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Note

Defensive Effects of Human Intestinal Antimicrobial Peptides against Infectious Diseases Caused by *Vibrio mimicus* and *V. vulnificus*

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Of human pathogenic *Vibrio* species, *V. mimicus* causes gastroenteritis whereas *V. vulnificus* causes fatal septicemia after consumption of contaminated seafood. These two pathogens produce hemolytic toxins termed *V. mimicus* hemolysin (VMH) and *V. vulnificus* hemolysin (VVH), respectively. These toxins elicit the cytolysis of various eukaryotic cells, as well as erythrocytes. The human intestine secretes cationic antimicrobial peptides (AMPs) to prevent infectious diseases. Paneth cells in the small intestine secrete α-defensin 5 (HD-5) and epithelial cells in the large intestine produce LL-37. In the present study, we examined the bactericidal activities of AMPs against *V. mimicus* and *V. vulnificus*. Although HD-5 showed no bactericidal activity, LL-37 revealed significant activity against both *Vibrio* species, suggesting that neither *V. mimicus* nor *V. vulnificus* can multiply in the large intestine. We also tested whether AMPs had the ability to inactivate the hemolytic toxins. Only HD-5 was found to inactivate VMH, but not VVH, in a dose-dependent manner through the direct binding to VMH. Therefore, it is considered that *V. mimicus* cannot penetrate the small intestinal epithelium because the cytolytic action of VMH is inactivated by HD-5.

Key words：*Vibrio mimicus* / *Vibrio vulnificus* / Hemolysin / Antimicrobial peptide.

The genus *Vibrio* consists of facultative anaerobic gram-negative bacteria that ubiquitously inhabit at aquatic environments; however, at least 12 species have been reported to be human pathogens (Janda et al., 1988; Chakraborty et al., 1997). Of the human pathogenic species, *Vibrio mimicus* causes gastroenteritis, whereas *Vibrio vulnificus* causes fatal septicemia after consumption of contaminated seafood (Shandera et al., 1983; Chakraborty et al., 1997; Strom and Paranjpye, 2000; Miyoshi 2006). These species produce extracellular hemolytic toxins termed *V. mimicus* hemolysin (VMH) (Miyoshi et al., 1997) and *V. vulnificus* hemolysin (VVH) (Shinoda et al., 1985; Miyoshi et al., 1993), respectively. Both VMH and VVH elicit the cytolysis of various eukaryotic cells, as well as erythrocytes (Yamanaka et al., 1990; Miyoshi et al., 1993; Kashimoto at al., 2010).

Defensins and cathelicidins are cationic antimicrobial peptides (AMPs) contributing to our innate immunity, the first defense system against microbial pathogens (Otte et al., 2003; Salzman et al., 2007; Burton and Steel, 2009). In the human small intestine, two α-defensins termed HD-5 and HD-6 (FIG. 1) are secreted from Paneth cells, and a β-defensin called HBD-1 (FIG. 1) is produced from epithelial cells. Among these defensins, HD-5 is known to be predominant (Otte et al., 2003). In the large intestine, HBD-1 and LL-37 (FIG. 1), which is the sole cathelicidine in humans, are produced from epithelial cells. The α-defensins are secreted constitutively, and their concentration in the crypt lumen is about 1-25 mg mL^{-1} (Bevins and Salzman, 2011). On

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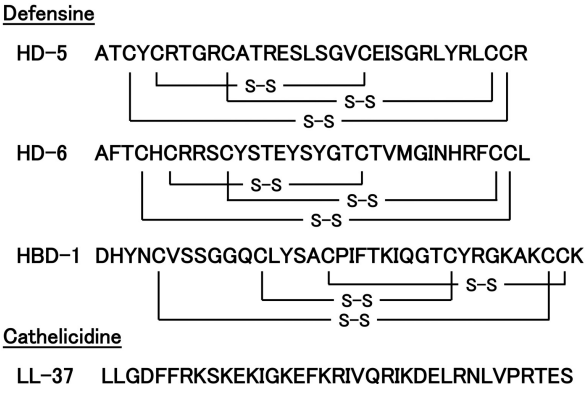

FIG. 1. Structures of human intestinal AMPs used in this study.

the other hand, production of the β-defensin and cathelicidin is induced by microbial infections.

AMPs are potent bactericidal factors, because they may bind directly to the bacterial cell membrane having negative charges and may cause disruption of the membrane by formation of a large hollow polymer (Hancock and Sahl, 2006; Salzman et al., 2007). In addition, recent studies have shown that human α-defensins including HD-5 can inactivate several bacterial extracellular toxins (Chun et al., 2005; Lehrer et al., 2009). In the present study, we examined whether human intestinal AMPs have bactericidal activity against *V. mimicus* and/or *V. vulnificus* and whether they can inactivate purified VMH and/or VVH. The results showed that LL-37 and HD-5 might act as host factors modulating infectious diseases caused by *Vibrio* species.

We assayed the bactericidal activities of the AMPs as described previously (Miyoshi et al., 2010) with some modifications. Human intestinal AMPs, such as HD-5, HD-6, HBD-1 and LL-37, were purchased from the Peptide Institute (Minoh, Osaka, Japan). Two clinical isolates, *V. mimicus* strain CS-66 and *V. vulnificus* strain L-180, were cultivated in TYE broth (0.5 % tryptone, 0.25 % yeast extract, 25 mM K_2HPO_4, pH 7.5) containing 2.0 % NaCl. The bacterial cells were harvested at the middle log-phase and suspended into S broth (0.5 % peptone, 0.6 % NaCl, 0.085 % KCl, 0.03 % calcium lactate, pH 7.5) at the cell density of 1×10^7 CFU mL^{-1}. Each AMP (0, 6.0 or 12 μg) was allowed to act on the bacterial cells (10^6 CFU) in a total volume of 120 μl of S broth at 37 ℃ for 2 h. After incubation, the number of the living bacterial cells was counted by the plating method, and the cell density (CFU mL^{-1}) was estimated. Thereafter, the ratio of the cell density before and after incubation (CFU_{t2}/CFU_{t0}) was calculated.

HD-5 is a predominant AMP in the human small intestine (Otte et al., 2003); however, this AMP showed

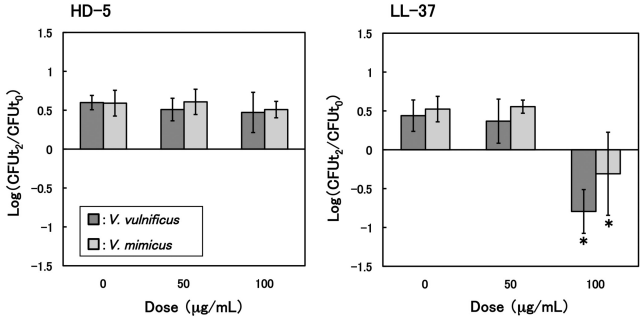

FIG. 2. The bactericidal activities of human intestinal AMPs against pathogenic *Vibrio* species.
HD-5 or LL-37 (0, 6.0 or 12 μg) was allowed to act on the living cells (10^6 CFU) of *V. mimicus* or *V. vulnificus* in a total volume of 120 μl of S broth at 37 ℃ for 2 h. After incubation, the number of the living bacterial cells was counted, and the cell density (CFU mL^{-1}) was estimated. Thereafter, the ratio of the cell density before and after incubation (CFU_{t2}/CFU_{t0}) was calculated (n = 4, *: $p<0.01$).

no bactericidal activity even when a dose of 100 μg ml^{-1} was allowed to act on *V. mimicus* or *V. vulnificus* cells (FIG. 2). Other small intestinal AMPs, HD-6 and HBD-1, were also found to have no vibriocidal activity against either species (data not shown). In contrast, LL-37, a major AMP in the human large intestine (Otte et al., 2003), revealed significant bactericidal activity against *V. minicus* and *V. vulnificus* at the same dose (FIG. 2). These results suggest that both *V. mimicus* and *V. vulnificus* are unable to multiply in the human large intestine.

We next tested whether human intestinal AMPs had the ability to inactivate the extracellular toxins produced by the *Vibrio* species. VMH and VVH were purified from the bacterial culture supernatants by combination of ammonium sulfate fractionation and column chromatography as described by Miyoshi et al. (1997) and Oh et al. (1993), respectively. The amounts of the toxins were determined by the Lowry method, and bovine serum albumin was used as a standard protein. The homogeneity of VMH and VVH was confirmed by SDS-PAGE. Each AMP (0-2.0 μg) was allowed to act on purified VMH or VVH (50 ng) in a total volume of 50 μL of PBS at 37 ℃ for 15 min. Thereafter, 5 % horse erythrocytes in PBS (50 μL) were added, and the mixture was allowed to incubate at 37 ℃ for 1 h. After incubation, the reaction was terminated by the addition of ice-cold PBS (400 μL), and the percentage of hemolysis was determined by measuring of the amounts of hemoglobin released from the disrupted erythrocytes (Shinoda et al., 1985).

As shown in FIG. 3, HD-5 was found to inactivate VMH in a dose-dependent manner, and the hemolytic

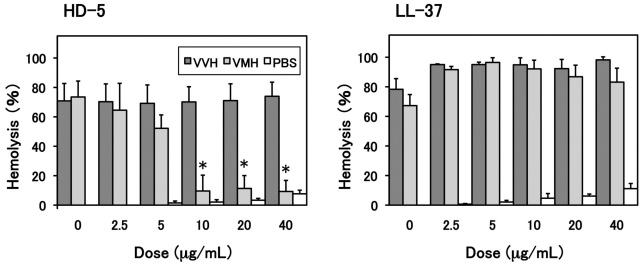

FIG. 3. Inactivation of the purified toxins by human intestinal AMPs.
HD-5 or LL-37 (0-2.0 μg) was allowed to act on VMH or VVH (50 ng) in a total volume of 50 μL of PBS at 37 ℃ for 15 min. Thereafter, 5 % horse erythrocytes in PBS (50 μL) were added, and the mixture was incubated at 37 ℃ for 1 h. After incubation, the percentage of hemolysis was measured (n = 4, *: p<0.01).

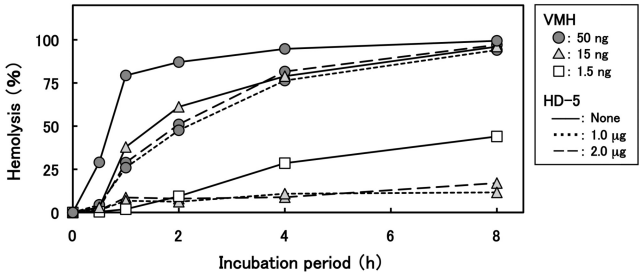

FIG. 4. Kinetics of hemolysis induced by VMH.
VMH (1.5, 15 or 50 ng) was treated with HD-5 (0, 1.0, or 2.0 μg) in a total volume of 50 μL of PBS at 37 ℃ for 15 min. Thereafter, 5 % horse erythrocytes in PBS (50 μL) were added, and the mixture was incubated at 37 ℃. At the indicated period, the percentage of hemolysis was measured (n = 2).

activity of VMH was reduced to 10-15 % with 10 μg mL^{-1} of HD-5. On the other hand, the activity of VVH was negligibly inhibited with HD-5 even when as high a dose as 40 μg mL^{-1} was added. The effects of HD-6, HBD-1, and LL-37 were also examined. However, LL-37 (FIG. 3), as well as HD-6 and HBD-1 (data not shown), showed no ability to inactivate VMH or VVH. FIG. 4 shows the kinetics of hemolysis induced by VMH with or without HD-5 treatment. The hemolytic reaction by VMH progressed time-dependently, but the reaction was apparently delayed by treatment with HD-5. At sub-cytolytic doses, VMH can function as an enterotoxin causing the secretion of the Cl^- ion and H_2O into the lumen of the small intestine (Takahashi et al., 2007). Therefore, although the hemolytic activity of VMH is apparently reduced, the enterotoxic activity may be not eliminated by incubation with HD-5. VMH has been documented to be a determinant of the putative virulence of diarrheal diseases (Miyoshi t al., 1997; Li et al., 2008). The findings shown herein may support the

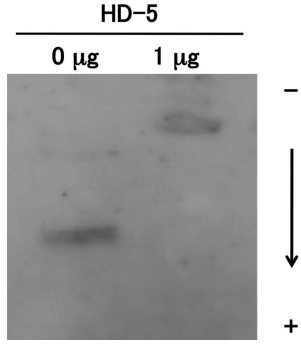

FIG. 5. Formation of the complex of HD-5 and VMH.
VMH (50 ng) was exposed or not exposed to HD-5 (1.0 μg) in a total volume of 50 μL of PBS at 37 ℃ for 15 min. Thereafter, the sample was subjected to native PAGE, and the VMH antigen was detected by Western blotting.

conclusion of the previous studies.

Taken together, it may be concluded that, in the case of *V. mimicus* infection, the bacterial cells remain in the small intestinal lumen because the cytolytic toxin VMH is inactivated by HD-5. On the other hand, in the case of *V. vulnificus* infection, the bacterial cells are able to invade the blood stream because VVH is resistant to the small intestinal AMPs including HD-5.

In order to clarify whether HD-5 directly binds to VMH or not, VMH (50 ng) was exposed or not exposed to HD-5 (1.0 μg) in a total volume of 50 μL of PBS at 37 ℃ for 15 min. After the treatment, each of the VMH samples was mixed with a half volume of the native PAGE buffer (192 mM glycine, 25 mM Tris, 70 % glycerol, 0.05 % bromphenol blue, pH 8.3) and was subjected to electrophoresis on the PhastSystem using the PhastGel High Density gel (GE Healthcare Bio-Sciences, Buckinghamshire, UK). After electrophoresis, the proteins separated were electrophoretically transferred to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare Bio-Sciences). Then, the membrane with the bound proteins was incubated with the rabbit IgG antibody against purified VMH, and the antigen-antibody complex was visualized using the anti-rabbit IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA) and ECL Western blotting detecting reagents (GE Healthcare Bio-Sciences). As shown in FIG. 5, formation of the complex of HD-5 with VMH was revealed. Namely, although free VMH migrated sufficiently into the separation gel, VMH treated with HD-5 showed little mobility in the native PAGE assay, which indicates the addition of the positive charge to VMH via the direct binding of HD-5, a cationic AMP.

We also tested whether the VMH-HD-5 complex can associate with the erythrocyte membrane or not. Horse

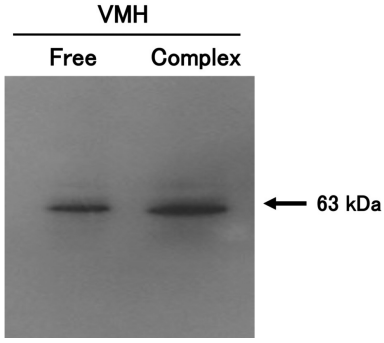

FIG. 6. Association of the VMH-HD-5 complex with horse erythrocyte ghosts.
Free VMH (50 ng) or the VMH-HD-5 complex, which was prepared by incubation of VMH (50 ng) with HD-5 (1.0 μg) at 37 ℃ for 15 min, was allowed to act on horse erythrocyte ghosts in a total volume of 100 μL of PBS at 37 ℃ for 30 min. Thereafter, the erythrocyte ghosts were collected by centrifugation (12000 x g, 10min) and subjected to SDS-PAGE, and the VMH antigen was detected by Western blotting.

erythrocyte ghosts were prepared as follows. In brief, the washed erythrocytes (10 mL) were disrupted by incubation with 120 mL of 10 mM phosphate buffer (pH 7.5) for 30 min in an ice-cold water bath, and the disrupted cells were collected by centrifugation (9600 x g, 40 min) and washed with 10 mM phosphate buffered saline (PBS; pH 7.5). Free VMH (50 ng) or the VMH-HD-5 complex, which was prepared by incubation of VMH (50 ng) with HD-5 (1.0 μg) at 37 ℃ for 15 min, was allowed to act on horse erythrocyte ghosts in a total volume of 100 μL of PBS at 37 ℃ for 30 min. Thereafter, the erythrocyte ghosts were collected by centrifugation (12000 x g, 10min), treated with an equal volume of the SDS-PAGE buffer (500 mM Tris-HCl, 2.0 % SDS, 70 % glycerol, 0.05 % bromphenol blue, pH 6.8) at 37 ℃ for 30 min and was subjected to electrophoresis on the PhastSystem using a PhastGel Gradient 10-15 (GE-Healthcare Bio-Sciences).

After SDS-PAGE, the proteins separated were transferred to a Hybond-P polyvinylidene difluoride membrane, and the VMH antigen was detected by Western blotting using the rabbit IgG antibody against purified VMH, the anti-rabbit IgG antibody conjugated with horseradish peroxidase and ECL Western blotting detecting reagents. As shown in FIG. 6, the complex was found to have the sufficient ability to associate with the erythrocyte membrane. Namely, the protein band of VMH antigen that steadily associates with the ghost membrane was detected by Western blotting. VMH is known to cause hemolysis through two processes, the membrane-binding process and the membrane-disrupting process (Miyoshi et al. 1997). The findings shown herein suggest that the binding of HD-5 to VMH block the later hemolytic process.

In conclusion, the present study revealed that human intestinal AMPs, such as HD-5 and LL-37, might be the modulating factors against infectious diseases caused by *Vibrio* species, because they can kill the bacterial cells or can inactivate the bacterial extracellular toxins.

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