± standard deviation of mean. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).
Supplementary Figure 2. mRNA expression of ACE2, HAT and TMPRSS2 in wd-Calu-3 and monolayer Calu-3 cells. Bar-charts show the relative mRNA expression of ACE2, HAT and TMPRSS2. Results are the calculated mean from three independent experiments ± standard deviation of mean. **, P <0.01.
Supplementary Figure 3. Production of cytokines and chemokines by coronaviruses and influenza A viruses at apical and basolateral sides of well-differentiated Calu-3 cells. Wd-Calu-3 cells were infected with SARS-CoV-2 (SCoV2), SARS-CoV (SCoV), MERS-CoV (MCoV), pandemic influenza A (H1N1pdm) and influenza A H5N1 (H5N1) viruses at MOI 2 from the apical side. At 96 h post-infection, concentrations of cytokines and chemokines in the culture supernatants from both apical and basal chamber were measured by cytometric bead assay. The bar charts were generated from the results in Figure 2 and presented in a way to show the statistical significances between apical and basolateral release of cytokines and chemokines. Graphs show the concentrations of cytokines and chemokines. Results are the calculated mean from three independent experiments ± standard deviation of mean. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Supplementary Figure 4. Caspase-3 staining in wd-Calu-3 infected with SARS-CoV-2.

Wd-Calu-3 cells were infected with mock or SARS-CoV-2 at MOI 0.1 (SCoV2, 0.1) or 2 (SCoV2, 2) from the apical side for 96 h. (a) Immunofluorescence staining of caspase-3 (in green) and nuclei (in blue) is shown. (b) Bar-charts show the percentages of cells with caspase-3 staining. Results are the calculated mean from three independent experiments ± standard deviation of mean. No statistical significances are found.
Supplementary Figure 5. mRNA expression of ACE2, HAT and TMPRSS2 after infection with coronaviruses in well differentiated Calu-3 cells. Wd-Calu-3 cells were infected with SARS-CoV-2 (SCoV2), SARS-CoV (SCoV), MERS-CoV (MCoV), pandemic influenza A (H1N1pdm) and influenza A H5N1 (H5N1) viruses at MOI 2 from the apical side. Bar-charts show the mRNA expression of ACE2, HAT and TMPRSS2 after (a to c) 24h and (d to f) 96h infection. Results are the calculated mean from three independent experiments ± standard deviation of mean. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. 
Supplementary Figure 6. Gene expression in HPMVECs treated with cytomix. HPMVECs were treated with cytomix or medium (mock) for 24h. Cell lysates were harvested for determination of mRNA expression of monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-1β, IL-6, IL-8, interferon gamma-induced protein 10 (IP-10), endothelial leukocyte adhesion molecule (E-selectin), vascular cell adhesion molecule-1 (VCAM-1), angiopoietin (Ang)-1 and Ang-2. Results are the calculated mean from three individual donors ± standard deviation of mean. *, $P <0.05$; **, $P <0.01$; ***, $P <0.001$. 
Supplementary Figure 7. Viral gene expression in HPMVECs treated with SCoV2-conditioned medium collected from wd-Calu-3 cells. HPMVECs were treated with conditioned medium from mock (CM-MK), conditioned medium from SARS-CoV-2 infection (CM-SARS2) or SARS-CoV-2 infection (SARS2). Bar-charts show the ORF-1b gene expression in HPMVECs treated with conditioned medium or infected for 24h. Results are the calculated mean from three individual donors ± standard deviation of mean. **, P <0.01.

Supplementary Figure 8. Viral gene and IP-10 expression in HPMVECs treated with SCoV2-conditioned medium and re-infection with SARS-CoV-2 virus. HPMVECs were treated with conditioned medium from mock (CM-MK), conditioned medium from SARS-CoV-2 infection (CM-SARS2) for 24 h and subsequently infected with SARS-CoV-2 (SARS2) for another 24 h. Bar-chart shows (a) ORF-1b gene expression and (b) IP-10 induction in HPMVECs after treatment with conditioned medium and subsequent infection with SARS-
CoV-2 for 24h. Results are the calculated mean from three individual donors ± standard deviation of mean. ***, P <0.001.

**Supplementary Figure 9. Cytotoxicity of imatinib in HPMVECs.** Bar-chart show the viability of HPMVECs treated with imatinib for 24 h at concentrations ranging from 5 µM to 40 µM. Vehicle treatment served as negative control. Results are the calculated mean from three individual donors ± standard deviation of mean.

**References**

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