ENHANCEMENT OF SARS-CoV INFECTION BY PROTEASES

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1. INTRODUCTION

The severe acute respiratory syndrome (SARS) is caused by a newly emergent coronavirus (SARS-CoV).[1, 2] This virus grows in a variety of tissues that express its receptor, but the mechanism of the severe respiratory illness is not well understood. SARS-CoV is supposed to enter cells via endosome, and its spike (S) protein, which is responsible for cell entry of this virus, is activated by a certain protease active only in acidic conditions in the endosome.[3] To see whether this is correct or not, we began to study the SARS-CoV entry mechanism. In the course of this study, we found that various proteases facilitated SARS-CoV entry from cell surface. This indicated that SARS-CoV has a potential to enter cells via two different pathways, endosomal and cell-surface pathways, depending upon the presence of proteases. Moreover, SARS-CoV entry from the cell surface mediated by proteases was a 100-fold more efficient infection than entry through endosomes. These results suggest that severe illness in the lung and intestine can be attributed to the proteases produced in these organs in inflammatory responses or physiological conditions.

2. MATERIALS AND METHODS

2.1. Viruses and Cells

The SARS-CoV Frankfurt 1 strain, kindly provided by Dr. J. Ziebuhr,[1] was propagated and titered using Vero E6 cells. Recombinant vaccinia virus harboring SARS-CoV S gene (DIs-S) was used to express S protein. This recombinant virus was made from highly attenuated vaccinia virus DIs.[4] Vesicular stomatitis virus (VSV) pseudotype bearing SARS-CoV S protein was produced as reported previously.[5]

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2.2. Proteases

Various proteases were dissolved in phosphate-buffered saline, pH 7.2 (PBS), and used at the indicated concentration in DMEM containing 5% FCS. The proteases used in this study were trypsin (Sigma, St Louis, MO, T-8802), thermolysin (Sigma, P1512), chymotrypsin (Sigma, C-3142), dispase (Roche, 1 276 921, Branchburg, NJ), papain (Worthington Biochemicals, 53J6521, Freehold, NJ), proteinase K (Wako, Tokyo, Japan), collagenase (Sigma, C-5183) and elastase (Sigma, E-0258).

2.3. Western Blot

S protein expressed in Vero E6 cells was analyzed by Western blotting. Preparation of cell lysates, electrophoresis in SDS-polyacrylamide gel, and electrical transfer of the protein onto a transfer membrane were described previously.[6] S protein was detected with anti-S antibody, IMG-557 (IMGENEX, San Diego, CA, USA).

2.4. Real-Time PCR

SARS-CoV entry or replication in VeroE6 cells was examined by real-time PCR to detect the copy number of mRNA9. The primers for amplification were complementary to the leader sequence (forward) and N gene (reverse) of SARS-CoV. The reaction was performed using a LightCycler instrument (Roche).

3. RESULTS

3.1. Proteases Induce Syncytia Formation and S Protein Cleavage

VeroE6 cells were infected with the Frankfurt-1 strain of SARS-CoV at a multiplicity of infection (MOI) of 0.5, and infected cells were treated with trypsin (200 μg/ml) at room temperature (RT) for 5 min after 20 h incubation. Cell fusion was detected from approximately 2 h after trypsin treatment. Fusion was also found after treatment with thermolysin or dispase. Little or no fusion occurred following treatment with papain, chymotrypsin, proteinase K, or collagenase. S proteins in cells treated with proteases that induce fusion were cleaved approximately in the middle, and a fragment corresponding to S2 of ca. 100 kDa protein was detected. However, no S2 band was detected in SARS-CoV infected cells treated with proteases that failed to induce fusion. VeroE6 cells were infected with DIs-S that harbors SARS-CoV S gene at MOI of 1, and these cells were also treated with various proteases as described above. Trypsin, thermolysin, and dispase induced fusion of S protein expressing cells, while other proteases failed to induce substantial cell fusion (Fig. 1). The results obtained using VeroE6 cells expressing S protein by recombinant vaccinia virus were very similar to those observed in VeroE6 cells infected with SARS-CoV. These results showed that various proteases activate the fusion activity of the SARS-CoV S protein by inducing its cleavage. It was also revealed that SARS-CoV infection was extensively inhibited by treatment of cells with bafilomycin (1 mM), which perturbs endosomal pH (Fig. 2). Collectively, these results suggest that SARS-CoV takes an endosomal pathway for its entry and that S protein cleavage is important for fusion activity, which is in good agreement with the observations of a previous report.[3]
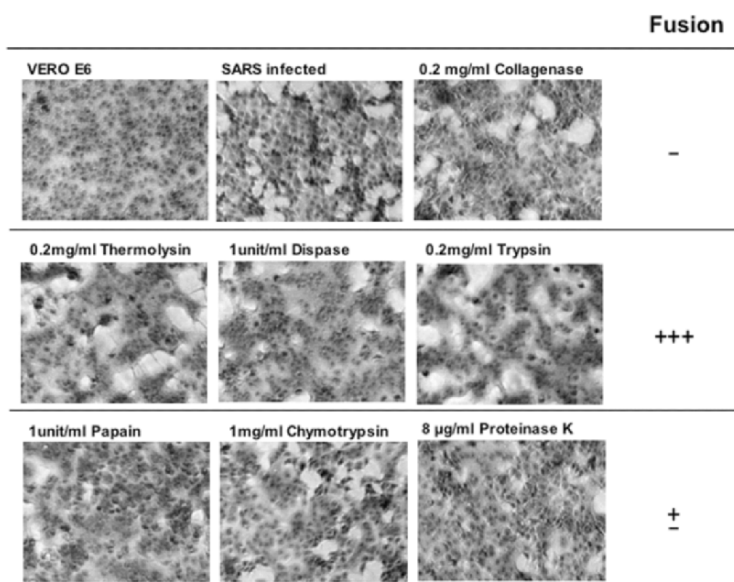
Figure 1. Fusion of SARS-CoV infected VeroE6 cells after treatment with various proteases.

3.2. Proteases Facilitate SARS-CoV Entry from the Cell Surface

The hypothesis proposed by Simmons et al.[3] stated that SARS-CoV is able to enter cells directly from their surface, if receptor-bound virus is treated with trypsin and other proteases that induce fusion. Treatment of VeroE6 cells with bafilomycin was shown to suppress SARS-CoV infection via the endosomal pathway to less than 1/100 (Fig. 2). Bafilomycin-treated cells were inoculated with SARS-CoV at an MOI of 1 and incubated on ice for 30 min. This allows virus binding to its receptor, but does not allow virus entry into cells. Cells were then treated with trypsin for 5 min at RT and incubated at 37°C for 6 h in the presence of bafilomycin. Virus entry was estimated by the newly synthesized mRNA9 measured quantitatively by real-time PCR. It was shown that trypsin with fusion-inducing activity extensively facilitated viral entry (Fig. 2). Thermolysin and dispase also facilitated entry into VeroE6 cells treated with bafilomycin. In contrast, two proteases that did not induce fusion, papain and collagenase, failed to do so. Pseudotype VSV bearing SARS-CoV S protein infection was also facilitated in bafilomycin-treated VeroE6 cells after treatment with proteases that induce fusion of SARS-CoV infected cells. Treatment of cells with trypsin before virus infection did not enhance viral entry (Fig. 2), indicating that the effects of trypsin on cells are not involved in this infection. Trypsin treatment of SARS-CoV prior to infection did not enhance infectivity, but reduced it by 10- to 100-fold (Fig. 3). These results demonstrate that SARS-CoV, when adsorbed onto the cell surface, fuses with the plasma membrane via the S protein after cleavage, suggesting a non-endosomal, direct entry of SARS-CoV into cells in the presence of proteases. Those findings also support the hypothesis drawn by Simmons et al.[3] that trypsin-like protease plays an important role in facilitating membrane fusion.

3.3. Various Proteases Enhance SARS-CoV Infection

Treatment with a high concentration of trypsin augmented virus entry or replication by approximately 10-fold during an early phase of the infection, from 3 to 6 h postinfection, compared with the standard infection. This implies that infection through the cell surface is approximately 10-fold more efficient than infection via the endosomal pathway. These data also imply that viral replication after entry via the cell surface proceeds approximately 1 h ahead of that via the endosomal pathway.

Because SARS-CoV replication was enhanced by trypsin treatment, we next assessed the efficiency of virus spread in the presence or absence of trypsin in a low MOI that mimics natural infection in humans. Ten pfu of virus were inoculated onto 10^5 confluent VeroE6 cells (MOI = 0.0001), and the cells were incubated at 37°C for 20 h in the media with or without various concentration of trypsin. The level of mRNA9 showed that virus replication was 100- to 1000-fold higher when cells were cultured in the presence of trypsin, compared to replication in the absence of trypsin. Viral infectivity also indicated that trypsin treatment enhanced viral growth by ca. 100-fold. This enhancement of viral replication observed in the presence of trypsin was also observed when infected VeroE6 cells were cultured in the presence of proteases, such as thermolysin and dispase, which induce fusion, but no enhancement was encountered when cultured in the presence of papain or collagenase, which fail to induce fusion. These observations suggest that proteases that facilitate SARS-CoV entry from the cell surface support efficient SARS-CoV infection. Thus, protease is likely to be responsible for the high multiplication of SARS-CoV in the target organs of SARS, such as the lungs, where various proteases are produced (e.g., by inflammatory cells), as well as in the intestines, where a number of proteases are physiologically secreted. Elastase is reported to be one of major proteases produced in inflammatory lungs.[7] Thus we examined whether elastase enhances SARS-CoV infection as do trypsin and thermolysin. Elastase enhanced SARS-CoV infection in cultured VeroE6 cells at low multiplicities of infection. This finding strongly suggests that SARS-CoV replication can be enhanced in the lungs by elastase. Thus, elastase is possibly a protease that is responsible for an acute severe illness caused by SARS-CoV.

4. DISCUSSION

SARS-CoV infection was evident in a number of organs, such as the liver, cerebrum, and kidneys, as well as in major target organs such as the lungs and intestines.[8, 9] In the latter organs, drastic tissue damage by SARS-CoV infection was observed, while the other organs were not so severely affected. Although the pathogenic mechanism of SARS has not been elucidated, the present study suggests that proteases secreted in major target organs play an important role in the high multiplication of virus in those organs, which could result in severe tissue damage. SARS-CoV may initially infect pneumocytes via an endosomal pathway. This would induce inflammation that generates a variety of proteases such as elastase. Once those proteases are present in the lungs, they may mediate a robust infection, which may result in enhanced replication. Although lung damage is reportedly mediated by cytokine storm,[8, 9] higher virus multiplication could also contribute to the cytokine storm by killing a large number of infected cells. Various proteases secreted in
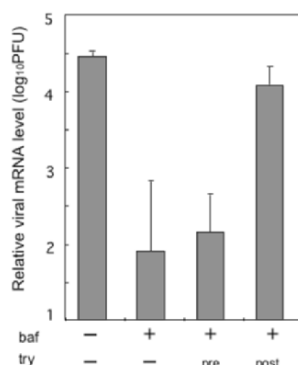
Figure 2. VeroE6 cells treated with bafilomycin were infected with SARS-CoV at 4°C for 30 min and then treated with trypsin at RT for 5 min (baf +, try post). Bafilomycin treated cells were treated with trypsin at RT for 5 min before SARS-CoV infection (baf +, try pre). Untreated cells (baf -, try -) or cells treated with bafilomycin alone (baf +, try -) were infected as controls. At 6 h postinfection, mRNA level was measured by real-time PCR and shown as $\log_{10}$PFU.

another target organ, the small intestines, could also contribute to the high viral titers detected in these tissues, which, in turn, may result in diarrhea.[10]

The present studies suggest that co-infection of SARS-CoV with nonpathogenic respiratory agents, such as *Chlamydia* or mycoplasma, could result in severe lung disease as a consequence of protease production or induction by the non-SARS-CoV agents, as has been shown by the enhancement of disease caused by influenza virus co-infected with nonpathogenic bacteria.[11, 12] Studies are in progress to examine whether co-infection exacerbates pneumonia in mice infected with SARS-CoV.
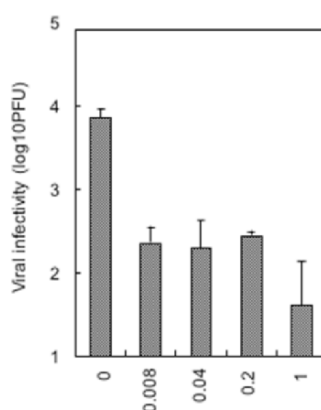

Figure 3. Treatment of SARS-CoV by trypsin. SARS-CoV was treated with various concentration of trypsin (from 0 to 1 mg/ml) in DMEM containing 5% FCS at RT for 30 min and infectivity was examined by plaque assay.

5. ACKNOWLEDGMENTS

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