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The Antimicrobial Peptide LL-37 Activates Innate Immunity at the Airway Epithelial Surface by Transactivation of the Epidermal Growth Factor Receptor[1]

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Antimicrobial peptides produced by epithelial cells and neutrophils represent essential elements of innate immunity, and include the defensin and cathelicidin family of antimicrobial polypeptides. The human cathelicidin cationic antimicrobial protein-18 is an antimicrobial peptide precursor predominantly expressed in neutrophils, and its active peptide LL-37 is released from the precursor through the action of neutrophil serine proteinases. LL-37 has been shown to display antimicrobial activity against a broad spectrum of microorganisms, to neutralize LPS bioactivity, and to chemoattract neutrophils, monocytes, mast cells, and T cells. In this study we show that LL-37 activates airway epithelial cells as demonstrated by activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and increased release of IL-8. Epithelial cell activation was inhibited by the MAPK/ERK kinase (MEK) inhibitors PD98059 and U0126, by the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor AG1478, by blocking anti-EGFR and anti-EGFR-ligand Abs, and by the metalloproteinase inhibitor GM6001. These data suggest that LL-37 transactivates the EGFR via metalloproteinase-mediated cleavage of membrane-anchored EGFR-ligands. LL-37 may thus constitute one of the mediators by which neutrophils regulate epithelial cell activity in the lung. ***The Journal of Immunology,*** **2003, 171: 6690–6696.**

The airway epithelium is frequently exposed to a variety of stimuli, including inhaled exogenous factors such as microorganisms and microbial products. Endogenous mediators that are released by inflammatory cells during infection and inflammation also affect the airway epithelium. Neutrophils play an important role in host defense by killing ingested microorganisms, and this mechanism includes the action of antimicrobial peptides. Various neutrophil-derived antimicrobial peptides have been identified, including the human cathelicidin cationic antimicrobial protein (hCAP)[3]-18 and the neutrophil defensins. Neutrophils that have been stimulated to degranulate may release these peptides and other granule components into the extracellular environment.

Human CAP-18, the only human cathelicidin identified to date, was first identified in neutrophils (1) and later shown to be expressed in various squamous epithelia (2), surface epithelial cells of the conducting airways, and serous and mucous cells of the submucosal glands (3), by keratinocytes in inflamed skin (4) and by specific lymphocyte and monocyte populations (5). It belongs to the cathelicidin family of antimicrobial peptides that are characterized by a conserved N-terminal cathelin domain and a variable C-terminal antimicrobial domain. This C-terminal domain can be cleaved off from the precursor by proteinases, releasing the active peptide. Exocytosed material from neutrophils contains hCAP-18 that has been proteolytically cleaved by proteinase-3 yielding the ~4.5 kD active α helical peptide LL-37 (6). LL-37 displays antimicrobial activity against a broad spectrum of microorganisms (7) and neutralizes LPS bioactivity (8, 9). The importance of cathelicidins for an effective host defense against infection is illustrated by a recent study showing that mice deficient in the murine cathelicidin-related antimicrobial peptide suffer from more severe bacterial skin infections (10). Overexpression of LL-37/hCAP-18 after pulmonary gene transfer in mice was shown to inhibit bacterial load and inflammatory response following pulmonary *Pseudomonas aeruginosa* challenge, whereas systemic gene transfer was shown to protect against endotoxemia (11). Also other activities were described for LL-37, including chemotactic activity toward neutrophils, monocytes, and T cells through binding to the G protein-coupled fMLP receptor, the formyl peptide receptor-like (FPRL) 1 (12), chemotactic activity toward mast cells (13), and stimulation of mast cell degranulation (14). Furthermore, a recent study by Scott et al. (15) showed that LL-37 activates lung epithelial cells and macrophages. Although a selective deficiency of CAP-18/LL-37 in humans has not been described, a recent study shows that the neutrophils from patients with morbus Kostmann, a severe congenital neutropenia, are deficient in CAP-18/LL-37 (16). This deficiency is accompanied by the occurrence of infections and periodontal disease, suggesting that CAP-18/LL-37 is involved in antimicrobial defense in humans.

Neutrophil-derived products, including antimicrobial peptides and serine proteinases, have been shown to activate airway epithelial cells (17–19). Neutrophil defensins are small cationic antimicrobial and chemotactic peptides (20) that activate host cells in

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[3] Abbreviations used in this paper: hCAP, human cathelicidin cationic antimicrobial protein; FPRL, formyl peptide receptor-like; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; EGFR, epidermal growth factor receptor; PBEC, primary bronchial epithelial cell; NAC, *N*-acetylcysteine; HB, heparin-binding; JNK, c-Jun N-terminal kinase; GPCR, G protein-coupled receptor; ADAM, a disintegrin and metalloprotease.

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vitro (17, 21) and cause lung injury in vivo (22). Neutrophil defensins induce IL-8 release from airway epithelial cells (17) and have been shown to increase airway epithelial cell proliferation, a process which is dependent on activation of the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK)1/2 (21). This suggests that these peptides may regulate cellular activity in the airway epithelium.

The aim of the present study was to investigate whether LL-37 activates airway epithelial cells. To this end ERK1/2 activation and IL-8 release in airway epithelial cells were determined. Given the key role of the epidermal growth factor receptor (EGFR) in growth factor-mediated ERK1/2 activation, we explored the involvement of EGFR in this process. Our results indicate that LL-37, in addition to its antimicrobial and chemotactic activities, may also contribute to innate immunity by activation of airway epithelial cells.

Materials and Methods

Peptide synthesis

LL-37 (amino acid sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFL RNLVPRTES) was synthesized by solid phase peptide synthesis on a TentagelS-AC (Rapp, Tübingen, Germany) using 9-fluorenylmethoxycarbonyl/t-Bu chemistry, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate/*N*-methylmorpholine for activation and 20% piperidine in *N*-methylpyrrolidone for fluorenylmethoxycarbonyl removal. The peptide was cleaved from the resin and deprotected with trifluoroacetic acid/water and purified on Vydac C18. The purified peptide was analyzed by RP-HPLC and the molecular mass was confirmed by MALDI-TOF mass spectrometry.

Human CAP-18 was isolated from neutrophils (6). Rabbit polyclonal anti-phosphorylated ERK1/2, p38 and c-Jun N-terminal kinase (JNK) Abs, and total ERK1/2, p38 and JNK Abs, were purchased from New England Biolabs (Beverly, MA). The inhibitors AG1478 (Calbiochem, La Jolla, CA), PD98059 (Alexis, Nottingham, U.K.), U0126 (Promega, Madison, WI), LY294002 (Stratagene, La Jolla, CA), and GM6001 (Chemicon International, Temecula, CA) were used to study pathways involved in activation of airway epithelial cells. Neutralizing Abs against amphiregulin, heparin-binding (HB)-EGF, and TGF-α were purchased from R&D Systems (Minneapolis, MN), and neutralizing anti-EGFR Abs from Lab Vision (Fremont, CA). Apocynin was obtained from Janssen (Beerschot, Belgium) and *N*-acetylcysteine (NAC), pertussis toxin, and TGF-α were obtained from Sigma-Aldrich (St. Louis, MO).

Generation of anti-LL-37 mAbs

Anti-LL-37 mAbs were generated using conventional hybridoma technology, and generation of these Abs has been described in part (6). Briefly, female BALB/c mice were s.c. immunized with a mixture of synthetic native and glutaraldehyde cross-linked LL-37 in Freund's complete adjuvant, and received a booster injection with LL-37 in Freund's incomplete adjuvant. Four days after an intrasplenal injection with LL-37, spleen cells were isolated and fused with cells from the SP2/0 mouse myeloma cell line. Hybridomas producing LL-37 specific IgG Abs were identified by ELISA and subcloned by limiting dilution. Reactivity and specificity were analyzed by dot blot analysis using LL-37 and by immunohistochemistry on formalin-fixed bronchial tissue. Abs were purified from culture supernatant by protein G affinity chromatography using the ÄKTAprime System (Amersham Pharmacia Biotech, Uppsala, Sweden).

Cell cultures

Cells from the mucoepidermoid lung carcinoma cell line NCI-H292 were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 20 U/ml penicillin, 20 μg/ml streptomycin (all from BioWhittaker, Walkersville, MD) and 10% heat-inactivated FCS (Life Technologies) at 37°C and 5% CO_2. Cells were passaged weekly using 0.25% (w/v) trypsin and 0.1% (w/v) EDTA.

Primary bronchial epithelial cells (PBEC) were isolated from macroscopically normal bronchial tissue that was obtained from patients who underwent lung surgery for lung cancer at the Leiden University Medical Center (Leiden, The Netherlands). PBEC were obtained from bronchial rings using enzymatic digestion of tissue as previously described (23). Cells were cultured in 6-well plates that were precoated with a combination of Vitrogen (30 μg/ml; Celtrix Laboratories, Palo Alto, CA), fibronectin (10 μg/ml; isolated from human plasma), and BSA (10 μg/ml; Boehringer Mannheim, Mannheim, Germany), in keratinocyte serum-free medium (Life Technologies), supplemented with EGF (0.2 ng/ml; Life Technologies), bovine pituitary extract (25 μg/ml; Life Technologies), isoproterenol (1 μM; Sigma-Aldrich), 20 U/ml penicillin, 20 μg/ml streptomycin at 37°C and 5% CO_2. Cyproxin (10 μg/ml; Bayer, Wuppertal, Germany) was included in the culture medium during the first week of culture. After trypsinizing (0.03% (w/v) trypsin, 0.01% (w/v) EDTA, 0.1% (w/v) glucose in PBS, pH 7.5), cells were stored in liquid nitrogen or subcultured.

Stimulation of NCI-H292 cells and subcultures of PBEC

NCI-H292 cells were cultured in tissue culture plates and after reaching near-confluence, the cells were rinsed with PBS and starved overnight in serum-free RPMI 1640 medium. The next day, the cells were stimulated in serum-free medium.

PBEC were used at passage 2 and cultured in tissue culture plates in DMEM/Ham's F12 (1:1) medium (Life Technologies) supplemented with 10 ng/ml EGF, 2% (v/v) Ultroser G (Life Technologies), 1 μM isoproterenol, 1 μM insulin (Sigma-Aldrich), 1 μM hydrocortisone (Sigma-Aldrich), 2 mM L-glutamine, 1 mM HEPES, 200 U/ml penicillin and 200 μg/ml streptomycin. After reaching near-confluence, cells were starved overnight in DMEM/Ham's F12 medium supplemented with 2 mM L-glutamine, 1 mM HEPES, 200 U penicillin and 200 μg/ml streptomycin, and subsequently stimulated in this medium. After stimulation, supernatant was collected, and either RNA was isolated or cellular lysates were prepared.

Cellular lysates

After washing airway epithelial cells in washing buffer (5 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$), cells were lysed in ice-cold lysis buffer (0.5% (v/v) Triton X-100, 1 mM Na_3VO_4 and mini-complete protease inhibitor mixture (Boehringer Mannheim, Basel, Switzerland) in washing buffer) for 15 min on ice. Cells were scraped from the wells and collected. After centrifugation for 5 min at 13,000 rpm at 4°C, the supernatant was collected and stored at −20°C. Protein concentrations of the lysates were measured by the bicinchoninic acid protein assay system (Pierce, Rockford, IL).

Gel electrophoresis and Western blotting

Gel electrophoresis and Western blotting were performed with Bio-Rad (Hercules, CA) systems according to the manufacturer's instructions. Samples were subjected to SDS-PAGE on a 10% glycine-based gel, and dissolved proteins were transferred to a polyvinylidene difluoride membrane (ERK1/2), or to nitrocellulose (p38 and JNK). Nonspecific binding sites were blocked, and the blots were incubated with rabbit polyclonal Abs against phosphorylated and total ERK1/2, p38 or JNK, and total ERK1/2, p38 or JNK, and secondary HRP-conjugated anti-rabbit IgG Abs. The ECL Western blotting detection system (Amersham Pharmacia Biotech) was used to reveal immunoreactivity. Densitometry was performed, and the ratio phosphorylated ERK1/2 and total ERK1/2 for each sample was calculated. Data from one experiment are representative of three individual experiments.

IL-8 ELISA

IL-8 levels were measured in cell-free supernatants using IL-8-specific sandwich ELISAs (CLB, Amsterdam, The Netherlands and BioSource International, Nivelles, Belgium).

Results

LL-37 increases IL-8 protein release

To study the effect of LL-37 on IL-8 release in airway epithelium, cells from the mucoepidermoid lung carcinoma cell line NCI-H292 and subcultures of PBEC were stimulated for different time periods with various concentrations of LL-37, and IL-8 release in cell-free supernatants was determined by IL-8-specific ELISA. LL-37 was shown to induce a concentration- and time-dependent increase in IL-8 release in both NCI-H292 cells (Fig. 1*A*) and PBEC (Fig. 1*B*). In contrast to LL-37, equimolar concentrations of the precursor protein hCAP-18 did not increase IL-8 release, indicating that hCAP-18 processing is required for activation of airway epithelial cells (data not shown). Cell viability after stimulation with various concentrations of LL-37 was studied by MTT mitochondrial activity assay (data not shown). At a concentration

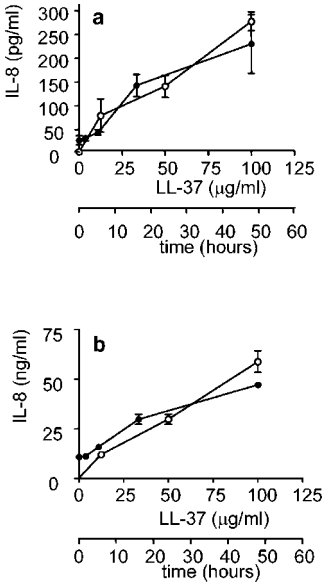

FIGURE 1. Effect of LL-37 on IL-8 release in NCI-H292 cells and PBEC. NCI-H292 cells and PBEC were stimulated with various concentrations of LL-37 for different time periods. IL-8 release in cell-free supernatant of NCI-H292 cells (*a*) and PBEC (*b*) was measured by ELISA. IL-8 release is shown as detected after various stimulation periods with 33.3 μg/ml LL-37 (○) or after stimulation with various concentrations of LL-37 for 24 h (●). Results are expressed as mean ± SEM of one representative experiment of three, each performed in triplicate.

of 100 μg/ml, LL-37 decreased mitochondrial activity of NCI-H292 cells, whereas concentrations of 33 μg/ml and lower did not affect mitochondrial activity.

LL-37 activates ERK1/2

Because ERK1/2 activation has been shown to be involved in IL-8 release, the effect of LL-37 on ERK1/2 activation in NCI-H292 cells was studied. Cells were stimulated with various concentrations of LL-37, or with TGF-α as a control, and both phosphorylated and total ERK1/2 levels were determined using Western blot analysis. LL-37 was shown to induce a concentration-dependent increase in ERK1/2 activation (Fig. 2*A*). After 15 min stimulation, ERK1/2 activation was most pronounced (data not shown), and therefore in additional experiments ERK1/2 activation was determined after 15 min stimulation. In contrast to LL-37, the precursor protein hCAP-18 used at equimolar concentrations, did not activate ERK1/2, indicating that only the mature peptide LL-37 activates airway epithelial cells (Fig. 2*B*). Furthermore, pertussis toxin did not inhibit LL-37-induced ERK1/2 activation, suggesting that G protein-coupled receptors (GPCR) may not be involved in this process (Fig. 2*C*). To study the specificity of LL-37-induced ERK1/2 activation, LL-37 was preincubated for 2 h with a monoclonal anti-LL-37 Ab before addition to cells. Preincubation of LL-37 with the mAb inhibited LL-37-induced ERK1/2 activation, whereas TGF-α-induced ERK1/2 activation was not affected by this monoclonal (Fig. 2*C*).

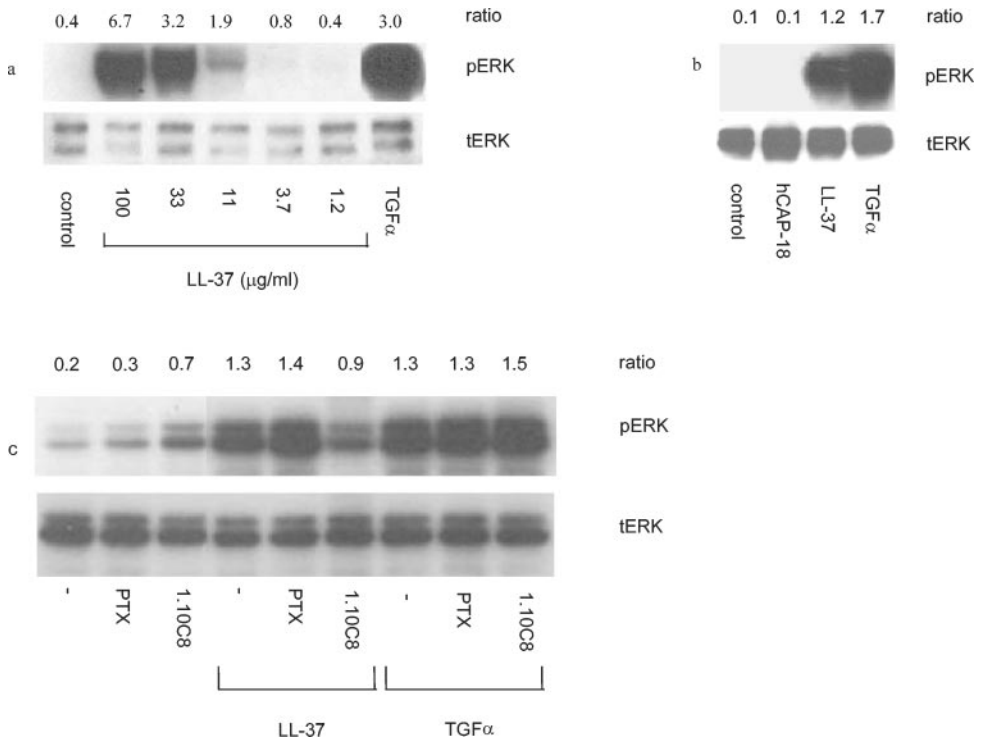

FIGURE 2. Effect of LL-37 on ERK1/2 activation in NCI-H292 cells. Cells were stimulated for 15 min with various concentrations LL-37, with 20 ng/ml TGF-α or without stimulus (*a*), or with LL-37 (30 μg/ml), an equimolar concentration of hCAP-18 (120 μg/ml), TGF-α (20 ng/ml) or without stimulus (*b*). The effect of pertussis toxin (PTX) was studied by preincubating the cells with pertussis toxin (160 ng/ml) for 24 h before stimulating the cells with 10 μg/ml LL-37. The effect of a monoclonal anti-LL-37 Ab on LL-37-induced ERK1/2 activation was studied by preincubating the following mixtures for 2 h before incubation with the cells for 15 min: LL-37 alone (10 μg/ml), LL-37 and monoclonal anti-LL-37 Ab 1.10C8 (600 μg/ml), 20 ng/ml TGF-α alone or combined with the mAb, the Ab alone or medium alone (*c*). Both phosphorylated (pERK) and total ERK1/2 (tERK) levels in cellular lysates were determined by Western blot analysis, and densitometry was performed. The ratio phosphorylated ERK and total ERK for each sample was calculated and shown at the *top* of each lane. Data are representative of three individual experiments.

EGFR is involved in LL-37-induced ERK1/2 activation

To study the involvement of EGFR signaling in LL-37-induced ERK1/2 activation, NCI-H292 cells were preincubated for 1 h with inhibitors of specific signaling molecules before adding LL-37 (Fig. 3). Both the EGFR tyrosine kinase inhibitor AG1478 and neutralizing anti-EGFR Abs were shown to inhibit LL-37-induced ERK1/2 activation, suggesting that EGFR signaling is involved in the process. The MAPK/ERK kinase (MEK) inhibitors U0126 and PD98059 blocked LL-37-induced ERK1/2 activation whereas the phosphatidylinositol 3-kinase inhibitor LY294002 had a minimal effect on LL-37-induced ERK1/2 activation. The antioxidants NAC and apocynin did not affect LL-37-induced ERK1/2 activation (data not shown), suggesting that reactive oxygen intermediates are not involved. Because metalloproteinases have been demonstrated to cleave EGFR ligands from their membrane-anchored forms and have in this way been shown to be involved in EGFR transactivation, the effect of the metalloproteinase inhibitor GM6001 was evaluated. GM6001 was shown to inhibit LL-37-induced ERK1/2 activation (Fig. 3), whereas it did not affect TGF-α-induced ERK1/2 activation (data not shown). To study involvement of EGFR ligands, neutralizing Abs against various EGFR ligands were tested for inhibition of LL-37-induced ERK1/2 activation. Anti-HB-EGF, anti-amphiregulin and anti-TGF-α neutralizing Abs alone did not significantly affect the process (data not shown). However, a mixture of the Abs blocked LL-37-induced ERK1/2 activation completely (Fig. 3).

LL-37-induced IL-8 release is mediated via EGFR and ERK1/2 and requires metalloproteinase activity

To study the involvement of ERK1/2 activation in LL-37-induced IL-8 release, NCI-H292 cells were incubated for 1 h with inhibitors of separate signaling molecules before adding LL-37 and after

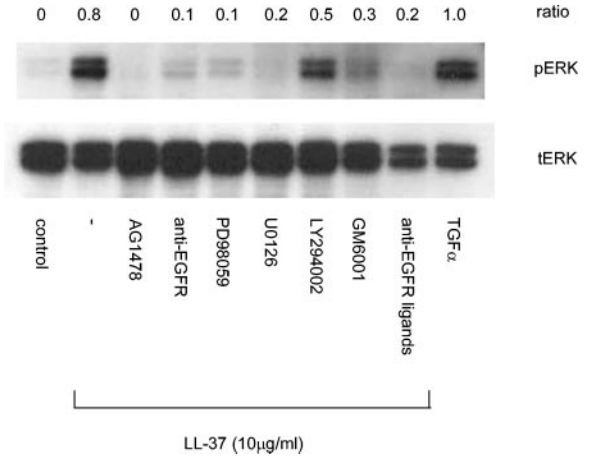

FIGURE 3. Effect of signaling pathway inhibitors on LL-37-induced ERK1/2 activation. NCI-H292 cells were preincubated for 1 h with the following inhibitors before adding 10 μg/ml LL-37: medium alone, the EGFR tyrosine kinase inhibitor AG1478 (1 μM), neutralizing anti-EGFR Abs (2 μg/ml), the MEK inhibitors PD98059 (50 μM) and U0126 (25 μM), the phosphatidylinositol 3-kinase inhibitor LY294002 (10 μM), the metalloproteinase inhibitor GM6001 (25 μM), or a combination of Abs against the EGFR ligands HB-EGF, TGF-α, and amphiregulin (AR; all at 5 μg/ml). As a control, cells incubated with medium or stimulated with 20 ng/ml TGF-α were used. After 15 min, cellular lysates were collected. Both phosphorylated (pERK) and total ERK1/2 (tERK) levels in cellular lysates were determined by Western blot analysis, and densitometry was performed. The ratio phosphorylated ERK and total ERK for each sample was calculated and shown at the *top* of each lane. Data are representative of three individual experiments.

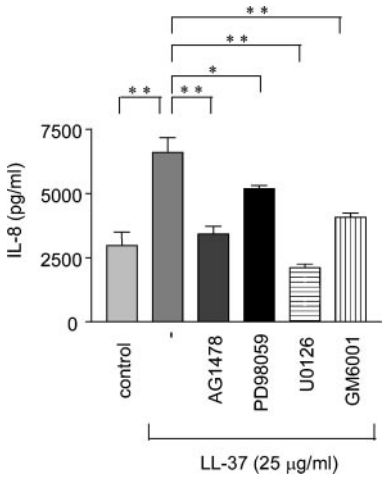

FIGURE 4. Effect of signaling pathway inhibitors on LL-37-induced IL-8 release in PBEC. Cells were preincubated for 1 h with the following inhibitors before adding 25 μg/ml LL-37: medium alone, the EGFR tyrosine kinase inhibitor AG1478 (1 μM), the MEK inhibitors PD98059 (50 μM) and U0126 (25 μM), and the metalloproteinase inhibitor GM6001 (25 μM). As a control, cells were incubated with medium alone. After 6 h, IL-8 levels in cell-free supernatant were determined by IL-8-specific ELISA. Results are expressed as $\pm$ SEM of one representative experiment of three, each performed in triplicate. Statistical analysis was performed using the unpaired Student *t* test; *, $p < 0.05$; **, $p < 0.01$.

6 h, IL-8 release in cell-free supernatants was determined by IL-8-specific ELISA (Fig. 4). The MEK inhibitors U0126 and PD98059 completely inhibited LL-37-induced IL-8 release, suggesting that ERK1/2 is centrally involved in LL-37-induced IL-8 release. The EGFR tyrosine kinase inhibitor AG1478 also blocked LL-37-induced IL-8 release, suggesting a role for EGFR signaling in LL-37-induced IL-8 release. To study the involvement of metalloproteinases in LL-37-induced IL-8 release, the effect of the metalloproteinase inhibitor GM6001 was studied. GM6001 inhibited LL-37-induced IL-8 release, suggesting involvement of metalloproteinases in LL-37-induced IL-8 release.

LL-37-induced activation of p38 and JNK

To study the effect of LL-37 on activation of the MAPKs p38 and JNK, NCI-H292 cells were incubated with various concentrations

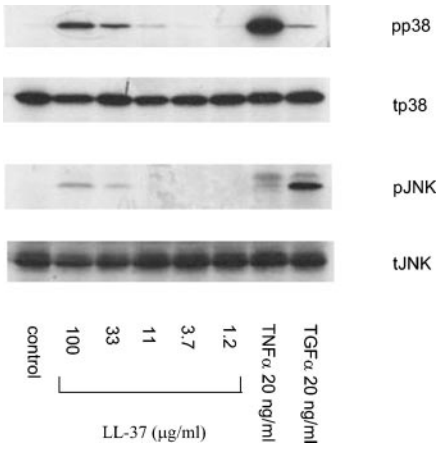

FIGURE 5. Effect of LL-37 on p38 and JNK activation in NCI-H292 cells. Cells were stimulated for 15 min with various concentrations LL-37, 20 ng/ml TGF-α, 20 ng/ml TNF-α, or without stimulus. Both phosphorylated p38 (pp38) and JNK (pJNK) and total p38 (tp38) and JNK (tJNK) levels in cellular lysates were determined using Western blot analysis.

of LL-37, or with TGF-α or TNF-α as a control. Both phosphorylated and total p38 and JNK levels were determined using Western blot analysis. LL-37 was shown to induce a concentration-dependent increase in p38 and JNK activation (Fig. 5). p38 Activation was demonstrated at 11 μg/ml LL-37, whereas JNK activation was demonstrated at a concentration of 33 μg/ml.

Discussion

We have shown that LL-37 activates airway epithelial cells as demonstrated by its ability to activate ERK1/2 and to increase the release of IL-8. This activation requires tyrosine kinase activity of the EGFR, and involves the action of metalloproteinases and EGFR ligands. This indicates that LL-37 activates epithelial cells by transactivation of the EGFR via metalloproteinase-dependent processing of EGFR ligands, as shown in the model depicted in Fig. 6. These results suggest that in addition to displaying antimicrobial and chemotactic activity, LL-37 is involved in innate immunity by activating airway epithelial cells.

LL-37 is not the first antimicrobial peptide that has been found to activate airway epithelial cells. Human neutrophil defensins increase IL-8 release in airway epithelial cells (17) and increase the proliferation of these cells in vitro (21). Also neutrophil-derived serine proteinases and lactoferrin activate epithelial cells (18, 24). Furthermore, a synthetic α helical peptide that shares structural and functional properties with LL-37 was shown to increase expression of selected genes, including various cell cycle inhibitors, in cells of a murine macrophage cell line (25). Finally, a very recent report showed that LL-37 increases chemokine production in mouse macrophages and the mouse lung, in the human lung alveolar epithelial cell line A549, and in whole blood (15). It therefore appears that the neutrophil may use various mediators to control the activity of epithelial and other cell types.

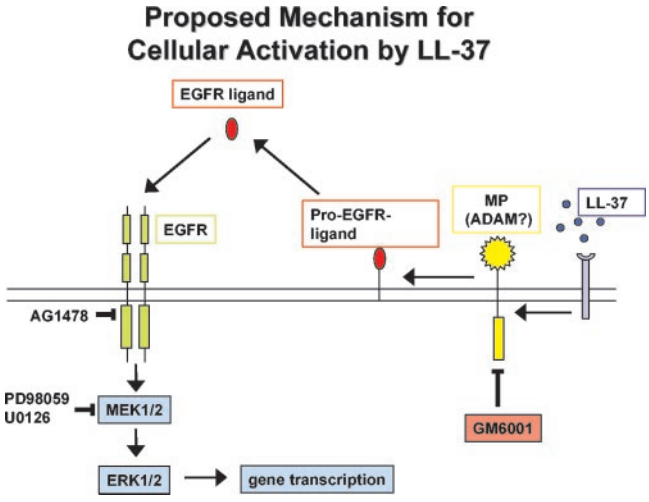

FIGURE 6. Proposed mechanism for cellular activation by LL-37. According to this model, exposure of airway epithelial cells to LL-37 results in activation of a metalloproteinase (MP), possibly a member of the ADAM family. The identity of the structures involved in mediating this metalloproteinase activation remains to be established. The metalloproteinase/ADAM subsequently cleaves membrane-anchored EGFR ligands that activate the EGFR resulting in ERK1/2 activation and gene transcription. Various selective inhibitors used in the present study to delineate this pathway, and their site of action, are shown: the metalloproteinase inhibitor GM6001, the EGFR tyrosine kinase inhibitor AG1478, and the MEK1/2 inhibitors PD98059 and U0126. Neutralizing antiamphiregulin (AR), anti-TGF-α, and anti-HB-EGF Abs and anti-EGFR Abs were used to inhibit the action of EGFR ligands and the EGFR, respectively.

The mechanisms involved in mediating the previously mentioned effects of neutrophil-derived antimicrobial polypeptides on epithelial cells are largely unknown. In the present study, we have partially elucidated the mechanism involved in LL-37-induced epithelial cell activation, by demonstrating the involvement of metalloproteinase activity, EGFR ligands and the EGFR itself in this process. In addition to direct activation of the EGFR by its ligands, various other mechanisms may control EGFR activation. These include ligand-independent activation by, e.g., reactive oxygen intermediates (26–28). Our finding that the antioxidants NAC and apocynin do not affect LL-37-induced ERK1/2 activation suggest that this pathway is not involved in the epithelial cell activation by LL-37. In addition, ligand-dependent transactivation of the EGFR by cleavage of membrane-anchored EGFR ligands has been described. This latter mechanism was found to be used by GPCR as demonstrated by studies such as those by Prenzel et al. (29). These studies showed that GPCR may cause ligand-dependent transactivation of the EGFR via metalloproteinase-mediated cleavage of the membrane-anchored EGFR ligand HB-EGF. Subsequent studies identified members of "a disintegrin and metalloprotease" (ADAM) family to be involved in the GPCR-mediated cleavage of membrane-anchored EGFR ligands (30, 31). ADAMs are membrane-anchored metalloproteinases present on a large variety of cell types that display various functions, including cleavage of cytokines and growth factors and their receptors (reviewed in Ref. 32). ADAM9, ADAM10, and ADAM12 have been shown to be involved in cleavage of HB-EGF (30, 31, 33), whereas ADAM17/TNF-α converting enzyme not only cleaves TNF-α, but is also involved in processing of precursor TGF-α (34). These data suggest that the metalloproteinase involved in LL-37-induced transactivation of EGFR in human bronchial epithelial cells is a member of the ADAM family. The mechanisms underlying ADAM activation and subsequent cleavage of membrane-bound ligands remain largely unknown. A recent study showed that the GPCR ligand bombesin increased ERK1/2 activation in COS7 cells, which was accompanied by complex formation between ADAM10 and HB-EGF (31). Therefore, because ERK1/2 activation by LL-37 takes place within minutes, LL-37 may regulate ADAM-metalloproteinase activation by promoting the association between ADAM-metalloproteinase and its EGFR ligand. The involvement of GPCR and/or G proteins in LL-37-induced transactivation of the EGFR remains to be established. Two studies have demonstrated the GPCR, FPRL1, as a receptor for LL-37. Both LL-37-induced chemotaxis (12) and endothelial proliferation (35) were shown to be mediated via the FPRL1. We studied the effects of a FPRL1-antagonistic peptide (tBoc peptide) on LL-37-induced activation of airway epithelial cells, and did not observe an effect of this peptide on LL-37-induced activation of airway epithelial cells. In addition, fMLP itself did not activate NCI-H292 cells. These data suggest that the FPRL1 is not involved in LL-37-induced activation of airway epithelial cells (data not shown). This conclusion is furthermore supported by our observation that the G protein inhibitor pertussis toxin did not inhibit LL-37-induced ERK1/2 activation. This indicates that GPCR and/or G proteins may not be involved in LL-37-induced epithelial cell activation.

Several recent studies have highlighted the potential importance of transactivation of the EGFR via a metalloproteinase-dependent mechanism in various animal models and clinical situations. PGE_2 activates EGFR and ERK2 mitogenic signaling pathways via metalloproteinase-dependent cleavage of TGF-α and was suggested to play a role in colon cancer growth and gastrointestinal hypertrophy (36). Keratinocyte migration in cutaneous wound healing was

shown to be mediated via cleavage of membrane-anchored HB-EGF by metalloproteinases (37). Furthermore, stimulation of cardiac myocytes with GPCR ligands resulted in shedding of HB-EGF by metalloproteinases and cardiac myocyte hypertrophy, and *in* vivo studies in mice showed that the metalloproteinase-inhibitor KB-R8895 inhibits HB-EGF shedding and cardiac hypertrophy. The metalloproteinase involved in this process was identified as ADAM12 (30).

The ERK/MAPK cascade has been shown to be involved in a large variety of cellular activities, ranging from cell survival and proliferation to expression of proinflammatory cytokines (reviewed in Refs. 38, 39). We demonstrated that LL-37-induced IL-8 production is at least partially mediated via ERK1/2 activation, as shown by the ability of the MEK inhibitors U0126 and PD98059 to inhibit LL-37-induced IL-8 release. In addition to activating ERK1/2, LL-37 was also found to cause phosphorylation of the MAPK p38 and JNK. Furthermore, the EGFR seems to be involved in LL-37-induced IL-8 release because the EGFR tyrosine kinase inhibitor AG1478 inhibited LL-37-induced IL-8 release. The involvement of ERK1/2 activation in IL-8 production has been demonstrated in studies investigating viral (40), IL-17 (41), or Utah Valley particulate matter (42) induced IL-8 release, and the EGFR has been shown to be involved in both ERK1/2 activation (36) and IL-8 release (43). In addition to the involvement of ERK1/2, we also found that metalloproteinase activity and EGFR are involved in LL-37-induced IL-8 release. We observed that both the EGFR tyrosine kinase inhibitor AG1478 and the metalloproteinase inhibitor GM6001 inhibit LL-37-induced IL-8 release. In addition, doxycyclin, an antibiotic reported to inhibit metalloproteinase activity and recently shown to inhibit cigarette smoke-induced mucin gene expression in bronchial epithelial cells (44), was found to inhibit LL-37-induced IL-8 release significantly (data not shown). These results suggest that the LL-37-induced cleavage of membrane-anchored EGFR ligands by metalloproteinases may increase IL-8 release from airway epithelial cells via activation of ERK1/2.

The effects of LL-37 on activation of airway epithelial cells may be relevant in vivo. Human CAP-18 has been shown to be present in neutrophils, and 10^6 neutrophils isolated from peripheral blood were shown to contain 0.627 μg hCAP-18 (1). Skin lesions from patients with psoriasis, a condition associated with increased expression of LL-37 in the skin, were found to contain LL-37 at a median concentration of 304 μM (45). Lung tissue and secretions may also contain substantial concentrations of LL-37. In situ hybridization studies of the conducting airways showed LL-37/hCAP-18 expression in the surface epithelia and in the submucosal glands (3). LL-37 was detected in bronchial alveolar lavage fluid (46), but its concentrations in bronchial alveolar lavage fluid were not determined. Finally, LL-37 was detected in tracheal aspirates of newborns at concentrations of $\sim$20 μg/ml, and LL-37 levels were demonstrated to be significantly increased in infants with pulmonary or systemic infections (47). These data indicate that epithelial cells in the skin and lung may be exposed to high concentrations of LL-37 in vivo. Based on these observations and our finding that LL-37 activates airway epithelial cells at concentrations $>$10 μg/ml, LL-37-induced epithelial cell activation may occur in vivo. Functionally, increased local release of LL-37 from neutrophils or epithelial cells following, for example, bacterial infection, may aid in killing microorganisms, but may also stimulate recruitment of other inflammatory cells. This recruitment may result from direct chemotactic activity of LL-37 or be mediated by its ability to stimulate chemokine release from epithelial cells. Previous data from our laboratory showed activation of airway epithelial cells by the antimicrobial and chemotactic neutrophil defensins (17, 21). This suggests that in addition to antimicrobial activity, antimicrobial peptides may communicate with host cells to regulate the immune reaction.

In summary, LL-37 was shown to activate airway epithelial cells as demonstrated by ERK1/2 activation and increased IL-8 release. Furthermore, this activation was shown to involve EGFR activation, metalloproteinase activity, and EGFR ligands. In addition to antimicrobial and chemotactic activity, LL-37 might play an important role in innate immunity by activating airway epithelial cells, suggesting that LL-37 plays a regulatory function in the antimicrobial and inflammatory response of airway epithelial cells. These observations and recent findings that show an association between LL-37 expression and human disease, suggest that LL-37 may form a target for new antimicrobial and anti-inflammatory treatment strategies for skin and lung disorders.

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