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Brief Communication

Wild-type human coronaviruses prefer cell-surface TMPRSS2 to endosomal cathepsins for cell entry

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ABSTRACT

Human coronaviruses (HCoVs) enter cells via two distinct pathways: the endosomal pathway using cathepsins to activate spike protein and the cell-surface or early endosome pathway using extracellular proteases such as transmembrane protease serine 2 (TMPRSS2). We previously reported that clinical isolates of HCoV-229E preferred cell-surface TMPRSS2 to endosomal cathepsin for cell entry, and that they acquired the ability to use cathepsin L by repeated passage in cultured cells and were then able to enter cells via the endosomal pathway. Here, we show that clinical isolates of HCoV-OC43 and -HKU1 preferred the cell-surface TMRRSS2 to endosomal cathepsins for cell entry, similar to HCoV-229E. In addition, the cell-culture-adapted HCoV-OC43 lost the ability to infect and replicate in air-liquid interface cultures of human bronchial tracheal epithelial cells. These results suggest that circulating HCoVs in the field generally use cell-surface TMPRSS2 for cell entry, not endosomal cathepsins, in human airway epithelial cells.

1. Introduction

Human coronaviruses (HCoVs) are causative agents of human common colds, and most people experience initial infection during childhood (Dijkman et al., 2008). To date, four HCoVs (229E, NL63, OC43 and HKU-1) have been identified. The first two belong to *alphacoronavirus* and the latter two to *betacoronavirus*.

HCoV-229E was first reported in 1966 (Hamre and Procknow, 1966) and the isolate of the day is widely used as the laboratory strain of the American Type Culture Collection (ATCC, VR-740). HCoV-229E enters cells via two distinct pathways: the endosomal pathway using cysteine protease to activate spike (S) protein and the cell-surface or early endosome pathway using extracellular proteases for activation. Studies using the ATCC isolate suggest that HCoV-229E enters cells via the late endosome using cathepsin L to cleave S protein, although it can enter cells via the cell surface or early endosome in the presence of transmembrane protease serine 2 (TMPRSS2) or trypsin (Bertram et al., 2013; Kawase et al., 2009). It has been thought that this is a general feature of HCoV-229E entry. However, we recently reported that clinical isolates of HCoV-229E preferred the cell-surface or early endosome pathway to the late endosome pathway for cell entry (Shirato et al., 2017). The ability of a clinical isolate of HCoV-229E to use cathepsin L was originally low, but it became able to use cathepsin after repeated passage in HeLa cells (Shirato et al., 2017). This suggests that in natural situations HCoV-229E enters respiratory epithelial cells via the cell-surface or early endosome route and that the character of ATCC VR-740 has been changed by repeated passage in cultured cells.

In recent years, a culture method for human bronchial tracheal epithelial (HBTE) cells that uses an air-liquid interface (ALI) has been developed and has come to be used as a model of the in vivo situation of human airway epithelium (HAE) (Fulcher et al., 2005). Using this system, HCoV-HKU1, which had previously been reported from sequence data, was first isolated (Pyrc et al., 2010), and it was later shown that all four HCoVs could be isolated upon HBTE-ALI cell culture (Dijkman et al., 2013). In the present study, we found that field isolates of HCoV-OC43 and HCoV-HKU1 could be isolated using HBTE-ALI cell culture, and we then used these clinical isolates to assess whether the mode of virus entry found in HCoV-229E was also in play in other HCoVs.

Abbreviations: ATCC, American Tissue Culture Collection; Cam, Camostat; CatL, Cathepsin L; DMEM, Dulbecco's modified Eagle's medium; HCoV, human coronavirus; S, spike; VHCR, very highly conserved region

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Table 1
Isolated viruses and RT-PCR results.

Name of isolate	Real-time RT-PCR (Cq Value)[a]		Accession No.
	OC43	HKU1	
Tokyo/SGH-15/2014		23.70	LC315650
Tokyo/SGH-36/2014	20.90		LC315646
Tokyo/SGH-61/2014	18.25		LC315647
Tokyo/SGH-06/2015	26.71		LC315648
Tokyo/SGH-18/2016		25.10	LC315651
Tokyo/SGH-65/2016	24.04		LC315649

[a] 140-μL volumes of specimens were used for RNA extraction (QIAamp Viral RNA Minikit) and eluted in 60 μL of kit buffer. Five microliters of the 60 μL were used for real-time RT-PCR.

2. Results

2.1. Isolation of clinical isolates of HCoVs by ALI culture

For isolation of HCoVs, nasal swabs were collected from outpatients who showed respiratory infection as a cardinal symptom when assessed at a hospital in Tokyo, Japan. Specimens diagnosed as containing HCoVs by real-time RT-PCR were used for virus isolation. Recently, the circulating HCoV strain in Japan has been biased toward HCoV-OC43 (Hara and Takao, 2015; Matoba et al., 2015; Yano et al., 2014). Therefore, we did not obtain specimens positive for HCoV-229E or HCoV-NL63; however, four HCoV-OC43 and two HCoV-HKU1 isolates were finally isolated successfully (Table 1). The sequences of S protein were analyzed and deposited in GenBank (accession nos. LC315646 to LC315651). The number of cycles of real-time RT-PCR to which virus-isolated specimens were subjected ranged from 18.25 to 26.71, and isolation was considered failed when more than 30 cycles were used (data not shown). This suggests that at least 10^4 copies of viral RNA are required for successful isolation by HBTE-ALI culture.

2.2. The replication kinetics of laboratory and clinical isolates of HCoV-OC43 in ALI culture

Similar to HCoV-229E, HCoV-OC43 was identified between the late 1960s and 1970 (Bruckova et al., 1970; McIntosh et al., 1967a, 1967b). The isolate at that time had been passed in suckling mouse brain and cell culture, and finally became widely used as a laboratory strain (ATCC VR-1558). As previously described, the entry mechanism of the clinical isolate of HCoV-229E differed from that of the cell-culture-adapted laboratory isolate, ATCC-VR740; the clinical isolate preferred the cell-surface TMPRSS2 route to the endosomal cathepsin route preferred by the ATCC isolate (Shirato et al., 2017). Therefore, we evaluated differences in infectivity and replication in culture cells (HCT-8) or HBTE-ALI culture using VR-1558 and the clinical isolate (SGH-36/2014) (Fig. 1). In HCT-8 cells, VR-1558 infected and replicated well, as expected. On the other hand, the amounts of SGH-36/2014 virus entering HCT-8 cells were much lower than the amounts of VR-1558, and replication of SGH-36/2014 was about 10^5 lower than that of VR-1558 (Fig. 1a). In HBTE-ALI culture, the result was completely reversed; SGH-36/2014 infected the cells and replicated well as expected. In contrast, VR-1558 could infect the HBTE-ALI culture. Though the amount of entered virus was much lower compared to SGH-36/2014, no viral RNA was detected in the cell wash 3 days after infection (Fig. 1b). This indicates that VR-1558 infected the HBTE-ALI culture with low efficiency, but it failed to produce progeny virus. In the previous report, a 20-times-passaged clinical isolate of HCoV-229E exhibited changes that allowed it to replicate well in HeLa cells, but its ability to replicate in HBTE-ALI culture decreased relative to that of the original isolate (Shirato et al., 2017). The present study indicates that the phenomenon seen in HCoV-229E is also present for HCoV-OC43;

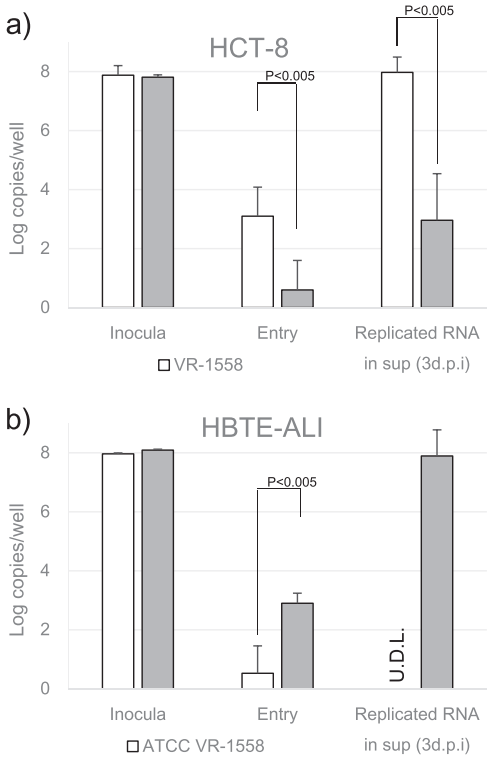

Fig. 1. Replication of laboratory and clinical isolates of HCoV-OC43 in HBTE-ALI cultures. Identical amounts (10^8 copies) of laboratory (VR-1558) and clinical (SGH-36/2014) isolates of HCoVOC43 were inoculated onto a) HCT-8 or b) HBTE-ALI cells. After incubation for 4 h, the cells were washed with DMEM twice and incubated at 34 °C. After 1 day of incubation, the cells were collected and used for analysis of virus entry. After 3 days of incubation, supernatants of HCT-8 cells and cell washes of HBTE-ALI were collected and used for analysis of viral replication. Data were expressed as log copies of RNA per well (n = 6 for HCT-8, n = 3 for HBTE-ALI).

namely, cell-culture-adapted virus cannot replicate in HBTE-ALI culture, which mimics human airway epithelial cells.

2.3. The entry route of clinical isolates of HCoVs in HBTE-ALI culture

In the case of HCoV-229E, the clinical isolate enters cells via the cell-surface or early endosome route using TMPRSS2, and the ATCC isolate VR-740 tends to enter cells via the late endosome pathway using cathepsin L (Bertram et al., 2013; Kawase et al., 2009; Shirato et al., 2017). The former route can be inhibited by the serine protease inhibitor camostat, and the latter by the cysteine protease inhibitor EST (E64D) (Kawase et al., 2012; Shirato et al., 2017, 2013). To evaluate the entry routes of clinical isolates of HCoVs, viruses were inoculated onto HBTE-ALI in the presence of EST or camostat (10 μM) and the amounts virus that entered were estimated by detecting subgenomic mRNAs using real-time RT-PCR (Fig. 2). First, the effect of inhibitor treatment on cell viability was determined in all cells. The treatments did not affect survival of the cell cultures at this concentration (Fig. 2a). Infection of HCoV-OC43, VR-1558, was inhibited only by EST and not by camostat (Fig. 2b). This indicates that VR-1558 enters cells via the late endosome pathway using cysteine proteases and it does not use TMRPSS2 for cell entry in HBTE-ALI culture. In contrast, clinical isolates of HCoV-OC43 were inhibited by camostat but were either not inhibited or were only partially inhibited by EST (Fig. 2c–f). In the case of SGH-36/2014, EST increased entry (Fig. 2c). For clinical isolates of HCoV-HKU1 (similar to HCoV-OC43), entry was inhibited only by camostat and EST tended to increase entry (Fig. 2g and h). As shown in Fig. 2a, treatment with inhibitors did not affect cell viability. This suggests that the reduced infection of VR-1558 by EST and clinical isolates by camostat were induced by the inhibition of proteases, not

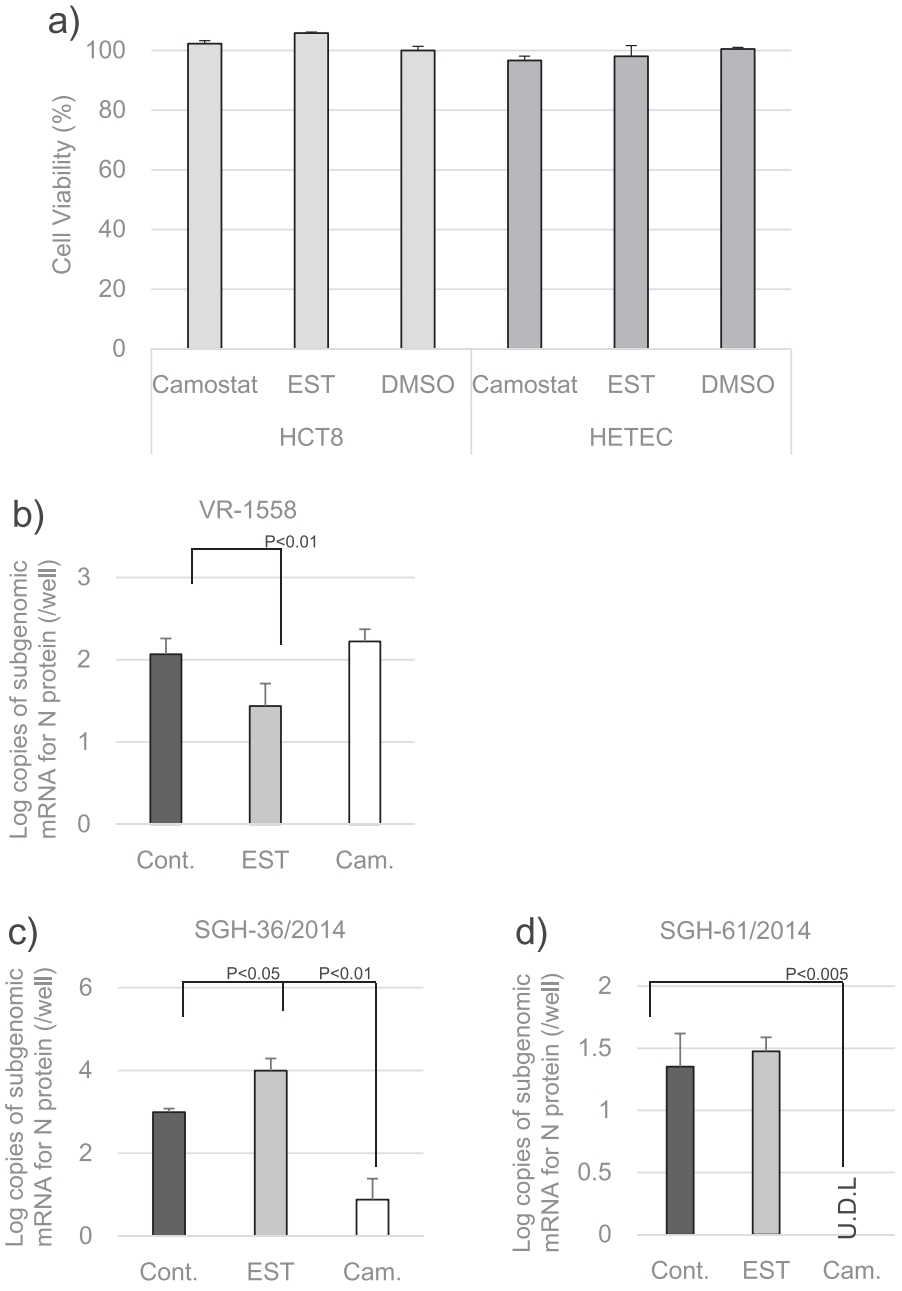

Fig. 2. Entry inhibition assay. Before virus inoculation, cells were treated with EST or camostat (10 μM) for 1 h. Then, 10^7 to 10^8 copies of the viruses were inoculated onto HBTE-ALI cultures and incubated in the presence of inhibitors. Cell viability after inhibitor treatment was determined using CellTiter-Glo and a GloMAX luminometer (a) (n = 3). Five HCoV-OC43 isolates [b) VR-1558, c) SGH-36/2014, d) SGH-61/2014, e) SGH-06/2015, and f) SGH-65/2016] and two HCoV-HKU1 isolates [g), SGH-15/2014 and h) SGH-18/2016] were examined. After incubation for 4 h, the cells were washed twice and incubated at 34 °C. After 1 day of incubation, the cells were collected and viral entry was calculated by quantifying subgenomic RNA containing nucleocapsid protein via real-time RT-PCR. DMSO was used as an inhibitor control. Data are shown as log copies RNA per well. (n = 3).

inhibitor treatment. These data are identical to those derived using HCoV-229E (Shirato et al., 2017) and suggest that the clinical isolates of HCoVs that cause human infections enter cells via the cell-surface or early endosome pathway using cell-surface proteases such as TMRSS2. The data also suggest that entry of cells via the late endosome using cathepsins is the case only for cell-culture-adapted or long-term-passaged isolates of HCoVs. Moreover, the increased entry of some clinical isolates in the presence of EST suggests that entry of cells via the late endosomal pathway induces some anti-viral host immunity.

The expression of TMRSS2 in HBTE-ALI and HCT-8 cultures was confirmed by mRNA quantitation (Fig. 3a). The expression of TMRPSS2 mRNA was found in Calu-3 and LoVo cells, which are known to be susceptible to Middle East Respiratory Syndrome (MERS) coronavirus (Shirato et al., 2013; Tao et al., 2013). TMRPSS2 expression was highly up-regulated and was prominent among cells tested in differentiated HBTE-ALI cultures. In HCT-8 cells, TMRPSS2 mRNA was detected, but the expression level was lower than in immature HBTE cells. Almost no TMRPSS2 was detected in HeLa cells. This suggests that the reduced infection and replication of HCoV-OC43 clinical isolates in HCT-8 cells were caused by the low level of TMPRSS2 expression relative to HBTE-ALI cultures.

That cathepsins use HCoV-OC43 for entry into HCT-8 cells was confirmed by an entry inhibition assay similar to that in HBTE-ALI cultures (Fig. 3b and c). The entry of VR-1558 in HCT-8 was inhibited only by EST treatment at both 10 and 100 μM (Fig. 3b). In SGH-36/2014 infection, a high concentration (100 μM) of EST inhibited entry (Fig. 3c). In contrast, camostat treatment did not show any inhibition in terms of VR-1558 and SGH-36/2014 in HCT-8 infection; however, HCT-

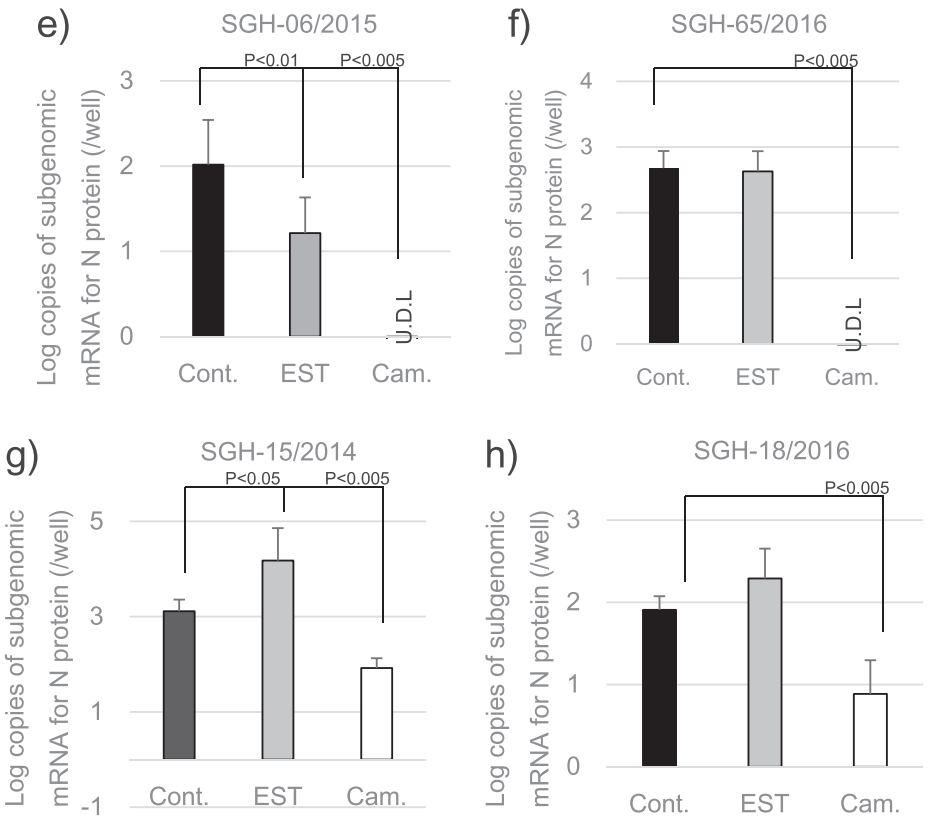

Fig. 2. (*continued*)

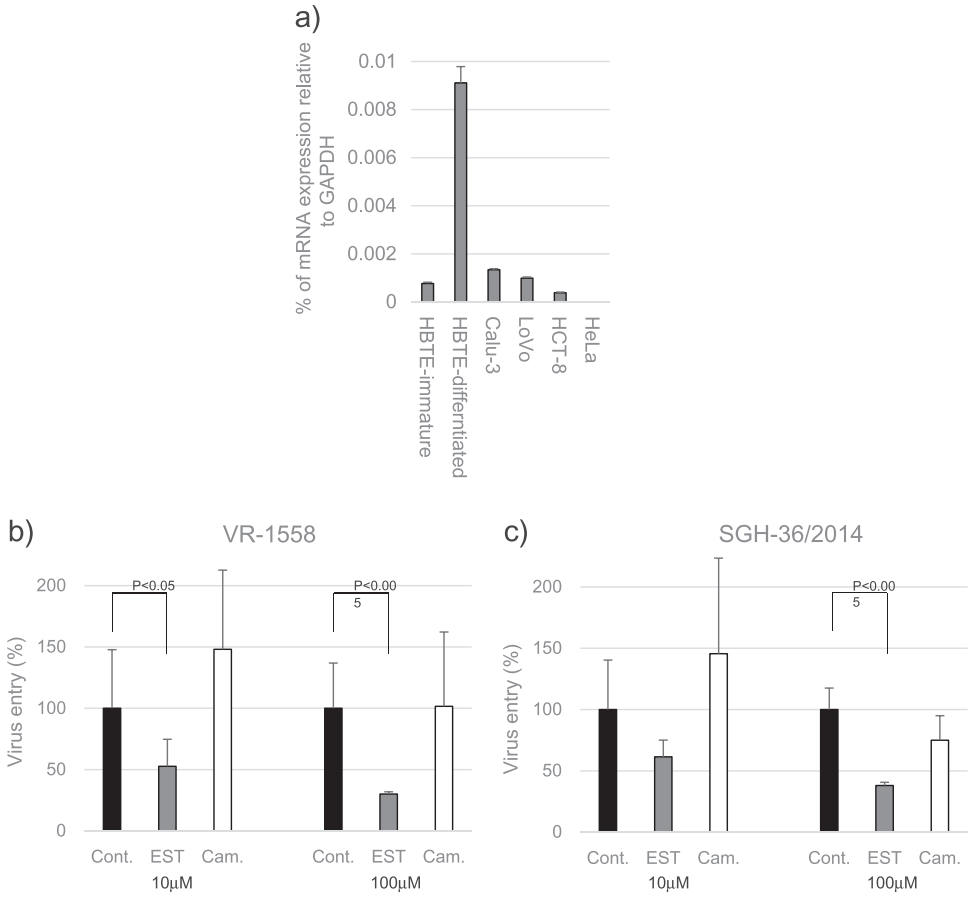

Fig. 3. Entry inhibition assay in HCT-8 cells. a) TMRPSS2 expression in HBTE-ALI and HCT-8 cells. Total RNAs were extracted by TRIzol reagent. The mRNA expression of TMPRSS2 was determined by real-time PCR; the data are presented as relative values to GAPDH expression (n = 4). A virus entry inhibition assay was performed using HCT-8 cells with b) VR-1558 and c) SGH-36/2014 according to the legend for Fig. 2. The inhibitor concentrations used were 10 or 100 μM. All data are shown as relative virus entry (%) as compared with that in the control case (DMSO treatment) (n = 4).

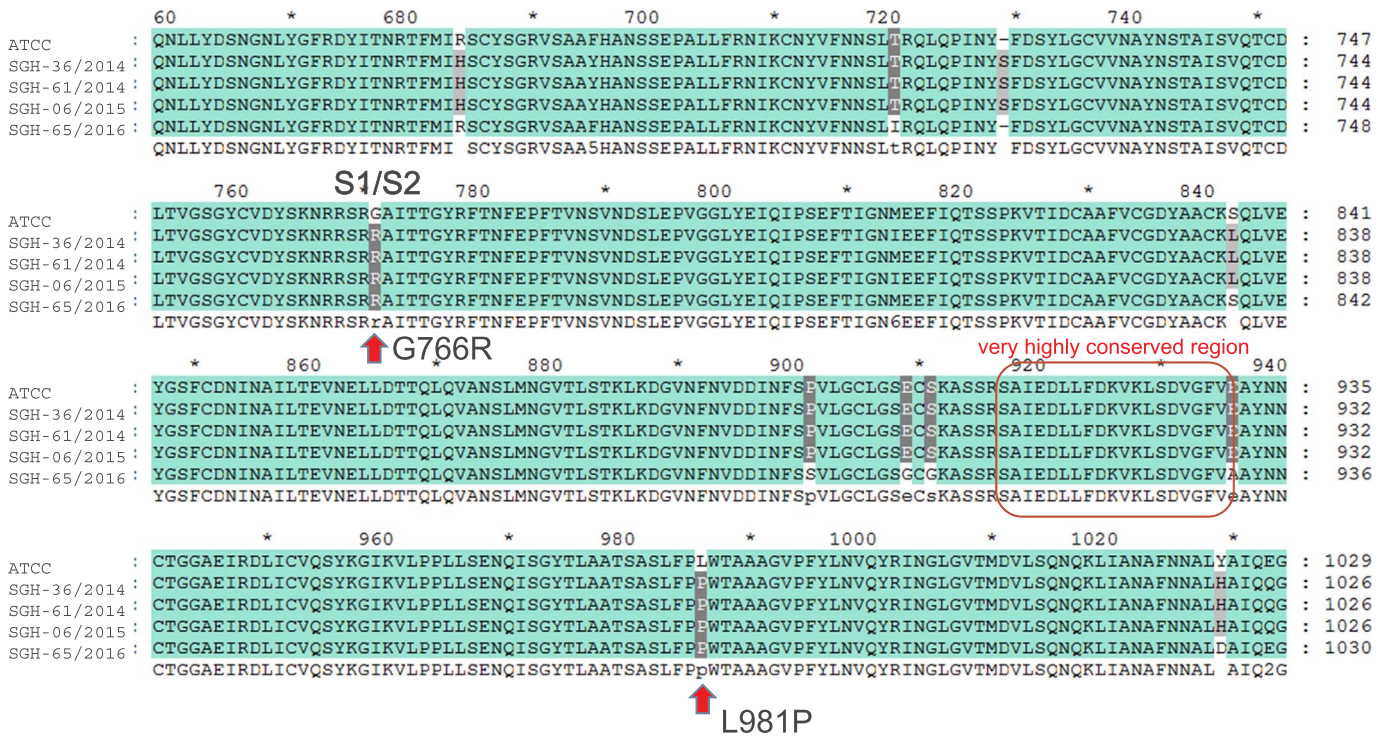

Fig. 4. Alignment of the amino acid sequence of the S protein of HCoV-OC43. The alignment was constructed using GeneDoc software. The amino acid sequence from 655 to 1029 of VR-1558 is shown. The accession numbers of the sequences are as follows: VR-1558, NC_005147; SGH-36/2014, LC315646; SGH-61/2014, LC315647; SGH-06/2015, LC315648; and SGH65-2016, LC315649.

8 cells expressed TMPRSS2 mRNA. These findings suggest that the expression of TMPRSS2 in HCT-8 cells is insufficient for HCoV-OC43 entry from the cell surface, and also suggest that cathepsins are used for HCoV-OC43 entry into HCT-8 cells.

3. Discussion

For many years, isolation of HCoVs was considered difficult (Peiris and Poon, 2009; Tyrrell and Myint, 1996). The first isolation of HCoV-229E and HCoV-OC43 was reported in the mid-1960s to 1970 using repeated passage in cultured cells or suckling mouse brains (Bruckova et al., 1970; Hamre and Procknow, 1966; McIntosh et al., 1967a, 1967b). Later, some studies reported isolation of HCoVs using LLC-MK cells, monkey kidney cells, and human embryo fibroblasts (Shirato et al., 2012; van der Hoek et al., 2004). Our group and others reported that addition of an external protease, such as trypsin, and a transmembrane serine protease, TMPRSS2, enhanced the entry and replication of HCoVs (Bertram et al., 2013; Kawase et al., 2009, 2012). For HCoV-229E, addition of these proteases resulted in successful isolation (Hirokawa et al., 2008; Matoba et al., 2016). However, no reliable cell line for isolation was available, unlike (for example) the MDCK cells used to isolate influenza viruses.

The results of our present study show that the reported difficulty in isolating HCoVs is a natural phenomenon because HCoVs circulating in the natural world do not enter cells via the late endosome pathway using cathepsins. It is difficult for the viruses to grow in commonly used cultured cells, which have only the late endosomal pathway. To date, some HCoVs have been isolated using cell culture as described above, but it may be that these results were rather accidental. Sometimes, particular features of the cultured cells may have affected the results, and, at other times, quasispecies may have been selected because of their ability to use cathepsins for cell entry. Such forced selection does indeed produce HCoVs, but the character of the viruses is changed from their natural state.

In this study, inhibition of the late endosomal pathway by EST increased the entry of some clinical virus isolates. This suggests that cell entry via the late endosomal pathway induces some anti-viral host immunity. Indeed, Bertram et al. (2013) reported that expression of interferon-induced transmembrane proteins (IFITMs) that inhibit diverse enveloped viruses also inhibited the entry of pseudotyped virus bearing HCoV-229E S protein, and that the reduced entry was rescued by co-expression of TMRPSS2. It is thought that IFITMs exert antiviral effects at the late stage of entry (Desai et al., 2014; Li et al., 2013; Munoz-Moreno et al., 2016). Thus, entering cells via endosomal cathepsins seems not to afford advantages for virus survival. Therefore, HCoVs circulating in the field may have evolved to not use the late endosomal route with cathepsins to avoid such unfavorable reactions by host cells. In addition, HCoV-OC43 used cathepsins for entry into the cultured cell line HCT-8. However, in this study EST treatment could not inhibit viral entry completely, even at a high concentration (100 μM). Previously, we reported that the siRNA-mediated gene knockdown of cathepsin L could not inhibit the entry of HCoV-229E completely, suggesting that other proteases are also used by HCoV-229E in the process of infection (Kawase et al., 2009). Moreover, treatment with EST, camostat, and leupeptin in WI-38 and MRC-5 cells (human fetal pulmonary fibroblasts without TMPRSS2 expression), did not inhibit infection by MERS-CoV and HCoV-229E. This suggested that proteases other than cysteine, serine, and threonine might be used for entry into these immature cells (Shirato et al., 2013). Therefore, although cathepsins are used during the entry process of HCoV-OC43 in HCT-8 cells, proteases other than cathepsins and TMRPSS2 are also used for the activation of S protein (like other human coronaviruses).

Development of the HBTE-ALI cell culture method allowed successful isolation of HCoVs, probably in their natural state (Dijkman et al., 2013; Pyrc et al., 2010). For the development of antiviral drugs, it is necessary to understand virus characteristics, and isolation of the virus is the first step. Therefore, it is important to isolate a virus that has natural characteristics.

Madu et al. (2009) reported that a common motif (very highly conserved region, VHCR) is found in the S protein of coronavirus and

may be a possible fusion peptide. It was thought that recognition sites for cleavable proteases might exist around the fusion peptide. In a previous study, we showed that the two amino acids that differ between laboratory and clinical isolates of HCoV-229E (R642M and N714K) were the determinants of the difference in cell entry route. These sandwich the VHCR, and were therefore not thought to be protease recognition sites. However, substitution of these two amino acids between the laboratory and clinical isolates completely changes (inverts) their cathepsin L use during cell entry (Shirato et al., 2017). This implies that some structural modification of S protein caused by the two amino acid mutations affects the accessibility of cathepsin L to its true recognition site. As shown in Fig. 4, only two remarkable amino acid differences were seen between the laboratory and clinical isolates of HCoV-OC43 (G766R and L981P). The G766R mutation is in the furin recognition site, and it may affect sensitivity to furin.

Park et al. (2016) reported that MERS coronavirus had different entry processes dependent on whether S protein was cleaved by furin. If S protein was cleaved by furin before cell entry, entry was sensitive to cell-surface proteases and MERS entered the cell by the early endosome. Yet the virus that had un-cleaved S protein was trafficked through cell-surface proteases and into the late endosome. They also reported that these un-cleaved viruses were less infectious to human airway epithelial and Calu-3 cell cultures (Park et al., 2016). Indeed, all clinical isolates of HCoV-OC43 retain the furin cleavage site (Fig. 4), and the data from this study show that their infectivity was dependent on the expression of TMPRSS2. This idea supports the findings in this study because the clinical isolates of HCoV-OC43 with cleaved S protein selected the cell-surface TMRPSS2 route for entry and were sensitive to cleavage by cell surface proteases.

Thus, although the mutation in the furin site may affect the use of cell-surface proteases, these two mutations sandwich the VHCR, similar to HCoV-229E. As reported previously, adaptation to TMPRSS2-negative cell cultures altered the protease sensitivity of HCoV-229E S protein from TMPRSS2 to cathepsin L (Shirato et al., 2017). Additional studies are required to elucidate the role of mutations in S protein in the infection events of HCoV-OC43.

4. Conclusion

We showed that the clinical isolates of HCoV-OC43 and -HKU1 preferred the cell-surface TMRRSS2 to endosomal cathepsins for cell entry, as did HCoV-229E. In addition, the cell-culture-adapted HCoV-OC43 lost the ability to infect and replicate in HBTE-ALI cell culture, which mimics the natural situation of HAE. These results indicate that clinical isolates of HCoVs from the field generally enter cells not via late endosomes with cathepsins but via the cell surface or early endosomes with TMPRSS2. In addition, this study suggests that virus adaptation in cultured cells after isolation alters the route of cell entry and that such modified isolates might not reflect the HCoVs of the natural world.

5. Materials and methods

5.1. Cells and viruses

HCT-8 cells (American Tissue Culture Collection [ATCC] CCL-244) were used. HCT-8 cells were maintained in DMEM (D5796; Sigma, St. Louis, MO, USA) containing 10% (v/v) horse serum. We used Cell Dissociation Solution- Non-enzymatic (C5914, Sigma) to exclude experimental bias induced by any trypsin remaining during cell preparation. Human bronchial tracheal epithelial (HBTE) cells were obtained from LIFELINE CELL TECHNOLOGY (FC-0035), and maintained in PneumaCult Ex medium (STEMCELL Technologies, Vancouver, Canada), according to the manufacturer's instructions. HBTE cells were generated by air-liquid interface (ALI) culture using Transwell Permeable Supports (Costar 3470) and PneumaCult ALI (STEMCELL Technologies).

The strain HCoV-OC43 (VR1558) was obtained from the ATCC and propagated and titrated using RD cells (ATCC CCL-136). Clinical isolates of HCoV-OC43 and HKU1 were isolated using HBTE-ALI culture. Nasal swabs were obtained from outpatients in a hospital in Tokyo, Japan between 2014 and 2016, after approval of the National Institute of Infectious Diseases Ethics Review Board for Human Subjects. The specimens were subjected to respiratory virus testing by real-time RT-PCR (Kaida et al., 2014). HCoV-positive specimens were inoculated onto HBTE-ALI cultures, and after 4 h incubation were washed with DMEM twice and incubated at 34 °C. After incubation for 3 days, cells were washed four times with DMEM containing 1% (v/v) fetal calf serum, which was then used as virus stocks. Virus replication was confirmed by calculating RNA copy numbers using real-time RT-PCR as described earlier (Dare et al., 2007; van Elden et al., 2004) and comparing them to those in the washings after virus adsorption. Isolated viruses and the relevant RT-PCR results are listed in Table 1. The sequences of the S proteins were obtained using Sanger's method and alignment analysis was performed using GeneDoc software (https://www.nrbsc.org/gfx/genedoc/ebinet.htm).

5.2. Virus replication and entry inhibition assay

To evaluate the replication kinetics of clinical and ATCC isolates of HCoV-OC43, 10^8 copies of virus were inoculated onto HBTE-ALI cultures or HCT-8 cells. After incubation for 4 h, the cells were washed twice and incubated at 34 °C. After 1 day of incubation, the cells were collected and used for analysis of virus entry. Cell supernatants or cell washings obtained after 3 days of incubation were used to evaluate virus replication as described below. The amounts of replicated virus were expressed as RNA copy numbers in cell supernatants or cell washings minus those remaining in cell washings after virus adsorption.

For the entry inhibition assay using HBTE-ALI cultures, 10^7 to 10^8 copies of viruses were inoculated onto cells that had been pre-treated with entry inhibitors [EST (23,25)trans-epoxysuccinyl-l-leucylamindo-3-methylbutane ethyl ester) (330005: Calbiochem, San Diego, CA, USA)] or camostat mesylate (3193: Tocris Bioscience, Bristol, UK) for 1 h. After incubation for 4 h in the presence of inhibitors, cells were washed twice and incubated at 34 °C. After 1 day of incubation, the cells were collected. Cell viability was determined using CellTiter-Glo and a GloMAX luminometer (Promega, Madison, WI, USA). Total cellular RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) with ribonucleic acid from baker's yeast (R6750, Sigma) as carrier RNA. To assess virus entry, subgenomic RNA containing nucleocapsid protein was amplified by real-time RT-PCR using the following primer and probe sets: For HCoV-OC43, forward, 5′- TCACTGATCTCTTGTTAGATCTTTTTGTA-3′; reverse, 5′- TTTGCTTGGGTTGAGCTCTT-3′; and probe, 5′- GGCCGATCAGTCCGACCAGTTT-3′ (FAM-labeled): for HCoV-HKU1, forward, 5′- TATCAGCTTACGATCTCTTGTCAGA-3′; reverse, 5′- TGGTCAGCCCAAGAAGTTTT-3′; and probe, 5′- GAAGCTCCTCTGGAAATCGTTCAGGA-3′ (FAM-labeled). RNA copy numbers were calculated from standard curves obtained with the aid of control RNA templates.

The data were statistically analyzed using the unpaired *t*-test and are presented as log RNA copies per well.

5.3. Determination of the expression levels of TMPRSS2 and cathepsin L mRNA

The expression level of TMPRSS2 mRNA was examined by a real-time RT-PCR assay. Total RNAs were extracted by TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Real-time PCR was performed using Fast Virus 1-step Master Mix (Thermo Fisher Scientific) and LightCycler96 (Roche, Basel, Switzerland). Commercial specific primers were used as follows: GAPDH, 4326317E (Thermo Fisher Scientific); and TMPRSS2, Hs00237175_m1 (Thermo Fisher Scientific). The data are presented as a relative value calculated based on each GAPDH value. Calu-3 cells

(ATCC, HTB-55) and LoVo cells (JCRB Cell Bank, Japan, IFO50067) were used as the positive control for TMPRSS2 expression, and HeLa cells (ATCC, CCL2.1) were used as the negative control.

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