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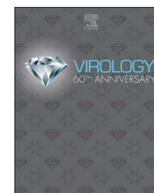

Brief Communication

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;

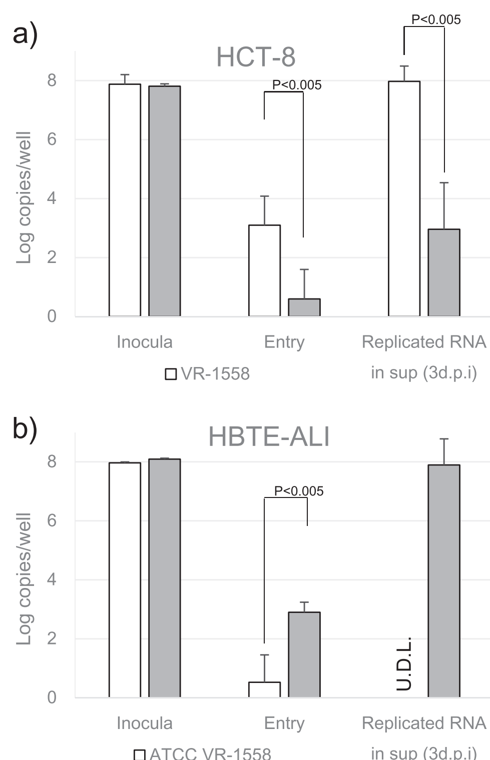

Fig. 1. Replication of laboratory and clinical isolates of HCoV-OC43 in HBTE-ALI cultures. Identical amounts (10^8 copies) of laboratory (VR-1558) and clinical (SGH-36/2014) isolates of HCoVOC43 were inoculated onto a) HCT-8 or b) HBTE-ALI cells. After incubation for 4 h, the cells were washed with DMEM 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

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