Porphyromonas gingivalis enzymes enhance infection with human metapneumovirus *in vitro*

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Relatively recently discovered, human metapneumovirus (HMPV) is a human pathogen with worldwide prevalence, accounting for a substantial percentage of respiratory tract diseases. Concurrent viral and bacterial infections enable intricate mechanisms of cooperation between pathogens, which complicate the symptoms and outcome of the disease. Such bilateral interactions are based on the modulation of bacterial growth on epithelium pathologically altered during viral illness and the modulation of immune responses, as well as the enhancement of virus replication by bacterial virulence factors. This study showed that proteases produced by *Porphyromonas gingivalis*, a Gram-negative bacterium implicated in the development of periodontitis, named gingipains, facilitated HMPV replication in LLC-MK2 cells and may contribute to HMPV pathogenicity in patients with periodontitis. Gingipains at low nanomolar concentrations enabled HMPV replication and allowed virus propagation *in vitro*. In contrast to previously published data for influenza virus, however, *Staphylococcus aureus* proteases and human neutrophil elastase did not affect virus replication.

INTRODUCTION

The excessive morbidity and mortality of bacterial infections occurring during or shortly after viral infection was first recognized during the Spanish influenza pandemic at the beginning of the 20th century. Epidemiological analysis following the pandemic showed that the incidence of bacterial pneumonia had increased and contributed substantially to mortality rates (Stone & Swift, 1919; Wilson & Steer, 1919). Today, comparisons of bacteriological and virological data from children hospitalized for respiratory diseases show a high percentage of viral and bacterial superinfections, which is even higher in groups suffering from severe illness (Ballinger & Standiford, 2010; Duttweiler et al., 2004; Kneyber et al., 2005; Kukavica-Ibrulj et al., 2009; Rothberg & Haessler, 2010; Thorburn et al., 2006). Several reports show that bacterial infection is promoted by a preceding viral illness, but, surprisingly, little is known about the opposite process. The idea of viral infection being promoted by bacteria has been proposed previously for influenza virus (Tashiro et al., 1987a, b). The mechanism apparently involves specific proteolysis of viral protein(s) involved in virus–cell fusion by bacterial extracellular proteases, which is a key step in virus entry and replication.

Human metapneumovirus (HMPV) is a pathogen that was first recognized in 2001 in the Netherlands (van den Hoogen et al., 2001). Since its identification, the virus has been isolated worldwide from patients suffering from respiratory tract infections, and clinical data suggest that it has been circulating in the human population for at least 50 years (van den Hoogen et al., 2001; Yang et al., 2009). HMPV infection has been associated with upper and lower respiratory illness, with 2–15 % prevalence among patients suffering from respiratory tract infections. Clinical manifestations include bronchitis, croup, pneumonia and otitis media, as well as asthma exacerbations (Banerjee et al., 2007; Bastien et al., 2003; Cilla et al., 2009; Falsey et al., 2003; Foulongne et al., 2006; Oliveira et al., 2009; Williams et al., 2004).

Successful infection with HMPV depends on two major determinants – receptor recognition and fusion of cellular and viral membranes. These actions are carried out by the F protein, which is a trimeric, transmembrane, type I viral fusion protein consisting of a short C-terminal cytoplasmic tail and an N-terminal extracellular domain. The F protein is expressed in the host cell as an inactive F0 form, which is subsequently activated by proteolytic cleavage, most probably carried out by eukaryotic endogenous proteases, such as transmembrane protease, serine 2 (TMPRSS2) and human airway trypsin-like protease (HAT). Such an event enables the virus to enter new target cells and initiate a new
As demonstrated previously for influenza virus, extracellular proteases produced by *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Haemophilus influenzae* may efficiently promote viral infection by direct processing and activation of viral proteins, or by the activation of endogenous host proteases (Scheiblauer et al., 1992; Tashiro et al., 1987a, b). For example, it has been shown that *Staphylococcus aureus* extracellular proteases may promote influenza disease progression in a mouse model and that inhibition of their proteolytic activity prevents the development of fatal pneumonia in mice (Scheiblauer et al., 1992; Tashiro et al., 1987a, b).

Periodontitis is one of the most common infectious diseases affecting humans (estimated prevalence of 4.2% in the human population). *Porphyromonas gingivalis* is considered to be one of the major players in the development of periodontitis, and proteolytic enzymes (gingipains) have been identified as major contributors to its pathogenicity. Arginine-specific (RgpA and RgpB) and lysine-specific (Kgp) gingipains attack several targets and are responsible – directly or indirectly – for the destruction of periodontal tissues and for interference with several host processes (Guo et al., 2010). At the clinical level, the disease is characterized by gingival inflammation and a loss of connective tissue and bone around the teeth roots (Griffen et al., 1998; Hajishengallis, 2009, 2010; Holt & Ebersole, 2005; Taylor, 2010). Additionally, periodontitis seems to be linked to several conditions, including rheumatoid arthritis, atherosclerosis and aspiration pneumonia (Kimizuka et al., 2001; Wegner et al., 2001; Terpenning et al., 2001; Scannapieco et al., 2003; Okuda et al., 2005; Scannapieco, 1999; Scannapieco et al., 2001). Moreover, clinical studies have shown a direct correlation between poor oral health and the risk of development of respiratory disease associated with influenza virus and possibly also other respiratory viruses (Abe et al., 2006; Adachi et al., 2007; Mojon et al., 1997; Okuda et al., 2005). Although no mechanistic explanation has been presented so far, it seems feasible that, in the case of HMPV, proteases contained in saliva may interact with the fusion proteins of viruses that require proteolytic cleavage, in the same manner as proteases derived from bacteria colonizing the respiratory tract in humans activate influenza virus (Tashiro et al., 1987a, b).

The current study provides a possible explanation for the increased frequency and enhanced severity of respiratory virus infection in patients with periodontitis. As a model system, HMPV was used as a relevant human pathogen that requires proteolytic processing for its activation.

### RESULTS

**HMPV requires proteases for efficient replication in LLC-MK2 cells**

It has been shown previously that some strains of HMPV, especially after extensive cell culture adaptation, may replicate in the absence of trypsin. This phenomenon is associated with the spontaneous appearance of a point mutation within the F gene cleavage site (S101P), affecting the canonical HMPV proteolysis (Schickli et al., 2005). We tested whether replication of the isolate used in this study was trypsin dependent. Clearly, protease presence was essential for replication of this clinical isolate, as only a minor increase in virus yield was observed for HMPV in medium deprived of trypsin (Fig. 1). The observed increase was consistent with previously published data on HMPV replication in the absence of trypsin in LLC-MK2 cells (Tollefson et al., 2010). Based on enzyme titration, we determined that the optimal trypsin concentration for HMPV replication that did not interfere with LLC-MK2 cell attachment and growth was 128 nM (3 μg ml⁻¹), with a 2–3 log increase in virus yield observed (Fig. 1). This concentration of trypsin was therefore used in all subsequent experiments.

**Extracellular proteases of Staph. aureus and human neutrophil elastase do not enhance replication of HMPV in LLC-MK2 cells**

For influenza virus, it has been shown that *Staph. aureus* extracellular proteases may promote viral infection and substantially contribute to virus-mediated pathology *in vitro* and *in vivo*. We evaluated the possible role of *Staph. aureus* extracellular proteases in promotion of HMPV infection in LLC-MK2 cells. It was clear that no HMPV replication was observed in the presence of the major *Staph. aureus* extracellular proteases, including V8 protease and staphopains A (ScpA) and B (SspB) at 100 nM concentration (highest non-cytotoxic concentration) (Fig. 2). To evaluate the function of bacterial co-infection further, we also tested whether human neutrophil elastase, commonly present at the inflammation site, affected HMPV replication. At the tested elastase

![Fig. 1. Replication of HMPV in the presence (+ Tryp) or absence (–Tryp) of trypsin. Data are presented as HMPV RNA copies ml⁻¹ (means ± sd).](http://vir.sgmjournals.org)
concentration (20 nM; highest non-cytotoxic concentration), we observed no effect on HMPV replication (Fig. 2).

**Extracellular proteases of *P. gingivalis* proteolytically activate HMPV virions**

As stated above, there is a clinical correlation between dental hygiene and the prevalence of influenza and influenza-like illnesses. This process has been correlated directly with protease activity in the saliva of patients with periodontal problems (Abe et al., 2006; Adachi et al., 2007; Mojon et al., 1997; Okuda et al., 2005). Here, we assessed the influence of gingipains, the major extracellular proteases of *P. gingivalis*, on the replication of HMPV. The arginine-specific gingipains HRgpA (a form of RgpA) and RgpB specifically enabled replication of HMPV (Fig. 3). HMPV replication in the presence of HRgpA or RgpB was similar to that observed in the presence of trypsin, with a 2–3 log increase in virus yield. Interestingly, however, in comparison with trypsin, the stimulation of virus replication by the arginine-specific gingipains required a 2 log lower concentration of the proteases. The lysine-specific Kgp also facilitated HMPV replication, but the increase in the virus yield was significantly lower than that exerted by the Rgps (Fig. 3). Furthermore, to test whether incubation with bacterial proteases led to the generation of fully viable virus particles, we assessed the virus titre in samples incubated with different proteases.

The results clearly showed that virions activated with gingipains were infectious and that the titre of infectious virus was comparable to that obtained with trypsin (Fig. 4).

To investigate whether virus activation was dependent on the proteolytic activity of gingipains, the effect of gingipain inhibition on virus replication was studied. As shown in Fig. 5, pre-treatment of gingipains with specific inhibitors basically abolished their ability to stimulate virus replication. No cytotoxicity was seen in the presence of inhibitors and no effect on replication of another human respiratory virus, human coronavirus NL63, was observed (data not shown). Therefore, it was clear that the observed in vitro enhancement of HMPV replication was dependent on the gingipain proteolytic activity. To confirm the observed phenomenon, we tested whether incubation of the virus with gingipains led to proteolytic processing of the F protein, which may in return result in activation of virions. As shown in Fig. 6, incubation with trypsin or gingipain led to the generation of a truncated form of the HMPV F protein. Surprisingly, after incubation with gingipains, we observed the appearance of an additional variant of the HMPV F protein, suggesting that, in addition to the canonical cleavage site, gingipains are able to generate alternative F1 and F2 variants.
Analysis of early events during HMPV infection

To elucidate further the mechanism of enhancement of HMPV replication, we tested at which stage of virus replication the presence of protease is essential. Pre-incubation of susceptible cells with the gingipains did not facilitate replication of HMPV (Fig. 7a), suggesting direct interaction of the proteases with virions, as shown previously for HMPV and trypsin (Biacchesi et al., 2004, 2006; Schickli et al., 2005; Schowalter et al., 2006). Pretreatment of the virus stock with gingipains did not enhance virus production (Fig. 7b). This finding argues that a protease applied at this stage damages virions, hampering virus infection and/or, as described previously, that the constant presence of a protease is required for maintenance of the multi-cycle replication pattern of HMPV. To test this hypothesis, we pre-incubated virions with a protease and used this material as the input sample for subsequent infection in medium supplemented with trypsin. As the trypsin concentration was identical for all samples, we assumed that virus degradation during protease pre-treatment would be reflected in decreased virus production in subsequent replication rounds. No decrease in virus replication was observed for the enzymes tested (Fig. 7c).

DISCUSSION

Replication of HMPV depends on the presence of extracellular proteases that enhance viral infection (Biacchesi et al., 2004, 2006). In this work, we studied the effect of proteases derived from host and bacteria on HMPV replication, which can increase the severity of respiratory tract infection by this virus. This included major proteases secreted by Staph. aureus (V8 protease and ScpA and SspB), a common colonizer of the nasal cavity (Shaw et al., 2004), gingipains routinely present in the oral cavity of patients infected with P. gingivalis (Johanson et al., 1980; Langmore et al., 1998; Leblebicioglu et al., 2009; Miyamoto et al., 2009) and endogenous elastase released from neutrophils at inflammatory sites. We observed a significant enhancement of HMPV replication by arginine-specific gingipains, suggesting the direct processing of HMPV F protein by these enzymes. Conversely, virus replication was not affected by human neutrophil elastase or Staph. aureus proteases that have been reported previously to enhance influenza virus infection in vitro and in vivo by proteolytic processing of the HA protein (Tashiro et al., 1987a, b).

The arginine-specific gingipains HRgpA and RgpB efficiently facilitated HMPV replication and enhanced viral infection. To rule out the possibility that the observed effect was due to non-specific interactions, we showed that selective inhibition of gingipain proteolytic activity completely negated their effect on HMPV replication. Virus
repetition was stimulated by trypsin and *P. gingivalis* enzymes to a similar extent; however, the optimal trypsin concentration was 128 nM, whilst for both gingipains the effective concentration was in the low nanomolar range. The stimulatory effect was apparently exerted exclusively by proteolysis of viral protein(s), as pre-incubation of cells with proteases had no effect on virus replication. Pre-incubation of HMPV virions with proteases affected neither HMPV levels nor the rate of virus replication (Fig. 7). We believe that, as presented previously, the constant presence of the protease is required for maintenance of multi-cycle replication. In addition, we considered the alternative that the premature activation of virions hampers HMPV cell entry. The former option was verified experimentally and the results in Fig. 7 clearly indicated that the continuous presence of a protease is a prerequisite for successful infection.

Activation of HMPV virions by gingipains HRgpA and RgpB is consistent with the substrate specificity of these enzymes. As with trypsin, HRgpA and RgpB cleave substrates after arginine at the P1 position, whilst the canonical cleavage site for HMPV is RQSR↓FVLG. Surprisingly, incubation with Kgp also resulted in a slight increase in virus yield. Kgp recognizes and hydrolyses peptide bonds after lysine at the P1 position, which is inconsistent with the canonical activation cleavage site for HMPV. Thus, it can be assumed that Kgp targets an alternative cleavage site, resulting in the generation of partially defective virus particles. These observations are consistent with the results obtained by Western blotting. Incubation of HMPV virions with gingipains appeared to result in proteolytic cleavage of the F protein. For arginine-specific proteases (HRgpA and RgpB), it seemed that the F protein was being processed to a product similar to that of trypsin, although a larger product was also generated, suggesting an alternative cleavage site. For Kgp, only a minor amount of protein was detectable by Western blotting, suggesting that, during infection, the amount of virions generated was much lower (as also seen with real-time RT-PCR) or that the F protein was fragmented by this lysine-specific enzyme. The lack of an effect with *Staph. aureus* proteases and neutrophil elastase can be attributed directly to the substrate specificity of these enzymes. SspB specifically cleaves after arginine in position P1 but also requires a hydrophobic amino acid at the P2 position. The other enzymes, ScpA, V8 and HNE, have different substrate specificities and are therefore unable to correctly recognize the HMPV canonical cleavage site. Taken together, these data suggested that proteolytic processing of the HMPV F protein outside of the conserved cleavage site, if it occurred, did not enable virus replication.

The clinical relevance of our findings is highlighted by the fact that *P. gingivalis* and increased levels of trypsin-like proteases are found in the saliva of patients with periodontal disease (Ingman *et al.*, 1993; Könönen *et al.*, 2007; Leblebicioglu *et al.*, 2009; Mättö *et al.*, 1998; Miyamoto *et al.*, 2009). One may assume that excretion of bacteria and bacteria-derived enzymes into the saliva from periodontal pockets infected with *P. gingivalis* may contribute to the development and/or severity of a viral respiratory disease by decreasing the minimal infectious dose of the virus and increasing the virus amplification rate. The presence of *P. gingivalis* in the oral cavity in patients lacking obvious symptoms of periodontitis further aggravates the problem. Moreover, anaerobic periodontal pathogens, including *P. gingivalis*, are associated with the development of aspiration pneumonia and lung abscess formation (Kimizuka *et al.*, 2003; Okuda *et al.*, 2005; Terpenning *et al.*, 2001).

In conclusion, it is reasonable to assume that proteases from *P. gingivalis*, a frequent inhabitant of dental plaque, may at least partially participate in the enhancement of viral respiratory disease. Further clinical studies are required to evaluate the actual extent of the contribution of bacterial infections to viral respiratory disease.

**METHODS**

**Cell culture.** LLC-MK2 cells were maintained in minimal essential medium (MEM), containing two parts Hank’s MEM and one part Earle’s MEM (PAA Laboratories) supplemented with 3% heat-inactivated FBS (PAA Laboratories), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. Cells were cultured in T25 flasks (Sarstedt) at 37 °C with 5% CO₂.
**Virus preparation, titration and infection.** HMPV stocks (clinical isolate, clade B2) were prepared by infecting LLC-MK2 cells in MEM supplemented with trypsin (3 μg ml⁻¹). No FBS was added to the medium. Cells were lysed after 7 days of infection by two freeze–thaw cycles. The virus-containing fluid was aliquotted and stored at −80 °C. A control HMPV cell lysate from mock-infected cells was prepared in the same manner as the virus stocks.

Virus yield was assessed by virus titration in fully confluent LLC-MK2 cells on 96-well plates, according to the method of Reed & Muench (1938). Plates were incubated at 37 °C for 7 days, and the cytopathic effect was scored using an inverted microscope. In subsequent experiments, fully confluent cells were exposed to HMPV at a TCID₅₀ of 2000 (m.o.i. of 0.01).

**Assessment of protease-mediated enhancement of HMPV replication.** LLC-MK2 cells were infected in 96-well plates. In a standard assay, virus stock was diluted and mixed with a protease. This mixture was applied to the cell monolayer. Cultures were incubated at 37 °C for 6 days and the cytopathic effect was scored using an inverted microscope. On day 6, the medium was collected and subjected to real-time RT-PCR analysis. To ensure that the observed replication was dependent solely on protease activity, specific protease inhibitors were used. For inhibition of the Kgp proteolytic activity, benzoyloxy carbonyl-Phe-Lys-CH₂OCO(2,4,6-Me3)phenyl.HCl was used, whilst H-D-Phe-Phe-Arg-chloromethylketone (Bachem Biosciences) was used for inhibition of RgpB and HRgpA.

In the pre-activation assay, an undiluted virus sample was mixed with a protease solution to yield the required final protease concentration. Samples were incubated for 2 h at 37 °C and then overlaid on the cell monolayer (preV). NC, Negative control: infection was conducted in protease-free medium with untreated virus stock; PC, positive control: cells were incubated with untreated virus stock in the presence of 128 nM trypsin. Data are presented as HMPV RNA copies ml⁻¹ (means ± SD). The statistical significance of the HMPV replication in comparison with the negative control (a, b) or the positive control (c) is indicated: NS, not significant; *, P<0.05; **, P<0.005; ***, P<0.0005.

**Virus detection by quantitative real-time RT-PCR.** HMPV total nucleic acids were isolated from cell culture supernatant using a total RNA mini kit (A&A Biotechnology), according to the manufacturer’s instructions. Reverse transcription was carried with High Capacity cDNA Reverse Transcription (Applied Biosystems), according to the manufacturer’s instructions. The HMPV virus yield was determined using real-time PCR. Five microlitres of cDNA was amplified in a 20 μl
reaction mixture, containing 1 × TaqMan Universal PCR Master Mix, with AmpErase UNG (Applied Biosystems), 50 nM specific molecular-groove binding (MGB) probe and 200 nM primers. Rox was used as the reference dye. The following primers were used for HMPV amplification: sense primer, 5′-GGCTGCAGCTCCAGTCAATTCAAA-3′; antisense primer, 5′-GTTATCCCTGACTGTGTCGAAAAT-3′; and probe, 5′-6-FAM-CGCACAATTGAGGACTCCTT-GMBNFQ-3′ (with 6-carboxyfluorescein at the 5′ end and MGB non-fluorescent quencher at the 3′ end). The reaction was monitored on a 7500 Fast Real-time PCR machine (Applied Biosystems) with the following settings: one cycle of 2 min at 50°C, 30 s at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C.

**Western blotting.** In order to determine whether gingipains were able to process the F protein of HMPV, LLC-MK2 cells were infected as described above in the presence of proteases (trypsin, HRGpA, RgpB and Kgp). After 2 h of incubation, virus was removed and the cells washed thoroughly with PBS. Fresh medium supplemented with proteases was added. After 6 days of infection, the cell culture supernatant was used as an input sample for Western blot analysis. Briefly, 10 μl culture medium was mixed with 10 μl lysis buffer, boiled for 5 min, chilled on ice and layered onto a 10% polyacrylamide gel. As a control, samples derived from cultures cultivated in the absence of trypsin for the last 48 h of infection were used. To assess the protein size, a PageRuler Plus protein size marker (Fermentas) was used. After electrophoresis, the proteins were transferred onto PVDF membrane (Millipore) by standard blotting (Bio-Rad) for 1 h at 4°C (100 V), in buffer containing 0.38 M Tris/HCl (pH 8.2), 0.31 M glycine, 3% SDS and 20% methanol. Non-specific binding sites were blocked with 3% skimmed milk (BioShop) in TBST (1× TBS supplemented with 0.1% Tween 20) overnight at 4°C. HMPV F protein was detected with mouse anti-HMPV F IgG (1:20000) and secondary anti-mouse antibody (1:20000; both from Pharmingen). All antibodies were diluted in 1% skimmed milk (Bioshop) in TBST (1× TBS supplemented with 0.1% Tween 20) overnight at 4°C. The signal was developed using Pierce ECL Western blotting substrate (Thermo Scientific). The signal was visualized by exposure of the membrane to an X-ray film (Kodak).

**Proteases.** Elastase was purchased from Elastin Products Company. Kgp, HRGpA and RgpB were isolated according to the methods described by Pike et al. (1998) and Potempa et al. (1998). The amount of active enzyme in each purified gingipain was determined by active site titration using H-D-Phe-Phe-Arg-chloromethylketone (Bachem) (Potempa et al., 1997). The concentration of active arginine-specific gingipains was calculated from the amount of inhibitor needed for complete inactivation of the protease.

Staphopains were purified from the culture media supernatants of *Staph. aureus* strain V8-BC10 or 8325-4, as described previously (Potempa et al., 1988). The active-site concentration of ScpA and SspB was determined by enzyme titration with E-64 and staphopastatin, respectively (Potempa et al., 1988; Raychom et al., 2003). The *Staph. aureus* V8 protease (glutamyldendopeptidase) was purified from the culture medium of the V8 strain, as described by Drapeau (1976). The protein concentration in each enzyme batch was determined using a bicinchoninic acid kit (Bio-Rad) with BSA as the standard. The homogeneity of purified gingipains was evaluated by SDS-PAGE.

**Activation of proteases.** Gingipains were activated prior to use with 20 mM L-cysteine in 0.2 M HEPES buffer (pH 8.0) supplemented with 5 mM CaCl2 at 37°C for 15 min. The activated protease was then diluted with Dulbecco’s MEM culture medium and stored on ice. ScpA and SspB were activated with 20 mM L-cysteine at room temperature for 15 min prior to use.

**Statistical analysis.** All experiments were repeated three times and results are expressed as means ± SD. To determine the significance of the results obtained, comparisons between groups were made using Student’s t-test. P<0.05 was considered significant.

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