An Antimicrobial Cathelicidin Peptide, Human CAP18/LL-37, Suppresses Neutrophil Apoptosis via the Activation of Formyl-Peptide Receptor-Like 1 and P2X7

Isao Nagaoka, Hiroshi Tamura and Michimasa Hirata

*J Immunol* 2006; 176:3044-3052; doi: 10.4049/jimmunol.176.5.3044

http://www.jimmunol.org/content/176/5/3044

**References**

This article cites 65 articles, 34 of which you can access for free at: http://www.jimmunol.org/content/176/5/3044.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
An Antimicrobial Cathelicidin Peptide, Human CAP18/LL-37, Suppresses Neutrophil Apoptosis via the Activation of Formyl-Peptide Receptor-Like 1 and P2X$_7$\(^1\)

Isao Nagaoka,\(^2,\)* Hiroshi Tamura,\(^\dagger\) and Michimasa Hirata\(^\ddagger\)

Peptide antibiotics possess the potent antimicrobial activities against invading microorganisms and contribute to the innate host defense. An antibacterial cathelicidin, human cationic antibacterial protein of 18 kDa/LL-37, not only exhibits potent bactericidal activities against Gram-negative and Gram-positive bacteria, but also functions as a chemoattractant for immune cells, including neutrophils. During bacterial infections, the life span of neutrophils is regulated by various pathogen- and host-derived substances. In this study, to further evaluate the role of LL-37 in innate immunity, we investigated the action of LL-37 on neutrophil apoptosis. Neutrophil apoptosis was assessed using human blood neutrophils based on the morphological changes. Of note, LL-37 dose dependently (0.01–5 pg/ml) suppressed neutrophil apoptosis, accompanied with the phosphorylation of ERK-1/2, expression of Bcl-x\(_L\) (an antiapoptotic protein), and inhibition of caspase 3 activity. Interestingly, LL-37-induced suppression of neutrophil apoptosis was attenuated by the antagonists for formyl-peptide receptor-like 1 (FPRL1) and P2X$_7$, nucleotide receptor. Of importance, the agonists for FPRL1 and P2X$_7$, apparently suppressed neutrophil apoptosis. Collectively, these observations indicate that LL-37 cannot only kill bacteria, but also modulate (suppress) neutrophil apoptosis via the activation of FPRL1 and P2X$_7$ in bacterial infections. Suppression of neutrophil apoptosis results in the prolongation of their life span, and may be advantageous for host defense against bacterial invasion. *The Journal of Immunology*, 2006, 176: 3044–3052.

Neutrophils play an important role as an effector of inflammation, tissue injury, and host defense against microbial infection (1). The lifetime of neutrophils, terminally differentiated blood cells, is relatively short, and they constitutively undergo apoptosis (2). Apoptotic neutrophils are phagocytosed by macrophages without release of proinflammatory mediators, leading to the limitation of tissue injury and resolution of inflammatory process (2). In this context, it is interesting to note that spontaneous apoptosis of neutrophils is inhibited in patients with sepsis, systemic inflammatory syndrome, and acute respiratory distress syndrome by the action of various pathogen- and host-derived substances, such as bacterial products (i.e., Gram-negative LPS; LPS), cytokines, and chemokines (i.e., IL-1\(\beta\) and IL-8) (3–7). The suppressed neutrophil apoptosis results in the prolongation of their life span and causes the uncontrolled release of cytotoxic metabolites and proinflammatory substances (i.e., reactive oxygen species and proteases), which leads to the amplification of systemic inflammation, tissue injury, and organ failure observed in those disorders (8, 9). In contrast, neutrophil apoptosis can be accelerated by Fas ligand, reactive oxygen species, immune complexes, and bacterial toxins (such as *Pseudomonas aeruginosa* exotoxin, pyocyanin) produced at the sites of inflammation and infection (10–13). Inappropriate induction of neutrophil apoptosis is likely to deplete neutrophil numbers and functions, thereby impairing host defense and favoring bacterial invasion and persistence.

Mammalian cells express a number of peptide antibiotics (such as defensins) that function as effector components in the innate host defense system. They are found in blood, secretions, epithelial tissues, and neutrophil granules, and exhibit potent antimicrobial activities against both Gram-positive and Gram-negative bacteria, fungi, and certain viruses (14–17). Among these peptides, cathelicidin is a novel family of antimicrobial peptides, characterized by the highly conserved cathelin-like prosequences and variable C-terminal sequences that correspond to the mature antibacterial peptides (18). Approximately 30 cathelicidin members have been identified from various mammalian species; however, only one cathelicidin, human cationic antibacterial protein of 18 kDa (hCAP18),\(^3\) has been found in humans, and its C-terminal mature antibacterial peptide, called LL-37, which comprises 37 aa residues (L\(^1\)LGDFRERKSKGKEGKEKRIVQRIKDFLRNLVPRTES\(^{37}\)), has been identified (19, 20). In addition to exhibiting potent antibacterial activities against both Gram-positive and Gram-negative bacteria (21), LL-37 can bind to LPS and blunt its biological activities (19, 22). Furthermore, LL-37 possesses the ability to chemoattract immune and inflammatory cells, including neutrophils, monocytes, and T cells via the action on a low affinity formyl-peptide receptor, formyl-peptide receptor-like 1 (FPRL1) (23). In addition, LL-37 is demonstrated to promote the processing and release of IL-1\(\beta\) from monocytes via the activation of P2X$_7$, a nucleotide receptor (24).

---

\(^{1}\)Department of Host Defense and Biochemical Research, Juntendo University, School of Medicine, Tokyo, Japan; \(^\dagger\)Seikagaku, Tokyo, Japan; and \(^\ddagger\)Institute of Ohtaka Enzyme, Hokkaido, Japan. Received for publication October 11, 2005. Accepted for publication January 3, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^{1}\)This work was supported in part by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science, and a Grant-in-Aid for 21st Century Centers of Excellence Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

\(^{2}\)Address correspondence and reprint requests to Dr. Isao Nagaoka, Department of Host Defense and Biochemical Research, Juntendo University, School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail address: nagaoka@med.juntendo.ac.jp

\(^{3}\)Abbreviations used in this paper: hCAP18, human cationic antibacterial protein of 18 kDa; Bz-ATP, benzoylbenzoyl-ATP; EGFR, epidermal growth factor receptor; FPRL1, formyl-peptide receptor-like 1; Ox-ATP, oxidized ATP.

Copyright © 2006 by The American Association of Immunologists, Inc.
Given that LL-37 is a multifunctional molecule (25, 26) and acts on neutrophils as a chemottractant (23), we hypothesized that LL-37 may have a potential to modulate the lifetime (apoptosis) of neutrophils. Thus, we investigated the effect of LL-37 on neutrophil apoptosis in vitro, and further evaluated the actions of antagonistic and agonistic agents for FPRL1 or P2X7 receptor, in this study, we provide the evidence that LL-37 can suppress neutrophil apoptosis possibly via the activation of FPRL1 and P2X7.

Materials and Methods

Reagents

LPS (from Escherichia coli 0111:B4), an Annexin VFITC apoptosis detection kit, a caspase 3 assay kit, 2′,3′-O-(4-benzoyl-benzoyl)-ATP (benzoylbenzoyl-ATP-0β-ATP) (BD Biosciences), GSH reductase, amylin (27), and FPRL1 agonists WKYMVm (Trp-Lys-Tyr-Met-Val-D-arginine) (28) or P2X7 inhibitors (50 μM Ox-ATP and 5 μM KN-93) (30–32). Moreover, neutrophils were directly incubated with FPRL1 agonists (0.1–10 μM WKYMVm and KM-1) (28) or a P2X7 agonist (50–500 μM Be-ATP) (30, 31) at 37°C for 18 h. After incubation, cells were cytospun (Cytospin 4; ThermoShandon) and stained with May-Grünwald-Giemsa. A minimum of 300 neutrophils/slide was examined by light microscopy on duplicate cytopsins, and apoptotic neutrophils were identified based on morphological changes characteristic of apoptosis, such as chromatid condensation, formation of rounded nuclear profiles, cell shrinking, membrane blebbing, and presence of cytoplasmic vacuolization (29, 33). Alternatively, cells were stained with Annexin VFITC and propidium iodide, according to the manufacturer’s instruction (Sigma-Aldrich). After a 10-min incubation in the dark, cells were analyzed by flow cytometry (FACSClariS). Apoptotic neutrophils were defined as annexin V-positive but propidium iodide-negative cells, and viable neutrophils as annexin V- and propidium iodide-negative cells (34).

Assessment of neutrophil apoptosis

Neutrophils (106 cells/ml) were incubated in the presence or absence of LL-37 (0.01–5 μg/ml) or LPS (E. coli 0111:B4, 10 ng/ml) at 37°C for 18 h in RPMI 1640–10% FBS in 5% CO2 in Falcon 2063 tubes. To further evaluate the involvement of FPRL1 and P2X7 in the LL-37-induced suppression of neutrophil apoptosis, neutrophils were incubated with 1 μM LL-37 at 37°C for 18 h in the presence or absence of an FPRL1 antagonist WRW4 (10 μM) (27) or P2X7 inhibitors (100 μM Ox-ATP and 5 μM KN-93) (30–32). Moreover, neutrophils were directly incubated with FPRL1 agonists (0.1–10 μM WKYMVm and KM-1) (28) or a P2X7 agonist (50–500 μM Be-ATP) (30, 31) at 37°C for 18 h. After incubation, cells were cytospun (Cytospin 4; ThermoShandon) and stained with May-Grünwald-Giemsa. A minimum of 300 neutrophils/slide was examined by light microscopy on duplicate cytopsins, and apoptotic neutrophils were identified based on morphological changes characteristic of apoptosis, such as chromatid condensation, formation of rounded nuclear profiles, cell shrinking, membrane blebbing, and presence of cytoplasmic vacuolization (29, 33). Alternatively, cells were stained with Annexin VFITC and propidium iodide, according to the manufacturer’s instruction (Sigma-Aldrich). After a 10-min incubation in the dark, cells were analyzed by flow cytometry (FACSClariS). Apoptotic neutrophils were defined as annexin V-positive but propidium iodide-negative cells, and viable neutrophils as annexin V- and propidium iodide-negative cells (34). Results were expressed as a percentage of apoptotic cells. Because the two methods for the assessment of neutrophil apoptosis (morphological changes and annexin V binding) closely correlated with each other (29, 33), 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 (29). In brief, after incubation with various agents in vitro, batches of 106 cells were lysed in 60 μl of lysis buffer (50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (pH 7.4), 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 5 mM DTT) and disrupted on ice by sonication. The sonicates were centrifuged (17,400 × g for 10 min), and the supernatants (10 μl; 5 × 106 cell equivalents, containing ~20 μg of protein) were incubated with 2 μM acetyl-Asp-Glu-Val-Asp-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 2 h in a total volume of 100 μl of assay buffer. Caspase 3 activity was measured at 405 nm in a model 3550-UV microplate reader (Bio-Rad), and expressed as nmol of p-nitroanilide liberated/106 cells/h.

The protein contents were determined with a bichromonic acid protein assay kit (Pierce).

Western blot analysis of the phosphorylation of ERK-1/2 and expression of Bcl-xL

Neutrophils (106 cells/ml) were incubated in the absence or presence of LL-37 (1 μg/ml) or LPS (10 ng/ml) for 4 h at 37°C in RPMI 1640–10% FBS. After washing with PBS containing 5 mM EDTA and 2 mM Na3VO4, cells were lysed in 30 μl of 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 Na3VO4, 10 mM p-nitrophenyl phosphate, and 1 mM diisopropyl phosphorylate) containing 1/25 v/v Complete (Roche Diagnostic Systems). The lysates were then mixed with 30 μl of SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.005% bromophenol blue, and 5% 2-ME), disrupted on ice by sonication, and centrifuged (14,700 × g for 10 min) at 100°C, and aliquots (μl; containing ~3 × 106 cell equivalents) were subjected to SDS-PAGE on a 7.5–20% linear gradient of polyacrylamide under reducing condition. The resolved proteins were electrotransferred to Immobilon-P polyvinylidene difluoride membrane (Millipore) using a Trans-Blot SD apparatus (Bio-Rad). The blots were blocked in Acine-Ponpon Pharmaceutical), and probed with mouse anti-phosphorylated ERK mAb (E-4 that recognizes both ERK-1 and ERK-2 (clone 8516.31), mouse anti-human TNF-α mAb (MAB11), mouse anti-human TNF-α mAb (MAB11), and HRP-conjugated avidin were purchased from eBioscience; mouse anti-phosphorylated ERK mAb (E-4 that recognizes both ERK-1 and ERK-2 phosphorylated at Tyr202/204), rabbit anti-ERK polyclonal Ab (K-23 that recognizes both ERK-1 p44 and ERK-2 p42), and mouse anti-Bcl-xL mAb (H-5) from Santa Cruz Biotechnology; mouse anti-human IL-1β mAb (clone 8516.31), mouse anti-human TNF-α mAb (clone 8251.12), mouse anti-human IL-8 mAb (clone 6217), and biontiniyated goat anti-human IL-8 polyclonal Ab from Genzyme Technine; mouse anti-human epidermal growth factor receptor (EGFR) mAb (clone LA1) from Upstate Biotechnology; HRP-conjugated goat anti-rabbit IgG from Organon Teknika; HRP-conjugated streptavidin from Zymed Laboratories; FITC-conjugated mouse anti-human CD3 δ mAb (SA14.1) and allophycocyanin-conjugated mouse anti-human CD20 mAb (H47) from Caltag Laboratories; PE-conjugated mouse anti-human CD14 mAb (MV4) from Beckman Coulter; and mouse purified control IgG from Jackson ImmunoResearch Laboratories. Western blot analysis of the phosphorylation of ERK-1/2 and expression of Bcl-xL

This study was approved by the Institutional Human Subject’s Review Board (Juntendo University, School of Medicine). Informed consent was obtained from all volunteers, and blood was drawn after 12 h fasting. Neutrophils and mononuclear cells were isolated from heparinized blood by dextran sedimentation of erythrocytes, followed by centrifugation over Ficoll-Paque Plus (Amersham Biosciences) density gradient (29). Purities were determined by differential cytopsin counts with May-Grünwald-Giemsa stain, and forward light scatter/side light scatter gating of cells stained with FITC-conjugated anti-CD3 mAb, PE-conjugated anti-CD14 mAb, and allophycocyanin-conjugated anti-CD20 mAb using a flow cytometer (FACSVerse; BD Biosciences). Neutrophil fraction contained 9.4 ± 2.0% of neutrophils, 5.7 ± 2.1% of eosinophils, 0.15 ± 0.36% of lymphocytes, and 0.15 ± 0.29% of monocytes (n = 24); 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 PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 0.5 mM KH2PO4 (pH 7.4)), cells were suspended at 106 cells/ml in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Sanko Junyaku); FBS contains <5/100 ml LPS, as certified by the manufacturer.
Assay for the effect of apyrase and quantification of ATP in the culture supernatants

To test the participation of extracellularly released ATP in the LL-37-induced suppression of neutrophil apoptosis, neutrophils (10⁶ cells/ml) were incubated with 1 μM LL-37 or 5 mM ATP (as a positive control) at 37°C for 18 h in the presence or absence of 10 U/ml apyrase, an enzyme that hydrolyzes ATP to AMP. Alternatively, neutrophils were incubated with exogenously added ATP (0.001–5 mM) at 37°C for 18 h, and then neutrophil apoptosis was quantitated.

In addition, extracellularly released ATP was quantified by the sensitive firefly luciferase assay, as described previously (34). To detect ATP, 10 μl aliquots of culture supernatants from LL-37-treated neutrophils (0.01–5 μg/ml; 37°C, 10 min and 18 h) or standard ATP were added to 0.1 ml of reaction buffer (50 mM Tris-acetate, 2 mM EDTA, 60 mM DTT, 0.072% BSA, and 10 mM magnesium acetate (pH 7.7)) containing 1 μM luciferase (Roche Molecular Biochemicals) and 0.1 mM N-luciferin (Wako Pure Chemical). Luminescence was measured with a Lumat LB9501 luminometer (Berthold Technologies), and light output was given as the integral relative light units over the 10-s measuring period. The concentration of ATP was calculated from a standard curve.

Measurement of cytokine production by neutrophils and mononuclear cells

Neutrophils or mononuclear cells (10⁶ cells/ml) were incubated in the absence or presence of LL-37 (0.01–5 μg/ml) for 18 h at 37°C in RPMI 1640–10% FBS. Culture supernatants were collected and used for quantification of IL-1β, TNF-α, and IL-8 by ELISA (29).

Microtiter plates (96-well half area flat bottom; Corning Glass) were coated with 25 μg/ml anti-human IL-1β (CRM56) or anti-TNF-α (MAB11) mAb (1/250 dilution in 1x coating buffer; eBioscience) overnight at 4°C. After washing with PBS-0.05% Tween 20, plates were blocked with 1x Assay Diluent (eBioscience) for 1 h at room temperature. The plates were then washed, added with culture supernatants (25 μl/well), and incubated for 2 h at room temperature. After washing, the plates were incubated with a combination of biotin-conjugated anti-human IL-1β (CRM57) or anti-TNF-α (MAB11) mAb (1/250 dilution in 1x Assay Diluent; 1 h) and HRP-conjugated avidin (1/250 dilution in 1x Assay Diluent; 30 min). For detection of IL-8, microtiter plates were coated with anti-IL-8 mAb (clone 6217; 2 μg/ml diluted in PBS) and blocked with Block Ace. After incubation with culture supernatants, the plates were further incubated with a combination of biotin-conjugated anti-IL-8 polyclonal Ab (20 ng/ml in Block Ace) and HRP-conjugated streptavidin (500 ng/ml in Block Ace). IL-1β, TNF-α, or IL-8 was finally detected by incubation with 3,3’,5,5’-tetramethylbenzidine liquid substrate (25 μl/well) for ~15 min. The reaction was terminated by the addition of 25 μl/well 2 M H₂SO₄, and absorbance at 450 and 570 nm was quantitated in a microplate reader. The detection limits of the ELISAs were <15 pg/ml for IL-1β, TNF-α, and IL-8.

To further evaluate a role of inflammatory cytokines in the LL-37-induced suppression of neutrophil apoptosis, neutrophils (10⁶ cells/ml) were incubated with LL-37 (0.01–5 μg/ml) at 37°C for 18 h in the presence or absence of 10 μg/ml neutralizing anti-IL-1β mAb (clone 8516.31), anti-TNF-α mAb (clone 1825.12), anti-human IL-8 mAb (clone 6217), or mouse control IgG, and neutrophil apoptosis was quantitated.

Statistical analysis

Data are expressed as mean ± SD, and analyzed for significant difference by one-way ANOVA with multiple comparison test (Prism 4; GraphPad). Differences were considered statistically significant at p < 0.05.

Results

Effects of LL-37 on neutrophil apoptosis and caspase 3 activity

Before looking at the actions of LL-37, we determined the spontaneous apoptosis of neutrophils. When neutrophils were incubated alone for 18 h, they exhibited characteristic features of apoptosis, such as chromatin condensation, formation of rounded nuclear profiles, cell shrinking, and presence of cytoplasmic vacuolization (Fig. 1B). Alternatively, neutrophil apoptosis was evaluated by flow cytometry using FITC-annexin V and propidium iodide staining. Incubation of neutrophils alone for 18 h substantially induced apoptosis defined as annexin V positive, but propidium iodide negative (Fig. 1E). Evaluation of neutrophil apoptosis based on the morphological changes revealed that >50% of neutrophils underwent apoptosis after incubation alone for 18 h (resting vs control; p < 0.001) (Fig. 2A). LPS (10 ng/ml) used as a control stimulus

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org)
Effects of LL-37 on neutrophil apoptosis and caspase 3 activity. 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 (0.01–5 μg/ml) or LPS (10 ng/ml). Neutrophils were also incubated for 18 h at 4°C in the absence of LL-37 or LPS (Resting). After incubation, apoptosis of neutrophils was quantitated, and expressed as a percentage of apoptotic cells (A). Alternatively, caspase 3 activity was assayed by incubating neutrophil lysates with acetyl-Asp-Glu-Val-Asp-al, a specific caspase 3 inhibitor. Caspase 3 activity was expressed as nmol of p-nitroanilide liberated/10^6 cells/h (B). Data are the mean ± SD of 4–18 separate experiments. Values are compared between the incubation at 37°C in the absence (Control) and presence of LL-37 or LPS. * p < 0.05; ** p < 0.001.

Effects of LL-37 on the phosphorylation of ERK and expression of Bcl-xL
To clarify the mechanism for the action of LL-37, we investigated the signaling molecules that mediate suppression of apoptosis.

First, we looked at the effect of LL-37 on the phosphorylation of ERK, a member of mitogen-activated kinase family. As shown in Fig. 3A, LL-37 (1 μg/ml) stimulation strikingly enhanced the phosphorylation of ERK-1/2. Furthermore, we evaluated the effect of LL-37 on the expression of Bcl-xL, an antiapoptotic protein. LL-37 (1 μg/ml) markedly induced the expression of Bcl-xL (Fig. 3B).

As expected (29), LPS (10 ng/ml) used as a control stimulus substantially augmented the phosphorylation of ERK and expression of Bcl-xL (Fig. 3).

Involvement of FPRL1 and P2X7 in the LL-37-induced suppression of neutrophil apoptosis
It has been demonstrated that LL-37 uses FPRL1 as a receptor to chemoattract neutrophils, monocytes, and T cells (23). In addition, LL-37 is reported to promote the processing and release of IL-1β from monocytes via the activation of P2X7 receptor (24). Thus, we determined the involvement of FPRL1 and P2X7 in the LL-37-induced suppression of neutrophil apoptosis.

First, we evaluated the effects of FPRL1 antagonist and P2X7 inhibitors. As shown in Fig. 4A, inhibitory agents for FPRL1 (WRW4) (10 μM) (27) and P2X7 (Ox-ATP, 100 μM; KN-93, 5 μM) (30–32) significantly reversed the LL-37-induced suppression of neutrophil apoptosis (LL-37 vs WRW4; LL-37 vs Ox-ATP; LL-37 vs KN-93; p < 0.01), although these agents did not induce neutrophil apoptosis by themselves (control vs WRW4; control vs Ox-ATP; control vs KN-93; p > 0.05). Similarly, FPRL1 antagonist (WRW4, 10 μM) and P2X7 inhibitors (Ox-ATP, 100 μM; KN-93, 5 μM) obviously attenuated the LL-37-induced inhibition of caspase 3 activity (LL-37 vs WRW4 + LL-37; Ox-ATP + LL-37; or KN-93 + LL-37; p < 0.0001), although these agents gave no effect on the enzyme activity by themselves (control vs WRW4; Ox-ATP, or KN-93; p > 0.05) (Fig. 4B). These observations apparently suggest that FPRL1 and P2X7 are involved in the LL-37-induced suppression of neutrophil apoptosis.

Next, to further determine the involvement of FPRL1 and P2X7 in the suppression of neutrophil apoptosis, neutrophils were directly incubated with the FPRL1 and P2X7 agonists, and apoptosis was evaluated. As shown in Fig. 5A, agonistic agents for FPRL1 (WKYMVM, 0.1–10 μM; MMK-1, 0.1–10 μM) (28) and P2X7 (Bz-ATP, 50–500 μM) (30, 31) dose dependently suppressed neutrophil apoptosis (control vs WKYMVM; MMK-1, or Bz-ATP; p < 0.001). Notably, the combinations of FPRL1 and P2X7 agonists cooperatively suppressed neutrophil apoptosis as well as the activation of caspase 3 (WKYMVM, MMK-1, or Bz-ATP vs
WKYMVm + Bz-ATP, or MMK-1; Bz-ATP; p < 0.001) (Fig. 5, B and C). These observations most likely indicate that the activation of FPRL1 and P2X7 in concert acts to induce the suppression of neutrophil apoptosis.

However, it is unclear whether both FPRL1 and P2X7 are activated in parallel to promote the survival of neutrophils. It is postulated that P2X7 activation is downstream of FPRL1, if a P2X7 antagonist inhibits the FPRL1 activation. In contrast, FPRL1 activation is assumed to be downstream of P2X7, if an FPRL1 antagonist inhibits the P2X7 activation. Thus, we evaluated the effects of FPRL1 and P2X7 agonists on the suppression of neutrophil apoptosis induced by FPRL1 and P2X7 agonists. As expected, FPRL1 antagonist (WRW4, 10 μM) and P2X7 inhibitors (Ox-ATP, 100 μM; KN-93, 1 μM) attenuated the FPRL1 (WKYMVm, 1 μM; MMK-1, 5 μM) and P2X7 (Bz-ATP, 100 μM) agonist-induced suppression of neutrophil apoptosis, respectively (WKYMVm vs WRW4, +WKYMVm, MMK-1 vs WRW4, +MMK-1, Bz-ATP vs Ox-ATP, or KN-93 +Bz-ATP; p < 0.001) (Fig. 6). Importantly, P2X7 inhibitors (Ox-ATP and KN-93) also abrogated the FPRL1 agonist (WKYMVm and MMK-1)-induced suppression of neutrophil apoptosis (WKYMVm vs Ox-ATP, +WKYMVm or KN-93, +WKYMVm, MMK-1 vs Ox-ATP, +MMK-1 or KN-93, +MMK-1; p < 0.001), although an FPRL1 antagonist (WRW4) did not affect the action of P2X7 agonist (Bz-ATP) (Bz-ATP vs WRW4, +Bz-ATP; p > 0.05). These observations suggest a possibility that P2X7 is likely to be activated downstream of FPRL1.

Roles of ATP and inflammatory cytokines in the LL-37-induced suppression of neutrophil apoptosis

Because LL-37 stimulation is shown to release ATP from monocytes (24), it is possible that ATP is released from neutrophils during incubation with LL-37 and acts on P2X7, thereby inducing...
FIGURE 6. Effects of FPRL1 antagonist and P2X7 inhibitors on the suppression of neutrophil apoptosis induced by FPRL1 and P2X7 agonists. 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 WKYMVm (1 μM), MMK-1 (5 μM), Bz-ATP (100 μM), WRW4 (10 μM), Ox-ATP (100 μM), KN-93 (1 μM), or their combination (WRW4 + WKYMVm, Ox-ATP + WKYMVm, KN-93 + WKYMVm; WRW4 + MMK-1, Ox-ATP + MMK-1, KN-93 + MMK-1; WRW4 + Bz-ATP, Ox-ATP + Bz-ATP, KN-93 + Bz-ATP). Neutrophils were also incubated for 18 h at 4°C in the absence of FPRL1 and P2X7 agonists, or FPRL1 and P2X7 antagonists (Resting). After incubation, apoptosis of neutrophils was quantitated, and expressed as a percentage of apoptotic cells. Data are the mean ± SD of 5–11 separate experiments. Values are compared between the incubation at 37°C in the absence (Control) and presence of WKYMVm, MMK-1, Bz-ATP, WRW4, Ox-ATP, KN-93, or their combination. **, p < 0.001.

suppression of apoptosis. Thus, we asked whether ATP participates in the observed effect of LL-37 on neutrophil apoptosis. Of note, exogenously added ATP exhibited an antiapoptotic activity at >0.1 mM (control vs 0.1 mM ATP; p < 0.05) (Fig. 7A). As expected, the addition of an ATP-degrading enzyme apyrase completely attenuated the action of exogenous ATP on neutrophil apoptosis (ATP vs ATP + apyrase; p < 0.001); however, apyrase never affected the LL-37-induced suppression of neutrophil apoptosis (LL-37 vs LL-37 + apyrase; p > 0.05) (Fig. 7B). Consistent with this, the levels of ATP detected in the culture supernatants of neutrophils (10^6 cells/ml) were only 10–50 nM after incubation with LL-37 (0.01–5 μg/ml), which were too much lower than those required to prevent neutrophil apoptosis. These observations apparently indicate that ATP, if any, released from neutrophils is unlikely to participate in the suppression of neutrophil apoptosis during incubation with LL-37.

Furthermore, it could be possible that proinflammatory cytokines (such as IL-1β, TNF-α, and IL-8) are produced and induce the suppression of neutrophil apoptosis during incubation with LL-37, because these cytokines are shown to repress neutrophil apoptosis (6, 7). To test this possibility, we measured the cytokine production by neutrophils and mononuclear cells (10^6 cells/ml) after incubation with LL-37 (0.01–5 μg/ml). The production levels of IL-1β, TNF-α, and IL-8 were <16, <16, and ~60 pg/ml for neutrophils, and <16 pg/ml, ~30 pg/ml, and 2 ng/ml for mononuclear cells, respectively. Furthermore, the effects of neutralizing mAbs (10 μg/ml) against IL-1β, TNF-α, and IL-8 were determined on the apoptosis of neutrophils during incubation with LL-37 (0.01–5 μg/ml); however, these mAbs gave no substantial effect on the LL-37-induced suppression of neutrophil apoptosis (data not shown). Given that IL-1β, TNF-α, and IL-8 exhibit antiapoptotic actions on neutrophils at >500 pg/ml (29), these findings suggest that the cytokine production by neutrophils and contaminated mononuclear cells (<0.3% in the neutrophil preparation) is too low to affect the apoptosis of neutrophils during incubation with LL-37.

Discussion
Peptide antibiotics, as the effectors in the innate host defense system, exhibit antimicrobial activities against a broad spectrum of microbes, including both Gram-positive and Gram-negative bacteria, fungi, and viruses (14–17). LL-37/HCAP18 is the only one human peptide that belongs to a cathelicidin family of antimicrobial peptides (18–20). LL-37 not only displays the potent antibacterial activities against Gram-positive and Gram-negative bacterial, but also can bind to LPS and neutralize its biological activities (19, 21, 22). Furthermore, LL-37 possesses the ability to chemottract neutrophils, monocytes, and T cells (23).

In this study, we determined the effect of LL-37, a multifunctional antimicrobial peptide (25, 26), on the apoptosis of neutrophils. The results indicated that LL-37 can suppress neutrophil apoptosis, accompanied with the phosphorylation of ERK-1/2, expression of Bcl-xL, and inhibition of caspase 3 activity. In addition, the present data suggest that the activation of FPRL1 and P2X7 is involved in the LL-37-induced suppression of neutrophil apoptosis. It has been shown that the activation of ERK, a member of mitogen-activated kinase family, generates the survival signals via the up-regulation of antiapoptotic proteins of Bcl-2 family (such as Bcl-xL) to prolong the life span of cells (7, 37). Moreover, apoptosis is modulated through the expression of Bcl-xL, that regulates the activation of caspase 3, one of death proteases functioning as the central executors of apoptosis (36, 38, 39). Of importance, the activation of FPRL1 or P2X7 is demonstrated to induce the phosphorylation of ERK-1/2 in human monocytic cells and neutrophils (40, 41). Thus, it is feasible to assume that the stimulation of neutrophils with LL-37 induces the phosphorylation of ERK-1/2 and the subsequent expression of antiapoptotic protein Bcl-xL, which inhibits caspase 3 activity and suppresses neutrophil apoptosis via the activation of FPRL1 or P2X7.

Cationic antimicrobial peptides (such as defensins and cathelicidins) kill the invaded microorganisms by perturbing their membranes; the action of those peptides is not receptor mediated, but involves a less specific interaction with microbial membrane components, because the peptides target cell surface anionic lipids such as phosphatidylglycerol and cardiolipin that are abundant in microbial membranes; the action of those peptides is not receptor mediated, but involves a less specific interaction with microbial membrane components, because the peptides target cell surface anionic lipids such as phosphatidylglycerol and cardiolipin that are abundant in microorganisms (42, 43). 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 (42). Interestingly, human β-defensin-2 is shown to chemotact T cells, immature dendritic cells, and TNF-α-primed neutrophils through the action on the cell surface CCR6 (44, 45). In addition, human neutrophil peptides
FIGURE 7. Effect of apyrase on the LL-37-induced suppression of 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 ATP (0.001–5 mM) (A). Alternatively, neutrophils were incubated for 18 h at 37°C in RPMI 1640–10% FBS in the absence (Control) or presence of LL-37 (1 μg/ml), ATP (5 mM), apyrase (10 U/ml), or their combination (+Apyrase; 1 μg/ml LL-37 and 10 U/ml apyrase, or 5 mM ATP and 10 U/ml apyrase) (B). Neutrophils were also incubated alone for 18 h at 4°C in the absence of LL-37, ATP, or apyrase (Resting). After incubation, apoptosis of neutrophils was quantitated, and expressed as a percentage of apoptotic cells. Data are the mean ± SD of 4–10 separate experiments. Values are compared between the incubation at 37°C in the absence (Control) and presence of LL-37, ATP, apyrase, or their combination. *, p < 0.05; **, p < 0.0001.

(defensins) are reported to induce IL-8 production from A549 lung epithelial cells via the activation of P2Y6 nucleotide receptor (46). In contrast, LL-37 functions as a chemoattractant for neutrophils, monocytes, and T cells via the interactions with FPRL1, a low affinity formyl-peptide receptor (23). Moreover, LL-37 is shown to promote the processing and release of IL-1β from monocytes via the activation of P2Xγ nucleotide receptor (24). In addition, it is known that neutrophils express both FPRL1 and P2Xγ on their surfaces (28, 47). Thus, we addressed the issue as to whether the LL-37-induced suppression of neutrophil apoptosis is mediated by FPRL1 and/or P2Xγ. The present data demonstrated that the suppressive effect of LL-37 on neutrophil apoptosis was inhibited by both an FPRL1 antagonist (WKYMvm) (27) and P2Xγ inhibitors (Ox-ATP and KN-93) (30–32). Furthermore, FPRL1 agonists (WKYMvm and MMK-1) (28) and a P2Xγ agonist (Bz-ATP) (30, 31) collaboratively suppressed the neutrophil apoptosis. Collectively, these observations suggest that the LL-37 induces the suppression of neutrophil apoptosis via the activation of both FPRL1 and P2Xγ. In addition, the suppression of FPRL1 activation by P2Xγ antagonists suggests that P2Xγ is likely to be activated downstream of FPRL1. Alternatively, LL-37 is reported to trans activate EGFR and release IL-8 from airway epithelial cells (48). In this study, however, we revealed that a neutralizing anti-EGFR mAb (clone LA1; 10 μg/ml) and a EGFR tyrosine kinase inhibitor AG1478 (1 μM) (48) never affected the action of LL-37 on neutrophil apoptosis (data not shown), suggesting that the EGFR activation is unlikely to be involved in the suppression of neutrophil apoptosis by LL-37.

To date, FPRL1 is proposed as the only receptor to which LL-37 can directly bind (28), whereas the direct interaction of LL-37 with a nucleotide receptor P2Xγ is not determined (24). Thus, LL-37 could be speculated to facilitate the extracellular release of ATP from the cells (24), which indirectly activates P2Xγ and subsequently suppresses neutrophil apoptosis. However, the present findings indicated that an ATP-hydrolyzing enzyme apyrase did not affect the LL-37-induced suppression of neutrophil apoptosis, and the released levels of ATP in the extracellular medium were too low to induce the suppression of neutrophil apoptosis. In another aspect, P2Xγ is shown to participate in the modulation of cellular reactions, such as macrophage responses to LPS, and the C-terminal portion of P2Xγ is thought to be essential for the functions of this receptor (49, 50). In fact, the P2Xγ C-terminal domain contains multiple protein-protein and protein-lipid interaction motifs with potential importance to the intracellular signaling and LPS action, and is able to directly bind with LPS, an amphipathic lipid-sugar molecule (51). However, LPS would have to be internalized in the cell before binding to P2Xγ, because the C-terminal domain of P2Xγ is predicted to be located inside the cell (50, 51).

Based on these observations, it is tempting to speculate that LL-37, an amphipathic peptide, may also penetrate into the cell membrane and bind with the P2Xγ C-terminal domain via the protein-protein interaction, thereby activating intracellular signaling to induce the suppression of neutrophil apoptosis. However, it cannot be ruled out that LL-37 interacts with the ligand-binding site of P2Xγ, because the action of LL-37 was inhibited by Ox-ATP, an irreversible P2Xγ blocker that reacts with unprotonated lysine residues located in the vicinity of the ATP binding site of the receptor (52). It remains to be clarified whether LL-37 can activate P2Xγ through a direct interaction or an as of yet-undefined indirect mechanism.

hCAP18/LL-37 was first identified in neutrophils and later shown to be expressed in various squamous epithelia, lung epithelial cells, keratinocytes in inflamed skin, specific lymphocytes (NK cells, γδT cells, and B cells) and monocytes, and skin mast cells (53–55). Interestingly, hCAP18/LL-37 can be locally induced at sites of inflammation and infection within epithelial cells, and invading neutrophils are likely to represent an additional source for the peptide (53, 56). In this context, it has been reported that the concentration of hCAP18/LL-37 increases up to 15 μg/ml in bronchoalveolar fluids of patients with pulmonary or systemic infections (56, 57). Moreover, the plasma level of hCAP18/LL-37 is shown to be 1.18 μg/ml in healthy individuals (58). Importantly, the present study indicated that LL-37 can suppress neutrophil apoptosis at >0.01 μg/ml in vitro. Thus, it is likely to be expected that the apoptosis of neutrophils could be modulated in vivo at the sites of inflammation or infection, or in the normal blood by LL-37 present in the local extracellular milieu. Consistent with this, it has been demonstrated that antiapoptotic genes are up-regulated, but proapoptotic genes are down-regulated in neutrophils that transmigrated to the inflammatory skin lesions in vivo in response to various chemotactic factors such as LL-37 (59).

Clearance of neutrophils from inflamed tissues is critical for the resolution of inflammation. Clinical studies have indicated that spontaneous apoptosis of neutrophils is inhibited in patients with sepsis, systemic inflammatory syndrome, and acute respiratory distress syndrome by the actions of various bacterial products, cytokines, and chemokines detected in these disorders (3–7). Activated neutrophils with prolonged survival are assumed to cause the amplified systemic inflammation, tissue injury, and organ failure via...
the uncontrolled release of cytotoxic metabolites and proinflammatory substances (8, 9). From this point of view, LL-37 is supposed to exert a harmful effect during inflammation by suppressing apoptosis and prolonging life span (survival) of neutrophils, which may lead to the amplification of inflammatory reactions. In contrast, physiological process of neutrophil apoptosis can be subverted by bacterial pathogens during infections (60). 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 apoptosis is accelerated and neutrophil-mediated host defense is impaired in vivo during infection with \textit{P. aeruginosa} by the action of pyocyanin, a predominant phenazine exotoxin (61). Considering its antiapoptotic actions, LL-37 is expected to exert an advantageous effect on host defense against bacterial infections by prolonging the life span of neutrophil, a major phagocyte that kills the invaded bacteria. It remains to be determined what effect(s) LL-37 can exhibit in the processes of inflammation and infection via its regulatory action on neutrophil apoptosis.

As for the effects of cathelicidin peptides on mammalian cell apoptosis, a porcine peptide PR-39 is shown to inhibit the apoptosis of murine RAW264.7 cells (62). In contrast, bovine peptides BMP-27/28 and a 27-mer peptide of hCAP18/LL-7 (corresponding to F^6-V^32) induce the apoptosis of tumor cells such as leukemia and squamous carcinoma cells (63, 64). However, the mechanisms and receptors involved in the antiapoptotic and proapoptotic actions of these peptides are not clarified.

LL-37 is originally identified as an antimicrobial peptide, which participates in the innate immune system, capable of protecting host from invasive microbial infections and neutralizing Gram-negative LPS (19–22, 65), and now regarded as a multifunctional host from invasive microbial infections and neutralizing Gram-negative LPS (19–22, 65), and now regarded as a multifunctional.

References


4. Jimenez, M. F., R. W. G. Watson, J. Parodo, D. Evans, D. Foster, M. Steinberg, O. D. Rotstein, and J. C. Marshall. 1997. Dysregulated expression of neutrophil apoptosis and prolonging life span (survival) of neutrophils, which may lead to the amplification of inflammatory reactions. In contrast, physiological process of neutrophil apoptosis can be subverted by bacterial pathogens during infections (60). 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 apoptosis is accelerated and neutrophil-mediated host defense is impaired in vivo during infection with \textit{P. aeruginosa} by the action of pyocyanin, a predominant phenazine exotoxin (61). Considering its antiapoptotic actions, LL-37 is expected to exert an advantageous effect on host defense against bacterial infections by prolonging the life span of neutrophil, a major phagocyte that kills the invaded bacteria. It remains to be determined what effect(s) LL-37 can exhibit in the processes of inflammation and infection via its regulatory action on neutrophil apoptosis.

As for the effects of cathelicidin peptides on mammalian cell apoptosis, a porcine peptide PR-39 is shown to inhibit the apoptosis of murine RAW264.7 cells (62). In contrast, bovine peptides BMP-27/28 and a 27-mer peptide of hCAP18/LL-7 (corresponding to F^6-V^32) induce the apoptosis of tumor cells such as leukemia and squamous carcinoma cells (63, 64). However, the mechanisms and receptors involved in the antiapoptotic and proapoptotic actions of these peptides are not clarified.

LL-37 is originally identified as an antimicrobial peptide, which participates in the innate immune system, capable of protecting host from invasive microbial infections and neutralizing Gram-negative LPS (19–22, 65), and now regarded as a multifunctional host from invasive microbial infections and neutralizing Gram-negative LPS (19–22, 65), and now regarded as a multifunctional.


