Clinical Isolates of Human Coronavirus 229E Bypass the Endosome for Cell Entry

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ABSTRACT Human coronavirus 229E (HCoV-229E), a causative agent of the common cold, enters host cells via two distinct pathways: one is mediated by cell surface proteases, particularly transmembrane protease serine 2 (TMPRSS2), and the other by endosomal cathepsin L. Thus, specific inhibitors of these proteases block virus infection. However, it is unclear which of these pathways is actually utilized by HCoV-229E in the human respiratory tract. Here, we examined the mechanism of cell entry used by a pseudotyped virus bearing the HCoV-229E spike (S) protein in the presence or absence of protease inhibitors. We found that, compared with a laboratory strain isolated in 1966 and passaged for a half century, clinical isolates of HCoV-229E were less likely to utilize cathepsin L; rather, they showed a preference for TMPRSS2. Two amino acid substitutions (R642M and N714K) in the S protein of HCoV-229E clinical isolates altered their sensitivity to a cathepsin L inhibitor, suggesting that these amino acids were responsible for cathepsin L use. After 20 passages in HeLa cells, the ability of the isolate to use cathepsin increased so that it was equal to that of the laboratory strain; this increase was caused by an amino acid substitution (I577S) in the S protein. The passaged virus showed a reduced ability to replicate in differentiated airway epithelial cells cultured at an air-liquid interface. These results suggest that the endosomal pathway is disadvantageous for HCoV-229E infection of human airway epithelial cells; therefore, clinical isolates are less able to use cathepsin.

IMPORTANCE Many enveloped viruses enter cells through endocytosis. Viral spike proteins drive the fusion of viral and endosomal membranes to facilitate insertion of the viral genome into the cytoplasm. Human coronavirus 229E (HCoV-229E) utilizes endosomal cathepsin L to activate the spike protein after receptor binding. Here, we found that clinical isolates of HCoV-229E preferentially utilize the cell surface protease TMPRSS2 rather than endosomal cathepsin L. The endosome is a main site of Toll-like receptor recognition, which then triggers an innate immune response; therefore, HCoV-229E presumably evolved to bypass the endosome by entering the cell via TMPRSS2. Thus, the virus uses a simple mechanism to evade the host innate immune system. Therefore, therapeutic agents for coronavirus-mediated diseases, such as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS), should target cell surface TMPRSS2 rather than endosomal cathepsin.

KEYWORDS TMPRSS2, cathepsin, coronavirus, endosomes

Human coronavirus 229E (HCoV-229E), which belongs to the genus *Alphacoronavirus*, is a causative agent of the human common cold. HCoV-229E was first reported in 1966 (1), and the isolate obtained at that time (VR-740) is still used as a laboratory strain by the American Type Culture Collection (ATCC). We previously reported that serological differences between VR-740 and Japanese clinical isolates (Sendai-H/

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1121/04 and Niigata/01/08) depend on the S1 subunit of the spike (S) protein (2). The genomic features of these clinical strains are similar to those of strains isolated in the United Kingdom, Ghana, and Australia, which are thought to be prevalent worldwide (2–4). The replication efficiency of these isolates in HeLa cells is 1 log unit less than that of the laboratory strain, suggesting that the inefficient replication of the isolates is due to nonadaptation to cultured cells (2).

Two major mechanisms are responsible for proteolytic activation of viral spike glycoproteins. For many enveloped viruses, such as human immunodeficiency virus (HIV) and influenza virus, cellular proteases (e.g., furin, trypsin, or transmembrane protease serine 2 [TMPRSS2]) cleave the glycoprotein during biogenesis, separating receptor binding and fusion subunits and converting the precursor glycoprotein to its fusion-competent state (5, 6). Alternatively, for other viruses, such as severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and Ebola virus, cleavage of the viral glycoprotein by cell surface or endosomal proteases (e.g., TMPRSS2, HAT, furin, trypsin, elastase, or cathepsin L) induces conformational changes during viral entry following receptor binding (7–14). After virus/receptor binding, HCoV-229E also utilizes host cellular proteases to trigger viral-membrane–cell membrane fusion. HCoV-229E enters cells at the cell surface in the presence of extracellular serine proteases, such as trypsin, but in their absence, the virus utilizes cathepsin L in the late endosome (15, 16). Despite these observations, the precise mechanism by which coronavirus penetrates the cell surface is unknown; however, it is possible that entry is via an early endosome, similar to that reported for HIV (17).

Zhou et al. reported the therapeutic effect of protease inhibitors against SARS-CoV. They showed that the pathogenesis of SARS-CoV in mice was effectively prevented by the serine protease inhibitor camostat (which inhibits TMPRSS2, HAT, and elastase), but not by cathepsin inhibitors (18). This suggests that SARS-CoV mainly utilizes cell surface proteases rather than endosomal cathepsin L *in vivo*. Here, we used protease inhibitors to examine the mechanisms used by laboratory-passaged and clinical isolates of HCoV-229E to enter cells. Finally, we discuss the preferred mechanism (the endosomal or cell surface pathway) of HCoV-229E in the human respiratory tract.

RESULTS

Clinical HCoV-229E isolates show reduced replication in HeLa cells. As previously reported, the replication of HCoV-229E clinical isolates 229E/clin-Sd (Sendai-H/1121/04) and 229E/clin-Ng (Niigata/01/08) in HeLa cells at 24 h postinfection was 1 log unit less than that of a laboratory strain, 229E/lab (ATCC VR-740) (2). Here, we compared the replication of these HCoV-229E strains in native HeLa cells and HeLa cells expressing TMPRSS2 (HeLa-TMPRSS2). Although the replication rates of both the laboratory strain and clinical isolates in HeLa-TMPRSS2 cells were almost equal (Fig. 1A), we confirmed that replication of clinical isolates in native HeLa cells was 1 log unit less than that of the laboratory strain (Fig. 1A). To clarify whether the decrease in replication was due to reduced infection of HeLa cells or to augmented infection of HeLa-TMPRSS2 cells, we performed a viral replication competition assay. Equal amounts of 229E/lab and 229E/clin-Sd (each at 10^3 PFU) were mixed and used to inoculate HeLa or HeLa-TMPRSS2 cells. The cells were then passaged three times (Fig. 1B). The ratio of 229E/lab and 229E/clin-Sd in the culture medium was measured by dual quantitative PCR using primers and probes specific for each strain. Although both 229E/lab and 229E/clin-Sd survived in HeLa-TMPRSS2 cells, 229E/clin-Sd disappeared after three passages in HeLa cells (Fig. 1B). These results suggest that the clinical isolate of HCoV-229E lacks the ability to grow in HeLa cells that do not express TMPRSS2.

Clinical isolates are less able to use cathepsin for cell entry. Viral entry into cells was quantified using pseudotyped vesicular stomatitis virus (VSV) bearing the S proteins of HCoV-229E. The green fluorescent protein (GFP)-positive cells were counted at 20 h postinoculation. As previously reported, protease inhibitors were used to determine the viral entry pathway because specific inhibitors of TMPRSS2 or cathepsin L

FIG 1 Replication of HCoV-229E in HeLa and HeLa-TMPRSS2 cells. (A) Viral replication. HeLa or HeLa-TMPRSS2 cells (10^5) were inoculated with 229E/lab, 229E/clin-Sd, and 229E/clin-Ng strains (10^3 PFU; $n = 12$). After 24 h, cells were collected and ultrasonicated, and the virus titer was determined in HeLa cells cultured in medium supplemented with trypsin. The bars and error bars indicate the means and standard deviations (SD), respectively. The data were analyzed using a two-tailed Student *t* test. (B) Viral replication competition. HeLa or HeLa-TMPRSS2 cells (10^5) were inoculated with a mixture of 229E/lab and 229E/clin-Sd (10^3 PFU of each virus) and incubated for 24 h. After passing three times, viral RNA was quantified in a dual quantitative PCR. The data are expressed as the means of three replicates ($n = 3$ independent culture wells). *** (very highly significant), $P \leq 0.001$.

block HCoV-229E infection via the cell surface or endosome, respectively (15, 16). Cells were treated for 30 min with E64d [(2S,25S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester], a broad inhibitor of cysteine proteases (including cathepsins), and camostat, a serine protease inhibitor that inhibits TMPRSS2, and then infected with pseudotyped viruses. The data were represented as percent blockade relative to that in cells not treated with inhibitors (Fig. 2A and B). As expected, camostat had no effect on viral entry into HeLa cells, whereas E64d blocked entry of both 229E/lab and 229E/clin (Fig. 2A), suggesting that these viruses use only cathepsin L in this cell line. In contrast, treatment of HeLa-TMPRSS2 cells with 5 μ M E64d blocked 50% of 229E/lab but only 10% of 229E/clin, whereas treatment with camostat blocked 30% of 229E/lab but 50% of 229E/clin (Fig. 2A). These data suggest that 229E/clin tends to use TMPRSS2 rather than cathepsin L and that 229E/lab does the opposite. Figure 2B also shows a similar effect in HeLa-TMPRSS2 cells when inhibitors were used at 10 μ M. Simultaneous treatment of HeLa-TMPRSS2 cells with 10 μ M camostat and 10 μ M E64d blocked the entry of both 229E/lab and 229E/clin almost completely (Fig. 2B), suggesting that both laboratory and clinical strains enter cells via two distinct pathways mediated by cathepsin L and TMPRSS2.

Next, we examined the cell entry kinetics of pseudotyped viruses. The viruses were adsorbed onto HeLa or HeLa-TMPRSS2 cells on ice for 1 h before being shifted to 37°C. Viral entry was prevented by treatment with 10 μ M E64d and 10 μ M camostat at the indicated times. Data were expressed as a percentage relative to virus-infected HeLa-TMPRSS2 cells in the absence of inhibitors (Fig. 2C). Entry of both the laboratory and clinical strains into HeLa-TMPRSS2 cells began immediately after the shift to 37°C; however, entry into HeLa cells was delayed by 1 h, suggesting that was the time it took for the virus to pass through the endosome. When we compared 229E/lab with 229E/clin in HeLa-TMPRSS2 cells, we found that 60% of 229E/lab had entered the cells at 1 h, by which time 80% of 229E/clin had already entered. This suggests that, by using cell surface TMPRSS2, 229E/clin entered cells faster than 229E/lab. In contrast, 7% of 229E/clin had entered HeLa cells at 1 h compared with about 30% of 229E/lab, suggesting 229E/lab entered cells faster than 229E/clin by using endosomal cathepsin L. Thus, reduced replication of clinical isolates in HeLa cells appears to be due to reduced activation of the S protein by cathepsin L, whereas the laboratory strain prefers to use cathepsin L.

Mutations in the S protein required for cathepsin use. As previously reported, amino acid differences between the laboratory strain (VR-740) and the clinical isolates

FIG 2 Blockade of pseudotyped-virus entry by protease inhibitors. HeLa or HeLa-TMPRSS2 cells were inoculated with the VSV-pseudotyped viruses bearing the 229E/lab, 229E/clin-Sd, and 229E/clin-Ng S proteins or the G protein of VSV. (A) Concentration dependency of inhibitors. HeLa or HeLa-TMPRSS2 cells were infected with VSV-pseudotyped viruses in the presence of a serially diluted cathepsin inhibitor, E64d, or a TMPRSS2 inhibitor, camostat. The error bars indicate SD. (B) Blockade of viral entry via two distinct pathways. Cells were infected with VSV-pseudotyped viruses bearing the S protein, as described above, in the presence of 10 μM E64d, 10 μM camostat, or a combination of the two. The GFP-positive cells were counted at 24 h postinfection ($n = 6$), and the data were expressed as percentages relative to those for the controls (absence of inhibitors). At least 200 GFP-positive cells were counted under control conditions. (C) Cell entry kinetics of pseudotyped HCoV-229Es. After incubation for the indicated times (0, 10, 20, 40, 60, 120, or 240 min), the cells were treated with 10 μM E64d and 10 μM camostat to stop viral entry. At least 200 GFP-positive cells/well were counted at 20 h postinfection ($n = 6$). The data are expressed as percentages relative to those in HeLa-TMPRSS2 cells cultured in the absence of inhibitors. The asterisks indicate the statistical significance of the data from 229E/clins compared with that from 229E/lab. * (significant), $P \leq 0.05$; ** (highly significant), $P \leq 0.01$.

have accumulated upstream of the receptor binding region (417 to 547) of the S1 subunit; otherwise, the amino acid sequences are 97% identical (2). A very highly conserved region (VHCR) in the S2 subunit of the coronavirus S protein is a possible fusion peptide (19). The protease cleavage site might be located on the N-terminal side of this VHCR. We hypothesized that amino acid differences around the VHCR of the laboratory and clinical strains (R642M, T681R, N714K, V765A, and A775S in the 229E/lab S protein), except a mutation (I700L) within the VHCR, might affect viral cathepsin usage. Expression plasmids bearing 229E/lab or 229E/clin-Sd S proteins harboring these mutations were constructed, and cell entry by pseudotyped VSV harboring the mutant S proteins was examined in HeLa cells. The pseudotyped viruses were adjusted to the same titer using HeLa-TMPRSS2 cells to enable comparison of their infectivity in HeLa cells. At least 200 GFP-positive cells/well were counted under control conditions. The number of GFP-positive cells induced by the nonglycoprotein pseudotype control

FIG 3 Cathepsin usage by the HCoV-229E spike protein. (A) Effects of amino acid substitutions in the S protein on virus entry. Five amino acid substitutions (R642M, T681R, N714K, V765A, and A775S) are present around the fusion peptide sequence in the S protein of 229E/lab and 229E/clin-Sd. VSV-pseudotyped viruses bearing mutated S proteins or VSV-G were inoculated onto HeLa or HeLa-TMPRSS2 cells, and the GFP-positive cells were counted at 24 h postinfection ($n = 6$). The data are expressed as percentages relative to those in HeLa-TMPRSS2 cells. (B) Effects of protease inhibitors on pseudotyped viruses bearing S proteins harboring R642M and N714K. HeLa or HeLa-TMPRSS2 cells were inoculated with VSV-pseudotyped viruses in the presence of inhibitors of cathepsin L (CatL) (cathepsin inhibitor III) or cathepsin B (CatB) (CA-074) (each at 10 μ M). DMSO-treated cells served as a negative control. At least 200 GFP-positive cells/well were counted at 20 h postinfection ($n = 6$). The data are expressed as percentages relative to those in HeLa cells treated with DMSO. n.s. (not significant), $P > 0.05$; * (significant), $P \leq 0.05$; ** (highly significant), $P \leq 0.01$. The error bars indicate SD.

(background) was substituted from the raw data. Of note, the S1 subunit of the 229E/clin-Sd S protein harbors three amino acid deletions compared with 229E/lab; therefore, the numerical position of the mutation in the S2 subunit is three less.

The above-described data (Fig. 1B) show that replication of the clinical strain in HeLa-TMPRSS2 cells was the same as that of the laboratory strain; however, replication of the clinical strain was lower in HeLa cells, indicating that it is less able to use cathepsin L than the laboratory strain. Among the five mutations, R642M or N714K in the S protein of 229E/lab caused a slight but significant reduction in virus entry into HeLa cells, whereas a combination of the two led to a further reduction. Similarly, M639R or K711N in the S protein of 229E/clin-Sd led to a slight increase in virus entry into HeLa cells, whereas a combination of the two led to a further increase (Fig. 3A). To clarify the effects of these mutations on cathepsin dependency, we next examined the cathepsin inhibitor sensitivity of the pseudotyped viruses, as was previously reported for filovirus (20). Exposure of 229E/lab to 10 μ M cathepsin L inhibitor III inhibited virus entry into HeLa cells by 90%; single mutations (R642M or N714K) in the 229E/lab S protein recovered entry by 30%, whereas a double mutant recovered entry by 80% (Fig. 3B). Exposure of 229E/clin-Sd to the cathepsin L inhibitor inhibited entry into HeLa cells by 24%. As previously reported, HCoV-229E may use other, as-yet-unidentified proteases in the endosome; therefore, the virus has the potential to infect HeLa cells in the presence of a cathepsin L-specific inhibitor (16). Single (M639R or K711N) or double mutations reduced viral entry by around 50%. Exposure of 229E/lab and 229E/clin-Sd

FIG 4 Replication and cell entry by the passaged HCoV-229E clinical isolate. (A) Replication of the passage 20 clinical isolate. HeLa or HeLa-TMPRSS2 cells (10^5) were inoculated with 229E/lab, 229E/clin-Sd-p1, and 229E/clin-Sd-p20 (10^3 PFU) ($n = 6$). After 24 h, the cells were collected and ultrasonicated, and virus titers were determined in HeLa cells cultured in medium supplemented with trypsin. P1, passage 1; P20, passage 20. (B) Effects of amino acid substitutions in the S protein on virus entry. There was one amino acid mutation in the S protein of 229E/clin-Sd at passage 20 (I577S). HeLa or HeLa-TMPRSS2 cells were inoculated with VSV-pseudotyped viruses bearing HCoV-229E S proteins or the VSV G protein in the presence or absence of 10 μ M cathepsin inhibitor III, and at least 200 GFP-positive cells/well were counted under conditions at 24 h postinfection ($n = 4$). The data are expressed as percentages relative to those in HeLa-TMPRSS2 cells. ** (highly significant), $P \leq 0.01$; *** (very highly significant), $P \leq 0.001$. The error bars indicate SD.

to the cathepsin B inhibitor CA-074 did not inhibit entry. These results suggest that the observed differences in the abilities of 229E/lab and 229E/clin-Sd to enter HeLa cells are due to the ability of the S protein to utilize cathepsin L. Also, two amino acid substitutions were required to attain sufficient use of cathepsin L. It would appear that these substitutions are not present in the protease cleavage region. The protease cleavage site is sandwiched between these mutations, suggesting that the mutations at these positions may affect access by cathepsin L.

Passage of clinical isolates results in increased cathepsin use. The above-described results suggest that HCoV-229E is less able to utilize cathepsin *in vivo* and that its ability to do so increases upon serial passage in cultured cells. To confirm this experimentally, first-passage 229E/clin-Sd-p1 was passaged a further 19 times in HeLa cells to generate 229E/clin-Sd-p20. The proliferation of 229E/clin-Sd-p1 in HeLa cells was 1 log unit less than that of 229E/lab, although the viruses grew equally well in HeLa-TMPRSS2 cells (Fig. 4A). After 20 passages, 229E/clin-Sd-p20 acquired a high capacity to replicate in HeLa cells (equivalent to that of 229E/lab) (Fig. 4A). Analysis revealed a single point mutation (I577S) in the S protein. Experiments using pseudotyped virus bearing a mutated S protein confirmed that I577S increased viral entry into HeLa cells (Fig. 4B). Entry of 229E/lab into HeLa cells was about 60% of that into HeLa-TMPRSS2 cells, whereas that of 229E/clin-Sd-p1 was about 20%; furthermore, a single mutation (I577S) in the 229E/clin-Sd S protein recovered virus entry by 60%; this recovered entry was inhibited by treatment with cathepsin L inhibitor III (Fig. 4B). However, when we passaged 229E/clin-Sd in HeLa-TMPRSS2 cells, we did not observe enhanced replication in HeLa cells or any mutations in the S protein.

The virus passaged in HeLa cells reduces infectivity in HBTE-ALI cells. We hypothesized that the cathepsin/endosomal pathway may be disadvantageous for HCoV-229E in that reliance on cathepsin would result in low replication in airway epithelial cells. Therefore, we prepared differentiated primary human bronchial/tracheal epithelial (HBTE) cells using an air-liquid interface (ALI) culture system, as described previously (15, 21). This is the best *in vitro* model of human airway epithelium (22). To confirm cell differentiation, HBTE cells were inoculated with human coronavirus HKU1 (HCoV-HKU1) (which replicates only in differentiated airway epithelial cells) (21). After 24 h, virus secreted on the apical surface was quantified by real-time PCR. The results revealed a 60,000-fold increase in the amount of viral RNA (Fig. 5A). We then

FIG 5 Replication of the passaged HCoV-229E clinical isolate in HBTE-ALI cells. (A) Characterization of HBTE cells by HCoV-HKU-1 infection. HBTE cells were cultured for 4 weeks in differentiation medium at an air-liquid interface (HBTE-ALI) in a 6.5-mm-diameter Transwell chamber. To confirm differentiation, HBTE cells, differentiated (incubated for 4 weeks) or undifferentiated (incubated for 0 weeks), were inoculated with HCoV-HKU1 ($n = 2$). After 2 h, the inoculated virus was removed and the cells were washed three times with medium. After 72 h of incubation at the ALI, RNA was collected from the medium, and real-time PCR was performed to quantify viral RNA. (B) Characterization of HBTE cells by measurement of cellular transcripts. Expression of cellular mRNAs encoding cell differentiation markers (E-cadherin, ZO-1, and MUC5AC) and factors associated with HCoV-229E infection (APN, TMPRSS2, and cathepsin L) in differentiated and undifferentiated HBTE cells was measured by real-time PCR ($n = 4$). The data are expressed as the fold change in transcript levels in differentiated cells relative to that in undifferentiated cells. (C) Replication of passaged HCoV-229E in HBTE cells. HBTE-ALI cells were inoculated with 229E/clin-Sd-p1 and 229E/clin-Sd-p20 (10^4 PFU), the titers of which were measured in HeLa-TMPRSS2 cells cultured in medium supplemented with trypsin ($n = 4$). HeLa cells were used as a control to confirm differential RNA expression due to the differing abilities of passage 1 and passage 20 virus to use cathepsin. After 1 h, the inoculated virus was removed, and the cells were washed three times in culture medium. After 24 h of incubation at the ALI, cellular RNA was collected, and viral RNA was measured by real-time PCR. ** (highly significant), $P \leq 0.01$; *** (very highly significant), $P \leq 0.001$. The error bars indicate SD.

measured the amounts of cellular mRNAs encoding cell differentiation markers and factors related to HCoV-229E entry in undifferentiated and differentiated HBTE cells by real-time PCR. All the transcripts examined in this study were detected in undifferentiated cells. However, mRNA encoding MUC5AC (a mucin secreted onto the airway surface) and aminopeptidase N (APN) (an HCoV-229E receptor) increased by 13-fold and 4-fold, respectively (Fig. 5B). There was no increase in mRNA encoding E-cadherin and ZO-1 (involved in the formation of tight junctions) or in mRNAs encoding TMPRSS2 or cathepsin L (Fig. 5B).

To compare the replication of 229E/clin-Sd at passages 1 and 20, differentiated HBTE cells and HeLa cells were inoculated with 10^4 PFU of virus, which was measured in a plaque assay using HeLa-TMPRSS2 cells cultured in trypsin-containing medium. After 24 h, cellular RNA was isolated, and viral RNA was quantified by real-time PCR. As expected, compared with passage 1 virus, there was significantly (15-fold) less RNA derived from passage 20 virus in differentiated HBTE cells but significantly more (27-fold) in HeLa cells (Fig. 5C). These results suggest that reliance on cathepsin leads to reduced viral replication in airway epithelial cells.

Locations of mutations in S protein. The amino acid substitutions in the S protein responsible for cathepsin use are shown in Fig. 6A, in which the amino acid sequences are aligned around a fusion peptide. To identify the positions of the amino acid substitutions within the tertiary structure, we modified the cryoelectron microscopic structures of the mouse hepatitis virus (MHV) and the HCoV-HKU1 S proteins (prefusion state) (23, 24) by homology modeling using the Molecular Operating Environment (MOE) software package (Chemical Computing Group). The topological features and the locations of the fusion peptide and the cleavage site are similar in both models. All the identified mutations that affect cathepsin use are scattered randomly throughout the S2 subunit; none are close to the protease cleavage site (Fig. 6B and C).

FIG 6 Homology modeling of HCoV-229E S protein. (A) Alignment of HCoV-229E S proteins around a fusion peptide. The intermediate regions between the S1 and S2 subunits of the HCoV-229E S glycoproteins were aligned using MAFFT software (CBRC, Japan). The trypsin cleavage site and the fusion peptide are indicated in green and yellow, respectively. The positions of the mutations causing an increase or decrease in cathepsin use are indicated in red or blue, respectively. (B and C) Theoretical structures of the S protein. The structures of HCoV-229E were constructed using MOE software based on the cryoelectron microscopic structure of the MHV S protein (B) or the HCoV-HKU1 S protein (C) in the prefusion state. The region around the fusion peptide in the S2 subunit (residues 572 to 953) is shown in orange. Mutations that cause increased or decreased cathepsin usage are shown in blue or red, respectively. The putative fusion peptide and the trypsin cleavage site are shown in yellow and green.

DISCUSSION

The results of this study suggest that HCoV-229E behaves differently in cell culture than in the human respiratory tract. We found that, compared with the strain reported in 1966 (229E/lab; ATCC VR-740), HCoV-229E clinical strains isolated in 2004 (229E/clin-Sd; Sendai-H/1121/04) and 2008 (229E/clin-Ng; Niigata/01/08) are less able to utilize endosomal cathepsin L for cell entry. In the original paper from 1966, the first cytopathic effects of HCoV-229E were observed 11 days after inoculation onto cultured cells in the absence of trypsin (1). The virus presumably acquired the ability to utilize cathepsin during the first isolation step or after passage over half a century. Here, virus passaged 20 times was also well able to use cathepsin. Interestingly, the passaged virus

was less able to replicate in differentiated airway epithelial cells, implying that the endosomal pathway is disadvantageous for virus replication *in vivo*. As shown in Fig. 2, in the absence of TMPRSS2, the virus must be exposed to the endosomal environment for 1 h during the entry process. Endosomal compartments are the main site of Toll-like receptor recognition, which triggers innate immune responses (25, 26); therefore, HCoV-229E may have evolved to bypass the endosome by entering via the cell surface using TMPRSS2, thereby evading the innate immune system. After virus infection through the endosome, epithelial cells are thought to produce cytokines, which then activate immune cells. The immune cells then trap the virus in the endosome, leading to an inflammatory reaction. Thus, viruses that enter cells through the endosome find it difficult to survive *in vivo*. However, induction of antiviral responses in cultured cells is thought to be weak; thus, viruses that prefer the endosomal pathway can survive.

Here, we did not assess the determinant responsible for high cathepsin L use by the laboratory strain, which might have acquired this ability half a century ago. Instead, we compared amino acid differences in the S proteins of the laboratory and clinical strains to identify the determinant responsible for low cathepsin L use. Of the 55 amino acid differences (46 in S1 and 9 in S2) between the S proteins of the laboratory and clinical strains, we selected 5 amino acid differences around the protease cleavage site in S2. Fortunately, we identified two amino acid substitutions (R642M and N714K) responsible for low cathepsin L use; furthermore, a single amino acid substitution (I577S) in the S protein of the clinical strain after 20 passages complemented the low cathepsin use caused by R642M and N714K. Examination of the theoretical structures of the S protein in its prefusion state suggests that the mutations occur randomly in the S2 subunit and are distant from the protease cleavage site. It is unclear how these mutations affect cathepsin cleavage by the S protein because receptor binding induces a conformation in the S protein that is distinct from that in the prefusion state, as reported for the MHV-2 and SARS-CoV S proteins (13, 27). We hypothesize that various point mutations in the S2 subunit induce a different conformational state at the cleavage site, thereby impeding access by cathepsin L. Further experiments are needed to clarify the mechanism(s) underlying proteolytic activation of the S protein by cathepsin L.

We and others previously reported that the serine protease inhibitor camostat specifically blocks TMPRSS2 and HAT, thereby inhibiting infection of cultured cells by HCoV-229E, MERS-CoV, SARS-CoV, and influenza virus (15, 16, 28–30). Furthermore, we recently reported that a serine protease inhibitor, nafamostat, inhibits TMPRSS2 and blocks MERS-CoV infection at a concentration 10 times lower than that of camostat (31). Another study clearly shows that the pathogenesis of SARS-CoV in a mouse model is ameliorated by camostat, but not by a cathepsin inhibitor (18). Furthermore, the results described here suggest that virus entry into airway epithelial cells via the endosomal pathway is associated with reduced virus replication. Therefore, pharmacological inhibition of endosomal cathepsin may not suppress virus replication. Thus, camostat and nafamostat, both of which are approved for the treatment of chronic pancreatitis (32, 33), may be suitable therapeutic candidates for respiratory coronavirus infections.

MATERIALS AND METHODS

Cells and viruses. HeLa cells (HeLa-229; ATCC CCL-2.1) and HeLa-TMPRSS2 cells (34), which were constructed by transfecting HeLa cells with a pcDNA3.1 plasmid containing the human TMPRSS2 gene (35), were used. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (D5796; Sigma, St. Louis, MO, USA) containing 5% fetal calf serum (FCS) (5% FCS-DMEM). To exclude experimental bias caused by the presence of trypsin during cell preparation, Cell Dissociation Solution Nonenzymatic (C5914; Sigma) was used to passage the cells. HBTE cells (FC-0035) were purchased from Lifeline Cell Technology (Frederick, MD, USA). The cells were plated on 6.5-mm-diameter Transwell permeable supports (Coaster; 3470). Human airway epithelium cultures were generated by growing the cells at an ALI for 4 weeks, resulting in well-differentiated, polarized cultures that resembled human pseudostratified mucociliary epithelium (22).

The ATCC strain of HCoV-229E (VR-740; designated 229E/lab in this study) was used (GenBank accession no. AB691763) (1). The clinical isolate Sendai-H/1121/04 (AB691764; designated 229E/clin-Sd) was isolated from a pharyngeal swab taken from a Japanese patient in 2004 and grown in LLC-MK2 cells cultured in trypsin-containing medium. Niigata01/08 (AB691767; designated 229E/clin-Ng) was isolated from a pharyngeal swab from a Japanese patient in 2008 and grown in CaCO2 cells cultured in

TABLE 1 Primers and probes employed for real-time PCR

Target mRNA	Method of detection	Primers and probes	
		Name	Sequence
229E/lab-S	TaqMan	229E-Lab-S-F	CGTTGACTTCAAACCTCAGA
		229E-Lab-S-R	ACCAACATTGGCATAAACAG
		229E-Lab-S-FAM	AGTTAAAGCACTTGCCACCGCC
229E/clin-S	TaqMan	229E-cS-S-F	CTATAACTGTCGCTCTGCTGT
		229E-cS-S-R	TACTAGCACTCCACCTATCAAAC
		229E-cS-S-HEX	TGACACCAACGAATTGGGTAGTGAAG
229E-N	Hybridization	229E-N-F	GTCGTCAGGGTAGAATACCTTA
		229E-N-R	CCCCTTTGCCCTTTCTAGT
		229E-N-FITC	GCCCTTTGCTTGTGATAGTGAACAACC
		229E-N-LC	TGGAAGGTGATACCTCGTAATTGGTACCC
HKU-1-replicase-1b	TaqMan	HKU1-Rep1b-F	CCTTGCGAATGAATGTGCT
		HKU1-Rep1b-R	TTGCATCACCAGTCTAGTACCAC
		HKU1-Rep1b-VIC	TGTGTGGCGGTTGCTATTATGTTAAGCCTG
Human TMPRSS2	Hybridization	TMPRSS2-F	CTCTACGGACCAAACCTTCATC
		TMPRSS2-R	CCACTATTCCTTGGCTAGAGTA
		TMPRSS2-FITC	TCAGAGGAAGTCCTGGCACCCCTGTGTG
		TMPRSS2-LC	CAAGACGACTGGAACGAGAATACGGGC
Human cathepsin-L	SYBR	CatL-F	GTGGACATCCCTAAGCAGGA
		CatL-R	CACAATGGTTTCTCCGGTC
MUC5AC	SYBR	MUC5AC-F	TACTCCACAGACTGCACCAACTG
		MUC5AC-R	CGTGTATTGCTTCCCGTCAA
E-cadherin	SYBR	E-cadherin-F	CCCACCACGTACAAGGGTC
		E-cadherin-R	CTGGGGTATTGGGGGCATC
Zo-1	SYBR	Zo-1-F	GCGGTGAGAGCCTTCTGATC
		Zo-1-R	CATGCTTACAGGAGTTGAGACAG
Human GAPDH	SYBR	GAPDH-F	AGAACATCATCCCTGCTCTACTG
		GAPDH-R	CCTCCGACGCCTGCTTCAC
Human APN	TaqMan	Applied Biosystems probe Hs00174265_m1	

trypsin-containing medium (2). All the viruses were propagated in HeLa cells and titrated on HeLa cells in the presence of supplemental trypsin, as described previously; titers were expressed as PFU (16).

The VSV-pseudotyped virus expressing GFP and harboring the HCoV-229E S protein or the VSV-G protein was prepared in 293/T17 cells as previously described (16). The VSV-pseudotyped virus harboring the VSV-G protein was used as a virus control. The VSV-pseudotyped virus harboring the mutant 229E S protein was prepared using the same method. Nucleotide mutations were inserted into a 229E S-expressing plasmid by site-directed-mutagenesis. The titer of VSV-pseudotyped viruses was determined in HeLa cells and expressed as focus-forming units (FFU).

Inhibitors. The following inhibitors were used: E64d (330005; Calbiochem, San Diego, CA, USA), camostat mesylate (3193; Tocris Bioscience, Bristol, UK), cathepsin L inhibitor III (219427; Calbiochem), and the cathepsin B inhibitor CA-074 (C5732; Sigma).

Pseudotyped-virus entry assay. HeLa or HeLa-TMPRSS2 cells were grown in 96-well plates (approximately 10^5 cells/well) and treated with protease inhibitors in 5% FCS-DMEM for 30 min at 37°C. The cells were inoculated with approximately 10^2 to 10^3 FFU of VSV-pseudotyped virus harboring the HCoV-229E S protein in the presence of the inhibitors, and the cells were incubated at 37°C for 20 h. Dimethyl sulfoxide (DMSO)-treated cells served as negative (no-inhibitor) controls. After incubation, images were captured under a BZ8000 microscope (Keyence Corporation, Osaka, Japan), and GFP-positive cells were counted using image measurement and analysis software (VH-H1A5 version 2.6; Keyence). The inhibitory effect was expressed as a percentage relative to control cells, as previously described (16).

To examine the kinetics of cell entry, HeLa or HeLa-TMPRSS2 cells were inoculated with VSV-pseudotyped viruses on ice for 1 h and then incubated at 37°C. After the indicated times (0, 10, 20, 40, 60, 120, or 240 min), cells were treated with E64d or camostat (final concentration, 10 μ M) to stop viral entry. After 24 h, the GFP-positive cells were counted, and the data were expressed as a percentage relative to those in HeLa-TMPRSS2 cells.

Growth competition of viruses. HeLa or HeLa-TMPRSS2 cells (10^6) in 24-well plates were simultaneously inoculated with 229E/lab and 229E/clin-Sd (10^3 PFU). After 1 h, the cells were washed three times with phosphate-buffered saline (PBS) and incubated at 37°C. After 20 h, the supernatants were collected and the amount of virus was titrated in a plaque assay using HeLa-TMPRSS2 cells. Next, newly prepared HeLa or HeLa-TMPRSS2 cells were inoculated with 10^3 PFU of virus. This process was repeated three times. Viral RNA in the culture supernatants was isolated using Isogen-LS reagent (Nippon Gene, Tokyo, Japan), together with 20 μ g of yeast RNA (Sigma) as a carrier. The amount of RNA derived from 229E/lab and 229E/clin-Sd was determined by real-time reverse transcription (RT)-PCR using a LightCycler (Roche, Basel, Switzerland). The primers and probes used for real-time PCR are described in Table 1. The RNA proportion of each virus was expressed as a per mille value.

Quantification of viral and cellular RNA. HBTE cells were cultured in differentiation medium (Lifeline Cell Technology) for 0 or 4 weeks at an ALI in a Transwell chamber, and cellular RNA was isolated by addition of Isogen reagent (Nippon Gene). HBTE cells were then inoculated with HCoV-229E (10^4 PFU) or HCoV-HKU1 (virus titer not quantified) for 2 h and washed three times prior to incubation at 37°C at the ALI. To detect HCoV-229 mRNA, Isogen reagent was added to the HBTE cells at 24 h postinoculation. To detect HCoV-HKU1 mRNA, culture medium was added to the apical surface at 72 h postinfection. The medium was then collected, and Isogen-LS reagent (Nippon Gene) was added. A real-time PCR assay was performed to quantify mRNA encoding HCoV-HKU1 replicase, 229E N protein, or cellular proteins. The primers and probes are listed in Table 1.

Structural model of the S protein. To construct the S protein model, the cryoelectron microscopic structure of the MHV S protein (23) was modified by homology modeling (36) using the MOE software package (Chemical Computing Group, Montreal, Canada). Structural figures were then generated using the PyMOL molecular visualization system.

Statistical analysis. A two-tailed Student *t* test was used to analyze statistical significance. A *P* value of <0.05 was considered statistically significant.

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