Proteolytic Inactivation of LL-37 by Karilysin, a Novel Virulence Mechanism of *Tannerella forsythia*

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Key Words

Innate immunity · Antimicrobial peptide · Cathelicidin · Metalloprotease · *Tannerella forsythia* · Inflammation · Infection · Periodontitis

Abstract

Tannerella forsythia is a gram-negative bacterium strongly associated with the development and/or progression of periodontal disease. Here, we have shown that a newly characterized matrix metalloprotease-like enzyme, referred to as karilysin, efficiently cleaved the antimicrobial peptide LL-37, significantly reducing its bactericidal activity. This may contribute to the resistance of *T. forsythia* to the antibacterial activity of LL-37, since their vitality was found not to be affected by LL-37 at concentrations up to 2.2 μ M. Furthermore, proteolysis of LL-37 by karilysin not only abolished its ability to bind lipopolysaccharide (LPS) to quench endotoxin-induced proinflammatory activity, but LL-37 cleavage also caused the release of active endotoxin from the LPS/LL-37 complex. Proteolytic inactivation of LL-37 bactericidal activity by karilysin may protect LL-37-sensitive species in the subgingival plaque and maintain the local inflammatory reaction driven by LPS from gram-negative bacteria. Conse-

quently, the karilysin protease may directly contribute to periodontal tissue damage and the development and/or progression of chronic periodontitis.

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Introduction

Antimicrobial peptides are components of innate immunity and are effective against gram-negative and gram-positive bacteria, parasites, fungi and some viruses [1, 2]. In mammals, antimicrobial peptides were also found to function as immunomodulators of the innate immune system, as they modulate expression of chemokines and cytokines, as well as eliciting or inhibiting proinflammatory responses [3, 4]. Since the recognition of their immunoregulatory functions, antimicrobial peptides are often referred to as host defense peptides. In the human oral cavity, several kinds of antimicrobial peptides are found, including α - and β -defensins, histatins and LL-37 [5].

Periodontal disease is a common bacteria-induced inflammatory disease in which the tooth-supporting connective tissues are progressively destroyed [6]. Antimi-

crobal peptides produced by mucosal epithelium appear to play a pivotal role in the host innate immune defense in the oral cavity. The best example is morbus Kostmann syndrome, in which deficiency of LL-37 in neutrophils and saliva has been correlated with the occurrence of severe periodontal disease [7].

Tannerella forsythia (previously *Bacteroides forsythus*) is a gram-negative bacterium strongly associated with periodontitis [8]. To date, only a few virulence factors of *T. forsythia* have been recognized, including a sialidase (SiaHI), a cysteine protease with hemolysin activity (PrtH), a trypsin-like protease and an extracellular protein, BspA, mediating the attachment to fibronectin and fibrinogen [9–11]. Analysis of the *T. forsythia* genome revealed the presence of numerous open reading frames encoding putative proteases. Recently, we showed that one of these genes (Los Alamos Oral Pathogens Database Accession No. TF0367) encodes for a proteolytically active matrix metalloprotease-like enzyme designated karilysin [12]. Metalloproteases derived from microbial pathogens have been documented as important virulence factors contributing to evasion of antimicrobial mechanisms of the innate immune system [13]. The latter strategy is often executed by proteolytic inactivation of antibacterial peptides, including LL-37. Therefore, in this study, we investigated the potential of karilysin to interfere with the antibacterial and anti-inflammatory functions of LL-37.

Materials and Methods

Peptide Synthesis

LL-37 was commercially synthesized by Peptide 2.0 (Chantilly, Va., USA) on an Applied Biosystems model 433A synthesizer on a scale of 0.1–0.25 mmol, using *tert*-butoxycarbonyl chemistry and HPLC purified on C18 silica columns. The structural integrity of the peptide was confirmed by mass spectrometry. The peptide concentration was determined by quantitative amino acid analysis, and then it was lyophilized in the form of trifluoroacetate salt and stored at 4°C. Stock solutions (6 mg/ml) were prepared in phosphate-buffered saline for the evaluation of antimicrobial activity and were stored frozen at –20°C in 1-ml aliquots to be ready for use.

Recombinant Karilysin Expression, Purification and Activity Determination

Karilysin expression (both wild-type and catalytic Glu136Ala mutant forms) in an *Escherichia coli* host and subsequent purification was performed as described previously [12]. The activity of the purified enzymes was examined by cleavage of the fluorescent-labeled azocasein. To block karilysin activity, EDTA or *o*-phenanthroline at 10 and 5 mM, respectively, were used to inhibit karilysin. Using the limulus test, karilysin preparations were shown to be free of lipopolysaccharide (LPS).

LL-37 Cleavage by Karilysin

LL-37 (5 µg) was incubated with karilysin (5–500 ng) at peptide/enzyme molar ratios from 100:1 to 10,000:1 in 20 µl of 100 mM Tris-HCl and 5 mM CaCl₂, pH 8.0, for 16 h at 37°C. For time dependency experiments, LL-37 was exposed to karilysin action for 0, 2, 4, 8, 12 and 16 h at a ratio of 1,000:1 (5 µg of LL-37 + 50 ng of karilysin) under the same conditions. Following incubation, the reaction was terminated by the addition of 1 M HCl (for HPLC experiments) or SDS-PAGE sample buffer, and after the denaturation step (95°C for 5 min), samples were subjected to SDS-PAGE. LL-37 alone and LL-37 incubated with karilysin in the presence of 5 mM *o*-phenanthroline served as controls. Degradation products of LL-37 were resolved by HPLC.

Reverse-Phase HPLC

Twenty microliters of karilysin-digested LL-37 from each time point (as indicated above) were separated on a Supelcosil LC-318 column (25 cm × 4.6 mm; Supelco). The elution was conducted using a 2-solvent system, i.e. 0.1% trifluoroacetic acid/H₂O (solvent A) and 80% acetonitrile/0.08% trifluoroacetic acid (solvent B), at a constant flow rate of 0.5 ml/min with a 10–70% gradient of solvent B over 70 min. UV absorbance was monitored at 215 nm, and fractions were collected at 0.5-min intervals.

Antimicrobial Assay

A colony reduction assay using *E. coli* (ATCC 33694) and *Porphyromonas gingivalis* W83, grown in LB and Schaedler Broth, respectively, was conducted to evaluate the antibacterial activity of LL-37. The *E. coli* and *P. gingivalis* cells were collected by centrifugation (5,000 g, 8 min), washed with phosphate-buffered saline and resuspended to a final concentration of 1×10^6 and 5×10^6 colony-forming units/ml (10% Hank's balanced salt solution/RPMI), respectively. LL-37 (22 µM), either intact or after preincubation with karilysin, was prepared in 100 mM Tris-HCl, pH 8.0, with 5 mM CaCl₂, and 20-µl aliquots were transferred to low-protein-binding polypropylene tubes. To each tube, 180 µl of the bacterial suspension was added. The plates were incubated at 37°C. After 1 h of incubation, aliquots were plated onto tryptic soy agar plates or Schaedler agar with 5% sheep blood for colony counting. All experiments were performed in triplicate.

Cell Culturing

Human monocyte-derived macrophages (hMDMs) were separated from fractions of peripheral blood mononuclear cells as described previously [14]. Peripheral blood mononuclear cells were seeded into 24-well plates and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated autologous human plasma, 2 mM L-glutamine and 50 µg/ml gentamicin (Sigma) in a humidified atmosphere of 5% CO₂. After 24 h, nonadherent cells were removed and adherent monocytes were differentiated to macrophages for 7–10 days, with fresh medium changes every second day. The hMDM phenotype was verified after nonenzymatic detachment of cells by immunofluorescent staining of CD14 (Dako Cytomation), CD16 (Dako Cytomation), CD11b (Becton Dickinson) and CD209 (Becton Dickinson) and subsequent flow cytometry analysis [14].

TNF-α Release

For TNF-α measurement, 0.3×10^6 hMDMs in 500 µl of RPMI-1640 supplemented with 0.2% autologous human plasma,

Fig. 1. Degradation of LL-37 by karilysin. **a** Dose-dependent degradation of LL-37 by karilysin (Kly). Karilysin (0.5–500 ng) was incubated with LL-37 (5 μ g) at molar ratios from 1:100 to 1:100,000 for 12 h. **b** HPLC chromatogram of karilysin-treated LL-37. A = Absorbance. Representative data from 3 independent experiments are shown.

2 mM L-glutamine and 50 μ g/ml gentamicin were stimulated with *E. coli* LPS (10 ng/ml) for 6 h in a total volume of 500 μ l in the absence or presence of LL-37 (2 μ M) or the same amount of LL-37 previously preincubated with karilysin. The experiment was performed in triplicate. After 6 h, media were harvested and kept at -20°C until analysis. The content of TNF- α was estimated by ELISA performed according to a protocol provided by the manufacturer (R&D Systems). Absorbance at 570 nm was subtracted from absorbance at 450 nm and the amount of cytokine was estimated according to the standard curve prepared from a known concentration of TNF- α .

Statistical Analysis

Results were analyzed for statistical significance using the Student's *t* test. Differences were considered significant when $p < 0.05$.

Results

Degradation of LL-37 by Karilysin

In humans, cathelicidin seems to play a key role in protecting the human periodontium against dental plaque bacteria. Therefore, we investigated if karilysin can degrade LL-37. To this end, the peptide was incubated with purified enzyme and samples were analyzed by SDS-PAGE. The analysis revealed that karilysin cleaves LL-37 in a concentration- and time-dependent manner

(fig. 1), leading to the accumulation of product(s) with a lower molecular mass than the native LL-37. After 12 h of incubation, a partial cleavage was observed at a 1:10,000 molar ratio of karilysin to LL-37 (fig. 1a). At a 1:1,000 ratio, the majority of LL-37 was cleaved after 2 h, but 12 h was needed for total conversion of the native peptide to lower-molecular-mass forms (fig. 1b). The degradation of LL-37 by karilysin was totally inhibited by EDTA (fig. 1a) and *o*-phenanthroline (data not shown). Significantly from the physiopathological point of view, karilysin also cleaved LL-37 in the presence of 10% human plasma. In this case, however, a higher concentration of the enzyme was needed to achieve the same degree of peptide cleavage as in the medium alone (data not shown).

Inhibition of Antibacterial Activity of LL-37 by Karilysin

T. forsythia is resistant to the antibacterial activity of LL-37 at concentrations up to 10 μ g/ml (2.2 μ M) (data not shown), and cleavage by karilysin may contribute to this resistance. Therefore, we analyzed the bactericidal activity of LL-37 after incubation with karilysin using *E. coli* and *P. gingivalis* as targets and found that karilysin efficiently inactivated the antibacterial activity of LL-37 in a concentration- and time-dependent manner (fig. 2). The loss of function correlated well with the level of

Fig. 2. Effect of karilysin treatment on antibacterial activity of LL-37 against *P. gingivalis* W83 and *E. coli* (ATCC 25922). LL-37 was incubated alone or with karilysin (Kly) at a molar ratio of 1,000:1 or 100:1 for 12 h (a) and at a molar ratio of 1,000:1 for 2, 4, 6, 8 and 12 h (b) then mixed with bacteria, incubated for 1 h at 37°C and plated onto agar plates to determine the numbers of

colony-forming units. Percentage survival was calculated and compared to bacteria grown without antimicrobial peptide LL-37 (100% survival). Mean values and standard deviations (error bars) of 3 independent experiments performed in duplicate are shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

cleavage of native LL-37 observed by SDS-PAGE (fig. 1a) and HPLC analysis (fig. 1b). Nevertheless, it should be noted that total inactivation of LL-37 bactericidal activity against *E. coli* was achieved only when the enzyme was incubated with the peptide at a 1:100 molar ratio. At a 1:1,000 ratio and with a long incubation time (12 h), the efficiency of *E. coli* killing by cleaved LL-37 was maximally reduced by 70% (fig. 2b).

Attenuation of Anti-Inflammatory Activity of LL-37 by Karilysin

Apart from being bactericidal, LL-37 possesses potent anti-inflammatory activity. This is mediated through direct binding of LL-37 to LPS [15]. Since stimulation of macrophages with LPS leads to the release of several pro-inflammatory mediators, including TNF- α , we used these systems to determine how cleavage of LL-37 by karilysin affected the peptide's ability to neutralize LPS. As expected, native LL-37 at 2 μ M totally abrogated LPS-induced release of TNF- α by hMDMs (fig. 3a). The peptide treatment with karilysin caused a dose-dependent inactivation of the ability of LL-37 to neutralize LPS. Even at very low concentrations of the enzyme (enzyme

to peptide ratio of 1:10,000), a significant decrease in LL-37 anti-inflammatory activity was observed, while LL-37 function was obliterated at the 1:100 molar ratio (fig. 3a). Inactivation of LL-37 was dependent on karilysin proteolytic activity, since the presence of *o*-phenanthroline protease inhibitor annihilated the effect of karilysin on LL-37 and a catalytically inactive karilysin mutant (karilysin^{E136A}) had no effect on neutralization of LPS by LL-37. Together, our findings indicated that the proteolytic truncation of LL-37 by karilysin effectively inactivates the biological activity of this peptide (fig. 3a).

The next obvious question to be asked was whether LL-37 in complex with LPS is susceptible to cleavage by karilysin and whether proteolysis of the peptide would release LPS from the complex to induce proinflammatory activity. To answer the question, we incubated the LL-37/LPS complex with karilysin and tested the reaction mixture for LPS-driven secretion of TNF- α . We observed a dose-dependent release of active LPS from the complex with LL-37 as manifested by TNF- α secretion by hMDMs (fig. 3b).

Fig. 3. Effects of LL-37 cleavage by karilysin on LPS-stimulated TNF- α secretion. **a** Macrophages were stimulated for 6 h with 10 ng/ml *E. coli* LPS, native LL-37, LL-37 digested with karilysin (Kly) at molar ratios from 1:100 to 1:100,000 for 12 h, LL-37 treated with the catalytically inactive form of karilysin (Kly^{E136A}) or karilysin inhibited by treatment with *o*-phenanthroline (OF). Supernatants were analyzed for TNF- α using ELISA. **b** LPS was first preincubated with LL-37 for 2 h, the complex was treated with

karilysin at molar ratios from 1:100 to 1:10,000 for 12 h and the digest was added to macrophages. As controls, macrophages were stimulated with 10 ng/ml *E. coli*-derived LPS, LL-37 or karilysin. Conditioned media were harvested after 6 h and the TNF- α concentration was determined using ELISA. Data are the mean $\pm$ SD values from 3 independent experiments, each performed at least twice. *** $p < 0.001$.

Discussion

In the oral cavity, especially on the tooth surface below the gum line, LL-37 seems to play a crucial role in controlling the colonization of the subgingival dental plaque by pathogenic species, since deficiency of LL-37, either genetic or locally induced, is associated with severe periodontitis [7]. In this study, we have shown that karilysin, a metalloprotease from *T. forsythia*, can inactivate the bactericidal activity of LL-37 in a time- and concentration-dependent manner through limited proteolysis. Since karilysin can cleave LL-37 in the presence of human plasma, the reaction may take place in vivo. In keeping with this, cathelicidin (the precursor of LL-37) is often found as a truncated 11-kDa form in chronic periodontitis lesions with a high load of *T. forsythia* [16]. Neverthe-

less, it must be kept in mind that in vivo, several proteases produced by other periodontopathogens can also degrade bactericidal peptides [17]. Finally, numerous other proteases are known to be encoded in the *T. forsythia* genome, which, if expressed in vivo, may themselves contribute to LL-37 inactivation. Taken together, the combined action of microbial proteases aimed at bactericidal peptides, including LL-37, may effectively protect *Fusobacterium nucleatum*, *Streptococcus mutans*, *Streptococcus sobrinus* and *Streptococcus salivarius*, which are highly susceptible to the bactericidal activity of LL-37 [18] but nevertheless thrive in dental plaque bathed in gingival crevicular fluid containing elevated amounts of these peptides.

Apart from its direct bactericidal activity, LL-37 possesses the ability to quench inflammatory reactions by

neutralizing LPS [19]. This function of LL-37 is most likely to be important in periodontitis sites heavily colonized by a large variety of gram-negative bacteria. The anti-inflammatory effect and the bactericidal activity of LL-37 were inactivated by karilysin with similar efficiencies. Karilysin was also capable of releasing LPS from the biologically inert LPS/LL-37 complexes, thus partly restoring the proinflammatory activity of the endotoxin. Therefore, proteolytic inactivation of LL-37 by karilysin and other host and periodontal-pathogen-derived proteases [20] may contribute to the maintenance of the local inflammatory reaction through LPS-induced release of proinflammatory cytokines. In summary, it is clear that by inactivating LL-37, karilysin may play an important

role in immune evasion of *T. forsythia* and contributes to the development of the chronic inflammatory response characteristic of periodontitis.

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