— Research Letter —

Defensive Effects of Human Antimicrobial Peptide α-Defensins against *Enterococcus faecalis*

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Cationic and amphiphilic antimicrobial peptides (AMPs) such as α-defensins and cathelicidins are factors related to innate immunity. In the present study, we examined the protective effects of two AMPs, human neutrophil peptide-3 and α-defensin-5, against the opportunistic pathogen *Enterococcus faecalis* (*E. faecalis*). The α-defensins had dose-dependent bactericidal activity, whereas they showed no synergistic effect on the antimicrobial actions of antibiotics. Although AMPs often neutralize bacterial bioactive products, neither α-defensin reduced the proteolytic activity of GelE, a toxic protease from *E. faecalis*. On the other hand, the α-defensins were found to be fairly stable even in the presence of excess amounts of GelE. These results indicate that α-defensins may be defensive factors against *E. faecalis* in humans.

Key words —— human α-defensin, antimicrobial peptide, innate immunity, *Enterococcus faecalis*

INTRODUCTION

Human defensins are members of the cationic and amphiphilic antimicrobial peptides (AMPs) contributing to innate immunity.[1–3] Defensins are 3.5–4.5-kDa small peptides containing several basic amino acid residues and six cysteine residues and are classified into α- and β-defensins based on the location of three intramolecular disulfide bonds.[1–3] Human α-defensins were first isolated from neutrophils, and four molecular species termed human neutrophil peptides (HNPs) 1–4 are currently known. Among them, HNP-4 is approximately 100-fold less abundant than the others. The primary structures of major HNPs 1–3 differ only at the *N*-terminal amino acid residue. The first residue is alanine (Ala) for HNP-1 and aspartic acid (Asp) for HNP-3 (Fig. 1), whereas HNP-2 lacks this position because it is generated from HNP-3 through proteolytic removal of Asp. On the other hand, the Paneth cells in the small intestine secrete two other α-defensins, human defensin-5 (HD-5) and HD-6; however, the expression level of HD-5 is greater than that of HD-6. By contrast, the β-defensins are secreted by various cells, such as leukocytes and epithelial cells.

AMPs are potent bactericidal factors because they bind to the bacterial cell membrane that has negative charges and disrupt the membrane by formation of large hollow polymers.[4,5] AMPs may also translocate across the cell membrane and may disturb the synthesis of DNA and/or proteins.[6] In addition, recent studies have shown that some AMPs can inactivate the bacterial extracellular products including toxins and enzymes. For example, the lethal factor produced by *Bacillus anthracis* is neutralized by HNP-1.[7] The cytolysins from Gram-positive bacteria are also inactivated by HNP-1 to -3 and HD-5.[8] However, it should be noted that human pathogens may resist the bactericidal actions of AMPs through modification of the phospholipid composition in the cell membrane,[9] production of peptidases that can degrade AMPs,[10,11] and expression of the AMP efflux pumps.[9]

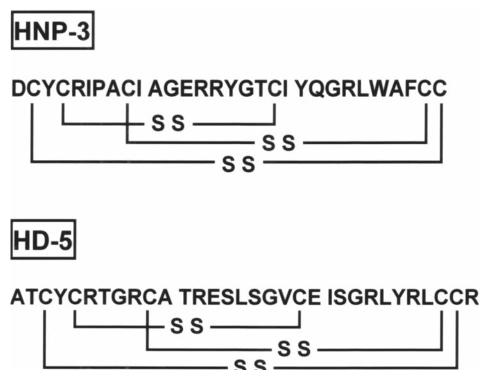

Fig. 1. Structures of Human α-Defensins, HNP-3 and HD-5

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Enterococcus faecalis (*E. faecalis*) is a Gram-positive bacterium inhabiting the gastrointestinal tracts of humans and other mammals,[12,13] and this species is also isolated from some probiotic foods.[14] Like other enterococci, *E. faecalis* causes life-threatening nosocomial infections because of its high multidrug resistantce.[15–17] Additionally, the production of toxic or virulent factors, such as adhensin, hemolysin, and gelatinase, may be related to the pathogenicity of this species.[15–17] In particular, the gelatinase GelE has been studied as an important toxic factor involved in bacterial invasion.[18,19] Additionally, this extracellular enzyme can digest many host proteins including fibrinogen, fibronectin, collagen, and laminin.[15,18] GelE is a metalloprotease containing one zinc ion in the active center and is classified into the thermolysin family.[19]

In this study, we examined whether two major human α-defensins, HNP-3 and HD-5 (Fig. 1), exert bactericidal activity against *E. faecalis* and whether they show inhibitory activity against purified GelE.

MATERIALS AND METHODS

Bacterial Strains —— *E. faecalis* strain NBRC12580 (*gelE*$^-$), NBRC12969 (*gelE*$^+$) and 677 (*gelE*$^+$) were used in the present study. Among them, strain NBRC12580 and NBRC12969 are environmental isolates, while strain 677 is a clinical isolate.

Purification of GelE —— Strain NBRC12969 precultured in de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Cambridge, U.K.) was inoculated into skim milk broth (10% skim milk, 0.5% glucose, pH 6.5) and cultivated at 37°C. After 24-hr cultivation, the culture supernatant was obtained by centrifugation at $7000 \times g$ for 60 min, and the proteins were precipitated by the addition of 60% saturated (390 g/l) ammonium sulfate and collected by centrifugation. The proteins collected were dissolved in Tris-HCl buffer 10 mM containing NaCl 0.15 M and $CaCl_2$ 1 mM (buffer A, pH 9.0) and dialyzed overnight against buffer A. The dialyzed preparation was applied to a HiLoad 26/60 Superdex 200 column (GE Healthcare, Piscataway, NJ, U.S.A.) equilibrated with Tris-HCl buffer 10 mM containing NaCl 1.5 M and $CaCl_2$ 1 mM (pH 9.0). Gel filtration was carried out at a flow rate of 2.0 ml/min, and the 5-ml fractions were collected. The fractions containing the high protease activity were collected and concentrated by using an Amicon YM10 membrane. Thereafter, ammonium sulfate was added until the concentration reached 0.5 M, and this crude preparation was applied to a Phenyl-Sepharose HP 5/5 column (GE Healthcare) equilibrated with Tris-HCl buffer 20 mM containing ammonium sulfate 0.5 M and $CaCl_2$ 1 mM (buffer B, pH 9.0). The bulk proteins were washed out with buffer B, and bound GelE was eluted with Tris-HCl buffer 20 mM containing $CaCl_2$ 1 mM (pH 9.0). Then 1-ml fractions were collected, and the fractions containing high protease activity were collected and concentrated. The preparation thus obtained was used as the purified GelE preparation.

Polyacrylamide Gel Electrophoresis —— Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on the PhastSystem (GE Healthcare) was carried out with a PhastGel Gradient 10–15 gel (GE Healthcare). After electrophoresis, the gel was stained with 0.25% Coomasie Briliant Blue R-250 and destained with 25% ethanol-8% acetic acid.

Protease Activity —— The protease activity of GelE toward azocasein (Sigma-Aldrich, St. Louis, MO, U.S.A.) was assayed using the method of Miyoshi *et al.*[20] Briefly, GelE was allowed to act on 1.0 mg of azocasein at 37°C for an appropriate period in a total of 0.6 ml of Tris-HCl buffer 100 mM (pH 7.5). The reaction was stopped by the addition of 1.4 ml of 5% trichloroacetic acid. After centrifugation at $1700 \times g$ for 10 min, 1.0 ml of the supernatant was withdrawn and mixed with 1.0 ml of NaOH 0.5 M, and absorbance at 440 nm was measured.

Bactericidal Activity —— The bactericidal activity toward *E. faecalis* was assayed using the method of Fernie-King *et al.*[21] The bacterial cells were harvested at the mid-log phase and suspended in 10 mM of Tris-HCl buffer supplemented with 52-fold diluted MRS broth (buffer C, pH 7.5) at the cell density of 2×10^5 CFU/ml. An aliquot of the bacterial cell suspension (15 µl) was mixed with an equal volume of HNP-3 (0–2.0 µM) or HD-5 (0–0.25 µM), and the mixture was incubated at 37°C for 2 hr. After incubation, the number of the living bacterial cells was counted using the plating method, and the percentage of surviving cells was estimated.

For the study on the antimicrobial activities of antibiotics (ampicillin, ciprofloxacin, streptomycin, and tetracycline), an aliquot of the cell suspension of strain NBRC12969 (15 µl) was added to MRS broth (2 ml) containing the test antibiotic. Then bacterial growth was observed after cultivation at 37°C

for 16–20 hr.

Inactivation of GelE with α-Defensins —— HNP-3 or HD-5 (0–500 nM) was allowed to act on purified GelE (10 nM) at 37°C for 30 min in a total of 40 µl of buffer C. Thereafter the residual protease activity of GelE was measured.

Inactivation of α-Defensins with GelE —— Purified GelE (0–300 nM) was allowed to act on HNP-3 (100 nM) or HD-5 (20 nM) at 37°C for 2 hr in a total of 40 µl of buffer C. Thereafter the residual bactericidal activity of HNP-3 or HD-5 was measured.

RESULTS AND DISCUSSION

Bactericidal Activity of Human α-Defensins against *E. faecalis*

When HNP-3 or HD-5 was allowed to act on *E. faecalis* strain NBRC12969 (an environmental isolate) or 677 (a clinical isolate), the number of living cells decreased in a dose-dependent manner (Fig. 2), indicating the significant bactericidal activity of human α-defensins. Although the strains all showed similar sensitivity to the defensins, they were more sensitive to HD-5, *i.e.*, the 50% effective doses (ED_{50}) of HNP-3 and HD-5 were 100 nM (350 ng/ml) and 20 nM (72 ng/ml), respectively. In contrast to our present study, Wilde *et al.*[22] previously reported that the ED_{50} of HNP-3 for *E. faecalis* was great than 10 µg/ml. However, it should be noted that the bactericidal activity of α-defensins is reduced in the presence of anionic ions. For the assay, we used Tris-HCl buffer, a cationic buffer, while Wilde *et al*. used phosphate buffer, an anionic buffer. The difference in the assay conditions might cause the varied results.

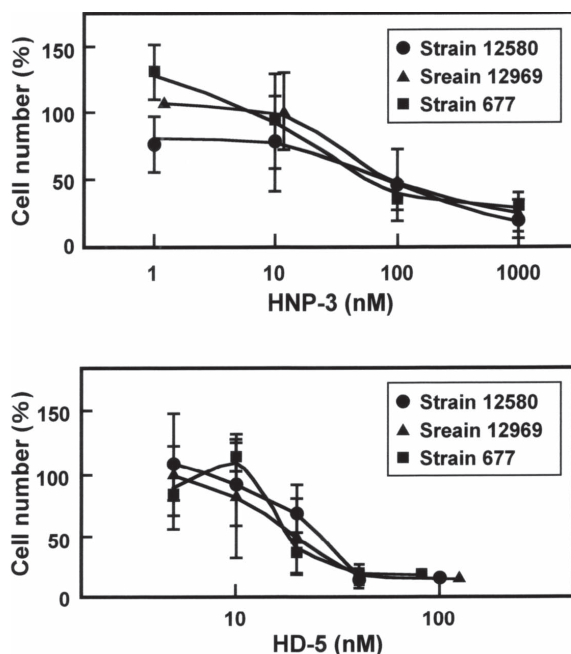

Fig. 2. Bactericidal Activity of Human α-Defensins against *E. faecalis*

HNP-3 (0–1.0 µM) or HD-5 (0–125 nM) was allowed to act on *E. faecalis* strain NBRC12580, NBRC12969, or 677 (1×10^5 CFU/ml) at 37°C for 2 hr. The number of the living bacterial cells was then counted, and the percentage of surviving cells was estimated ($n = 3$).

To test whether the α-defensins show synergistic effects on the antimicrobial activities of antibiotics, ampicillin, ciprofloxacin, streptomycin, and tetracycline were allowed to act on *E. faecalis* strain NBRC12969 in the presence or absence of a low concentration of HNP-3 (10 nM) or HD-5 (2 nM). Neither α-defensin affected the antimicrobial activities of the antibiotics (data not shown). These results suggest that the bactericidal actions of HNP-3 and HD-5 are due to direct attack on the bacterial cell membrane, but not due to the inactivation of the cytoplasmic substance(s) related to the targets of antibiotics.

Purification of *E. faecalis* GelE

E. faecalis is known to secrete a thermolysin-like metalloprotease GelE as an extracellular toxic factor.[18,19] To study the interaction of α-defensins with GelE, we first purified GelE from the culture supernatant of strain NBRC12969. The bacterium was cultivated in skim milk broth, and the culture supernatant was collected at the early stationary phase and fractionated by the addition of 60% saturated ammonium sulfate. Thereafter the crude preparation obtained was applied to a HiLoad 26/60 Superdex 200 column, and the protease eluted at molecular mass of around 35 kDa was collected and concentrated. Our preliminary experiments revealed that, similar to the thermolysin-like metalloproteases from human pathogenic *Vibrio* species,[23] the enzyme produced by *E. faecalis* was markedly hydrophobic. Therefore the concentrated preparation was applied to a Phenyl-Sepharose HP 5/5 column. As expected, after elution of the other proteins, the protease was recovered as a single but slightly broad peak.

When the final preparation was subjected to SDS-PAGE, a single protein band with a molecular mass of 35 kDa was detected (Fig. 3). The *N*-terminal amino acid sequence of the 35-kDa protein was also analyzed. As shown in Fig. 3, a single sequence, NH_2-Val-Gly-Ser-Glu-Val-Thr-

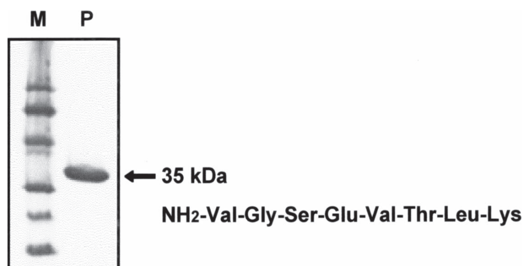

Fig. 3. SDS-PAGE Profile of the Final Preparation

The preparation (5 μg) was treated with 2% SDS at 100°C for 3 min and subjected to SDS-PAGE on the PhastSystem using the PhastGel Gradiant 10–15 gel. After electrophoresis, the gel was stained with 0.25% Coomasie Brilliant Blue R-250. Lane M: molecular weight marker proteins (97, 66, 45, 30, 20.1, and 14.4 kDa), and lane P: final preparation.

Leu-Lys, corresponding to Val[192] to Lys[199] of the GelE precursor of *E. faecalis* strain V583[19)] was determined. Phosphoramidon is a well-known competitive inhibitor of thermolysin-like metalloproteases.[24)] When the purified protease was treated with this inhibitor, the proteolytic activity was lost completely (data not shown). These results clearly demonstrate that the protease purified was GelE.

Interaction of Human α-Defensins with *E. faecalis* GelE

To determine whether human α-defensins can neutralize GelE, HNP-3 or HD-5 (0–500 nM) was allowed to act on GelE (10 nM) at 37°C for 30 min, and the residual activity of GelE was measured. However, no significant reduction in the proteolytic activity was observed (data not shown). This result may indicate no neutralizing effect of the α-defensins on Gel, although it may be possible that GelE digested and inactivated the α-defensins. To test this possibility, purified GelE (0–300 nM) was allowed to act on HNP-3 (100 nM) or HD-5 (20 nM) at 37°C for 2 hr. The bactericidal activity of either α-defensin was significantly reduced only when incubated with the high dose of 300 nM of GelE (Fig. 4), indicating that α-defensin is fairly resistance to GelE. It should be emphasized that the concentration of GelE in the culture supernatant was about 10 nM, suggesting no inactivation of HNP-3 and HD-5 in the human body. In other words, because both HNP-3 and HD-5 are fairly resistant to GelE, the sensitivity of each strain of *E. faecalis* to the α-defensins is independent of the ability to produce GelE. To clarify this, the sensitivity of strain NBRC12580 (*gelE*$^-$) to HNP-3 or HD-5 was compared with that of strains NBRC12969 and 677 (*gelE*$^+$). As shown in Fig. 2, the dose-response curve for strain NBRC12580 was similar to that for the other two strains. Taken together, the bactericidal actions of HNP-3 and HD-5 may be due to the direct attack on and disruption of the bacterial cell membrane.

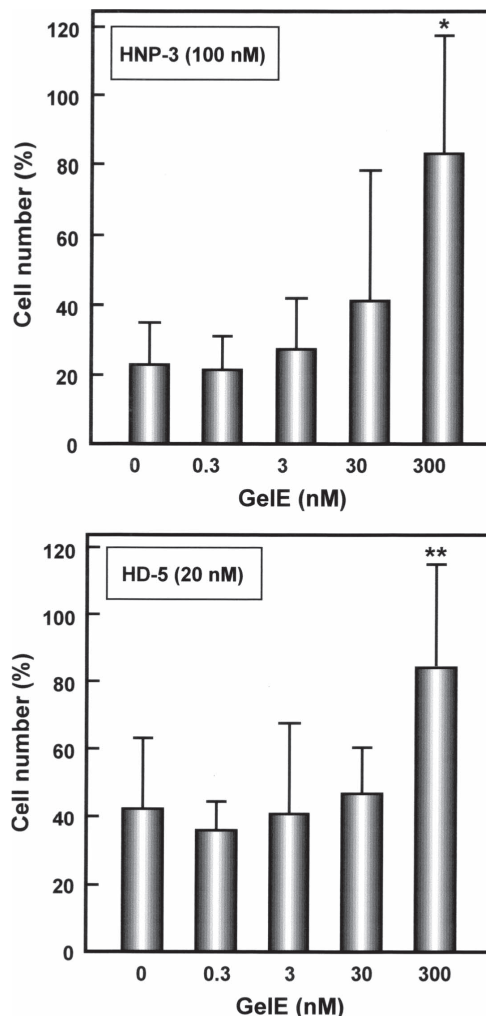

Fig. 4. Inactivation of Human α-Defensins with GelE

Purified GelE (0–300 nM) was allowed to act on HNP-3 (100 nM) or HD-5 (20 nM) at 37°C for 2 hr. The residual bactericidal activity was then measured ($n = 4$). $^{*}p = 0.014$, $^{**}p = 0.049$.

Human α-defensins have been documented to function as the primary defensive factors against invading microorganisms. The present study indicated that two α-defensins have the bactericidal activity against *E. faecalis*, an opportunistic human pathogen. Therefore it may be concluded that the α-defensins are factors in the innate immunity to *E. faecalis*.

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