Human Cathelicidin CAP18/LL-37 Changes Mast Cell Function toward Innate Immunity

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The antimicrobial peptide LL-37 is generated from skin keratinocytes during infection of Gram-negative bacteria and exerts a microbicidal effect. LL-37 also causes functional changes in mast cells. Mast cells in the skin are involved in the innate immune system response against microbial infections *via* Toll-like receptors (TLRs), such as TLR4, which that is known to recognize lipopolysaccharide (LPS), a bacterial component. Thus, in the present study, we examined the effects of LL-37 on the expression of TLRs and the generation of cytokines on mast cells, and considered functional changes in the host defense system against bacteria. We observed that LL-37 increased the level of TLR4 mRNA and TLR4 protein, and that LL-37 induced the release of IL-4, IL-5 and IL-1 β from mast cells. Cross-interaction between LL-37-triggered TLR4 augmentation and LL-37-inducible cytokine generation was also examined. Although the up-regulation of LL-37-inducible Th2 cytokines was cancelled by LPS, the augmentation of pro-inflammatory cytokine production was still observed. These findings indicate that LL-37 co-existing with the bacterial component switches mast cell function and directs human mast cells toward innate immunity. In conclusion, LL-37 may be a candidate modifier of the host defense against bacterial entry by serving as an alarm for sentinels such as mast cells.

Key words cathelicidin; LL-37; Toll-like receptor; mast cell

Antimicrobial peptides generated in response to bacterial infection play a pivotal role in the first line of host defense systems against the invasion of pathogens. These peptides possess bactericidal activity against a broad spectrum of microbes, including Gram-positive bacteria, Gram-negative bacteria, fungi, and certain viruses.¹⁾ Cathelicidins, which are one of the major antimicrobial peptide families, are composed of the cathelin domain and the C-terminal antimicrobial domain.²⁾ The cathelin domain is highly conserved, but the C-terminal antimicrobial domain is a less conserved peptide that corresponds to mature antimicrobial peptides.²⁾ Among cathelicidin family members found in various mammals, only one cathelicidin, hCAP18 (human cationic anti-bacterial protein of 18 kDa), has been found in humans. hCAP18 has 37 amino acid residues, termed LL-37, which are responsible for the antimicrobial effect at its C-terminal.³⁾ In addition to its bactericidal activity, LL-37 is a chemo-attractant for immune and inflammation cells, including T cells, neutrophils, monocytes, and mast cells.^{4,5)} Thus, it appears that LL-37 may serve not only as a bactericidal substance but also as an alarm signal when pathogens invade.

Toll-like receptors (TLRs) expressed on various types of immune cells also play a pivotal role in innate immunity. TLR1, TLR2, TLR4, TLR5, and TLR9 are especially important for host defense against bacteria because these TLRs recognize bacterial components and then promote cytokine secretion.⁶⁾ Mast cells express functional TLRs^{7–10)} and contribute to host defense or the initiation of an innate immune response by releasing cytokines and chemokines following TLR stimulation.^{11–14)} For example, the *W/W^v* mouse, a mast cell-deficient mouse, has an increased incidence of mortality from cecal ligation and puncture; however, the increased mortality is reduced in mast cell-reconstituted *W/W^v* mice. Mast cell-derived TNF- α released after TLR4 stimulation may contribute to this attenuated mortality.^{11,15)}

Mast cells are located in the epithelial tissue in which LL-37 is generated; therefore, it is possible that LL-37 sends an alarm signal to mast cells to reinforce the body's defenses against infectious disease. In the present study, we demonstrate that LL-37 up-regulates both TLR4 mRNA and protein expression, and observed that LL-37 induced the release of Th2-type cytokines and pro-inflammatory cytokine from mast cells. Further, the increased expression of Th2 cytokines selectively diminished by LPS treatment. These observations may indicate that LL-37 is a candidate modifier of the host defense against bacterial entry by serving as an alarm for sentinels such as mast cells.

MATERIALS AND METHODS

Cell Culture The LAD2 human mast cell line was maintained in StemPro-34 serum-free medium (Gibco, Grand Island, NY, U.S.A.) supplemented with StemPro nutrient supplement (13 ml/500 ml; Gibco), penicillin–streptomycin (1 : 100; Gibco), 2 mM L-glutamine (Gibco), and 0.1 μ g/ml of human stem cell factor (PeproTech Inc., Rocky Hill, NJ, U.S.A.) at 37 °C in an atmosphere containing 5% CO₂. The culture media was replaced every 2 weeks, and the cells were used after at least 3 d of culture.

β -Hexosaminidase (Hex) Release Assay LAD2 mast cells were incubated with the indicated concentrations of LL-37 (HyCult Biotechnology, Uden, Netherlands) for 30 min at 37 °C. Reactions were stopped by placing them on ice. To measure β -Hex release, 50 μ l of the cell culture supernatant was transferred to the wells of 96-well plates. The remainder of the cell culture supernatant was removed, and the bound cells were lysed by mixing well with 200 μ l of UltraPure distilled water (Gibco) and then freezing and thawing three times. The β -hex in supernatants and cell lysates was incubated with 26 mM citrate buffer (pH 4.5) and 2.3 mg/ml *p*-ni-

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trophenyl *N*-acetyl- α -D-glucosaminide for 60 min at 37 °C. The reaction was developed by adding 0.4 M glycine (pH 10.7), and absorbance was measured at 405 and 570 nm by using a multiplate reader. The percent β -Hex release was calculated as follows: % β -Hex release = [β -Hex in supernatant/(β -Hex in supernatant + total β -Hex in pellet)] $\times$ 100%.

Immunoprecipitation Normal rabbit IgG and mAbs to TLR1 (H-90) or TLR9 (H-100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) were used for immunoprecipitation. Protein G Sepharose 4 Fast Flow (Amersham Biosciences, Uppsala, Sweden) was incubated with normal rabbit IgG (IgG-beads) or specific Ab (specific Ab-beads) overnight at 4 °C. LAD2 cells were lysed in 4 $\times$ lysis buffer containing a mixture of sodium orthovanadate (Sigma, St. Louis, MO, U.S.A.), sodium pyrophosphate tetra-basic decahydrate (Sigma), sodium fluoride (Sigma), CompleteTM protease inhibitor cocktail tablet (Roche), protease inhibitor cocktail (Sigma), 3,4-dichloroisocoumarin (Sigma), benzamidine (Sigma), and octyl-beta-glucoside (Pierce, Rockford, IL, U.S.A.). The cell lysate was incubated with IgG beads for 30 min at 4 °C for to remove non-specific protein. The supernatant was incubated with specific Ab beads overnight at 4 °C, and the target protein was separated from the beads by boiling for 4 min with sample buffer including NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, U.S.A.), NuPAGE sample reducing agent (Invitrogen), 2-mercaptoethanol (Kanto Chemical, Tokyo, Japan), and 4 $\times$ lysis buffer.

Western Blot The following primary Abs were used for Western blotting: mAbs to TLR1, TLR2, TLR5, and TLR9 (Imgenex, San Diego, CA, U.S.A.), polyclonal Ab to TLR4 (H-80) (Santa Cruz Biotechnology, Inc.), and mAbs to the extracellular signal regulated kinase (ERK)1/2 phosphorylated on Thr 202 and Tyr 204, to ERK1, to p38 phosphorylated on pT180 and pY182, and to p38 (BD Bioscience, San Jose, CA, U.S.A.). HRP-conjugated anti-mouse IgG (for TLR2, TLR5, TLR9, phosphorylated ERK1/2, ERK1, phosphorylated p38, and p38) and HRP-conjugated anti-rabbit IgG (for TLR1 and TLR4) were purchased from Amersham Bioscience and used as secondary Abs. For the detection of TLR2, TLR4, TLR5, ERK, and p38, LAD2 cells were lysed in a mixture of protease inhibitor cocktail (Sigma), NuPAGE sample reduction agent (Invitrogen), and NuPAGE LDS Sample Buffer (Invitrogen) and then heated for 10 min at 70 °C. Protein extracts were separated on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and then transferred onto a nitrocellulose or polyvinylidene difluoride membrane (Invitrogen). Western blotting was performed according to the instructions for the Immobilon detection reagents (Millipore Corp., Billerica, MA, U.S.A.). Immunoreactive bands were visualized using an LAS-1000 imaging system (Fujifilm, Tokyo).

Isolation of RNA and Generation of cDNA by Reverse Transcription TRIzol (Invitrogen) was used to isolate total RNA from LAD2 cells incubated in culture medium with or without 1 μ g/ml LL-37 for 1, 3, 6, 12 or 24 h. Isolated RNA was purified using an RNeasy mini kit (Qiagen, Valencia, CA, U.S.A.) and was reverse-transcribed to cDNA. For each reaction, 2 μ g of RNA was diluted to 11 μ l with UltraPure distilled water (Gibco) and mixed with 1 μ l of oligo (dT)_{12–18} primer (Invitrogen) and 1 μ l of 10 mM dNTP mix (Invitrogen), heated to 65 °C for 5 min, and quickly chilled on ice.

Next, 4 μ l of 5X first-standard buffer and 2 μ l of 0.1 M dithiothreitol (DTT) were added, and the mixture was incubated for 2 min at 42 °C. To each reaction, 1 μ l of SuperScript II RNase H[−] Reverse Transcriptase (Invitrogen) was added, and the mixture was incubated for 60 min at 42 °C. Finally, each reaction was mixed with 0.5 μ l of RNase H (Invitrogen) and incubated for 20 min at 37 °C.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) PCR was carried out using AccuPrime SuperMix I (Invitrogen). Amplification was carried out for the following number of cycles: 48 for formylpeptide receptor like 1 (FPRL1) and P2X₇, and 25 for β -actin. Each cycle included denaturation for 30 s at 94 °C, annealing for 30 s (63 °C for FPRL1, 57 °C for P2X₇, or 61 °C for β -actin), and extension for 1 min at 68 °C. After the final cycle, the samples were incubated for 10 min at 68 °C. PCR products were visualized by agarose gel electrophoresis, followed by ethidium bromide staining. The primers were as described previously.^{16,17} All primers were synthesized by Hokkaido System Science.

Real-Time PCR Total RNA was isolated from LAD2 cells, and cDNA was synthesized as described above. To examine the expression of TLR4 and β -actin mRNA, 0.5 μ l (for β -actin) or 1 μ l (for TLR4) of cDNA were mixed with 1 μ l of TaqMan primer/probe set (Applied Biosystems, Foster City, CA, U.S.A.), 10 μ l of TaqMan Universal Master Mix (Applied Biosystems), and UltraPure distilled water (Gibco) to bring the volume to 20 μ l. Real-time PCR was performed with a model 7300 real-time PCR system (Applied Biosystems).

FlowCytomix LAD2 cells were treated with or without 1 μ g/ml of LL-37 for 6 h, and then LAD2 was washed with the culture medium twice after incubation with LL-37. They were incubated for 48 h in culture medium that included 5% fetal bovine serum (FCS), in the presence or absence of 150 ng/ml of LPS-binding protein (LBP) and lipopolysaccharide (LPS). The supernatant was collected for cytokine and chemokine determination by a Th1/Th2 10plex FlowCytomix kit (Bender Medsystem, Burlingame, CA, U.S.A.) according to the manufacturer's instructions.

Statistical Analysis Two-tailed unpaired Student's *t*-test was used to determine statistical significance. Differences were considered significant when the probability (*p*) was <0.05. Data are expressed as the mean $\pm$ S.E.M.

RESULTS

LAD2 Express FPRL1 and P2X₇, and Induces Phosphorylation of ERK1/2 or p-38 by LL-37 Treatment LL-37 is known to directly bind to only FPRL1,¹⁸⁾ a receptor for *N*-formylmethionyl-leucyl-phenylalanine (fMLP), and is also reported to activate P2X₇ through the production of ATP.¹⁹⁾ And some studies have reported that LL-37 activates mitogen-activated protein kinase (MAPK), such as ERK1/2 and p38^{20,21)}; therefore, to confirm whether LAD2 cells can recognize LL-37, we first examined the expression of FPRL1 and P2X₇ using RT-PCR and evaluated the phosphorylation of ERK1/2 and p38 in LAD2 cells treated with LL-37. Both FPRL1 and P2X₇ were expressed by LAD2 (Fig. 1A), and ERK1/2 and p-38 were phosphorylated by LL-37 treatment for 5 min and 30 min, respectively, as shown in Fig. 1B. Furthermore, LL-37 induced β -Hex release from LAD2 cells

Fig. 1. Activation of LAD2 Cells by LL-37

(A) LAD2 expressed mRNA for FPRL-1 and P2X₇. Data are representative of 3 independent experiments. (B) LAD2 cells were treated with or without LL-37 (1 μ g/ml) for the indicated times. The levels of phosphorylated ERK1/2 (p-ERK1/2) and p38 (p-p38) were analyzed with Western blotting. Membranes were then stripped and reprobed with an antibody against total ERK1 or total p38. Data are representative of 3 independent experiments. (C) LL-37 induced β -Hex release from LAD2 cells. LAD2 cells were incubated for 30 min with the indicated concentration of LL-37 at 37 °C or 4 °C. **: $p < 0.01$ versus no added LL-37. Data represent the means $\pm$ S.E.M.s of 3 independent experiments.

Fig. 2. LL-37 Up-Regulates TLR4 Expression by LAD2 Cells

(A) LAD2 cells were treated with or without LL-37 (1 μ g/ml) for the indicated times, and the levels of TLRs were analyzed by Western blotting. Data are representative of 3 independent experiments. (B) After 6 h of incubation with LL-37, TLR4 levels in LAD2 cells were analyzed by Western blotting. Data are representative of 3 independent experiments.

(Fig. 1C), consistent with previous studies of rat mast cells.²² These results indicate that LAD2 cells can recognize LL-37 and are activated by LL-37.

The Effect of LL-37 on the Expression of TLRs on LAD2 Mast Cells In addition to its bactericidal activity, LL-37 could activate immunocytes such as keratinocytes and mast cells^{4,20}; therefore, we hypothesized that LL-37 might enhance mast cell capability to detect invading bacteria by modulation of TLR expression. TLR1 and TLR2 recognize peptidoglycan,²³ and TLR4, TLR5, and TLR9 recognize LPS,²⁴ flagellin,²⁵ and CpG-DNA,²⁶ respectively. We examined whether the expressions of these TLRs were changed in mast cells after incubation with 1 μ g/ml of LL-37. Immunoprecipitation was used to detect TLR1 and TLR9. Western blot study showed that TLR4 was up-regulated after 6 and 12 h of incubation with LL-37, but TLR1, TLR2, TLR5, and TLR9 were not up-regulated (Fig. 2A). Furthermore, LL-37 increased TLR4 expression in a concentration-dependent manner (Fig. 2B).

LL-37 Up-Regulates TLR4 mRNA on LAD2 Mast Cells We further investigated the effect of LL-37 on TLR4 by using real-time PCR. As shown in Fig. 3, TLR4 mRNA was increased after 6 and 12 h of incubation with LL-37.

Effect of LL-37 on the Release of Cytokines and

Fig. 3. LL-37 Up-Regulates TLR4 mRNA in LAD2 Cells

LAD2 cells were stimulated with or without LL-37 (1 μ g/ml) for 1, 3, 6, 12, or 24 h. TLR4 mRNA expression was assessed using real-time PCR. Results are the means $\pm$ S.E.M.s of 3 independent experiments.

Chemokine from LAD2 Mast Cells LL-37 has been reported to stimulate chemokine and cytokine secretion from a variety of cell types.^{18,19,27} Thus, we examined the release of various humoral factors caused by LL-37 treatment. LAD2 cells were treated with LL-37 for 54 h. Cell culture supernatants were assayed by FlowCytomix to measure Th1 cytokines (IFN- γ , IL-2 and TNF- β), Th2 cytokines (IL-4, IL-5 and IL-10), pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) and chemokine (IL-8). LL-37 tended to increase IL-2 and TNF- α , and significantly increased IL-1 β release from LAD2 cells (Fig. 4). Interestingly, LL-37 also significantly induced Th2 cytokines, IL-4 and IL-5 release (Fig. 4).

LPS Negatively Regulates LL-37-Inducible Th2 Cytokine Release from LAD2 Cells LL-37 generally co-exists with the bacterial component at a local infection site because microbe infection induces the generation of antimicrobial peptide. To explore the cooperative effect of LL-37 with bacterial components on mast cells, we next investigated the effect of LPS with LL-37 on the generation of cytokines from mast cells.

LAD2 cells were treated with LL-37 for 6 h, and washed free of peptide before addition of the TLR4 agonist LPS, to avoid inactivation of LPS by LL-37 in solution.²⁸ We then measured IL-4, IL-5 and IL-1 β in supernatant using FlowCytomix. LL-37 significantly increased IL-4, IL-5 and IL-1 β release from LAD2 cells, as shown in Fig. 5. LL-37-induced IL-5 generation was cancelled by LPS and increased release of IL-4 was also partly inhibited by LPS, although IL-1 β

Fig. 4. LL-37 Induces the Release of IL-1 β , IL-4 and IL-5 from LAD2 Mast Cells

LAD2 cells were stimulated with or without LL-37 (1 μ g/ml) for 54 h. Cytokine and chemokine releases were measured by FlowCytomix assay in cell culture supernatants. Results are the means $\pm$ S.E.M.s of 3 independent experiments. **: $p < 0.01$ versus no added LL-37 (Medium). *: $p < 0.05$ versus no added LL-37 (medium).

Fig. 5. Up-Regulation of Th2-Type Cytokines by LL-37 is Selectively Suppressed by LPS

LAD2 cells were stimulated with or without LL-37 (1 μ g/ml) for 6 h. After washing free of peptide, these cells were incubated with or without LPS for 48 h. The release of IL-1 β , IL-4 and IL-5 in supernatants was then measured using FlowCytomix. Results are the means $\pm$ S.E.M.s of 3 independent experiments. **: $p < 0.01$ versus LL-37(-) and LPS (-). *: $p < 0.05$ versus LL-37(-) and LPS (-). #: $p < 0.05$ versus LL-37(+) and LPS (+).

production was not diminished (Fig. 5), whereas, LPS alone had no effect on cytokine release from LAD2 cells.

DISCUSSION

It is well known that mast cells express Fc ϵ RI, a high-affinity IgE receptor, and induce allergic inflammation through aggregation of the receptor by antigen-antibody reaction.²⁹ Moreover, mast cells also express various functional TLRs and contribute to host defense or initiation of the innate immune response *via* TLRs.^{11–13}

Antimicrobial peptides secreted by epithelial and other cells possess bactericidal activity against a broad spectrum of microbes,¹ and they have chemotactic activity against immune and inflammatory cells.^{4,5} In addition to its chemotactic activity to mast cells, this peptide has other effects, such as the production of prostaglandins and interleukins,²² implying that LL-37 is an immune coordinator for mast cells. Thus, we examined the effects of LL-37 on TLR expression and humoral factor release from human mast cells, and investigated the role of LL-37 in the functional change of mast cells.

LL-37 is known to directly bind to only FPRL1¹⁸; however, FPRL1 is not functional on rat mast cells.⁵ LL-37 promotes the processing and releasing of IL-1 β from monocytes *via* the activation of P2X₇, which is a nucleotide receptor,¹⁹ but it is known that mast cells do not express this receptor³⁰; therefore, we first confirmed the expression of these recep-

tors on LAD2. Messenger RNA experiments revealed that LAD2 expressed these receptors. Next, to validate whether LAD2 cells can recognize LL-37, we examined the phosphorylation of ERK1/2 and p38 in LAD2 cells treated with LL-37. We confirmed that LL-37 could activate LAD2 mast cells. Next, we examined the change in TLR expression in mast cells following stimulation with fMLP, a FPRL1 ligand, ATP or LL-37. In addition to its bactericidal activity, LL-37 could activate immunocytes such as keratinocytes and mast cells.^{4,20} Thus, we hypothesized that LL-37 might enhance mast cell capability to detect invading bacteria by modulation of TLR expression. As expected, Western blot analysis showed that LL-37 up-regulated TLR4 expression in a concentration- and time-dependent manner but did not affect other TLRs on LAD2 mast cells. We further investigated the effect of LL-37 on TLR4 expression by using real-time PCR. LL-37 caused about a 1.6-fold induction of TLR4 mRNA at 6 hours' incubation. Kubo *et al.* demonstrated that about a 1.5-fold increase of TLR4 mRNA by LPS treatment induces functional TLR4 protein expression using LAD2 mast cells,³¹ suggesting that 1.6-fold increase of TLR4 mRNA by LL-37 may be involved in the up-regulation of TLR4 protein expression. We next examined the mechanisms of increased expression of TLR4 by LL-37. Whereas, neither fMLP nor ATP affected the expression of TLR4, and they also did not cause cytokine release (Supplemental Figs. 1 and 2). Speculating that these receptors are not involved in the effect of LL-37: LL-37 may affect LAD2 *via* unknown pathway. Okumura *et al.* and Yoshikawa *et al.* revealed that IFN- γ up-regulated the TLR4 expression using mouse bone marrow-derived mast cells and human bone marrow-derived mast cells, respectively,^{14,32} and Talreja *et al.* demonstrated that histamine induced TLR4 expression in endothelial cells.³³ We examined IFN- γ generation by LL37, and assessed histamine release involved in the degranulation of mast cells; however, 1 μ g/ml of LL-37 caused neither IFN- γ release nor degranulation of mast cells, as shown in Figs. 1B and 4; therefore they might not participate in the mechanism of TLR4 up-regulation. In fact, TLR4 expression was not changed by histamine treatment (Supplemental Fig. 3). Unfortunately, we could not indicate the mechanisms of the increased expression of TLR4 by LL-37 because details of the TLR4 expression mechanisms are still unknown. Further investigation is therefore needed.

It has been reported that LL-37 could mediate both pro-inflammatory and anti-inflammatory effects by modulating humoral factor release.³⁴ We found that LL-37 stimulates IL-1 β

release in addition to augmentation of IL-4 and IL-5 release. The release of IL-1 β would contribute to host defense by neutrophil filtration. IL-5 induces the growth, maturation and survival of eosinophils,³⁵⁾ which play an essential role in the development of allergic inflammatory response. IL-4 induces IgE production by B cells and promotes the preferential differentiation of T cells into the Th2 subset³⁶⁾; therefore, our results suggested that LL-37 can increase not only host defensive inflammatory response but also allergic inflammatory response. At local infection sites, bacterial components such as LPS would co-exist with LL-37 because massive production is observed by bacterial infection although only a little LL-37 is constantly produced on the skin surface. We therefore investigated the change in cytokine generation on mast cells by costimulation of LL-37 and LPS. LL-37-induced IL-5 release was significantly suppressed by LPS, and LL-37-induced IL-4 release also tended to decrease with LPS. IL-4 and IL-5 are produced through the IL-4/STAT6-dependent pathway.^{37,38)} IL-4/STAT6 signaling has been reported to be inhibited by SOCS-1, a suppressor of cytokine signaling,³⁹⁾ whereas, it has been reported that SOCS-1 is induced by LPS stimulation.⁴⁰⁾ Therefore, we now speculate that SOCS-1 is induced in response to LPS and this may cause the inhibition of LL-37-induced IL-4 and IL-5 release, although further investigation is needed to clarify this point. Whereas, LL-37-induced IL-1 β was not suppressed by LPS. These observations might provide evidence that, in the presence of LPS, LL-37 selectively induces host defensive inflammatory response in mast cells.

In conclusion, this is the first report to reveal that LL-37 up-regulates TLR4 and Th2-type and pro-inflammatory cytokine production, and that LL-37 selectively induce host defensive inflammatory response in mast cells in the presence of LPS, and alter mast cell function by LPS stimulation. These observations may indicate that LL-37 is a candidate modifier of the host defense against bacterial entry by serving as an alarm for sentinels such as mast cells.

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