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Antimicrobial Peptides Human β-Defensins and Cathelicidin LL-37 Induce the Secretion of a Pruritogenic Cytokine IL-31 by Human Mast Cells

François Niyonsaba,* Hiroko Ushio,* Mutsuko Hara,* Hidenori Yokoi,† Mitsutoshi Tominaga,‡ Kenji Takamori,‡ Naoki Kajiwara,§ Hirohisa Saito,§ Isao Nagaoka,¶ Hideoki Ogawa,* and Ko Okumura*

In addition to their microbiocidal properties, human β-defensins (hBDs) and cathelicidin LL-37 stimulate a number of mammalian cell activities, including migration, proliferation, and cytokine/chemokine production. Because hBDs and LL-37 cause mast cells to release pruritogens such as histamine and PGs, we hypothesized that these peptides would stimulate the secretion of a novel pruritogenic mediator IL-31, predominantly produced by T cells. hBDs and LL-37 enhanced IL-31 gene expression and IL-31 protein production and release in the human mast cell line LAD2, as well as in peripheral blood-derived cultured mast cells, suggesting that mast cells are another source of IL-31. Moreover, the expression of IL-31 was elevated in psoriatic skin mast cells, and hBD-2–4 and LL-37, but not hBD-1, enhanced its expression in vivo in rat skin mast cells. hBDs and LL-37 also induced the release of other pruritogenic mediators, including IL-2, IL-4, IL-6, GM-CSF, nerve growth factor, PGE_2, and leukotriene C_4, and increased mRNA expression of substance P. hBD– and LL-37–mediated IL-31 production/release was markedly reduced by pertussis toxin and wortmannin, inhibitors of G-protein and PI3K, respectively. As evidenced by the inhibitory effects of MAPK-specific inhibitors, hBD-2–4 and LL-37 activated the phosphorylation of MAPKs p38, ERK, and JNK that were required for IL-31 production and release. The ability of hBDs and LL-37 to stimulate the production and release of IL-31 by human mast cells provides a novel mechanism by which skin-derived antimicrobial peptides/proteins may contribute to inflammatory reactions and suggests a central role of these peptides in the pathogenesis of skin disorders. ***The Journal of Immunology*****, 2010, 184: 000–000.**

The skin generates a number of antimicrobial peptides/proteins (AMPs) that provide a front-line component in innate immunity and inhibit microbial invasion; however, characterization of their activity as solely antimicrobial might be an oversimplification of the diverse functions of these molecules. In fact, there is a growing body of evidence suggesting that, apart from exhibiting a broad spectrum of microbiocidal properties, AMPs display additional activities that are related to stimulation and modulation of the cutaneous immune system. These diverse functions include chemoattraction and activation of immune and/or inflammatory cells, enhancement of the production of cytokines and chemokines, acceleration of angiogenesis, promotion of wound healing, neutralization of harmful microbial products, and bridging of both innate and adaptive immunity (1).

The major AMPs found in humans are defensins and cathelicidins. Human defensins are divided into α- and β-defensins based on gene organization, cellular location, expression pattern, and disulfide bond connectivity (1, 2). In contrast to α-defensins, which are distributed in neutrophils and intestinal Paneth cells (3, 4), human β-defensins (hBDs) are mainly generated by the epithelia of several organs, including skin (1, 5). To date, four hBDs (hBD-1, -2, -3, and -4) have been identified in human skin. The first, hBD-1, is constitutively expressed by various epithelial tissues, particularly in the terminal layers of skin, urogenital tissue, and respiratory tissue (6, 7). hBD-2 was initially identified in psoriatic lesions in human epidermis, and has been shown to be inducible in activated normal keratinocytes (8, 9). Expression of hBD-1 and hBD-2 can be detected in monocytes, macrophages, and monocyte-derived dendritic cells, indicating that these peptides are not exclusively epithelial cell-associated AMPs (10). Like hBD-2, hBD-3 was first isolated from lesional psoriatic scales (11); however, it is also abundant in nonepithelial tissues (11). The expression of hBD-4 has been identified at the mRNA level, but the isolation of natural hBD-4 peptide has not yet been reported (12). hBD-4 is constitutively expressed in testis and gastric antrum, and it is inducible in differentiated human primary keratinocytes (13) and respiratory epithelial cells (12). Both hBD-1 and hBD-2 are overexpressed in monocytes on exposure to bacteria, LPS, or IFN-γ (10, 14), whereas hBD-2–4 are highly detected in keratinocytes stimulated by bacteria, TNF-α, IL-1β, IL-17 (for hBD-2), or IL-22 (for hBD-2 and hBD-3) (13, 15–17). Among hBDs, only hBD-3 expression is regulated by growth factors such as insulin-like growth factor-1 and TGF-α in keratinocytes (18). In respiratory epithelial cells, hBD-4 mRNA expression is amplified by exposure to bacteria or to PMA (12).

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Abbreviations used in this paper: AMP, antimicrobial peptide/protein; Ctrl, control; EIA, enzyme immunoassay; hCAP18, human cationic antibacterial protein of 18 kDa; hBD, human β-defensin; FPRL1, formyl peptide receptor-like 1; LT, leukotriene; Med, medium; NGF, nerve growth factor; PTx, pertussis toxin; SCF, stem cell factor; Sub P, substance P; Sup, supernatant; Wort, wortmannin.

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The name cathelicidin defines a diverse group of cationic peptides based on their evolutionarily highly conserved cathelin-like N-terminal domain and a structurally variable cationic AMP at the C terminus (19). The unique human cathelicidin is designated human cationic antibacterial protein of 18 kDa (hCAP18) because of its very close relationship to the CAP18 found in rabbits (20). Mature AMP derived from hCAP18 is termed LL-37; it begins with two leucine residues and has 37 aa residues. LL-37 is found in specific granules of neutrophils, but is also expressed in NK cells, γδT cells, B cells, and monocytes (21), mast cells (22), keratinocytes (23), and various epithelial cells (24, 25). 1,25-Dihydroxyvitamin D_3 is thought to be a potent inducer of hCAP18/LL-37 mRNA transcription, and it seems that the presence of vitamin D_3 is essential for cathelicidin induction in skin infection and wounds (26–28). In contrast to colon epithelium, LL-37 expression is enhanced in keratinocytes by insulin-like growth factor-1 and TGF-α (29). Similar to hBDs, the expression of LL-37 is augmented in various skin disorders (1); however, these AMPs are downregulated in atopic dermatitis (30). This explains why patients with atopic dermatitis often demonstrate increased susceptibility to bacterial and viral infections, particularly *Staphylococcus aureus*.

Apart from their direct antimicrobial functions, hBDs and LL-37 activate several types of cells, including neutrophils, keratinocytes, and monocytes (1). Furthermore, hBDs and LL-37 have been reported to chemoattract and degranulate murine, rat and human mast cells (31–35), subsequently increasing vascular permeability via mast cell activation (35, 36). Although the mechanism by which hBDs and LL-37 activate mammalian cells is not yet well understood, it has been shown that these peptides bind to functional receptors to stimulate various types of cells. For example, hBD-1–3 likely bind to the chemokine receptor CCR6 on dendritic cells, T cells, and monocytes (37, 38), and LL-37 reportedly activates formyl peptide receptor-like 1 (FPRL1) (39), purinergic receptor $P2X_7$ (40), and transactivates epidermal growth factor receptor (41). Moreover, hBDs and LL-37 are reported to trigger MAPK pathways during the secretion of cytokines and chemokines in various cell types (1).

Because hBDs and LL-37 cause mast cells to release histamine and PGD_2, both of which are known as pruritogenic mediators (42, 43), we speculated that hBDs and LL-37 might stimulate IL-31 expression and/or secretion. IL-31 is a newly discovered member of the gp130/IL-6 cytokine family, and it is produced mainly by activated $CD4^+$ T cells (44). Recently, IL-31 has been shown to be involved in the development of chronic dermatitis through the induction of severe pruritus in transgenic mice with lymphocyte-specific overexpression of IL-31 (44). Skin IL-31 mRNA levels are significantly higher in NC/Nga mice with scratching behavior (45), and this cytokine is overexpressed in pruritic atopic dermatitis but not in nonpruritic psoriatic lesions compared with human healthy skin (46). Moreover, acute allergic contact dermatitis, a skin disease featuring skin inflammation and pruritus, has also been associated with higher IL-31 mRNA levels than those seen in healthy skin (47). Together, these observations provide evidence for a pivotal role of IL-31 in the induction of pruritus in skin disorders.

In this study, we show that human mast cells constitute another source of IL-31, and that secretion of IL-31 from mast cells is increased upon stimulation with hBDs and LL-37. These peptides also induced the release of several pruritogenic mediators, including cytokines, nerve growth factor (NGF), PGE_2, and leukotriene (LT) C_4, from mast cells. We also demonstrate that hBDs and LL-37 activate MAPKs and that the secretion of IL-31 is controlled by G protein, PI3K, and MAPK pathways. Thus, hBDs and LL-37 may contribute to skin inflammatory responses by causing mast cells to secrete IL-31 and other pruritogenic factors.

Materials and Methods

Reagents

Antimicrobial peptides hBD-1, hBD-2, hBD-3 and hBD-4 were obtained from the Peptide Institute (Osaka, Japan). LL-37 (L^1LGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES^{37}) was synthesized by the solid-phase method on a peptide synthesizer (model PSSM-8; Shimadzu, Kyoto, Japan) by fluoroenylmethoxycarbonyl chemistry, and the molecular mass was confirmed on a mass spectrometer (model TSQ 700; Thermo Quest Finnigan, Manchester, U.K.). Human myeloma IgE was obtained from Calbiochem (La Jolla, CA); mouse anti-human IgE and mouse anti-rat mast cell (clone AR32AA4, 1:100) Abs were from BD Pharmingen (San Diego, CA). Rabbit anti-rat IL-31 Ab (1:100) was purchased from Abcam (Cambridge, MA). Mouse anti-human IL-31 Ab (clone 308202, 1:50) was from R&D Systems (Minneapolis, MN), and mouse anti-human mast cell tryptase Ab (clone G3, 1:1500) was obtained from Chemicon (Temecula, CA). Secondary Abs conjugated with Alexa Fluor 488 or Alexa Fluor 594 were from Invitrogen (Carlsbad, CA). Rabbit polyclonal anti-phosphorylated p38, ERK and JNK Abs, and p38, ERK and JNK Abs were purchased from Cell Signaling Technology (Beverly, MA). The inhibitors SB203580 (Sigma-Aldrich, St. Louis, MO), PD98059 (Cell Signaling Technology) and SP600125 (Calbiochem) were used to study the MAPK pathways involved in the activation of mast cells. Pertussis toxin and wortmannin were obtained from Sigma-Aldrich.

Cell culture and stimulation

The LAD2 cell line isolated from the bone marrow of a patient with mast cell leukemia was a gift from Dr. Arnold Kirshenbaum (National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, MD) (48). These cells were grown in Stem Pro-34 medium containing nutrient supplements (Invitrogen), supplemented with 2 mM L-glutamine (Invitrogen), 100 IU/ml penicillin and 100 μg/ml streptomycin (Meiji Seika, Tokyo, Japan), and 100 ng/ml human stem cell factor (SCF) (Wako, Osaka, Japan). Cell culture medium was hemidepleted every week with fresh medium. Human peripheral blood-derived cultured mast cells were obtained using previously described methods with some modifications (49). G-CSF-mobilized human peripheral bloods $CD34^+$ cells (Veritas Corporation, Tokyo, Japan) were cultured in serum-free Iscove's methylcellulose medium (Stem Cell Technologies, Vancouver, BC, Canada) containing 200 ng/ml SCF, 50 ng/ml IL-6 and 2.5 ng/ml IL-3 (PeproTech, London, U.K.), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY). At 6 wk, the methylcellulose medium was dissolved in PBS, and the cells were then resuspended and cultured in IMDM supplemented with 100 ng/ml SCF, 50 ng/ml IL-6, 5% FCS, 55 μM 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Hemidepletions of media were performed weekly by adding fresh media. The final purity of mast cells always exceeded 95%. Following incubation with various doses of hBDs or LL-37, mast cells at a final concentration of $1–2 \times 10^6$ cells/ml were centrifuged; the supernatants were used for ELISA, and the pellets were used for total RNA extraction or Western blotting analysis.

ELISA

IL-2, IL-4, IL-6, IL-8, IL-31, GM-CSF, TNF-α, NGF, LTC_4, and PGE_2 released into cell-free supernatants from cultures of mast cells stimulated with hBD-1–4 or LL-37 or from nonstimulated control cultures were measured with ELISA or enzyme immunoassay (EIA). The ELISA kits specific for IL-2, IL-4, IL-6, IL-8, IL-31, GM-CSF, NGF, and TNF-α were purchased from R&D Systems, whereas EIA kits for LTC_4 and PGE_2 were obtained from Cayman Chemical Company (Ann Arbor, MI). Supernatants were stored at −20°C until used for ELISA or EIA, according to the manufacturer's instructions. In some experiments, mast cells were pretreated with different inhibitors for 2 h before stimulation with hBDs and LL-37.

Treatment of mast cells with pertussis toxin, wortmannin, and MAPK inhibitors

The effects of G-protein inhibitor pertussis toxin, PI3K inhibitor wortmannin, and MAPK specific inhibitors SB203580 (p38 inhibitor), PD98059 (ERK inhibitor), and SP600125 (JNK inhibitor) were investigated by incubating LAD2 mast cells with pertussis toxin (200 ng/ml), wortmannin (20 μM), SB203580 (10 μM), PD98059 (10 μM), or SP600125 (20 μM) for 2 h at 37°C in culture medium. Cells were then stimulated with hBDs or LL-37 for 12 h, and ELISA for IL-31 was performed as above.

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Immunofluorescence staining

All animal procedures were approved by the institutional Animal Care and Use Committee of Juntendo University School of Medicine. Sprague-Dawley rats weighing 300–400 g were injected intradermally with 50 μl hBDs or LL-37 (500 ng) into one side of the ear and with vehicle (0.9% normal saline) into the other ear. After 12 h, rats were sacrificed, and small pieces of the ears were embedded in optical cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen. Cryosections (5 μm) were cut using a CM1850 cryostat (Leica, Wetzlar, Germany) and mounted on silane-coated glass slides. After blocking in PBS with 5% normal goat serum (Chemicon) and 2% BSA (Sigma-Aldrich), cryosections were double-labeled with anti-rat IL-31 and anti-rat mast cell Abs for 2 h at room temperature. After washing with PBS, the sections were incubated with secondary Abs for 1 h at room temperature.

Human skin donors provided written informed consent for participation in this study that was conducted with the approval of the medical ethical committee of the Juntendo University Urayasu Hospital. As described previously (50), 3-mm punch biopsies were taken from normal abdominal skins of three healthy volunteers and from lesional abdominal skins of three patients with clinical appearance of psoriasis. Cryosections (7 μm) from 4% paraformaldehyde-fixed skins were cut, fixed with ice-cold acetone, and then blocked in PBS with 5% normal donkey serum (Chemicon) and 2% BSA. The sections were double-stained with Abs against human IL-31 at 4°C overnight and human mast cell tryptase for 1 h at room temperature, and then the secondary Abs were added to the sections for 1 h at room temperature. Immunolabeling controls were performed by either omitting primary Abs in the procedure or replacing them with normal IgG. Sections were mounted in Vectashield mounting medium with DAPI (Vector Laboratories, Peterborough, U.K.), and viewed with a confocal laser-scanning microscope DMIRE2 (Leica).

Western blot analysis

LAD2 cells (2×10^6 cells/ml) were incubated with hBDs, LL-37, or IgE/anti-IgE for 5–60 min. Following stimulation, cell lysates were obtained by lysing cells in lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.02% NaN_3, 0.1% SDS, 1% NP 40) containing protease inhibitor mixture, phosphatase inhibitor mixture 1 and mixture 2 (Sigma-Aldrich) prepared according to the manufacturer's specifications. Equal amounts of total protein were subjected to 12.5% SDS-PAGE. After nonspecific binding sites were blocked, the blots were incubated with polyclonal Abs against phosphorylated or unphosphorylated p38, ERK, and JNK overnight. The membrane was developed with an ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). To quantify band intensity, densitometry using the software program Image Gauge (LAS-4000plus; Fujifilm, Tokyo, Japan) was performed to allow correction for protein loading.

Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from mast cells using Trizol reagent (BRL; Life Technologies, Rockville, MD), according to the manufacturer's instructions. First-strand cDNA was synthesized from 3 μg total RNA with oligo$(dT)_{12\text{-}18}$ primers using Superscript II RNase H^- reverse transcriptase (Life Technologies), as described previously (51). Real-time PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ). Amplification and detection of target mRNA were analyzed using a 7500 Real-Time PCR System (Applied Biosystems), according to the manufacturer's instructions. IL-31 and substance P primer/probe sets were obtained from Applied Biosystems Assays-on-Demand. To standardize mRNA concentrations, transcript levels of the housekeeping gene β-actin were determined in parallel for each sample, and relative IL-31 or substance P transcript levels were corrected by normalization based on β-actin transcript levels.

Statistical analysis

Statistical analysis was performed using Student *t* test or one-way ANOVA with multiple comparison test (Prism 4, GraphPad Software, San Diego, CA), and $p < 0.05$ was considered to be significant. The results are shown as the mean ± SD.

Results

Antimicrobial hBDs and LL-37 induce IL-31 gene expression in human mast cells

Antimicrobial hBDs and LL-37 have been reported to induce the release of inflammatory mediators such as histamine and PGD_2 that are known to contribute to pruritus (32, 35). We therefore asked whether hBDs and LL-37 could also stimulate the expression of the novel pruritus-promoting cytokine IL-31, which has been reported to be produced predominantly by T cells (44). Incubation of LAD2 cells with various concentrations of hBD-1–4 or LL-37 resulted in significant, dose-dependent increases in IL-31 mRNA expression as analyzed by quantitative real-time PCR (Fig. 1). The effect of hBD-3 was stronger than that of other AMPs, with concentrations of hBD-3 as low as 1 μg/ml inducing significant levels of IL-31 mRNA expression. In preliminary experiments, we observed that the expression of IL-31 mRNA reached a peak within 6 h after exposure to 20 μg/ml hBD-1, and that on exposure to 10 μg/ml hBD-2–4 or LL-37 it reached a peak within 3 h; in both cases, the IL-31 mRNA level then gradually decreased and returned to baseline by 12 h (data not shown). We also confirmed by trypan blue exclusion and measurement of lactate dehydrogenase activity that hBDs and LL-37 were not cytotoxic at the concentrations used in our study (data not shown).

hBDs and LL-37 stimulate IL-31 production/release by human mast cells

Because hBDs and LL-37 increased IL-31 mRNA levels in mast cells, we investigated whether they could also boost the production of IL-31 protein. After stimulation of LAD2 cells with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37 for 3–24 h, the release of IL-31 protein in cell-free supernatants was determined using a specific ELISA kit. As shown in Fig. 2*A*, hBD-2–4 and LL-37 significantly induced the release of IL-31. Although 20 μg/ml hBD-1 markedly increased IL-31 mRNA expression, as seen in Fig. 1, the same concentration had no significant effect on IL-31 release. Doses of hBD-1 >20 μg/ml did not further enhance IL-31 release (data not shown). The augmentation of IL-31 release by mast cells after hBD and LL-37 activation raised the question of

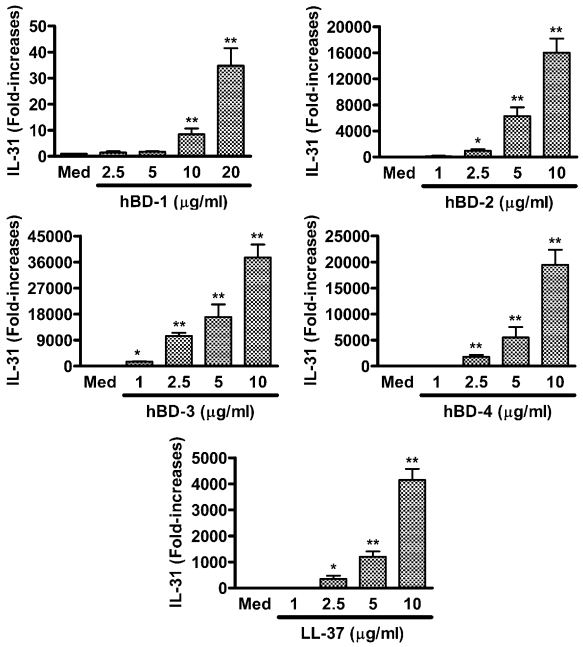

FIGURE 1. hBDs and LL-37 increase gene expression of IL-31 in LAD2 mast cells. LAD2 cells (2×10^6) were stimulated with the indicated concentrations (1–20 μg/ml) of hBD-1–4 or LL-37 for 6 h for hBD-1, and for 3 h for hBD-2–4 and LL-37. After incubation, total RNA was extracted and converted into cDNA, and real-time PCR was performed to analyze changes in IL-31 gene expression. Each bar shows the mean ± SD from four to six independent experiments, each of which was run in triplicate. Values represent fold increases in gene expression compared with cells incubated with medium alone. $*p < 0.01$; $**p < 0.001$. Med, medium.

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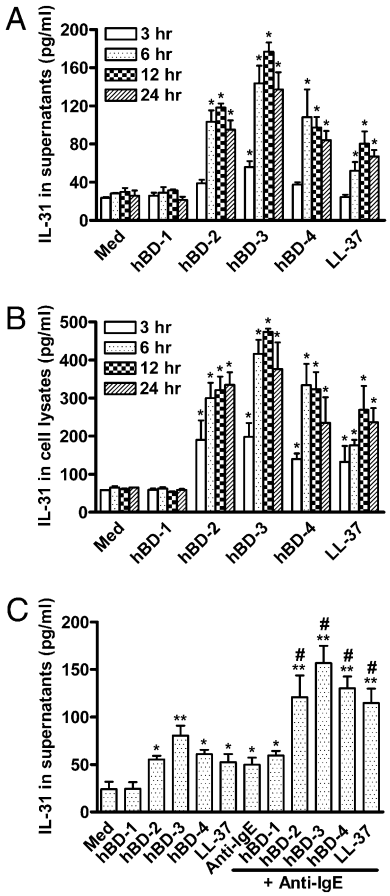

FIGURE 2. hBDs and LL-37 induce IL-31 production and release from LAD2 mast cells. *A*, LAD2 cells (2×10^6) were stimulated with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4 or 10 μg/ml LL-37 for 3–24 h, and the amounts of IL-31 in cell-free supernatants were determined by ELISA. *B*, hBD– and LL-37–stimulated cell lysates were obtained by lysing mast cells in lysis buffer containing protease inhibitor mixture. The IL-31 content of cell lysates was analyzed by ELISA. *C*, Cells (2×10^6) were sensitized with 1 μg/ml IgE overnight and stimulated with 1 μg/ml anti-IgE, 5 μg/ml hBD-1–4 or 5 μg/ml LL-37 alone or in combination for 12 h. The amounts of IL-31 in the cell-free supernatants were determined by ELISA. $*p < 0.05$ and $**p < 0.01$, when values were compared with the untreated group (Med, medium); $^{\#}p < 0.05$ when values were compared between each combination and the corresponding peptide used alone. Each bar represents the mean ± SD of four separate experiments.

whether the increased release was due to increased membrane permeability or to increased IL-31 synthesis. We therefore examined IL-31 amounts in cell lysates. As seen in Fig. 2*B*, large amounts of IL-31, almost 2-fold higher than the amounts extracellularly released into the respective cell culture supernatants, were detected in hBD- and LL-37-stimulated cell lysates. This finding suggests that hBDs and LL-37 induce both the production and release of IL-31.

Because IgE/anti-IgE also stimulated IL-31 gene expression and protein production in LAD2 cells (unpublished observation), we investigated whether IgE/anti-IgE and hBDs or LL-37 could cooperate to further increase IL-31 release. Mast cells were sensitized with 1 μg/ml myeloma IgE overnight and stimulated with 1 μg/ml anti-IgE alone or in combination with 5 μg/ml hBDs or LL-37. Combined treatment with anti-IgE and hBDs or LL-37 was found to significantly augment IL-31 release into the culture supernatant compared with the amounts released after treatment with each peptide alone (Fig. 2*C*). However, the simultaneous stimulation with anti-IgE and peptides showed additive but not synergistic effect. In preliminary experiments, we titrated anti-IgE, hBDs and LL-37, determined the lowest concentration required to maximally increase IL-31 release, and used this concentration for combination experiments. An additive effect was defined as a combined activity that equaled the sum of individual activities, whereas a synergistic effect was defined as a combined activity greater than the sum of individual activities.

The ability of hBDs and LL-37 to stimulate IL-31 secretion by human mast cells was not unique to the LAD2 cell line. In fact, these peptides also significantly increased both IL-31 gene expression (Fig. 3*A*) and protein production/release (Fig. 3*B*) in human peripheral blood-derived cultured mast cells. However, peripheral blood-derived mast cells had a weaker response to hBDs and LL-37 than LAD2 cells, suggesting different characteristics of these two cell types.

hBDs and LL-37 enhance the expression of IL-31 in mast cells in vivo

To verify that mast cells express IL-31 and that hBDs and LL-37 enhance this expression in vivo, we performed double-immunolabeling studies in rat skin using Abs to mast cells and to IL-31. We detected a feeble constitutive expression of IL-31 in rat skin mast cells; this constitutive expression was not affected by the presence of hBD-1. However, in the presence of hBD-2–4 or LL-37, the expression of IL-31 in skin mast cells was prominent (Fig. 4). Most of the mast cells showed positive immunostaining for IL-31, implying that this cell population forms a key source of IL-31 in the skin. The omission of primary Abs resulted in loss of specific staining (data not shown).

The expression of IL-31 in mast cells was also confirmed in human skin by double-staining with IL-31 and mast cell tryptase

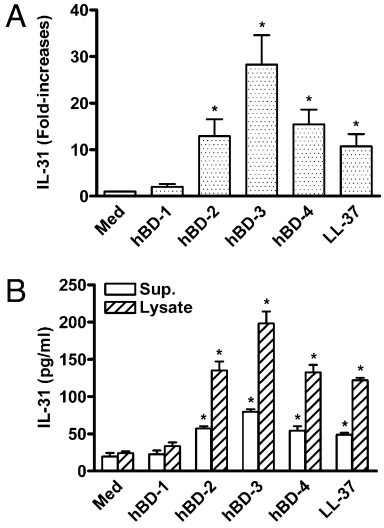

FIGURE 3. hBDs and LL-37 enhance IL-31 gene expression and protein production and release from human peripheral blood-derived cultured mast cells. *A*, Cells (1×10^6) were stimulated with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37 (6 h treatment with hBD-1 and 3 h treatment with hBD-2–4 or LL-37). Real-time PCR was performed to analyze changes in IL-31 gene expression. Each bar shows the mean ± SD from four separate experiments, each of which was run in triplicate. Values represent fold increases in gene expression compared with cells incubated with medium alone. Med, medium. $*p < 0.01$. *B*, Cells were stimulated with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37 for 12 h, and the concentrations of IL-31 in cell lysates (Lysate) and cell-free supernatants (Sup.) were determined by ELISA. Values are the mean ± SD of four separate experiments compared with the untreated group. $*p < 0.05$. Med, medium.

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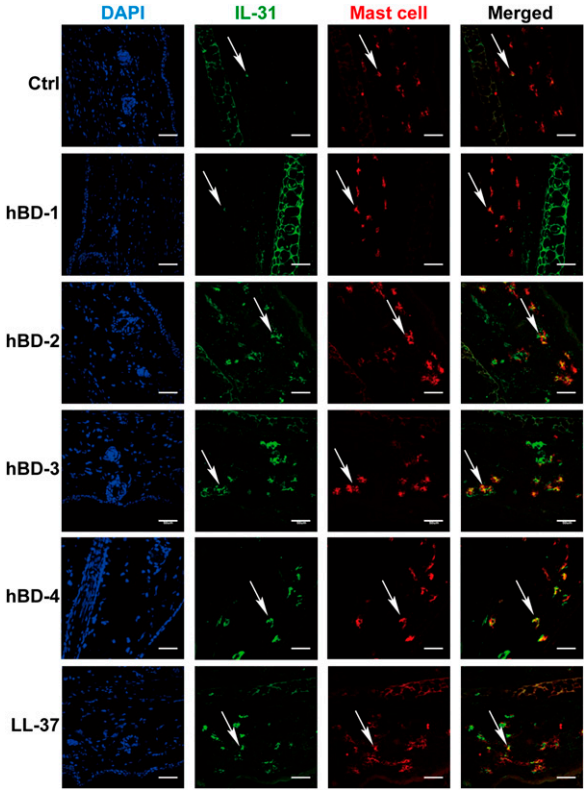

FIGURE 4. hBDs and LL-37 increase expression of IL-31 in mast cells in vivo. Rat ears were injected intradermally with 50 μl hBDs or LL-37 (500 ng) or with vehicle (0.9% normal saline) for 12 h. Frozen sections (5 μm) were incubated with either IL-31 (green) or mast cell (red) specific Abs, and immunofluorescence staining was performed using goat anti-rabbit Alexa Fluor 488 or goat anti-mouse Alexa Fluor 594. Arrows indicate IL-31 positive cells (green), mast cells (red), or colocalization of IL-31 and mast cells (merged). Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI, blue). Results are representative of five independent experiments. Original magnification ×40. Scale bar, 50 μm. Ctrl, control.

Abs. In comparison with healthy volunteers, the expression level of IL-31 was greater in patients with psoriasis, and mast cells from psoriatic skin showed colocalization with IL-31 (Fig. 5). Similarly, skin mast cells from chronic atopic dermatitis patients also highly expressed IL-31 (data not shown).

Mast cells induce the release of various pruritus-promoting mediators upon stimulation with hBDs and LL-37

We next tested whether hBDs and LL-37 stimulate the release of other pruritogenic mediators in addition to IL-31. The results shown in Fig. 6*A* reveal that hBD-2–4 and LL-37, but not hBD-1, significantly induced the release of various cytokines, including IL-2, IL-4, IL-6, and GM-CSF, which have been reported to contribute to itching (42, 52–55). In addition, we verified that hBD-2–4 and LL-37 augmented the production of other cytokines, such as IL-8 and TNF-α, that also likely play a role in itching (56, 57) (data not shown). We also examined the effects of hBDs and LL-37 on the expression or release of the important pruritus-inducing factors substance P, NGF, PGE_2 and LTC_4. The results revealed that although hBD-2–4 and LL-37 drastically induced substance P mRNA expression (Fig. 6*B*), they failed to stimulate its protein production (data not shown). Increased release of both PGE_2 and LTC_4 was observed within 30 min, after which it decreased gradually, whereas NGF release remained high even after 6 h of stimulation by hBDs and LL-37 (Fig. 6*B*).

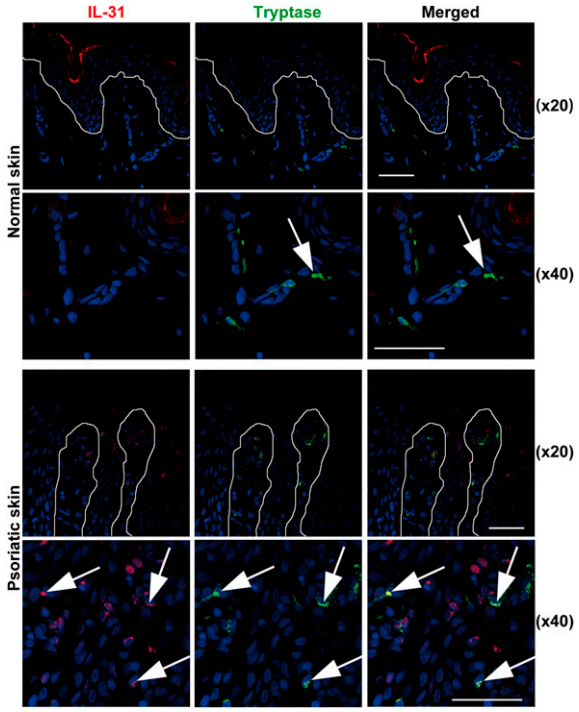

FIGURE 5. Expression of IL-31 protein in mast cells from human skin. Frozen sections (7 μm) of skins from healthy volunteers (normal skin) and inflamed lesional psoriasis (psoriatic skin) were stained with either IL-31 (red) or mast cell tryptase (green) specific Abs, and immunofluorescence staining was performed using Alexa Fluor 488 or Alexa Fluor 594. Arrows indicate IL-31 positive cells (red), mast cells (green), or colocalization of IL-31 and mast cells (Merged). Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI, blue). Results are representative of three independent experiments from three healthy volunteers and three psoriatic patients. The white dotted line in each panel indicates the border between the epidermis and the dermis. Scale bar, 50 μm.

Effects of pertussis toxin and wortmannin on hBD- and LL-37–mediated IL-31 production/release

hBDs and LL-37 are known to activate rat mast cells via G-protein coupled receptors (32, 35). We therefore investigated the effects of pertussis toxin, an inhibitor of G-protein, on hBD- and LL-37-induced IL-31 secretion. Pretreatment of mast cells with pertussis toxin significantly decreased the production/release of IL-31 (Fig. 7). The role of PI3K in hBDs and LL-37 activities was examined by preincubating mast cells with a PI3K inhibitor, wortmannin. Like pertussis toxin, wortmannin noticeably reduced the production/release of IL-31 induced by hBDs and LL-37 (Fig. 7). These observations indicate that hBDs and LL-37 stimulate human mast cells via G-protein-coupled receptors and PI3K pathways.

Activation of MAPKs by hBDs and LL-37 is necessary for the production/release of IL-31

Because hBDs and LL-37 have been reported to induce phosphorylation of MAPKs in various cell types (35, 36, 51), we reasoned that they might activate MAPKs in human mast cells. As seen in Fig. 8, hBD-2–4 and LL-37 markedly induced phosphorylation of p38, ERK, and JNK. In preliminary experiments, the maximal activation of p38, ERK, or JNK induced by hBDs and LL-37 was observed after 30 min.

The activation of MAPKs was required for the secretion of IL-31 by hBDs and LL-37. This is shown by the noteworthy suppression of IL-31 production and release by specific inhibitors of p38, ERK, and JNK (Fig. 9). The presence of SB203580, PD98059, or

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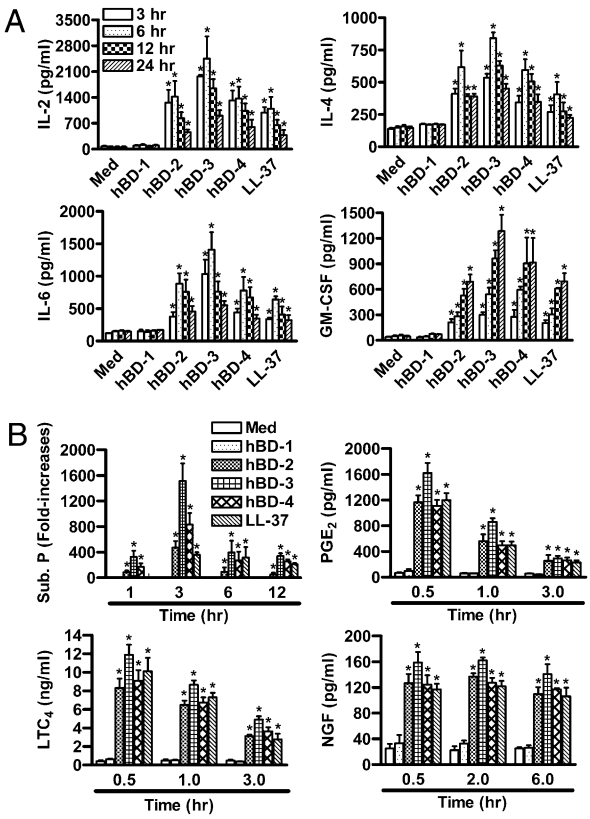

FIGURE 6. hBDs and LL-37 induce the expression or release of various pruritogenic mediators by LAD2 mast cells. *A*, Cells (2×10^6) were stimulated with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37 for 3–24 h, and the concentrations of IL-2, IL-4, IL-6, and GM-CSF released into cell-free supernatants were determined by ELISA. Values were compared between stimulated and nonstimulated groups. Each bar represents the mean ± SD of four to six separate experiments. $*p < 0.01$. *B*, Cells were stimulated with hBDs or LL-37 for the indicated times. Real-time PCR was performed to analyze changes in substance P gene expression (Sub. P). Values represent fold increases in gene expression above cells incubated with medium alone. $*p < 0.01$. After cell stimulation, enzyme immunoassays were used to analyze the amounts of PGE_2 and leukotriene C_4 (LTC_4) in cell-free supernatants, and NGF levels were detected using an ELISA. Values were compared between stimulated and non-stimulated cells. Med, medium. $*p < 0.01$. Each bar represents the mean ± SD of three to five separate experiments.

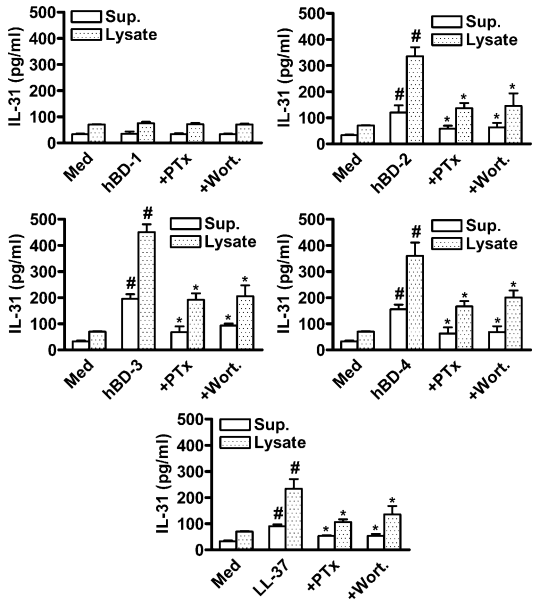

FIGURE 7. Effects of pertussis toxin and wortmannin on hBD– and LL-37–induced IL-31 production and release by LAD2 mast cells. Cells (2×10^6) were pretreated with 200 ng/ml pertussis toxin (PTx), 20 μM wortmannin (Wort.), or solvent (Med, medium) for 2 h, and were then challenged for 12 h with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37. The concentrations of IL-31 in cell lysates (Lysate) and cell-free supernatants (Sup.) were then determined by ELISA. Values are the mean ± SD of five separate experiments. Med, medium with solvent. $^{\#}p < 0.05$ as compared between stimulated group without inhibitor and untreated group; $*p < 0.05$ as compared between the presence and absence of each MAPK inhibitor.

SP600125 partially inhibited the production/release of IL-31 caused by hBD-2–4 and LL-37. Interestingly, the combination of all MAPK inhibitors further strikingly decreased the production and release of IL-31. The doses of MAPK inhibitors used in this study were not toxic to mast cells, as analyzed by trypan blue exclusion and lactate dehydrogenase activity (data not shown).

We further showed that IgE/anti-IgE treatment, which elevated IL-31 expression at both the gene and protein levels in mast cells, significantly stimulated p38, ERK, and JNK phosphorylation; this activation reached a peak at 5 min (Fig. 10).

Discussion

In the current study, we investigated the ability of antimicrobial hBDs and LL-37 to stimulate human mast cells to secrete the novel pruritogenic cytokine IL-31. We demonstrated that hBDs and LL-37 induced both IL-31 mRNA expression and IL-31 protein production and release, and that they also stimulated the release of other pruritogens, including cytokines, NGF, PGE_2, and LTC_4. The production and release of IL-31 was under the control of G-protein, PI3K, and MAPK pathways. Thus, our data suggest a novel mechanism for hBDs and LL-37 in inflammatory and/or allergic reactions.

The human skin is permanently exposed to a variety of potentially harmful microorganisms, but usually remains free of infection. This is not only due to the physical barrier of the stratum corneum, but also to the production of AMPs that form an innate epithelial chemical shield and inhibit microbial invasion (1). Among these AMPs, hBDs, and LL-37 have been reported to exhibit a broad spectrum of microbiocidal properties; they also display various stimulatory activities in mammalian cells (1). We and other investigators have demonstrated that hBDs and LL-37 recruit and stimulate mast cells to release inflammatory mediators such as histamine and PGD_2 (32, 35), which promote pruritus (42, 43). In this study, we show that, in addition to T cells, mast cells constitute another source of a pruritogenic factor, IL-31 (44). Upon stimulation with hBD-2–4 or LL-37, IL-31 was highly expressed in the LAD2 mast cell line and in rat skin mast cells. In addition, compared with cells from healthy human skin, the expression of IL-31 was notably elevated in skin mast cells from patients with psoriasis, a skin disease in which pruritus is observed in 70–90% of patients (42), and that has been associated with markedly enhanced levels of hBDs and LL-37 (1, 30). However, the fact that hBDs and LL-37 are downregulated in atopic dermatitis (30), in which the expression of IL-31 in mast cells was also augmented (data not shown), implies that these peptides are not exclusive IL-31 inducers in mast cells. We observed that IgE/anti-IgE also induced IL-31 production in mast cells and that it, like hBDs and LL-37, could also activate MAPKs. However, given that the combination of IgE/anti-IgE with hBDs or LL-37 resulted in additive but not synergistic stimulation of IL-31 secretion, our results suggest that the receptor signaling pathways involved in the stimulation of IL-31 release by IgE/anti-IgE and by hBDs or

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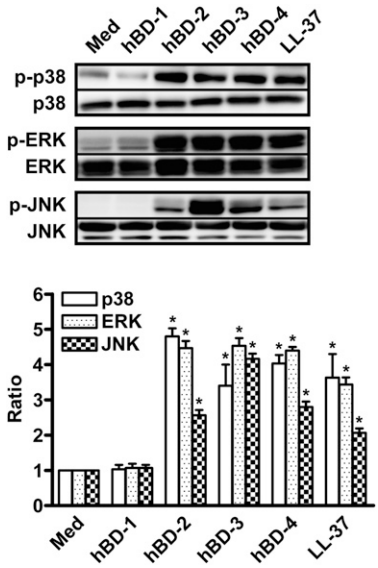

FIGURE 8. hBDs and LL-37 induce the phosphorylation of MAPKs in LAD2 mast cells. Cells (2×10^6) were stimulated for 30 min with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37. The levels of phosphorylated and unphosphorylated p38 (p-p38, p38), ERK (p-ERK, ERK) and JNK (p-JNK, JNK) in cellular lysates were then determined by Western blot analysis. *Upper panel*, Representative of four separate experiments with similar results. *Lower panel*, Bands were quantified by densitometry using the software program Image Gauge (LAS-4000plus) to allow correction for protein loading. Data represent the ratio of the intensity of each phosphorylated protein (p-p38, p-ERK or p-JNK) divided by the amount of the respective unphosphorylated protein (p38, ERK or JNK). Values are the mean ± SD of four independent experiments. $*p < 0.05$ as compared between stimulated and non-stimulated cells. Med, medium.

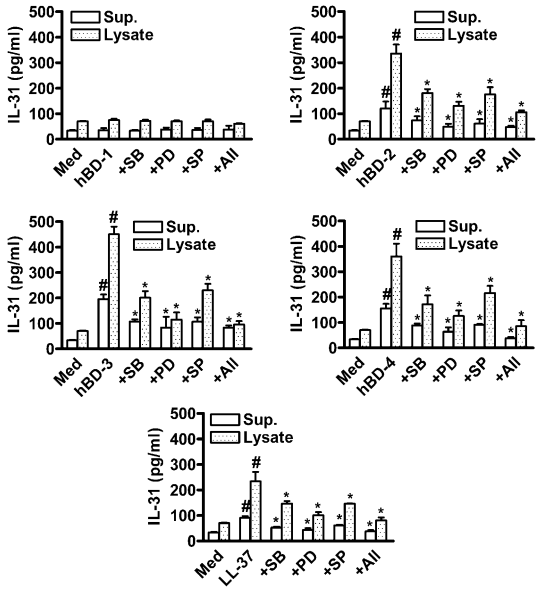

FIGURE 9. Effects of MAPK inhibitors on hBD- and LL-37-induced IL-31 production and release by LAD2 mast cells. Cells (2×10^6) were preincubated with 10 μM SB203580 (SB), 10 μM PD98059 (PD), 20 μM SP600125 (SP), or with a combination of 10 μM SB203580, 10 μM PD98059, and 20 μM SP600125 (All), or solvent (Med, medium) for 2 h. Cells were then stimulated for 12 h with 20 μg/ml hBD-1, 10 μg/ml hBD-2–4, or 10 μg/ml LL-37. The concentrations of IL-31 in cell lysates (Lysate) and cell-free supernatants (Sup.) were determined using an ELISA. Values are the mean ± SD of five separate experiments. $^{\#}p < 0.05$ as compared between stimulated group without inhibitor and the untreated group (Med, medium with solvent); $*p < 0.05$ as compared between the presence and absence of each MAPK inhibitor.

LL-37 may not interact. The observation that all hBDs except hBD-1 stimulated the production and release of IL-31 is probably not that the hBD-1 used was biologically inactive, because hBD-1 significantly enhanced IL-31 gene expression (Fig. 1); also, the same reagent markedly activated differentiated keratinocytes, as previously reported (51). Further investigations to determine whether the secondary structure of hBD-1 may affect its stimulatory ability are necessary.

Apart from the secretion of IL-31, hBDs and LL-37 also enhanced the release of numerous cytokines, including IL-2, IL-4, IL-6, IL-8, GM-CSF, and TNF-α, which are known to play a role in the elicitation of itching (52–57). However, several experimental studies have suggested an indirect pruritogenic effect of above cytokines via other mediators (55, 58). Furthermore, hBDs and LL-37 elicited the expression or release of the most potent pruritogens, including substance P, PGE_2, LTC_4, and NGF (42, 59–61). These observations provide novel evidence that hBDs and LL-37 may participate in inflammatory and/or allergic reactions via the activation of mast cells.

To gain insight into the cellular signaling mechanism by which hBDs and LL-37 stimulate IL-31 secretion in mast cells, the receptor-mediated process was examined. The results revealed that hBD- and LL-37-induced IL-31 production/release is controlled by a G-protein pathway. Until now, the receptors through which hBDs activate mast cells have not been well characterized. It has been reported that hBD-1 and hBD-2 chemoattract dendritic cells and T cells via a G-protein coupled chemokine receptor, CCR6 (37). Furthermore, hBD-3 reportedly chemoattracts monocytes and CCR6-transfected human embryonic kidney 293 cells (38), and suppresses neutrophil apoptosis through the activation of CCR6 (62). However, Soruri et al. (31) recently demonstrated that hBD-1–4 and mBD-8 were unable to induce the migration of dendritic cells, T cells or RBL-2H3 and 300.19 cells stably expressing CCR6. Likewise, a specific CCR6 ligand, MIP-3α/CCL20, could not compete with hBD-2 binding to rat mast cells (33). Similarly, a functional receptor for LL-37 on mast cells has not yet been identified. LL-37 likely activates FPRL1 (39) and purinergic receptor $P2X_7$ (40), and transactivates epidermal growth factor receptor in various cells (41). However, it is not clear whether these receptors are involved in LL-37-mediated mast cell activation. The failure of MMK1, an FPRL1-specific agonist, to compete with LL-37 binding to mast cells suggests that FPRL1 may not be a functional LL-37 receptor in mast cells (34). Other studies have shown that LL-37 activation of airway epithelial cells and human keratinocytes does not occur via FPRL1 (41, 63). Further studies are required to identify the functional receptors of hBDs and LL-37 on mast cells. We found that, in addition to G-protein coupled pathway,

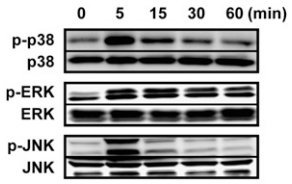

FIGURE 10. IgE/anti-IgE induces the phosphorylation of MAPKs in LAD2 mast cells. Cells (2×10^6) were sensitized with 1 μg/ml IgE overnight and stimulated with 2 μg/ml anti-IgE for 5–60 min; the levels of phosphorylated and unphosphorylated p38 (p-p38, p38), ERK (p-ERK, ERK), and JNK (p-JNK, JNK) in cell lysates were then determined by Western blot analysis. One representative experiment of three separate experiments with similar results is shown.

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hBDs and LL-37 also activated the PI3K pathway. However, given that neither G-protein nor PI3K pathways were completely suppressed by their specific inhibitors, the presence of additional pathways for hBDs and LL-37 (e.g., another activating receptor or transactivation) cannot be excluded.

This study also showed that MAPK activation is involved in hBD- and LL-37-mediated mast cell activation. The mammalian MAPK family mainly consists of p38, ERK, and JNK, which are activated by different stimuli, and target different downstream molecules, thereby performing different functions, including regulation of growth, differentiation, inflammation, and production of cytokines and chemokines (51). We demonstrated that hBDs and LL-37 induced the activation of p38, ERK, and JNK, and that the activation of these molecules was further required for mast cell stimulation, as their specific inhibitors significantly suppressed IL-31 secretion caused by hBDs and LL-37.

IL-31 is a recently identified pruritogenic factor mainly produced by activated T cells (44); its mRNA expression is upregulated in pruritic skin from humans with atopic dermatitis and other pruritic skin lesions (46, 47). In NC/Nga mice, increased IL-31 mRNA significantly coincides with increased scratching counts (64). In human keratinocytes, IL-31 induces the expression of chemokine genes associated with atopic skin inflammation (44). Hence, through the induction of chemokines, IL-31 may recruit inflammatory cells, which become activated and in turn produce more IL-31, thereby aggravating skin inflammation and pruritus. Besides its pruritogenic properties, IL-31 has been shown to be involved in the regulation of cell proliferation in lung epithelial cells and colorectal cancer cells, and it plays an important role in regulating hematopoiesis via the activation of STAT-3 and STAT-5 (44, 65). Moreover, IL-31 stimulates the secretion of proinflammatory cytokines, chemokines, and matrix metalloproteinases, and it likely plays a crucial role in the regulation of inflammation and the immune response (65–67). Although the concentrations of IL-31 produced by hBDs and LL-37 are low (pg/ml level) compared with the doses of IL-31 (ng/ml level) needed to exert its stimulatory functions (66, 67), IL-31 may cooperate with other molecules in stimulating its biologic functions. For instance, the combination of IL-31 with IL-17 and of IL-31 with IL-4 or IL-13 has resulted in increased production of numerous cytokines and chemokines in epithelial cells (66, 67). Thus, the ability of hBDs and LL-37 to stimulate IL-31 secretion by mast cells suggests a key role of these AMPs in the immune and inflammatory responses mediated by IL-31.

In the skin, mast cells are distributed within the dermis and are involved in the pathogenesis of several skin disorders, including wounding and lichen planus, in which concentrations of AMPs are abundantly enhanced (1, 68). Because the basal membrane between the dermis and epidermis is impaired in wounding and lichen planus, this may lead to direct contact between skin-derived AMPs and mast cells, resulting in subsequent activation of mast cells. Human epithelium contains elevated amounts of hBDs and LL-37 at sites of infection or inflammation. For example, the concentrations of hBD-2 and LL-37 have been estimated at ~157 μM and ≥1605 μM, respectively, in a pruritic skin disease—psoriasis (30). This observation supports the pathophysiologic relevance of the doses of hBDs and LL-37 (ranging 1–20 μg/ml, equivalent to 0.2–4.0 μM) used in this study.

In conclusion, our study demonstrated novel functions of hBDs and LL-37 in human mast cells and showed that mast cells constitute another source of IL-31. The hBD– and LL-37–mediated stimulation of the production of pruritogenic factors by mast cells provides a novel mechanism by which human AMPs may contribute to inflammatory reactions and suggests a role for these AMPs in the pathogenesis of skin disorders.

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Disclosures

The authors have no financial conflicts of interest.

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