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Two Arginine Residues in the COOH-Terminal of Human β-Defensin-3 Constitute An Essential Motif for Antimicrobial Activity and IL-6 Production

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Abstract

Human β-defensin-3 (HBD-3) possesses antimicrobial activities and the potential to induce proinflammatory cytokines. HBD-3 contains a unique motif of two arginine residues (Arg. R) in the COOH-terminal region. To understand the bioactive properties of the Arg residues of HBD-3, we examined antimicrobial activities against *S. aureus* and *P. aeruginosa* using synthetic HBD-2, HBD-3, and two variant peptides of HBD-3: the Arg-truncated variant designated desR HBD-3, and NRR HBD-3, in which both Arg residues were shifted to the N-terminal region. IL-6 production from keratinocytes was studied using the peptides. HBD-3 possessed approximately 5-fold more potent antimicrobial activities, evaluated as the minimum inhibitory concentration, against *S.aureus* compared with desR and NRR HBD-3, while no significant activity was observed in HBD-2. The antimicrobial activity of HBD-3 against *S. aureus* was well preserved even at high sodium chloride concentrations, but was attenuated in desR and NRR HBD-3. All the peptides exhibited similar antimicrobial activities against *P. aeruginosa*, but HBD-2 and desR HBD-3 showed diminished antimicrobial activities against *P. aeruginosa* at high salt concentrations. IL-6 production was significantly induced in keratinocytes with HBD-3, but not remarkably with stimulation by other peptide. These Arg residues are essential for the antimicrobial and biological properties of HBD-3.

Key words.
antimicrobial peptide - *S. aureus* - *P. aeruginosa* – salt insensitivity- IL-6

Introduction

Defensins are small cationic antimicrobial peptides that play important roles in the innate immunity of organisms ranging from molds and plants to vertebrate and invertebrate animals (1-3). Defensins contain six cysteine residues forming three conserved disulfide bridges. These bridges seem to play a very important role in the tertiary structure of defensins, which consist of triple-stranded twisted antiparallel β-sheets on which an α-short helix may be grafted (4-6). Few residues are conserved in their sequences, apart from the six cysteine residues. Although the number, spacing and connectivity of the cysteine residues in different defensin families vary, all structures determined to date conform quite closely to this characteristic disulfide bridges (7-9). Both α- and β-defensins have been isolated in humans. Human α-defensins are found in neutrophils and intestinal Paneth cells, whereas human β-defensins (HBDs) are mainly produced by epithelial cells of several organs (1, 10, 11). To date, four HBDs (HBD-1, -2, -3 and -4) have been identified in human skin. HBD-1 is constitutively produced by various epithelial tissues, including urogenital and respiratory tracts and skin (12). In contrast, HBD-2, -3, and -4 are described as inducible β-defensins(13). HBD-3 was detected by screening human genomic sequences (14, 15), and was successively isolated from human lesional psoriatic scales (16) like HBD-2 (17). HBD-4 has not yet been isolated, but has been identified solely by genomics (13).

The β-defensin family has shown broad-spectrum antimicrobial activities against gram-positive and -negative bacteria, fungi and enveloped viruses in vitro (2, 18). HBD-1, -2, and -4 have been reported to exhibit potent activity predominantly against gram-negative bacteria such as *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (18, 19) , although they exert little to no activity against gram-positive

bacteria such as *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pyogenes* (*S. pyogenes*) (17, 19, 20) . In comparison to other members of the β-defensin family, HBD-3 possesses broad-spectrum antimicrobial activity against both gram-negative and -positive bacteria and shows activity at much lower concentrations. It was also reported that, among several antimicrobial peptides, only HBD-3 accumulated at levels sufficient to account for killing when *S. aureus* were exposed to human skin explants and blocking peptide binding of HBD3 inhibited killing of the bacteria (21). Furthermore, HBD-3 maintains its activity in the presence of salt, although HBD-1, -2, and -4 are salt sensitive (18, 19, 22, 23). Consequently, HBD-3 seems to be the most efficacious antimicrobial peptide of the β-defensin family (24).

The mechanism of this potent, broad and salt-insensitive antimicrobial activity of HBD-3 has not been fully investigated. Studies of peptide analogues of HBD-3 (9, 13, 25) have pointed to the distribution of the positively charged amino acids and hydrophobic side chains as important parameters of antimicrobial activity, while the overall hydrophobicity may correlate with cytotoxicity to mammalian cells.

In addition to their antimicrobial activities, HBDs exhibit chemotactic ability, including migration, proliferation, and cytokine/chemokine production and function to induce the adaptive immune system (26). It has also been reported that HBDs induce proinflammatory cytokines including IL-6, IL-10, IP-10 (27) and IL-18 (28) in primary human keratinocytes.

As a bioactive property of HBD-3, we focused on two arginine residues (Arg. R), Arg^{42} and Arg^{43}, in the COOH (C) -terminal region of HBD-3, which constitutes a unique structure of HBD-3. We prepared two recombinant variants of HBD-3: “desR HBD-3”, in which Arg^{42} and Arg^{43} in the C-terminal were truncated, and “NRR HBD-3”, in

which the two Arg residues were shifted from the C-terminal to the NH_2 (N) –terminal region, maintaining completely equal positive charges as in HBD-3. Primary structures of HBD-3, desR HBD-3, NRR HBD-3 and HBD-2 are shown in Fig. 1a. 3-D cartoon protein structure homology modelling of desR HBD-3 and NRR HBD-3 were generated by SWISS-MODEL (Fig. 1b) (29) . We assayed the antimicrobial activity and salt-insensitivity of synthetic HBD-3, and compared the results with those of synthetic HBD-2, desR HBD-3 and NRR HBD-3. We examined IL-6 production from cultured human keratinocytes with the stimulation of each peptide.

Methods

Expression and purification of recombinant desR HBD-3 and NRR HBD-3

Complementary DNA encoding a mature peptide of HBD-3 was obtained by reverse transcriptase-PCR from our frozen stock of total RNA from psoriatic skin and used as a template for the following site-directed mutagenesis of desR HBD-3. For cloning desR HBD-3, a pair of primers was synthesized. The forward primer was the 43-mer (5'-ATATGAATTCATCGAAGGTCGTGGAATCATAAACACATTACAG-3') containing a FXa recognition site together with an *Eco*RI cutting site in the 5' region just before the peptide sequence. Similarly, the reverse primer was the 30-mer (5'-GTCGACTTATTTCTTGCAGCATTTTCGGCC-3') with a stop codon before the SalI cutting site in the 5' region and excluding the two codons coding Arg^{42} and Arg^{43} of HBD-3.

Following PCR amplification, products were cloned in pCR2.1-TOPO (Invitrogen). This construct was digested with *Eco*RI and *Sal*I and gel-purified. EcoRI/SalI inserts were ligated into EcoRI and SalI-digested pET-28a plasmid DNA and transformed into BL21 (DE3) Codon Plus cells (Stratagene Cloning Systems, Inc., La Jolla, CA, USA) to produce N-terminal His6-tagged fusion proteins.

To prepare NRR HBD-3, the construct of desR HBD-3 was used as a template and the forward primer was the 43-mer (5'-ATATGAATTCATCGAAGGTCGTCGAAGAGGAATCATAAACACA -3') containing two codons for two Arg residues, the FXa recognition site and the EcoRI cutting site in the 5' region just before the sequence of desR HBD-3. The reverse primer was the same one used for desR HBD-3. The PCR product of NRR HBD-3 was finally cloned in pET-28a and transformed into BL21 (DE3) Codon Plus cells, similarly to that of desR

HBD-3. All mutations were verified by DNA sequencing prior to expression.

The expression protocol using a pET-28a expression system was previously reported (30).

Recombinant proteins were expressed for 6 h at 37℃ in *E. coli* BL21 (DE3) codon Plus cells growing exponentially in Terrific Broth medium by induction with 0.2mM isopropyl-1-thio-β-D-galactopyranoside under kanamycin selection. Bacterial cells were harvested by centrifugation and lysed in 6 M guanidine HCl in 100 mM Tris-HCl (pH 8), and clarified by centrifugation.

His-tagged fusion proteins were eluted from nickel-nitorilotriacetic acid (Ni-NTA, Qiagen) resin with 1M imidazole, 6 M guanidine-HCl, and 100 mM Tris-HCl (pH6.0). Fusion proteins were dialyzed against 5% acetic acid, lyophilized and dissolved in an aliquot of 5% acetic acid. They were desalted by C-18 RP-HPLC, followed by cleavage with Factor Xa (Novagen, Darmstadt, Germany). The cleaved proteins were purified by C-18 RP-HPLC. Peptide concentrations were determined using bicinchoninic acid assay (Pierce, Rockford, IL). The identities of recombinant desR and NRR HBD-3 molecules were verified by NH_2-terminal sequencing and Tris-Glycine SDS PAGE with SimplyBlue^{TM} SafeStain staining in which ≧ 0.5 μg peptides were incubated in 1X NuPAGE Sample Reducing Agent (ThermoFisher scientific) containing 50mM dithiothreitol (DTT) at 85℃ for 2 min and electrophoresed (Fig. 1c). Commercially available synthetic HBD-2 and HBD-3 (sHBD-2 and -3) (PEPTIDE INSTITUTE, INC., Osaka, Japan) were co-migrated as controls. The bands of all the peptides, even sHBD-2 and sHBD-3, were larger than the theoretical value. However, molecular weights of both sHBD-2 and sHBD-3 were confirmed by ESI-MS analysis, conducted by the manufacturer, and matched with theoretical value .

Bacterial Assays

Recombinant desR and NRR HBD-3 and sHBD-3 and sHBD-2 were tested for microbial activity against *S. aureus* FDA209P and *P. aeruginosa* NBRC12582, which were kindly provided by Dr. Mineshiba. Clinically isolated wild-type *S. aureus* and *P. aeruginosa* were also used for this assay. Three strains of *S. aureus* (MSSA 1 ~ 3) were isolated from a 4-year-old male (4M) with impetigo, a 37-year-old female (37F) with atopic dermatitis and a 51M with abdominal skin ulcer. Three strains of *P. aeruginosa* (P.ae 1 ~ 3) were isolated from a 67M with leg ulcer, a 66F with herpetic gangrene and a 54M with mycosis fungoides. Bactericidal assays were performed according to the previous report with some modifications (31). Briefly, bacteria growing exponentially at 37℃ in trypticase soy broth were deposited by centrifugation at 1700 × g for 10 min, washed and resuspended in 10 mM PBS, with 1% TSB (pH 7.4). The microorganisms were incubated at 37 ℃ with peptides in a total volume of 50 µl at a concentration of ~1 × 10^6 CFU/ml for 2 h in a shaking incubator. To investigate the salt sensitivity of each peptide, 10 µg/ml of each peptide was incubated with ~1 × 10^6 CFU/ml of either *S. aureus* FDA209P or *P. aeruginosa* NBRC12582 in 50 µl of 10 mM PBS containing 1% TSB (pH 7.4) in the presence of 0-250 mM NaCl for 2 h at 37℃. Following microbial exposure to peptides, 20 µl of the 1:100 diluted samples were plated on trypticase soy agar plates. The surviving microorganisms were counted as colony-forming units/ml after incubation at 37℃ for 12-18 h. To confirm the reproducibility, the experiments were performed at least three times. The representative results are shown in figures.

Keratinocyte culture and stimulation

Keratinocytes were cultured and stimulated according to the previous reports (27, 32). The conditions of the culture and stimulation of keratinocytes were made reference to other's previous report. Normal human epidermal keratinocytes purchased from Kurabo Industries (Osaka, Japan) containing 0.06 mM Ca^{2+} and 1 ×Epilife Defined Growth Supplement (EDGS, Cascade logics/Invitrogen) at 37 ℃ under standard tissue culture conditions. Cultures were maintained in this media with the addition of 100 U/ml penicillin and 50 μg/ml streptomycin. After cells were serially passaged at 60-70% confluence, experiments were conducted with subconfluent cells at passage three or four in the proliferative phase at 60-80% confluence. For stimulation, keratinocytes were cultured in 24-well tissue culture plates. After removal of growth medium, cells were washed twice with phosphate-buffered saline (PBS) (-) before culture in EpiLife cell culture medium supplemented with only antibiotics for 24 hours. Three-well of the keratinocytes were subsequently stimulated with 30 μg/ml of one of each peptide, HBD-2, -3, desR HBD-3 and NRR HBD-3 respectively. The concentration of peptides was determined according to the previous report by Niyonsaba et al. (27).

Real-time quantitative polymerase chain reaction for IL-6

Total RNA was extracted from cultured and stimulated keratinocytes using an RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. cDNA transcription was carried put in 20 μl reverse transcriptase (RT) reaction mixtures containing 1 μg RNA, 0.5 mM deoxyribonucleoside triphodphates, RNase inhibitor (Promega, Madison, WI, U.S.A.) , 150 pmol random hexamer primer (Invitrogen, Tokyo, Japan) . The obtained cDNA was used in real-time quantitative polymerase chain reaction (qPCR)

and reaction mixtures containing 1 µl each of (10 pmol/µl) forward and reverse primers, 10.0 µl 2x iQ SYBR green Supermix (Promega), and 5µl cDNA template, 30 mM MgCl2. The protocol used included a denaturation step (95 ℃ for 15 min) followed by amplification repeated 55 times (94℃ for 15 s, 60℃ for 20 s, and 72℃ for 20 s). A melting curve analysis performed following every run confirmed the amplification of a single product and no primer dimer. In each experiment, reactions were carried out in triplicate for each sample and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization.

ELISA for IL-6

IL-6 released in the cell-free supernatants from non-stimulated or stimulated cultures with 30 µg/ml HBD-2, -3, desR HBD-3 and NRR HBD-3 for 48 hours were measured with ELISA kits, Quantikine Human IL-6 Immunoassay (R&D Systems, Minneapolis, MN, U.S.A.). Supernatants were stored at -20℃ until use for ELISA according to the manufacturer's instructions.

Results

The antibacterial activities of HBD-2, HBD-3 and its mutant peptides of HBD-3 against *S. aureus* FDA209P and *P. aeruginosa* NBRC12582.

We examined the antibacterial activities of sHBD-2, -3, desR HBD-3 and NRR HBD-3 against *S. aureus* FDA209P and *P. aeruginosa* NBRC12582. Against *S. aureus* FDA209P, sHBD-2 did not show any antimicrobial activity until 20 μg/ml, while sHBD-3 killed all the cells at 2.0μg/ml. Meanwhile, desR HBD-3 showed attenuated activity and its minimum inhibitory concentration (MIC) was between 5 and 10 μg/mL. Unexpectedly, the activity of NRR HBD-3, which contains the same positive net charges as the native HBD-3, did not improve from that of desR HBD-3. All the peptides showed very potent antimicrobial activities against *P. aeruginosa* NBRC12582 and the MICs of all peptides were less than 5 μg/ml.

The representative data for activities against *S. aureus* and *P. aeruginosa* are shown in Fig. 2a and 2b, respectively.

Salt insensitivity of antimicrobial activity against *S. aureus FDA209P* and *P. aeruginosa* NBRC12582.

We analyzed bacterial killing of the defensins at a fixed concentration (10 μg/ml) in the presence of increasing concentrations of sodium chloride against *S. aureus* FDA209P and *P. aeruginosa* NBRC12582. Against *S. aureus* FDA209P, HBD-3 maintained potent antimicrobial activity against *S. aureus* FDA209P in a wide range of NaCl concentrations including a physiological level, while the antimicrobial activities of desR and NRR HBD-3 were attenuated at a low salt concentration of 50 μM. HBD-2 did not show any activity even in a salt-free condition (Fig. 2c). Against *P. aeruginosa*

NBRC12582, HBD-3 and NRR HBD-3 maintained potent microbial activity at up to 250 mM of salt, while desR HBD-3 and HBD-2 could only maintain their activities up to 150 mM and 50 mM of NaCl (Fig. 2d).

The antibacterial activity of the defensins against wild-type *S. aureus* and *P. aeruginosa*.

We also examined the antimicrobial activities of the defensins against three wild-type strains of *S. aureus* and *P. aeruginosa*, respectively. Bacterial survival curves against the defensins (representative data) are shown in Fig. 3a, b. Against wild-type *S. aureus*, HBD-3 possessed the most potent activities and desR HBD-3 lost its antimicrobial activity, as did HBD-2. NRR HBD-3 possessed lower activity than HBD-3. The MICs of HBD-3 and NRR HBD-3 were 2~4 μg/ml and 10~20 μg/ml, respectively (Fig. 3a). On the other hand, all the peptides possessed activities against wild-type strains of *P. aeruginosa*. The MICs of HBD-2 and HBD-3 were 4~10 μg/ml and 5 μg/ml, respectively. The activities of desR and NRR HBD-3 were slightly inferior to those of HBD-2 and 3, and their MICs were 10~20 μg/ml (Fig. 3b) while their activities against *P. aeruginosa* NBRC12582 were the same as those of HBD-2 and -3.

IL-6 gene expression of keratinocytes stimulated with HBD-2,-3, and the mutant peptides.

IL-6 gene expression was examined by real-time quantitative PCR assay to investigate the role of Arg^{42} and Arg^{43} of HBD-3 in stimulating normal human epidermal keratinocytes to produce IL-6. The increase of mRNA expression, which began at 3 h, persisted until 12 h before decreasing (data not shown) and we analyzed IL-6 mRNA

expression of keratinocytes stimulated by each peptide for 12 h. HBD-3 revealed a very strong induction of IL-6 mRNA, 5.8 $\times 10^3$ fold greater than in the control. In contrast, HBD-2 did not increase IL-6 mRNA expression. Two arginine modified mutants, desR and NRR HBD-3, still had the ability to stimulate mRNA expression, however this ability was significantly attenuated in both cases. The expressions of IL-6 mRNA stimulated by desR and NRR HBD-3 were about 0.1% of that, stimulated by HBD-3 (Fig. 4a).

IL-6 secretion of keratinocytes stimulated with HBD-2,-3, and the mutant peptides

After stimulation of keratinocytes with each peptide for 48 h, the production of IL-6 in cell-free supernatant was determined by using commercially available ELISA kits. As shown in Fig. 4b, HBD-3 was the most powerful in inducing the production of IL-6. Both desR HBD-3 and NRR HBD-3 induced much less secretion of IL-6, which means that the induction ability depended not only on the total net charge but also on the positioning of these two unique Arg residues.

Discussion

Among the defensin family, especially HBD-3 has a broad antibacterial activity and maintains a potent activity even at high NaCl concentration

. Thus, HBD-3 is expected to be a new therapeutic candidate for multi-drug-resistant strains, and understanding the potent and broad antimicrobial mechanisms of HBD-3 is very important so that modifications can be made to improve its pharmaceutical efficacy. Our two mutant peptides of HBD-3 were designed based on the hypothesis that the two Arg residues, Arg^{42} and Arg^{43}, in the C-terminal region of HBD-3, which other human beta defensins do not possess, play an essential role in its activity. In accordance with our hypothesis, desR HBD-3, in which two Arg residues were truncated, did not maintain potent activity against *S. aureus* and had only feeble activity against *P. aeruginosa*.

Interestingly, NRR HBD-3, in which two Arg residues were shifted from the C- to the N-terminal of HBD-3 and whose net charges were equal to those of HBD-3, revealed attenuated antimicrobial activities against both strains. NRR HBD-3 showed more potent antimicrobial activity against *P. aeruginosa* than desR HBD-3 only at the conditions in which NaCl concentrations were above physiological level.

HBD-3 contains two positively charged patches: Patch 1 consists of R14, R17, K26, R42, R43, K44, and K45, and patch 2 consists of K32, K36, R38, and K39. It is suggested that truncation of these Arg residues might cause the loss of antimicrobial activities, and that this loss could not be restored by transferring these two residues to other positions. Interestingly, even though desR HBD-3 possesses more net charges than HBD-2, the antimicrobial activities of desR and NRR HBD-3 against *P. aeruginosa* are inferior to those of HBD-2. This suggests that patch 1 of HBD plays a

greater role in killing *P. aeruginosa* than *S. aureus*: however, this point has not yet been researched and should be elucidated. Although the antimicrobial activities of both desR and NRR HBD-3 against *S. aureus* under an NaCl environment were almost equally attenuated, suggesting the importance of the positions of the two Arg residues, the antimicrobial activity of NRR HBD-3 against *P. aeruginosa* under an NaCl environment was the same as that of HBD-3, indicating that the salt insensitivity of HBD-3 against gram-negative strains is regulated chiefly by total charges or depends on other components besides the positively charged patch 1 of HBD-3. Moreover, it is possible that the mechanisms of the antimicrobial activities of the defensins under an NaCl environment differ between *S. aureus* and *P. aeruginosa*.

HBD also has immunomodulative functions as well as antimicrobial activities and has attracted much attention in the field of dermatology because it can promote proinflammatory cytokines secretion in primary human keratinocytes (16). We examined the effect of the modulation of two Arg residues in HBD-3 upon the production of one of the proinflammatory cytokines, IL-6, by human epidermal keratinocytes. We showed that the modification of two unique Arg residues in HBD-3 resulted in suppressing IL-6 expression by keratinocytes at both the transcriptional and translational levels, and demonstrated that these two Arg residues might also be the structural determinant in the pro-inflammatory process. For these assays, we referred to the previous report (21, 28) and decided the concentration of HBDs as 30 μg/ml. Although the concentrations of antimicrobial peptides in human body are not defined clearly, it has been proven that these concentration in the epithelial tissues at sites where an infection or an inflammation has occurred were high. In the past report (33), HBD-2 has been estimated at ~157 μM in psoriatic lesions. We think that the

concentrations of HBDs (30 μg/ml ; equivalent to 5.8 μM of HBD-3 and 6.9 μM of HBD-2) in our study were adequate for evaluating the physiological roles of HBDs in keratinocytes.

On the other hand, it was also reported that HBD-3 does not induce TNF-α or IL-6 in macrophages and effectively inhibits TNF-α and IL-6 accumulation in the presence of LPS (34). Studying the association between the modification of these two Arg residues in HBD-3 and immunosuppressive activity in macrophages, will be topic of our future research.

In summary, two unique Arg residues in the C-terminal region of HBD-3 play an essential role for the salt-resistant and broad antimicrobial activities of HBD-3 and should be positioned at the C-terminal to maintain the activity of HBD-3. These two residues also contribute to the production of IL-6, one of the proinflammatory cytokines from keratinocytes.

We hope that the investigation of the structural determinants of these remarkable features of HBD-3 leads to a novel therapeutic approach to infectious diseases in the future.

Conflict of Interest

The authors state no conflict of interest.

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Yoko Sakagami-Yasui performed the research and wrote the paper.

Yoshinori Shirafuji designed the research study and performed the research.

Osamu Yamasaki contributed clinically isolated bacteria and analyzed the data.

Shin Morizane designed the research study about proinflammatory cytokine expression.

Toshihisa Hamada and Hiroshi Umemura analyzed the data.

Keiji Iwatsuki supervised the research and assisted to write the paper.

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Figure legends

Figure 1.

The primary structures of HBD-2, HBD-3, desR-HBD-3 and NRR HBD-3 (a) and 3-D cartoon structures of desR-HBD-3 and NRR HBD-3 (b). The 3-D structures were generated by SWISS-MODEL. HBD-3 was used as a template (pdb acquisition code, 1KJ6).

Peptides were electrophoresed in Tris-Glycine SDS PAGE and stained with Coomassie Blue (c). Lanes: *1*, HBD-2; *2*,HBD-3; *3*,desR HBD3; *4*,NRR HBD3.

Figure 2.

To examine antimicrobial activities of the defensins against *S. aureus* FDA209P and *P. aeruginosa* NBRC12582, the indicated concentrations of the defensins were combined with exponentially growing *S. aureus* FDA209P cells (a) and *P. aeruginosa* NBRC12582 cells (b) in 10 mM PBS with 1% TSB (pH 7.4) for 2 h at 37 °C. Following exposure, bacteria were plated onto TSA plates, incubated for overnight at 37 °C, and surviving bacteria were quantitated as colony forming units per ml (*CFU/ml*).

To examine salt dependence of antibacterial activity of the defensins, *S. aureus* FDA209P cells (c) and *P. aeruginosa* NBRC12582 cells (d) were incubated with the defensins at 10 μg/ml in 10 mM PBS, with 1% TSB (pH 7.4) containing different concentrations of NaCl for 2 h at 37 °C , and surviving bacteria were quantitated as described above.

Figure 3.

Antibacterial activities of the defensins against wild-type *S. aureus* (a) and *P. aeruginosa* (b). Exponentially growing bacterial cells were exposed to the indicated concentrations of the defensins for 2 h, and surviving bacteria were quantitated as described in the legend of Fig. 2. Backgrounds of wild-type strains are described in Methods.

Figure 4.

IL-6 gene expression and secretion of keratinocytes stimulated with HBD-3, desR HBD-3, NRR HBD-3 and HBD-2.

Cultured keratinocytes (passage 3 or 4) were stimulated with 30 μg/ml of HBD-2, -3, desR HBD-3 and NRR HBD-3, respectively for 12 h and IL-6 gene expression of stimulated keratinocytes were examined by real-time quantitative PCR assay. Data represent the mean±SEM of triplicate determinations. HBD-3 induced IL-6 mRNA significantly stronger than any other peptide and control ($p<0.01$) (a).

IL-6 released in the cell-free supernatants from non-stimulated or stimulated cultures with 30 μg/ml HBD-2, -3, desR HBD-3 and NRR HBD-3, respectively for 48 h were measured. Data represent the mean±SEM of triplicate determinations. HBD-3 induced IL-6 secretion significantly stronger than any other peptide and control ($p<0.01$) (b).

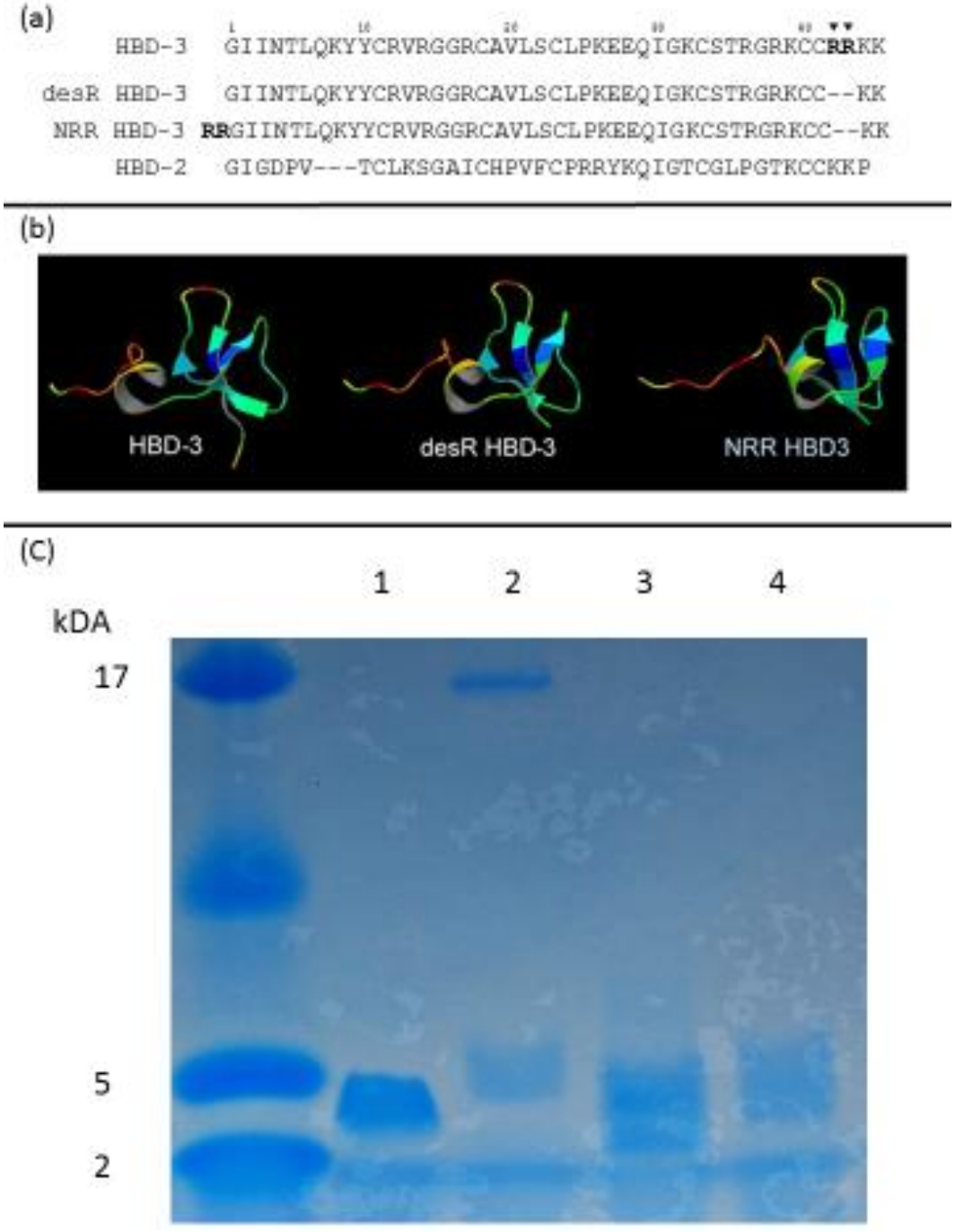
(a)
HBD-3 GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK
desR HBD-3 GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCC--KK
NRR HBD-3 RRGIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCC--KK
HBD-2 GIGDPV---TCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP
(b)
HBD-3
desR HBD-3
NRR HBD3
(C)
1
2
3
4
kDA
17
5
2

Fig. 1

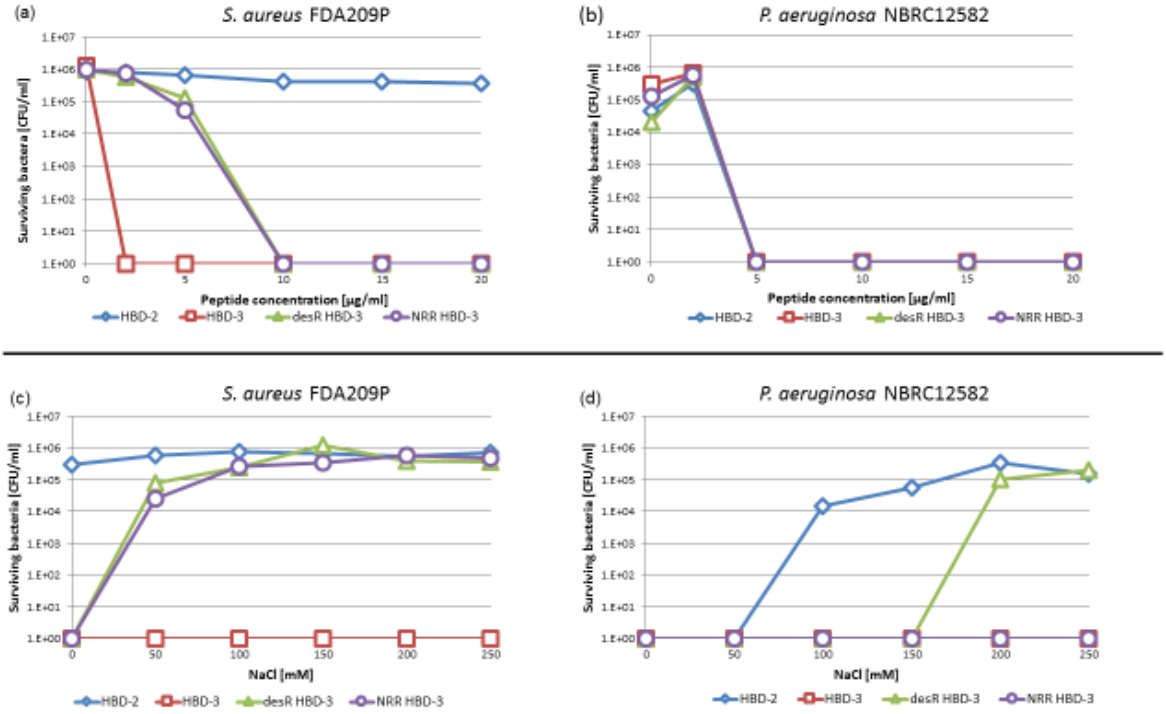
(a)
S. aureus FDA209P
Surviving bacteria [CFU/ml]
Peptide concentration [μg/ml]
HBD-2
HBD-3
desR HBD-3
NRR HBD-3
(b)
P. aeruginosa NBRC12582
Surviving bacteria [CFU/ml]
Peptide concentration [μg/ml]
HBD-2
HBD-3
desR HBD-3
NRR HBD-3
(c)
S. aureus FDA209P
Surviving bacteria [CFU/ml]
NaCl [mM]
HBD-2
HBD-3
desR HBD-3
NRR HBD-3
(d)
P. aeruginosa NBRC12582
Surviving bacteria [CFU/ml]
NaCl [mM]
HBD-2
HBD-3
desR HBD-3
NRR HBD-3

Fig. 2

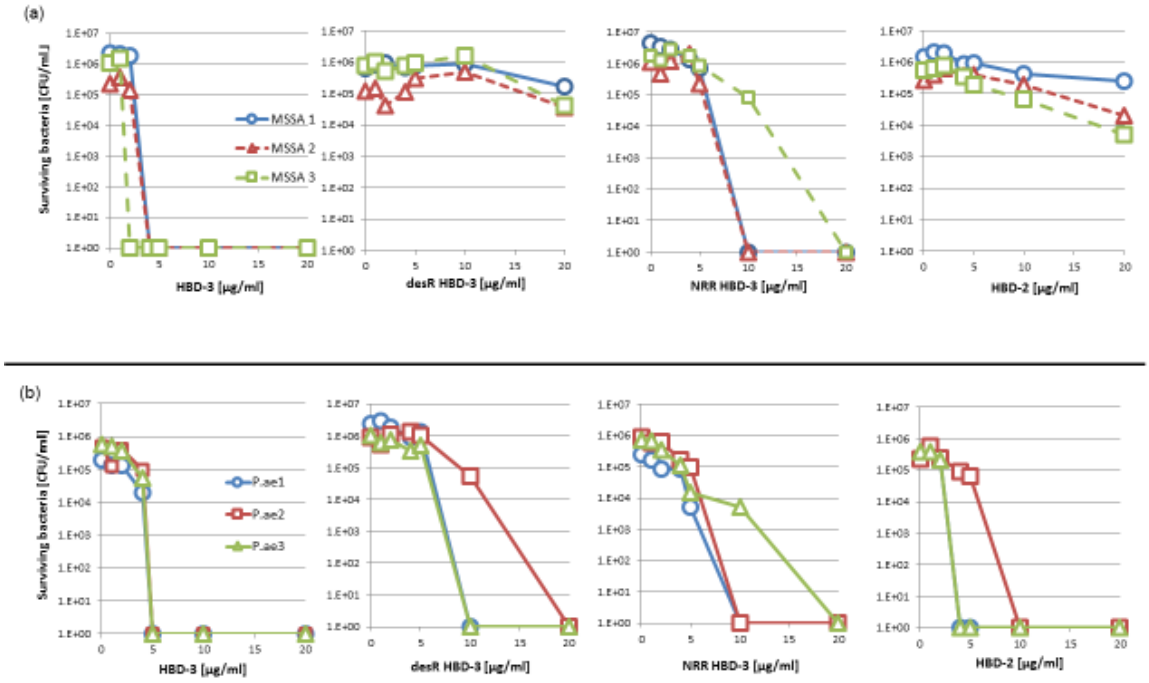
(a)
Surviving bacteria [CFU/ml]
MSSA 1
MSSA 2
MSSA 3
HBD-3 [μg/ml]
desR HBD-3 [μg/ml]
NRR HBD-3 [μg/ml]
HBD-2 [μg/ml]
(b)
Surviving bacteria [CFU/ml]
P.ae1
P.ae2
P.ae3
HBD-3 [μg/ml]
desR HBD-3 [μg/ml]
NRR HBD-3 [μg/ml]
HBD-2 [μg/ml]

Fig. 3

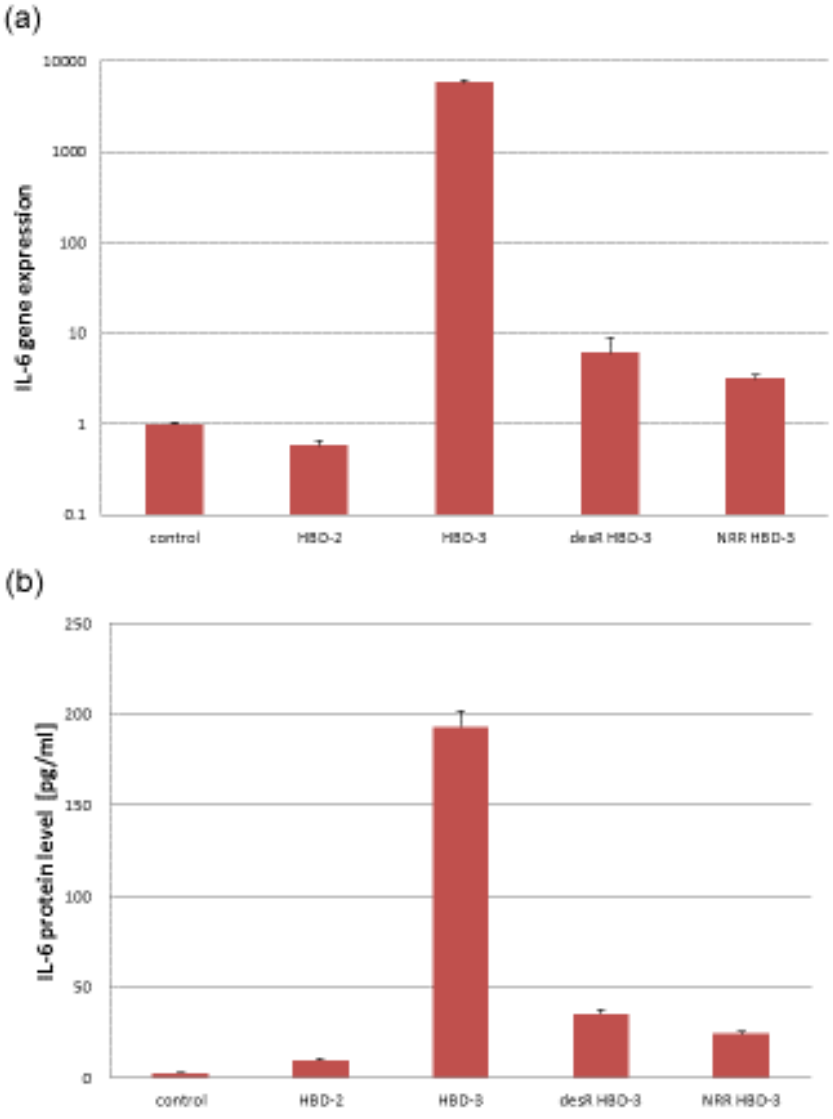
(a)
IL-6 gene expression
10000
1000
100
10
1
0.1
control
HBD-2
HBD-3
desR HBD-3
NRR HBD-3
(b)
IL-6 protein level [pg/ml]
250
200
150
100
50
0
control
HBD-2
HBD-3
desR HBD-3
NRR HBD-3

Fig. 4