SPANDIDOS PUBLICATIONS

Evaluation of the effect of α-defensin human neutrophil peptides on neutrophil apoptosis

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Abstract. Peptide antibiotics possess potent antimicrobial activities against invading micro-organisms and contribute to the innate host defense. Antimicrobial α-defensin human neutrophil peptides (HNPs) not only exhibit potent bactericidal activities against Gram-negative and -positive bacteria but also function as immunomodulatory molecules by inducing cytokine and chemokine production, as well as inflammatory and immune cell activation. Neutrophil is a critical effector cell in host defense against microbial infection, and its lifespan is regulated by various pathogen- and host-derived substances. Here, in order to further evaluate the role of HNPs in innate immunity, we investigated the action of HNPs-1 to -3 on neutrophil apoptosis. Neutrophil apoptosis was assessed using human blood neutrophils based on the morphological changes. Of note, HNP-1 most potently suppressed neutrophil apoptosis among HNPs-1 to -3, accompanied by the down-regulation of truncated Bid (a pro-apoptotic protein), the up-regulation of Bcl-x_L (an anti-apoptotic protein), and the inhibition of mitochondrial membrane potential change and caspase 3 activity. It should be noted that, a selective $P2Y_6$ antagonist, MRS2578, abolished the suppression of neutrophil apoptosis elicited by HNP-1 as well as UDP (a $P2Y_6$ ligand). Collectively, these observations suggest that HNPs, especially HNP-1, can not only destroy bacteria but also modulate (suppress) neutrophil apoptosis via the $P2Y_6$ signaling pathway. The suppression of neutrophil apoptosis results in the prolongation of their lifespan and could be advantageous for the host defense against bacterial invasion.

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Introduction

Neutrophils play a critical role as effector cells in inflammation, tissue injury and in host defense against microbial infection (1). The lifespan of neutrophils, which are terminally differentiated blood cells, is relatively short and they constitutively undergo apoptosis (2-4). In this context, it should be noted that the spontaneous apoptosis of neutrophils is inhibited in patients with sepsis, systemic inflammatory syndrome (SIRS) and acute respiratory distress syndrome (ARDS) by the action of various pathogen- and host-derived substances such as bacterial products [i.e., Gram-negative lipopolysaccharide (LPS)], cytokines and chemokines (i.e., IL-1ß and IL-8) (3,5-8). The suppressed neutrophil apoptosis results in the prolongation of their lifespan and could be beneficial for the host defense against bacterial invasion. However, the prolonged survival of activated neutrophils in patients with the above disorders, can cause the uncontrolled release of cytotoxic metabolites and pro-inflammatory substances (i.e., reactive oxygen species and proteases), which leads to the amplification of systemic inflammation, tissue injury and organ failure (9,10). In contrast, neutrophil apoptosis can be accelerated by the Fas ligand, reactive oxygen species, immune complexes and bacterial toxins (such as *Pseudomonas aeruginosa* exotoxin, pyocyanin) produced at the sites of inflammation and infection (11-14). The inappropriate induction of neutrophil apoptosis likely depletes neutrophil numbers and functions, thereby impairing host defense and favoring bacterial invasion and persistence.

Mammalian cells express a number of peptide antibiotics that function as effector components in the innate host defense system (15). They are found in blood, secretions, epithelial tissues as well as in neutrophil granules, and exhibit potent antimicrobial activities against a broad spectrum of invading micro-organisms, including both Gram-positive and -negative bacteria, fungi and viruses (16-20). Among these peptides, defensins and cathelicidins are considered as the 2 major classes of antimicrobial peptides in humans (16-20). Defensins are characterized by the 6-cysteine residues forming 3 intramolecular disulfide bridges, and are divided into α- and ß-defensins based on the distribution of cysteines and the linkages of disulfide bonding (16,17,19,20). α-defensins are

found in neutrophils and in the Paneth cells of the small intestine, whereas human ß-defensins (hBDs) are mainly expressed by epithelial tissues. In contrast, cathelicidins are a family of antimicrobial peptides characterized by the highly conserved cathelin-like pro-sequence and variable C-terminal sequences that correspond to the mature antibacterial peptides (18). To date, 6 different human α-defensin molecules have been described (16,17,19,20). Human α-defensins-1, -2, -3 and -4 are also termed as human neutrophil peptides (HNPs)-1, -2, -3 and -4, as they are mainly expressed in neutrophils. HNPs-1, -2 and -3, which differ only in the first amino acid, account for 5-7% of the total neutrophil proteins, whereas HNP-4, with an amino acid sequence distinct from other HNP sequences, comprises <1% of the total defensins in neutrophils. Since no gene that encodes HNP-2 has been identified, it is regarded as a proteolytic product of HNP-1 and/or HNP-3. The other 2 human α-defensins, human defensins (HDs)-5 and -6, are constitutively expressed in the Paneth cells within the epithelium in the small intestine. Thus, they are called enteric defensins. In contrast, hBDs-1 to -6 are produced by various epithelial tissues, including the urogenital and respiratory tracts, and the skin (16,17,19). Approximately 30 cathelicidin members have been isolated from various mammalian species. However, only 1 cathelicidin, the human cationic antibacterial protein of 18 kDa (hCAP18), has been identified in humans, and its C-terminal mature antibacterial pepetide, called LL-37, has been identified (21,22).

In addition to their antimicrobial properties, HNPs, hBDs and LL-37 have the potential to modulate several host cell functions, such as pro-inflammatory mediator production, as well as immune and inflammatory cell activation (19,23). In this context, LL-37 and hBDs have been reported to activate neutrophils as chemoattractants (24,25). Based on these findings, we previously evaluated the potential effects of LL-37 and hBDs on neutrophil apoptosis, and revealed that LL-37 and hBD-3 suppress neutrophil apoptosis via the actions on a low affinity formyl-peptide receptor, formyl-peptide receptor-like 1 (FPRL1), and the nucleotide receptor, $P2X_7$ (26), as well as the CC chemokine receptor (CCR)-6 (27), respectively. Of importance, it has been demonstrated that HNPs utilize the $P2Y_6$ signaling pathway to stimulate human lung epithelial cells to produce IL-8 (28), and that human neutrophils express the $P2Y_6$ receptor (29). Thus, we hypothesized that HNPs could also have a potential to activate neutrophils to modulate apoptosis via the action on $P2Y_6$. In this study, we investigated the effects of commercially available human α-defensins (HNPs-1 to -3 and HD-5) on neutrophil apoptosis, and provide evidence that HNP-1 can most potently suppress neutrophil apoptosis among the α-defensins examined, accompanied by the down-regulation of truncated Bid (a pro-apoptotic protein), the up-regulation of Bcl-x_L (an anti-apoptotic protein), and the inhibition of mitochondrial membrane potential change and caspase 3 activity, possibly via the action on the $P2Y_6$ nucleotide receptor.

Materials and methods

Reagents. HNPs-1, -2, -3, HD-5 and hBD-3 were purchased from the Peptide Institute (Osaka, Japan). UDP (a $P2Y_6$ agonist), MRS2578 [a $P2Y_6$ antagonist, 1,4-di-(phenylthioureido) butane], the annexin V-FITC apoptosis detection kit, and the caspase 3 assay kit were from Sigma-Aldrich (St. Louis, MO, USA). A 37-mer peptide of hCAP18 (LL-37) was synthesized and purified, as previously described (26). Tissue culture supplies were obtained from Becton-Dickinson Labware (Franklin Lakes, NJ, USA).

Antibodies. Mouse anti-Bcl-x_L monoclonal antibody (H-5) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), goat anti-human/mouse Bid polyclonal antibody (AF860) from R&D Systems (Minneapolis, MN, USA), rabbit anti-$P2Y_6$ monoclonal antibody from Epitomics (Burlingame, CA, USA), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (MAB374) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG from Chemicon International (Temecula, CA, USA), HRP-conjugated goat anti-mouse IgG/IgM, HRP-conjugated rabbit anti-goat IgG and mouse purified control IgG from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3 monoclonal antibody (S4.1) and allophycocyanin-conjugated mouse anti-human CD20 monoclonal antibody (HI47) from Caltag Laboratories (Burlingame, CA, USA), and phycoerythrin (PE)-conjugated mouse anti-human CD14 monoclonal antibody (MY4) from Beckman Coulter (Fullerton, CA, USA).

Cell preparation. This study was approved by the Institutional Human Subject's Review Board (School of Medicine, Juntendo University). Informed consent was obtained from healthy volunteers, and blood was drawn from the cubital vein. Neutrophils and mononuclear cells were isolated from heparinized blood by the dextran sedimentation of erythrocytes followed by centrifugation over Ficoll-Paque™ Plus (GE Healthcare, Buckinghamshire, UK) density gradient (26). Purities were determined by differential cytospin counts with May-Grünwald-Giemsa stain, and forward light scatter/side light scatter gating of cells stained with FITC-conjugated anti-CD3 monoclonal antibody, PE-conjugated anti-CD14 monoclonal antibody and allophycocyanin-conjugated anti-CD20 monoclonal antibody using a flow cytometer (FACSVantage; BD Biosciences, San Jose CA, USA). The neutrophil fraction contained 94±2% neutrophils, 5.7±2.1% eosinophils, 0.15±0.36% lymphocytes and 0.15±0.29% monocytes (n=24). The mononuclear cell fraction contained 85.8±2.3% lymphocytes, 11.4±1.1% monocytes and 2.8±0.6% neutrophils (n=4). After washing with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), the cells were suspended at 10^6 cells/ml in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA), unless otherwise noted. The FBS contained <0.03 ng/ml of endotoxin as certified by the manufacturer.

Assessment of neutrophil apoptosis. Neutrophils (10^6 cells/ml) were incubated in the absence or presence of HNPs-1 to -3, HD-5 (5-40 μg/ml) or hBD-3 (1-10 μg/ml) at 37˚C for 18 h in RPMI-1640-10% FBS in 5% CO_2 in Falcon® 2063 tubes.

ore, in order to assess the role of $P2Y_6$, the neutro-
e incubated with HNP-1 (20 μg/ml) or UDP (a $P2Y_6$ agonist, 30-3000 μM) at 37˚C for 18 h in the absence or presence of 1 μM MRS2578 (a $P2Y_6$ antagonist) (30,31). After incubation, the cells were cytocentrifuged (Cytospin 4; ThermoShandon, Cheshire, UK) and stained with May-Grünwald-Giemsa. A minimum of 300 neutrophils/slide was examined by light microscopy on duplicate cytospins, and apoptotic neutrophils were identified based on the morphological changes characteristic of apoptosis, such as chromatin condensation, the formation of rounded nuclear profiles, cell shrinking, membrane blebbing, and the presence of cytoplasmic vacuolization (26,32). Alternatively, the cells were stained with annexin V-FITC and propidium iodide, according to the manufacturer's instructions (Sigma-Aldrich). After a 10-min incubation in the dark, the cells were analyzed by flow cytometry (FACSVantage). Apoptotic neutrophils were defined as annexin V-positive but propidium iodide-negative cells, and viable neutrophils as annexin V- and propidium iodide-negative cells (32). The results were expressed as the percentage of apoptotic cells. As the two methods for the assessment of neutrophil apoptosis (morphological changes and annexin V-binding) closely correlated with each other (26,32), neutrophil apoptosis was evaluated essentially based on the morphological changes.

Measurement of caspase 3 activity. Caspase 3 activity was measured with a colorimetric assay kit (Sigma-Aldrich), as previously reported (26). In brief, neutrophils (10^6 cells/ml) were incubated in the absence or presence of HNPs -1 to -3, HD-5 (40 μg/ml) or hBD-3 (10 μg/ml) at 37˚C for 18 h in RPMI-1640-10% FBS. After washing with PBS, the cells ($3x10^6$ cells) were lysed in 60 μl lysis buffer [50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, pH 7.4, 5 mM 3-[(3-cholamidopropyl)dimethlyammonio]-1-propane-sulfonate and 5 mM dithiothreitol] and disrupted on ice by sonication. The sonicates were centrifuged (17,400 x g for 10 min), and the supernatants (10 μl; $5x10^5$ cell equivalents, containing ~20 μg protein) were incubated with 2 mM acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide substrate in the absence or presence of 200 μM acetyl-Asp-Glu-Val-Asp-al, a specific inhibitor for caspase 3, at 37˚C for 1 h in a total volume of 100 μl assay buffer. Caspase 3 activity was measured at 405 nm in a Model 3550-UV microplate reader (Bio-Rad Laboratories, Hercules, CA, USA), and expressed as nmol of *p*-nitroanilide liberated/10^6 cells/h. The protein contents were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA).

Western blot analysis. In the preliminary experiments, an optimal incubation period was determined for the expression of truncated Bid and Bcl-x_L following the HNP-1-stimulation. Neutrophils (10^6 cells/ml) were incubated in the absence or presence of 40 μg/ml HNP-1 at 37˚C for 4 h in RPMI-1640-10% FBS. After washing with PBS containing 5 mM EDTA and 2 mM Na_3VO_4, the cells (10^6 cells) were lysed in 30 μl lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM Na_3VO_4, 10 mM *p*-nitrophenyl phosphate and 1 mM diisopropyl fluorophosphate) containing 1/25 v/v Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). The lysates were then mixed with 30 μl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue and 5% 2-mercaptoethanol), disrupted on ice by sonication, and centrifuged (17,400 x g for 10 min). The supernatants were denatured for 3 min at 100˚C, and aliquots (20 μl; containing ~$3x10^5$ cell equivalents) were subjected to SDS-PAGE on a 7.5-20% linear gradient of polyacrylamide under reducing condition. The resolved proteins were then electrotransferred to Imobilon™-P polyvinylidene difluoride membrane (Millipore, Bedford, MA) using a Trans-Blot® SD apparatus (Bio-Rad Laboratories). The blots were blocked in Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan), and probed with mouse anti-Bcl-x_L monoclonal antibody (H-5; 1:5000) or goat anti-human/mouse Bid polyclonal antibody (AF860; 1:5000). The blots were further probed with HRP-conjugated goat anti-mouse IgG/IgM (1:10000) or HRP-conjugated rabbit anti-goat IgG (1:10000), and Bcl-x_L and Bid/truncated Bid were finally detected with SuperSignal® West Pico Chemiluminescent substrate (Pierce).

Thereafter, the blots were stripped by incubating in WB Stripping Solution Strong (Nacalai Tesque, Kyoto, Japan) at room temperature for 15 min, and GAPDH contained in each sample was detected by reprobing with mouse anti-GAPDH monoclonal antibody (MAB374; 1:40000) and HRP-conjugated goat anti-mouse IgG/IgM (1: 40000).

Detection of mitochondrial membrane potential change. In order to analyze mitochondrial depolarization, neutrophils were stained with the mitochondrial membrane potential-dependent lipophilic dye, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) by using a DePsipher™ kit (Trevingen, Gaithersburg, MD, USA) (36). In brief, the neutrophils (10^6 cells/ml) were incubated in the absence or presence of HNP-1 (40 μg/ml) at 37˚C for 18 h in RPMI-1640-10% FBS. Thereafter, the cells were harvested by centrifugation (500 x g for 5 min at room temperature), and the pelleted cells (10^6 cells) were suspended in 1 ml diluted DePsipher solution (1X reaction buffer containing 5 μg/ml JC-1 dye) and incubated at 37˚C for 20 min in the dark. The stained cells were washed once in 1X reaction buffer, re-suspended in the same buffer and quickly analyzed for the intensities of green (FL1) and red fluorescence (FL2) by flow cytometry using a 488-nm argon laser (FACScan, BD Biosciences). In the cells with intact mitochondrial membrane potential, the dye was concentrated in the mitochondrial matrix due to the electrochemical potential gradient and fluoresced bright red in its multimeric form. In contrast, in the cells with disrupted mitochondrial membrane potential, the dye was dispersed throughout cytoplasm, where it fluoresced green in its monomeric form. The results were expressed as the percentage of cells with disrupted (enhanced green but diminished red fluorescence) or intact (enhanced red but diminished green fluorescence) mitochondrial membrane potential.

Measurement of cytokine production by neutrophils and mononuclear cells. In order to determine the cytokine production from HNP-1-stimulated cells, neutrophils or

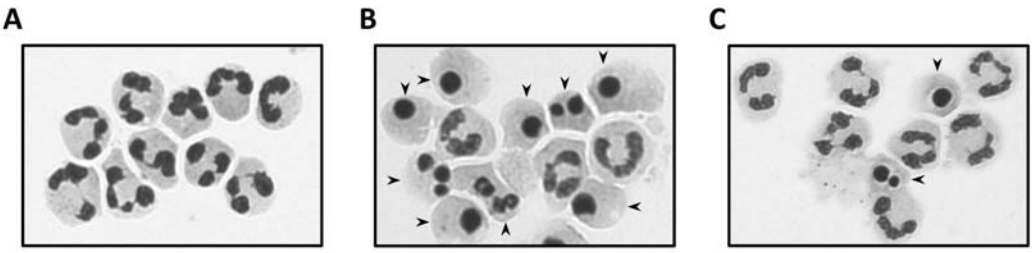

Figure 1. Assessment of neutrophil apoptosis. Neutrophils were incubated at 4˚C for 18 h in the absence of HNP-1 (A). Neutrophils (10^6 cells/ml) were also incubated at 37˚C for 18 h in RPMI-1640-10% FBS in the absence (B) or presence of HNP-1 (20 μg/ml) (C). After incubation, neutrophil apoptosis was assessed by the morphological changes. Apoptotic neutrophils exhibit characteristic features of chromatin condensation, the formation of rounded nuclear profiles, cell shrinking and the presence of cytoplasmic vacuolization (as indicated by the arrowheads). Representative data are shown.

mononuclear cells (10^6 cells/ml) were incubated in the absence or presence of HNP-1 (1-40 μg/ml) at 37˚C for 18 h in RPMI-1640-10% FBS. Culture supernatants were collected and used for the quantification of MIP-3α by enzyme-linked immunosorbent assay (ELISA) using DuoSet ELISA Development kits (R&D Systems), and the quantification of IL-8, IL-1ß, TNF-α, IL-6, IL-10 and IL-12p70 by BD™ Cytometric Bead Array using the human inflammatory cytokine kit (BD Biosciences). The detection limits were <16 pg/ml for MIP-3α and <20 pg/ml for IL-8, IL-1ß, TNF-α, IL-6, IL-10 and IL-12p70.

Expression of $P2Y_6$ in neutrophils. The expression of $P2Y_6$ was detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. Total RNA was isolated from the neutrophils using an RNeasy Plus mini kit (Qiagen, Valencia, CA, USA), and RT-PCR was performed with a high fidelity RT-PCR kit (ReverTra-Plus-™, Toyobo, Osaka, Japan). First-strand cDNA synthesis was carried out using total RNA, oligo(dT)20 primer and ReverTra Ace reverse transcriptase at 42˚C for 60 min, followed by termination at 85˚C for 5 min. Then, PCR was performed by denaturization at 94˚C for 2 min followed by 30 cycles of amplification (98˚C for 10 sec, 58˚C for 30 sec and 68˚C for 45 sec) and extension at 68˚C for 5 min, using KOD-Plus DNA polymerase and a set of primers specific for $P2Y_6$ (sense primer, 5'-CCGCTGAACATCTGTGTC-3'; antisense primer, 5'-AGAGCCATGCCATAGGGC-3'; 465 bp) and a house-keeping gene GAPDH (sense primer, 5'-ACCACAGT CCATGCCATCAC-3'; antisense primer, 5'-TCCACCACC CTGTTGCTGTA-3'; 450 bp). PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

In addition, neutrophil lysates (~$3x10^5$ cell equivalents) were subjected to SDS-PAGE, and the resolved proteins were electrotransferred to the membrane, as described above. The blots were blocked, and probed with rabbit anti-$P2Y_6$ monoclonal antibody (1:5000) and HRP-conjugated goat anti-rabbit IgG (1:10000) to detect $P2Y_6$ with a chemiluminescent substrate. Furthermore, the blots were stripped, and GAPDH contained in each sample was detected by reprobing with mouse anti-GAPDH monoclonal antibody (1:40000) and HRP-conjugated goat anti-mouse IgG/IgM (1:40000).

Statistical analysis. Data were expressed as the means ± standard deviation (SD), and analyzed for significant difference by a one-way analysis of variance (ANOVA) with the multiple comparison test (Prism 4, GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $P<0.05$.

Results

Effects of HNPs on neutrophil apoptosis and caspase 3 activity. Prior to studying the actions of HNPs, we determined the spontaneous apoptosis of neutrophils. When the neutrophils were incubated alone for 18 h at 37˚C, they exhibited characteristic features of apoptosis, such as chromatin condensation, the formation of rounded nuclear profiles, cell shrinking, and the presence of cytoplasmic vacuolization (Fig. 1B), compared to the resting cells incubated at 4˚C (Fig. 1A). The evaluation of neutrophil apoptosis based on the morphological changes revealed that >50% of neutrophils underwent apoptosis after incubation alone for 18 h at 37˚C (resting vs. control; $P<0.001$) (Fig. 2). hBD-3 (1-10 μg/ml) used as the control stimulus reduced neutrophil apoptosis from 52.7±1.4% to 12.9±1.9% at 10 μg/ml (control vs. hBD-3; $P<0.001$). The spontaneous neutrophil apoptosis was inhibited by incubation with HNP-1 (Fig. 1C). HNP-1 (5-40 μg/ml) dose-dependently suppressed neutrophil apoptosis, and neutrophil apoptosis was reduced to 31.5±3.5% by 40 μg/ml hBD-3 (control vs. hBD-3; $P<0.001$) (Fig. 2). Of note, neither HNP-2, -3 (Fig 2) nor HD-5 (data not shown) significantly influenced neutrophil apoptosis at the concentrations examined (5-40 μg/ml).

Next, we evaluated the activation of caspase 3, a key executor of apoptosis (34). Consistent with the changes in the number of apoptotic cells, caspase 3 activity was increased from 0.04±0.05 to 5.95±0.56 nmol/10^6 cells/h after incubation alone at 37˚C for 18 h (resting vs. control, $P<0.001$) (Fig. 3), and the activity was reduced to 2.56±0.19 nmol/10^6 cells/h by hBD-3 (10 μg/ml), the control stimulus (control vs. hBD-3; $P<0.001$). Of importance, HNP-1 but not HNP-2, or -3 (Fig. 3) and HD-5 (data not shown) significantly suppressed the activation of caspase 3 at 40 μg/ml. Caspase 3 activity was reduced to 3.88±0.39 nmol/10^6 cells/h by HNP-1 (control vs. HNP-1; $P<0.001$).

Effects of HNP-1 on the expression of truncated Bid and Bcl-x_L, and mitochondrial membrane potential change. In order to clarify the mechanism for the HNP-1-induced

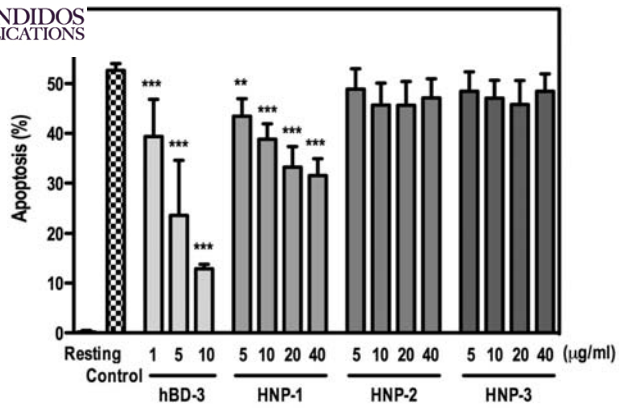

Figure 2. Effects of HNPs on neutrophil apoptosis. Neutrophils (10^6 cells/ml) were incubated at 37˚C for 18 h in RPMI-1640-10% FBS in the absence (control) or presence of HNP-1, -2 and -3 (5, 10, 20 and 40 μg/ml), or hBD-3 (1, 5 and 10 μg/ml). Neutrophils were also incubated at 4˚C for 18 h in the absence of HNPs or hBD-3 (resting). After incubation, neutrophil apoptosis was quantified, and expressed as the percentage of apoptotic cells. Data are expressed as the means ± SD of 3-12 separate experiments. Values are compared between the incubation at 37˚C in the absence (control) and presence of HNPs or hBD-3. **P<0.01, ***P<0.001.

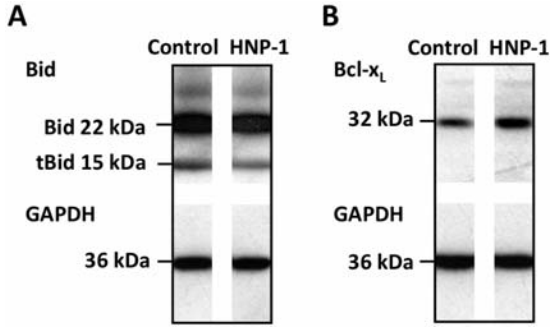

Figure 4. Effects of HNP-1 on the expression of truncated Bid and Bcl-x_L. Neutrophils (10^6 cells/ml) were incubated at 37˚C for 4 h in RPMI-1640-10% FBS in the absence (control) or presence of HNP-1 (40 μg/ml). The expression of truncated Bid (A) and Bcl-x_L (B) was detected by probing with goat anti-human and mouse Bid polyclonal antibody or mouse anti-Bcl-x_L monoclonal antibody and HRP-conjugated rabbit anti-goat IgG or HRP-conjugated goat anti-mouse IgG/IgM, respectively. In order to confirm that equal amounts of proteins were analyzed in each sample, the blots were stripped, and GAPDH was detected by reprobing with mouse anti-GAPDH monoclonal antibody and HRP-conjugated goat anti-mouse IgG/IgM. Anti-human/mouse Bid polyclonal antibody can recognize the 15-kDa truncated Bid (tBid) as well as the 22-kDa Bid (upper half of panel A). Data are from 1 of 4 separate experiments.

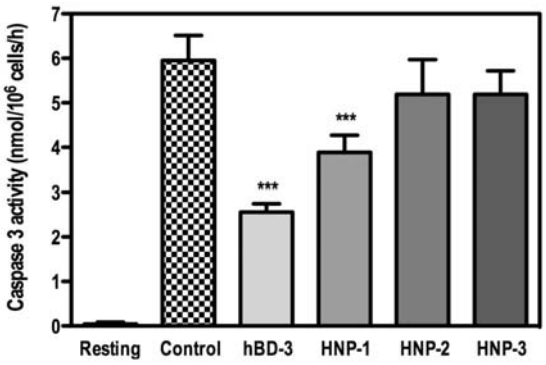

Figure 3. Effects of HNPs on caspase 3 activity. Neutrophils (10^6 cells/ml) were incubated at 37˚C for 18 h in RPMI-1640-10% FBS in the absence (control) or presence of HNP-1, -2 and -3 (40 μg/ml), or hBD-3 (10 μg/ml). Neutrophils were also incubated at 4˚C for 18 h in the absence of HNPs or hBD-3 (resting). After incubation, caspase 3 activity was assayed by incubating neutrophil lysates with acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide substrate in the absence or presence of acetyl-Asp-Glu-Val-Asp-al, a specific caspase 3 inhibitor at 37˚C for 1 h. Caspase 3 activity is expressed as nmol of *p*-nitroanilide liberated/10^6 cells/h. Data are expressed as the means ± SD of 4-8 separate experiments. Values are compared between the incubation at 37˚C in the absence (control) and presence of HNPs or hBD-3. ***P<0.001.

suppression of neutrophil apoptosis, we evaluated the expressions of apoptosis-associated proteins, such as truncated Bid (a pro-apoptotic protein) and Bcl-x_L (an anti-apoptotic protein). Of note, consistent with its suppressive action on neutrophil apoptosis, HNP-1 (40 μg/ml) down-regulated truncated Bid, whereas it up-regulated Bcl-x_L (Fig. 4).

The dissipation of mitochondrial electrochemical potential gradient is known as an early event of apoptosis (33). Thus, we investigated the effect of HNP-1 on the mitochondrial membrane potential change in apoptotic neutrophils. The assessment of the mitochondrial membrane potential change with the cationic lipophilic dye, JC-1 (33) revealed that the percentage of cells with intact mitochondrial membrane potential (enhanced red but diminished green fluorescent cells) was 40.6±9.1%, whereas that with disrupted mitochondrial membrane potential (enhanced green but diminished red fluorescent cells) was only 4.6±1.4% among the resting neutrophils incubated at 4˚C for 18 h (resting; Fig. 5). In contrast, the percentage of cells with intact mitochondrial membrane potential decreased to 28.5±4.9%, whereas that with disrupted mitochondrial membrane potential increased up to 18.4±9.8% after incubation at 37˚C for 18 h (control) (resting vs. control; P<0.05). Importantly, HNP-1 suppressed the mitochondrial membrane potential change; HNP-1 (40 μg/ml) slightly increased the number of neutrophils with intact mitochondrial membrane potential, but significantly decreased that with disrupted mitochondrial membrane potential even after incubation at 37˚C for 18 h (control vs. HNP-1; P<0.05).

Inflammatory cytokine production during incubation with HNP-1. It could be possible that pro-inflammatory cytokines (such as IL-1ß, TNF-α, IL-8, IL-6, IL-12p70 and MIP-3α) are produced and suppress neutrophil apoptosis during incubation with HNP-1, as these cytokines have the potential to repress neutrophil apoptosis (5,7,27,35,36). In order to test this possibility, we measured the cytokine production by neutrophils and mononuclear cells (10^6 cells/ml) after incubation with HNP-1 (40 μg/ml). The production levels of IL-1ß, TNF-α, IL-8, IL-6, IL-12p70 and MIP-3α were <20 pg/ml, <20 pg/ml, <20 pg/ml, <20 pg/ml, <20 pg/ml and <16 pg/ml for the neutrophils, and <20 pg/ml, <20 pg/ml, ~30 pg/ml, <20 pg/ml, <20 pg/ml and <16 pg/ml for the mononuclear cells, respectively. Given that IL-1ß, TNF-α, IL-8, IL-6, IL-12p70 and MIP-3α exhibit anti-apoptotic actions on neutrophils at >500 pg (IL-1ß, TNF-α, IL-8) (35),

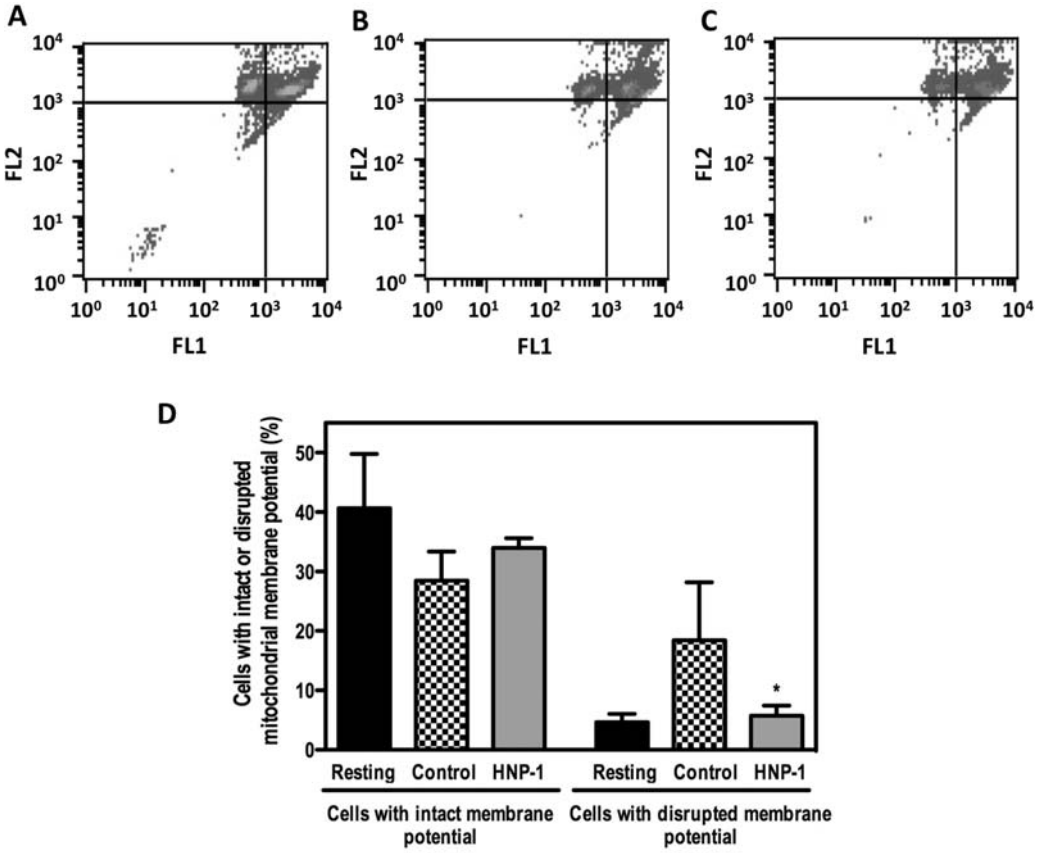

Figure 5. Effects of HNP-1 on the mitochondrial membrane potential change. Neutrophils were incubated at 4˚C for 18 h in the absence of hBDs (A) (resting). Neutrophils (10^6 cells/ml) were also incubated at 37˚C for 18 h in RPMI-1640-10% FBS in the absence (B) (control) or presence of 40 μg/ml HNP-1 (C) (HNP-1). After incubation, the cells were harvested and stained with the mitochondrial membrane potential-dependent lipophilic dye, JC-1. The stained cells were analyzed for the intensities of green (FL1) and red fluorescence (FL2) by flow cytometry. Results are expressed as the percentage of cells with intact (enhanced red but diminished green fluorescence) or disrupted (enhanced green but diminished red fluorescence) mitochondrial membrane potential (D). Data are expressed as the means ± SD of 5-8 separate experiments. Values are compared between the incubation at 37˚C in the absence (control) and presence of 40 μg/ml HNP-1. *P<0.05.

>1 ng/ml (IL-6) (36), >20 ng/ml (IL-12p70) (7) or >0.01 μg/ml (MIP-3α) (27), these findings suggest that the cytokine production by neutrophils and contaminated mononuclear cells (<0.3% in the neutrophil preparation) is too low to affect neutrophil apoptosis during incubation with HNP-1.

Role of $P2Y_6$ in the HNP-1-induced suppression of neutrophil apoptosis. Although $P2Y_6$ is known as a purinergic receptor for UDP (30), it has been reported that HNPs utilize the $P2Y_6$ signaling pathway to stimulate human lung epithelial cells to produce IL-8 (28). Thus, we determined the involvement of $P2Y_6$ in the HNP-1-induced suppression of neutrophil apoptosis.

First, we examined the expression of $P2Y_6$ in neutrophils. RT-PCR analysis using $P2Y_6$-specific primers revealed that $P2Y_6$ mRNA transcripts are expressed in neutrophils (Fig. 6A), as previously reported (29). Furthermore, Western blot analysis using a $P2Y_6$-specific antibody demonstrated that the $P2Y_6$ protein is expressed in neutrophils (Fig. 6B).

Next, in order to determine the involvement of $P2Y_6$ in the suppression of neutrophil apoptosis, the neutrophils were directly incubated with the $P2Y_6$ agonist, UDP (30), and its effect on neutrophil apoptosis was evaluated. As shown in Fig. 6C, UDP (30-3000 μM) dose-dependently suppressed neutrophil apoptosis (control vs. UDP; P<0.01). Furthermore, we evaluated the effects of the $P2Y_6$ antagonist, MRS2578 (31). Of importance, MRS2578 (1 μM) significantly reversed the HNP-1-induced as well as the UDP-induced suppression of neutrophil apoptosis (UDP vs. UDP + MRS2578, and HNP-1 vs. HNP-1 + MRS2578; P<0.001), although this agent did not affect the apoptosis of neutrophils incubated without HNPs (control) or with HNP-2 or -3 (control vs. MRS2578, HNP-2 vs. HNP-2 + MRS2578, and HNP-3 vs. HNP-3 + MRS2578; P>0.05)(Fig. 6D). These observations suggest that $P2Y_6$ is involved in not only the UDP-induced but also the HNP-1-induced suppression of neutrophil apoptosis.

Co-operative actions of HNP-1, hBD-3 and LL-37 on the suppression of neutrophil apoptosis. We demonstrated that the human antimicrobial peptides, LL-37, hBD-3 and HNP-1, can suppress neutrophil apoptosis (26,27, the present study). Thus, we determined whether LL-37, hBD-3 and HNP-1 could co-operatively suppress the neutrophil apoptosis by incubating neutrophils with low concentrations of LL-37, hBD-3, HNP-1 or their combinations. As shown in Fig. 7, 0.1 μg/ml LL-37, 1 μg/ml hBD-3 and 10 μg/ml HNP-1 slightly reduced the neutrophil apoptosis (control vs. HNP-1;

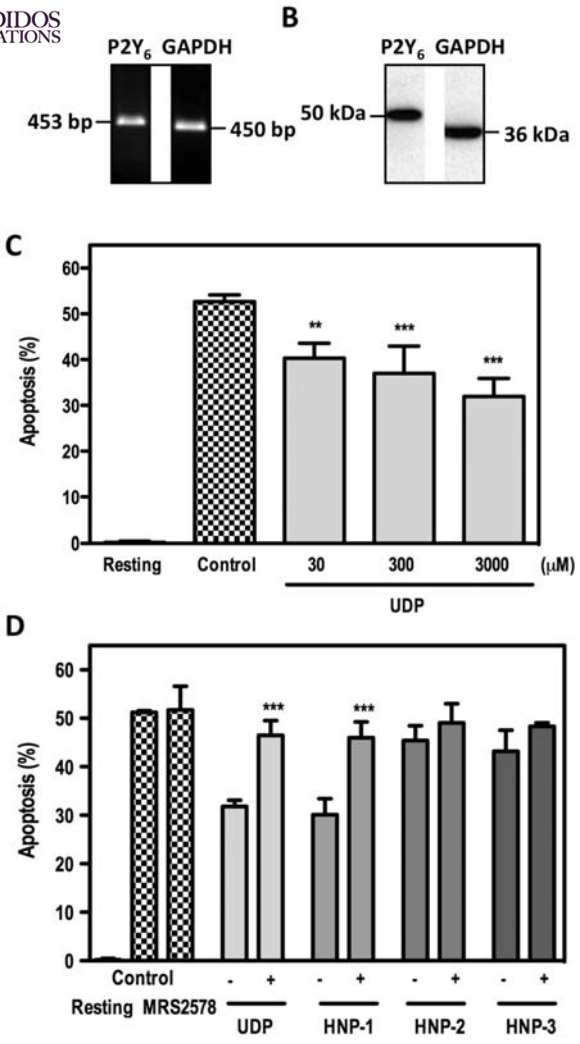

Figure 6. Expression of $P2Y_6$ and effect of a $P2Y_6$ antagonist on the HNP-1-induced suppression of neutrophil apoptosis. Total RNA was isolated from the neutrophils, and the cDNA products of $P2Y_6$ (453 bp) and GAPDH (450 bp) were detected by RT-PCR (A). Neutrophil lysates were subjected to SDS-PAGE, and $P2Y_6$ (50 kDa) and GAPDH (36 kDa) were detected by Western blotting using rabbit anti-$P2Y_6$ monoclonal antibody or mouse anti-GAPDH monoclonal antibody and HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG/IgM, respectively (B). Data are from 1 of 4 separate experiments. Neutrophils (10^6 cells/ml) were incubated for 18 h at 37˚C in RPMI-1640-10% FBS in the absence (control) or presence of UDP (30-3000 μM) (C). Alternatively, neutrophils were incubated for 18 h at 37˚C in RPMI-1640-10% FBS in the absence (control) or presence of HNP-1, -2, -3 (20 μg/ml each), UDP (300 μM), MRS2578 (1 μM), or their combination (+; 20 μg/ml HNP-1, -2, or -3 and 1 μM MRS2578, or 300 μM UDP and 1 μM MRS2578) (D). Neutrophils were also incubated alone for 18 h at 4˚C in the absence of HNPs, UDP or MRS2578 (resting). After incubation, the apoptosis of the neutrophils was quantified, and expressed as the percentage of apoptotic cells. Data are expressed as the means ± SD of 3-5 separate experiments. Values are compared between the incubation at 37˚C in the absence (control) and presence of UDP (C), or between the incubation at 37˚C with HNPs or UDP in the absence (-) and presence (+) of MRS2578 (D). $^{**}P<0.01$, $^{***}P<0.001$.

P<0.05). Of note, the combinations of 2 peptides co-operatively decreased apoptosis (LL-7, hBD-3 or HNP-1 vs. LL-37 + hBD-3, hBD-3 + HNP-1 or HNP-1 + LL-37; P<0.05), and the combination of 3 peptides further reduced apoptosis (LL-37 + hBD-3, hBD-3 + HNP-1 or HNP-1 + LL-37 vs. LL-37 + hBD-3 + HNP-1; P<0.01). These observations

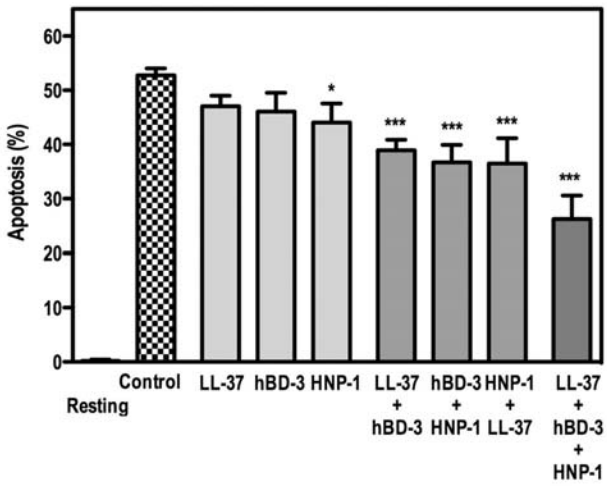

Figure 7. Effects of LL-37, hBD-3 and HNP-1 on neutrophil apoptosis. Neutrophils (10^6 cells/ml) were incubated for 18 h at 37˚C in RPMI-1640-10% FBS in the absence (control) or presence of LL-37 (0.1 μg/ml), hBD-3 (1 μg/ml), HNP-1 (10 μg/ml) or their combination (0.1 μg/ml LL-37 and 1 μg/ml hBD-3, 1 μg/ml hBD-3 and 10 μg/ml HNP-1, 10 μg/ml HNP-1 and 0.1 μg/ml LL-37, or 0.1 μg/ml LL-37, 1 μg/ml hBD-3 and 10 μg/ml HNP-1). Neutrophils were also incubated alone for 18 h at 4˚C in the absence of LPS (resting). After incubation, neutrophil apoptosis was quantified, and expressed as the percentage of apoptotic cells. Data are expressed as the means ± SD of 4-5 separate experiments. Values are compared between the absence (control) and presence of LL-37, hBD-3, HNP-1 or their combination. $^{*}P<0.05$, $^{***}P<0.001$.

clearly indicate that LL-37, hBD-3 and HNP-1 can act in concert on neutrophils to suppress apoptosis.

Discussion

Peptide antibiotics, as the effectors of the innate host defense system, exhibit antimicrobial activities against a broad spectrum of microbes, including both Gram-positive and -negative bacteria, fungi and viruses (15). HNPs, which belong to the α-defensin family of antimicrobial peptides, not only display potent antibacterial actions but also stimulate various host cell types to induce cytokine and chemokine production (19,23). Furthermore, HNPs possess the ability to chemoattract monocytes, naïve T cells and immature dendritic cells, and exhibit adjuvant activity (37-39).

In the current study, we investigated the effect of human α-defensins, multifunctional antimicrobial peptides, on the apoptosis of neutrophils, principal effector cells in innate immunity against microbial infection. The results indicate that HNP-1 most potently suppressed neutrophil apoptosis among the α-defensins examined (HNP-2, HNP-3 and HD-5) (Fig. 2). During the process of apoptosis, truncated Bid (a pro-apoptotic protein) is cleaved from Bid and translocates to the mitochondria to perturb the mitochondrial functions, by disrupting mitochondrial membrane potential and promoting cytochrome c release, which results in the activation of effector caspases and finally induces apoptosis (34,40). In contrast, Bcl-x_L acts as an anti-apoptotic protein to preserve mitochondrial integrity, thereby suppressing the activation of caspase cascade and apoptosis (34,41). Thus, we determined the effects of HNP-1 on the expression of truncated Bid and

Bcl-x_L. Of note, consistent with its anti-apoptotic action, HNP-1 down-regulated truncated Bid and up-regulated Bcl-x_L (Fig. 4). Furthermore, HNP-1 inhibited the dissipation of mitochondrial membrane potential and the activation of caspase 3, one of the death proteases functioning as the central executioners of apoptosis (34) (Figs. 3 and 5). In addition, we evaluated the involvement of the $P2Y_6$ nucleotide receptor in the HNP-1-induced suppression of neutrophil apoptosis by using a selective $P2Y_6$ antagonist (MRS2578) (31), since it has been demonstrated that HNPs utilize the $P2Y_6$ signaling pathway to stimulate human lung epithelial cells to produce IL-8 (28), and that human neutrophils express the $P2Y_6$ receptor (29). RT-PCR and Western blot analyses indicated that neutrophils express the $P2Y_6$ receptor (Fig. 6A and B). Moreover, MRS2578 abolished the suppression of neutrophil apoptosis elicited by HNP-1 as well as by UDP (a $P2Y_6$ ligand) (Fig. 6C and D). Together, these observations suggest that HNP-1 is able to induce the down-regulation of pro-apoptotic truncated Bid and the up-regulation of anti-apoptotic Bcl-x_L, which inhibits the mitochondrial membrane potential change and caspase 3 activity, thereby suppressing neutrophil apoptosis via the $P2Y_6$ signaling pathway. In this context, it is noteworthy that UDP activates the $P2Y_6$ receptor signaling pathway to attenuate the apoptosis of astrocytes and skeletal muscle cells (42,43), and induce the survival of osteoclasts and mast cells (44,45). Based on these findings, it can be speculated that the activation of the $P2Y_6$ signaling pathway not only by nucleotides (such as UDP) but also by α-defensins (especially HNP-1) is likely to play a role in the suppression of apoptosis or cell survival in various types of cells, including neutrophils.

Cationic antimicrobial peptides (such as defensins and cathelicidins) destroy the invading micro-organisms by perturbing their membranes. The action of these peptides is not receptor-mediated but involves a less specific interaction with microbial membrane components, since the peptides target cell surface anionic lipids such as phosphatidyl glycerol and cardiolipin that are abundant in micro-organisms (44). In contrast, the mammalian cell membrane is mainly composed of electrically neutral phospholipids such as phosphatidylcholine and sphingomyelin, for which the affinity of antimicrobial peptides is generally low (44). Antimicrobial peptides have been shown to act on the cell surface receptors to modulate various host cell functions (i.e., pro-inflammatory mediator production, and immune and inflammatory cell activation). For instance, human cathelicidin LL-37 activates neutrophils, monocytes and T cells via the interactions with FPRL1, a low-affinity formyl-peptide receptor (24,26) and $P2X_7$, a nucleotide receptor (26,45,46). In addition, HNPs induce IL-8 production from lung epithelial cells via the $P2Y_6$ signaling pathway (28), and hBD-2 chemoattracts immature dendritic cells and T cells and TNF-α-primed neutrophils via the action on CCR6 (23,25). Moreover, we have previously revealed that hBD-3 induces the suppression of neutrophil apoptosis by acting on CCR6 (27). The current study further demonstrates that among the α-defensins, HNP-1 but not HNP-2, and HNP-3 and HD-5 suppress the apoptosis of neutrophils via the $P2Y_6$ signaling pathway. Of interest, the amino acid sequences of HNP-1, -2 and -3 differ only in the N-terminal end, whereas HD-5 is distinct from the other HNPs. Thus, it can be postulated that the difference in the N-terminal sequences of HNPs-1 to -3 can be recognized by the $P2Y_6$ signaling pathway to induce the suppression of neutrophil apoptosis. Supporting this sequence-specific action of HNPs, it has been reported that HNP-1 most potently exhibits the chemotactic activity for monocytes among HNPs-1 to -3 (37).

HNPs are synthesized in neutrophils, whereas hBDs are mainly produced in epithelial tissues, including the respiratory and urogenital tracts, and the skin (16,17,19,20). Furthermore, LL-37 is expressed in keratinocytes and lung epithelial cells as well as in neutrophils (19,47). The expression of these antimicrobial peptides is locally induced at the sites of inflammation and infection within epithelial cells, and invading neutrophils represent an additional source of peptides (47,48). In this context, it has been reported that the concentrations of HNPs, hBDs and LL-37 are increased up to 40 μg/ml in bronchoalveolar and nasal fluids from patients with inflammation and infection (48-50). Importantly, we have demonstrated that LL-37, hBD-3 and HNP-1 can suppress neutrophil apoptosis *in vitro* at the concentrations (0.01-40 μg/ml) comparable to those at the sites of inflammation and infection (26,27) (Fig. 2), and that LL-37, hBD-3 and HNP-1 can co-operatively suppress neutrophil apoptosis *in vitro* (Fig. 7). Thus, it can be speculated that the antimicrobial peptides, LL-37, hBD-3 and HNP-1, in concert modulate neutrophil apoptosis *in vivo* in the local milieu at the sites of inflammation or infection by utilizing different receptors (such as FPRL1, $P2X_7$, CCR6 and $P2Y_6$). Furthermore, HNPs and LL-37 are expected to exert their actions on neutrophils in a paracrine/autocrine fashion, as they are stored in the azurophil and specific granules of neutrophils, respectively, and are extracellularly released from activated neutrophils (51,52). In this context, it should be noted that anti-apoptotic genes are up-regulated, and pro-apoptotic genes are down-regulated *in vivo* in neutrophils that transmigrated to the inflammatory skin lesions and were challenged with inflammatory/immunomodulatory molecules, including LL-37, hBDs and HNPs (53).

The clearance of neutrophils from inflamed tissues is critical for the resolution of inflammation. Clinical studies have indicated that spontaneous neutrophil apoptosis is inhibited in patients with severe inflammation (such as sepsis, SIRS and ARDS) by the actions of various bacterial products, cytokines and chemokines, detected in these disorders (3,5-8). Activated neutrophils with prolonged survival cause the amplification of inflammation and tissue injury via the uncontrolled release of cytotoxic metabolites and pro-inflammatory substances (9,10). From this point of view, antimicrobial peptides (LL-37, hBD-3 and HNP-1) exert a harmful effect during inflammation by suppressing apoptosis and prolonging the lifespan (survival) of neutrophils, which could lead to the augmented inflammatory reactions. In contrast, the physiological process of neutrophil apoptosis can be subverted by bacterial pathogens during infections (54). The inappropriate or premature apoptosis of neutrophils could deplete cell numbers and functions, impairing host defense and favoring bacterial persistence in infections. In this context, it has been reported that neutrophil

is accelerated and neutrophil-mediated host defense ed *in vivo* during infection with *Pseudomonas aeruginosa* by the action of pyocyanin, a predominant phenazine exotoxin (14). Considering their anti-apoptotic action, antimicrobial peptides (LL-37, hBD-3 and HNP-1) exert an advantageous effect on host defense against bacterial infections by prolonging the lifespan of neutrophils, major phagocytes engaged in the killing of invading bacteria.

As for the effects of α-defensins on mammalian cell apoptosis, HNPs have been shown to induce the apoptosis of human bronchial BEAS-2B and alveolar A549 epithelial cells, as well as Jurkat T-cells associated with caspase activation and mitochondrial injury (55,56). In contrast, HNPs can induce the proliferation of A549 lung epithelial cells and human lung fibroblasts possibly via the mitogen-activated protein (MAP) kinase signaling pathway (57,58). However, except for our current study, no reports exist on the anti-apoptotic actions of HNPs on neutrophils, terminal non-proliferating cells. HNPs were originally identified as antimicrobial peptides, which participate in the innate immune system, capable of protecting the host from invasive microbial infections (16,17), and are now regarded as multifunctional molecules that link the innate immune response to the adaptive immune system by exerting various immunomodulatory actions, such as cytokine and chemokine production, and immune and inflammatory cell migration (19,23). The present study demonstrates for the first time an additional function of HNPs, particularly HNP-1, in prolonging the lifespan of neutrophils via the action on $P2Y_6$. This finding provides novel insight into the role of HNPs in the regulation of neutrophil lifespan (apoptosis) as well as the innate and adaptive host defense systems.

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