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Short communication

Bovine respiratory coronavirus enhances bacterial adherence by upregulating expression of cellular receptors on bovine respiratory epithelial cells

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ABSTRACT

Bovine coronavirus (BCoV) is one of the agents causing bovine respiratory disease complex (BRDC), with single infection tending to be mild to moderate; the probability of developing pneumonia in BRDC may be affected by viral and bacterial combinations. Previously, we reported that bovine respiratory syncytial virus (BRSV) infection enhances adherence of Pasteurella multocida (PM) to cells derived from the bovine lower respiratory tract but that BRSV infection in cells derived from the upper respiratory tract reduces PM adherence. In this study, we sought to clarify whether the modulation of bacterial adherence to cells derived from the bovine upper and lower respiratory tract is shared by other BRDC-related viruses by infecting bovine epithelial cells from the trachea, bronchus and lung with BCoV and/or PM. The results showed that cells derived from both the upper and lower respiratory tract were susceptible to BCoV infection. Furthermore, all cells infected with BCoV exhibited increased PM adherence via upregulation of two major bacterial adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and platelet-activating factor receptor (PAF-R), suggesting that compared with BRSV infection, BCoV infection differentially modulates bacterial adherence. In summary, we identified distinct interaction between bovine respiratory viruses and bacterial infections.

1. Introduction

Bovine coronavirus (BCoV) is a member of the family Coronavirus, genus Betacoronavirus, species Betacoronavirus 1. BCoV is a pneumoenteric virus that infects the upper and lower respiratory tract as well as the intestine (Saif, 2010). Some studies have reported that BCoV is among the most frequently detected viruses in calves with respiratory symptoms (Neill et al., 2014; Castells et al., 2019) and multiple infection with Pasteurella multocida (PM) were found in farms with respiratory symptoms (Kishimoto et al., 2017). The pathogenicity of BCoV infection in the bovine respiratory tract is considered milder than that of other bovine respiratory disease complex (BRDC)-related viruses, such as bovine respiratory syncytial virus (BRSV) (Beaudeau et al., 2010).

As bacterial infection of the lower respiratory tract can lead to severe pneumonia (Bryson, 1985), clarifying the mechanism of bacterial invasion from the upper to lower respiratory tract is of great importance. Our previous study revealed that PM adherence to BRSV-infected bovine respiratory epithelial cells (BRECs) is dependent on the site of origin from the upper or lower part of the respiratory tract (Sudaryatma et al., 2019). Interestingly, the number of adhering bacteria decreased and increased in BRSV-infected cells derived from the upper and lower respiratory tract, respectively. These changes in the number of adhering bacteria were caused by down- and upregulation, respectively, of two bacterial adhesion molecules (Sudaryatma et al., 2020a, 2020b). Reduced and enhanced bacterial adherence to BRSV-infected upper and lower BRECs would permit bacteria to pass from the upper to lower respiratory tract, inducing severe pneumonia. The tendency to cause pneumonia differs markedly among BRDC-related viruses. Thus,
The BCoV used in this study was isolated from nasal swab of cattle showing mild respiratory symptoms. Swab samples were tested for BCoV by qRT-PCR, as described by our group (Mekata et al., 2020). The samples were inoculated onto a monolayer of human rectal tumor cells (HRT-18 G) in serum-free Opti-MEM (Thermo Fisher Scientific, Wal-tham, MA, USA) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (FUJIFILM Wako Pure Chemical, Osaka, Japan). After 60 min. of adsorption, the cells were washed, and fresh serum-free Opti-MEM with 0.5 μg/mL TPPCK trypsin (Sigma-Aldrich, St. Louis, MO, USA) was added. The cultures were maintained for 5 days at 37 °C in 5% CO2. Three blind passages were conducted by collecting the supernatant via centrifugation at 3000 rpm for 10 min at 4 °C and inoculating onto fresh monolayer cells following the methods described above. Further passage for virus propagation was conducted using Dubbecco's Modified Eagle Medium (DMEM; Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS) without the addition of TPPCK trypsin. Virus isolation was confirmed by qRT-PCR. The virus titer was measured based on the number of adhering bacteria (MOI 100) after 60 min. of adsorption, the cells were washed, and fresh serum-free Dulbecco's modified Eagle's medium:Nutrient Mixture F-12 (DMEM/F12), GlutaMAX (Thermo Fisher Scientific) to achieve a multiplicity of infection (MOI) of 100, respectively.

2.2. Culture of bovine respiratory epithelial cells

Bovine respiratory epithelial cells (BRECs) from the trachea (bTEC), bronchus (bBEC), and lung (bLEC) used in this study were previously established by our group (Sudaryatma et al., 2019). For infection, the virus and bacterium were diluted in antibiotic- and serum-free Dulbecco’s modified Eagle’s medium: Nutrient Mixture F-12 (DMEM/F12), GlutaMAX (Thermo Fisher Scientific) to achieve a multiplicity of infection (MOI) of 100, respectively.

2.3. BCoV and/or PM infection of BRECs

Each type of BREC (5 × 10^5 cells/mL) was seeded and then inoculated with BCoV (50,000 TCID 50); cells without the virus served as a negative control. The supernatant and cell lysate were collected at 1, 3, 6, and 12 h postinfection (hpi) and at 3, 5, and 7 days postinfection (dpi). BCoV-infected and uninfected cells were exposed to PM (MOI 100) for 1 h at 37 °C, and the number of adhering bacteria was measured in colony forming units (CFUs) as described previously (Sudaryatma et al., 2019). The number of adhering bacteria was also counted at 1, 2, and 3 dpi. For microscopic observation, PM was labeled with fluorescein isothiocyanate (FITC; Dojindo Laboratories, Kumamoto, Japan), as described previously (Sudaryatma et al., 2018). BCoV-infected and uninfected cells were exposed to FITC-labeled PM for 1 h and observed by fluorescence microscopy (DP-74, Olympus, Tokyo, Japan); cell nuclei were visualized by Hoechst 33342 staining (ab145597; Abcam, Cambridge, UK).

2.4. Real-time RT-PCR to quantify viral RNA and cytokine mRNA

Viral or cellular RNA was extracted from supernatants or cells using a NucleoSpin Virus (TaKaRa Bio, Kusatsu, Japan) or an RNAasy Mini Kit (Qiagen, Venlo, Netherlands), respectively. All qRT-PCR assays were performed in triplicate using a LightCycler 96 system (Roche Diagnostics, Rotkreuz, Switzerland) and a One Step PrimeScript RT-PCR Kit (TaKaRa Bio). Synthetic control DNA was designed based on the BCoV Kakegawa strain (Accession No. AB354579). The primers (forward: 5'-GGACCCAAGTACGAGTAGG-3'; reverse: 5'-GACCTTCTGACGCTTTCAAATA-3'), probe (5'-FAM/ATTCCGACT/Zen/AGGTTCGCTG/IBFQ-3') and amplification conditions used in this study were previously reported (Kishimoto et al., 2017; Mekata et al., 2020). To analyze mRNA expression of bovine cytokines, qRT-PCR was performed using One Step TB Green PrimeScript RT-PCR Kit II (TaKaRa Bio). The primers used to quantify mRNA levels of bovine IL-1β, IL-6, TNF-α, ICAM-1 and GAPDH have been described previously (Sobotta et al., 2017; Sudaryatma et al., 2020b). The primers for PAF-R (Forward: 5'-CACGGACAGGCTGAAATAC-3' and Reverse: 5'-TGGGGCAGAAA CAGATG-3') were designed based on the reference sequence in this study (Accession No. NM001040538). Relative mRNA expression between the infected sample and uninfected control was calculated using the 2^ΔΔCT method, and the result is expressed as the fold change.

2.5. Western blot analysis

BCoV-infected cells in a 6-well plate were lysed with 300 μl lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.6, 1% NP-40, protease inhibitor, and phosphatase inhibitor) and sonicated twice for 10 s each. The protein concentrations of the samples were determined using a TaKaRa BCA Protein Assay Kit (TaKaRa Bio). Protein samples (10 μg per well) were loaded into each lane of a 4–15 % SDS polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA), separated by electrophoresis, and transferred to a PVDF membrane (Bio-Rad Laboratories) using a Transblot semidry system (Bio-Rad Laboratories). The membranes were blocked overnight at 4 °C with blocking buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1 % Tween 20, and 3% bovine serum albumin) and then incubated with either mouse anti-ICAM-1 (ab22123; Abcam), rabbit anti-PAF-R (ab104162; Abcam) or mouse anti-GAPDH (ab9482; Abcam) primary antibodies at room temperature for 1 h. After vigorous washing, the membranes were incubated with a secondary goat anti-mouse IgG (ab205719; Abcam) or anti-rabbit IgG (ab205718; Abcam) antibody conjugated to hors eradish peroxidase at room temperature for 1 h. The chemiluminescent signals were detected using the western blot hyper HRP substrate (TaKaRa Bio) and a ChemiDoc Touch Imaging System (Bio-Rad Laboratories).

2.6. Statistical analysis

Data are reported as the mean and standard error of the mean (SEM) of at least three independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey’s Multiple Comparison Test. A p-value <0.05 was considered statistically significant. The data analysis was performed using R Studio software (RStudio, Boston, MA USA).
3. Results

3.1. BCoV isolation and the viral susceptibility of BRECs

BCoVs were isolated from 7 nasal swab samples of 32 qPCR-positive cattle. The seven virus isolates were named C13, C19, CS5, U39, S5, S13 and S15. Because the C13 strain produced the highest number of viral copies in the culture medium (data not shown), we used this strain for further experiments. Although viral copy numbers increased or remained stable during viral passages, the cytopathic effect (CPE) in BCoV-infected HRT-18 G cells was not clear. Thus, the virus titer was determined by using MDBK cells.

To assess the susceptibility of BRECs to BCoV, qRT-PCR was performed to measure the viral copy number in the culture medium at different time points. Viral RNA was detected from 3 to 7 dpi in bTECs, bBECs, and bLECs (Fig. 1A), with significant differences in viral copy number among the three cell types (p < 0.05). bLECs produced the greatest amount of viral RNA compared with bTECs and bBECs. In addition, we observed a clear CPE in all BCoV-infected BRECs, including floating cells and circular cells (Fig. 1B). BCoV-infected BRECs expressed different levels of mRNA associated with inflammation (Fig. 1C). For example, the peak of IL-1β expression was observed at 3 hpi in all three cell types. However, the highest level of IL-6 expression was observed at 3 hpi in bTECs and bBECs and at 1 hpi in bLECs. TNF-α expression in bTECs and bLECs peaked at 3 hpi, but it reached a peak at 6 hpi in bBECs.

3.2. BCoV infection of BRECs enhances PM adherence

To examine whether bacterial adherence to BRECs can be enhanced...
by BCoV infection, cells were infected with BCoV for three days and then inoculated with PM. Compared to cells not infected with the virus, the number of adhering bacteria increased significantly for all BCoV-infected BRECs at 2–3 dpi (p < 0.05) (Fig. 2A and B). Specifically, rates of increasing numbers of adhering bacteria were 1.7- and 2.2-fold in bTECs, 1.7- and 2.5-fold in bBECs, and 2.0 and 2.4-fold in bLECs at 2 and 3 dpi compared to virus-uninfected cells, respectively. Moreover, coinfection of BCoV with bacteria stimulated expression of inflammatory cytokines (Fig. 2C), and the levels of IL-6 and TNF-α mRNA expression in bTECs coinfected with BCoV and PM were significantly higher than those in cells infected with PM alone (p < 0.05). In contrast, expression of IL-1β in bTECs coinfectd with BCoV and PM was lower than that in cells infected with only PM. Nevertheless, mRNA expression of IL-6, TNF-α and IL-1β in bBECs and bLECs coinfectd with BCoV and PM was significantly higher than that in cells only infected with PM (p < 0.05).

3.3. BCoV infection upregulates ICAM-1 and PAF-R expression in BRECs

We recently demonstrated that expression of two cell surface receptors, platelet-activating factor receptor (PAF-R) and intercellular adhesion molecule-1 (ICAM-1), determines the adherence of PM to BRSV-infected BRECs (Sudaryatma et al., 2020a, 2020b). Based on this finding, we measured the expression levels of these receptors by

![Fig. 2. Bovine respiratory coronavirus (BCoV) infection enhances the adherence of Pasteurella multocida (PM) to bovine respiratory epithelial cells (BRECs). (A) BRECs preinfected with 5000 TCID<sub>50</sub> of BCoV for 1, 2, and 3 days were exposed to PM (MOI = 100) for 1 h. The number of PM adhering to BRECs was counted on Brucella agar. Data are expressed as the mean (±SEM; n = 3; *p < 0.05 compared to uninfected cells). (B) FITC-labeled PM (green) adherence to BCoV-infected and -uninfected BRECs at 3 dpi was observed with a fluorescence microscope; nuclei were stained with Hoechst-33342 (blue) (scale bar: 30 μm). (C) Expression of bovine IL-1β, IL-6, and TNF-α mRNA in BRECs infected with BCoV alone at 3 dpi, PM alone at 1 hpi, or BCoV 3 dpi plus PM 1 hpi. Data represent the mean of relative mRNA expression between the virus and/or bacteria infected samples and uninfected controls (±SEM; n = 3; * p < 0.05 compared to PM alone).]
qRT-PCR and western blotting. As depicted in Fig. 3A, BCoV infection stimulated mRNA expression of PAF-R and ICAM-1 in BRECs. We observed a peak of PAF-R expression at 12 hpi in all cell types infected with BCoV (3.2-, 2.1- and 3.8-fold increase, respectively). In bTECs, ICAM-1 expression reached its peak (3.4-fold increase) at 6 h after BCoV infection, and the highest level of ICAM-1 expression in bBECs and bLECs was observed at 12 hpi (2.1- and 3.8-fold increase, respectively). Consistent with the results for mRNA expression, levels of PAF-R and ICAM-1 protein expression increased significantly after BCoV infection in all cells compared with uninfected cells (Fig. 3B and C), and this difference was statistically significant ($p < 0.05$).

4. Discussion

Bovine respiratory coronavirus infection tends to induce milder respiratory symptoms than BRSV infection (Beaudeau et al., 2010; Gershwin et al., 2015; Oma et al., 2016). In general, understanding the mechanism of differences in pathogenicity between BCoV and BRSV is important to predict the prognosis of affected animals and minimize damage due to BRDC. In this study, we found that BCoV infection induced the adherence of PM to bovine upper and lower respiratory epithelial cells through upregulation of ICAM-1 and PAF-R. This result was in contrast to the outcome of BRSV infection, which reduced PM...
adherence and ICAM-1 expression in cells derived from the upper respiratory tract (Sudaryatma et al., 2019, 2020b). PM is one of the major bacteria in the upper respiratory tract and microbiota in the upper respiratory tract is an important defense mechanism to prevent invasion of bacteria into the lower respiratory tract (Allen et al., 1992; Man et al., 2017). Hence, this study provides a possible reason for the difference in pathogenicity between BRSV and BCoV infection in BRDC.

Here, we show that all BREC's from three respiratory tissues were susceptible to BCoV. Indeed, we observed time-dependent replication of BCoV in bTECs (representative of the upper respiratory tract), bBECs (representative of the upper part of the lower respiratory tract), and bLECs (representative of the lower part of the lower respiratory tract). Moreover, robust viral replication in bLECs correlated with a clear CPE and high mRNA expression of inflammatory cytokines (Fig. 1B and C). Although most of BCoV infection induce mild respiratory symptoms, invasion of BCoV into the lower respiratory tract appears to induce severe inflammation and pneumonia (Amoroso et al., 2020). In general, HRT-18 G cells are used for BCoV isolation due to their high susceptibility as well as the high productivity of the virus in these cells. BCoV-infected HRT-18 G cells exhibited a slight CPE in this study, though it was not a clear effect. Therefore, we used MDBK cells to determine the virus titer. Recent field isolates of BCoV in Japan have reportedly lost hemagglutination activity in chicken red blood cells (Kanno et al., 2018). We also confirmed the same feature in our isolates (data not shown). Accordingly, the characteristics of BCoVs circulating in Japan might differ from those of prior strains, explaining why the CPE in HRT-18 G cells was difficult to observe.

This study showed that PM adherence to upper and lower BREC's was significantly enhanced by preinfection with BCoV. Furthermore, we confirmed upregulation of ICAM-1 and PAF-R in BCoV-infected bovine upper and lower respiratory epithelial cells. A similar tendency has been observed in human respiratory cells infected with mildly pathogenic viruses such as rhinovirus and coronavirus NL63 (Ishizuka et al., 2003; Golda et al., 2011). Infection with these viruses enhanced expression of cellular receptors in respiratory epithelial cells and bacterial adherence. Upregulation of bacterial adhesion molecules in the upper respiratory tract during viral infection can be read in two ways. One is associated with more severe damage in the upper respiratory tract. Another is an important defense system for the host to prevent infection in the lower respiratory tract. BRSV infection downregulates expression of these molecules in the upper respiratory tract and would enhance bacterial invasion into the lower respiratory tract (Sudaryatma et al., 2019, 2020b). We do not know why BCoV and BRSSV infection induce different type of cell response. BRSSV NS1 and NS2 proteins function to inhibit the interferon responses and the virus evades the host immunity (Schlender et al., 2000; Bossert et al., 2003). These kinds of protein might affect to the expression of bacteria adhesion molecules and induce bacterial invasion into the lower respiratory tract. Thus, it might be important to clear the virus-mediated modulation of bacterial adhesion molecule expression to identify the pathogenicity of BRDC-related virus.

Under our conditions, BCoV infection enhanced adherence of bacteria to BREC's, inducing overproduction of inflammatory cytokines. Although mRNA expression levels of IL-6 and TNF-α were increased in coinfected upper respiratory cells (bTECs), IL-1β was decreased. Regardless, all three inflammatory cytokines were increased in coinfected lower respiratory cells (bBECs and bLECs). IL-1β is responsible for neutrophil recruitment and inflammation in the lungs (Ichinohe et al., 2009). The enhanced expression of this cytokine has been associated with several autoinflammatory diseases (McDermott and Tchopp, 2007). Thus, control of IL-1β expression in upper respiratory epithelial cells coinfected with BCoV and PM might be important to maintain a homeostasis in the upper respiratory tract.

In conclusion, our data demonstrate that BCoV infection enhances PM adherence and triggers inflammatory responses in cells derived from the upper and lower respiratory tracts. BCoV infection upregulated both ICAM-1 and PAF-R expression, enhancing bacterial adherence to BCoV-infected cells. The study presents evidence for a distinct mechanism of BRDC-related infections in response to viral infections in respiratory epithelial cells. Understanding this complex interplay will help in the development of new therapeutic strategies for BRDC.

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Data availability

The data that support the findings of this study are available from the corresponding author, H.M., upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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