Neutrophil Defensins Enhance Lung Epithelial Wound Closure and Mucin Gene Expression In Vitro

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Human airways are frequently exposed to potentially harmful agents that cause tissue injury. Upon such injury, a repair process is initiated that comprises cell migration, proliferation, and differentiation. We have previously shown that human neutrophil defensins (human neutrophil peptides 1–3 [HNP1–3]) induce airway epithelial cell proliferation. Because of the role of cell proliferation in epithelial wound repair, we investigated the effect of HNP1–3 on airway epithelial wound closure and mucin gene expression in vitro. Using NCI-H292 airway epithelial cell cultures, we demonstrated that HNP1–3 cause a dose- and time-dependent increase of wound closure as well as increased cell migration. Furthermore, HNP1–3 caused a biphasic activation of the mitogen-activated protein kinase extracellular-regulated kinase 1 and 2 (ERK1/2). Both the effects of HNP1–3 on wound closure and ERK1/2 activation were blocked by specific inhibitors of the mitogen-activated protein kinase kinase MEK, whereas inhibitors of epidermal growth factor receptor tyrosine kinase, phosphatidylinositol 3-kinase, and Src did block defensin-enhanced wound closure but not ERK1/2 activation. Finally, HNP1–3 increased mRNA encoding the mucins MUC5B and MUC5AC, suggesting a role for defensins in mucous cell differentiation. These results indicate that neutrophil defensins increase epithelial wound repair in vitro, which involves migration and proliferation, and mucin production. Neutrophil defensin-enhanced wound repair appears to require epidermal growth factor receptor activation and downstream signaling pathways.

The integrity of the airway epithelium is an important prerequisite for an efficient host defense system. Epithelial injury, as observed in various inflammatory lung diseases (1), is followed by a repair process that serves to restore epithelial integrity. During this repair process inflammatory cells such as neutrophils are recruited to the site of injury, and these cells may contribute to host defense, injury, and the repair process itself (2–5). The functional effects of neutrophils during inflammation and subsequent wound repair are dependent on the extent of their activation and the products that are released. Neutrophil defensins constitute one group of products released from stimulated neutrophils that kill microorganisms as well as host cells at the site of inflammation, but recent data suggest that they may also contribute to epithelial repair (6, 7).

Defensins are small, arginine-rich cationic peptides that contain six highly conserved cysteine residues, forming a compact looped structure. Depending on the positions of the cysteine residues that participate in disulphide linkages, defensins are divided in α- and β-defensin families (8). The defensins that are released by stimulated neutrophils (called human neutrophil peptides 1–4 [HNP1–4]) are members of the α-defensin subfamily that are stored in large amounts in the azurophilic granules (9). Whereas they are mainly present in neutrophils (hence the name neutrophil defensins), their production has also been detected in certain lymphocyte subsets (10). Neutrophil defensins, originally identified as broad-spectrum antimicrobial peptides, have more recently been implicated in the regulation of inflammatory and immunologic processes, including complement activation; cytotoxicity; chemotaxis of immature dendritic cells, T cells, and monocytes; induction of epithelial cytokine release; and enhancement of humoral and cellular immune responses (reviewed in Ref. 11). Finally, a possible role of defensins in wound repair is suggested by results from in vitro studies showing that neutrophil defensins enhance proliferation of human lung epithelial (7) and renal carcinoma cells (12), and murine fibroblasts and retinal epithelial cells (6).

Epithelial injury is normally followed by a complex repair process that comprises subsequent epithelial migration, proliferation, and differentiation (13). This process is mediated predominantly via growth factors and their receptors, including the epidermal growth factor (EGF) receptor that plays a pivotal role in epithelial repair (14). Activation of the EGF receptor is followed by stimulation of various signaling pathways, including the mitogen-activated protein kinases (MAPKs) p38, c-jun N-terminal kinase (JNK), extracellular–regulated kinase 1 and 2 (ERK1/2), and big MAPK (BMK, ERK5), that subsequently result in activation of various transcription factors (e.g., activator protein-1 and nuclear factor-kB) and gene transcription. In particular, ERK1/2 and ERK5 have been shown to be required for epithelial cell growth (15).

Although neutrophil defensins display growth-promoting activities toward cells of different origin, their role in wound repair and the mechanism underlying this activity...
have not been clarified. The aim of this study was primarily to delineate the role of neutrophil defensins in airway epithelial wound repair by assessment of their effect on epithelial wound closure. In addition, we determined the effect of defensins on epithelial mucin gene expression, as mucin expression is a feature associated with differentiation of mucus epithelial cells. Furthermore, we aimed to unravel the mechanism underlying these activities. Because of the importance of EGF receptor signaling in epithelial wound repair and the observation that neutrophil defensin–mediated lung epithelial cell proliferation is inhibited by a specific inhibitor of the ERK1/2 signaling pathway (7), we focused on the involvement of this pathway.

Material and Methods

Defensin Isolation

Human neutrophil defensins were isolated from neutrophil granules as a mixture of HNP-1, -2, and -3 as previously described (7, 16).

Cell Culture

Cells from the muco-epidermoid lung carcinoma cell line NCI-H292 (ATCC, Manassas, VA) were cultured at 37°C in a 5% CO₂–humidified atmosphere in RPMI 1640 medium (Gibco, Grand Island, NY), supplemented with 2 mM L-glutamine, 20 μg/ml penicillin, 20 μg/ml streptomycin (Bio Wittaker, Walkersville, MD), and 10% heat-inactivated fetal calf serum (FCS; Gibco).

Subcultures of primary bronchial epithelial cells (PBEC) were obtained from resected lung tissue, derived from patients that underwent a pneumectomy or lobectomy for lung cancer at the Leiden University Medical Center. Bronchial epithelial cells were isolated essentially as previously described (17) using enzymatic digestion with 0.1% (wt/vol) proteinase XIV (Sigma Aldrich, St. Louis, MO). Epithelial cells were gently stripped, washed in keratinocyte serum-free medium (Gibco), and incubated submerged in keratinocyte serum-free medium, supplemented with 10 ng/ml EGF, 2% (vol/vol) bovine pituitary extract (Gibco), 30 μg/ml cyproxin (Bayer, Leverkusen, Germany). PBEC were cultured in tissue culture plates precoated with 10 μg/ml fibronectin (isolated from human plasma), 30 μg/ml Vitrogen (Cohesion Technologies Inc., Palo Alto, CA), and 10 μg/ml bovine serum albumin (Sigma Aldrich). For the experiments, cells from passage two were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F12 (1:1) medium (Gibco) supplemented with 10 ng/ml EGF, 2% (vol/vol) Ultroser G (Gibco), 1 μM isoproterenol, 1 μM insulin (Sigma Aldrich), 1 μM hydrocortisone (Sigma Aldrich), 2 mM L-glutamine, 1 mM Heps (Gibco), 20 μM penicillin, and 20 μg/ml streptomycin.

Wound Repair Model

NCI-H292 cells were cultured to confluence in 6-well tissue culture plates. After overnight serum deprivation, three circular wounds (3 mm in diameter) were scraped in each well using a sharpened silicone tube attached to a Pasteur pipette and a microscope. After washing with PBS and allowing the cultures to recover for 1 h in serum-free medium, the wounded monolayers were incubated in serum-free medium alone or supplemented with transforming growth factor (TGF)-α (Sigma Aldrich), HNP1-3, or FCS at concentrations as indicated. To study the role of selected signaling pathways, wounded monolayers were incubated with AG1478 (1 μM; Calbiochem, La Jolla, CA), PD98059 (50 μM; Alexis, Nottingham, UK), U0126 (25 μM; Promega, Madison, WI), LY294002 (10 μM; Stratagene, La Jolla, CA) or PPI (25 μM; Alexis) 1 h before addition of HNP1-3 or TGF-α. Possible cytotoxic effects of the inhibitors at concentrations used in these experiments were excluded by Trypan Blue assays (data not shown). Images were collected using a digital camera and analyzed using Axiovision software (Carl Zeiss Vision, München-Hallbermoos, Germany) at various time points by determining the percentage remaining wound area as compared with the time point of stimulation (t = 0).

Cell Migration Assay

Cell migration was assessed using a modified Boyden chamber assay. NCI-H292 cells were cultured overnight in serum-free medium and subsequently detached using 5 mM EDTA. Next, the cells were added to the upper compartment of Transwell filters (8 μm pore-size; Costar, Cambridge, MA) in serum-free medium. Following addition of medium alone or supplemented with 8 μg/ml HNP1-3 or 10% FCS to the lower, the upper, or both compartments, the cells were allowed to migrate for 6 h. After removal of the cells on the topside of the filters and fixation with 4% paraformaldehyde for 20 min, cells were stained with hematoxylin and migrated cells were counted in three high-power fields (×400 magnification).

MAPK Activation Assays

For the detection of native and activated MAPKs in NCI-H292, cells were cultured to 80–90% confluence. After overnight starvation in serum-free RPMI medium, the cells were incubated with HNP1-3, TGF-α or tumor necrosis factor (TNF)-α (PeproTech, Rocky Hill, NJ) in serum-free medium. The effects of the various inhibitors of the EGF receptor signaling pathway were assessed by incubating NCI-H292 cells with these inhibitors using concentrations as described for wound closure experiments. In addition, the involvement of ligand binding to the EGF receptor in ERK1/2 activation was evaluated by preincubating cells with 2 μg/ml mouse monoclonal anti-EGF receptor antibodies (Ab-10; NeoMarkers, Fremont, CA) for 1 h before addition of 8 μg/ml HNP1-3 or 20 ng/ml TGF-α and subsequent incubation for 15 min or 1 h. Further experiments with PBEC cells were started and subsequently incubated with various stimuli in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium supplemented with 2 mM L-glutamine, 1 mM Heps, 200 μM penicillin, and 200 μg/ml streptomycin.

Next, the cells were washed and lysed with sample buffer (for p38 and JNK detection: 0.2 M Tris-HCl pH 6.8, 4% [wt/vol] SDS, 16% [vol/vol] glycerol, 4% [vol/vol] 2-mercaptoethanol, 0.003% [wt/vol] bromophenol blue), or lysis buffer (for ERK1/2 detection: 0.5% [vol/vol] Triton X-100, 0.1 M Tris-HCl pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM NaVO₃, complete protease inhibitor cocktail [Roche, Basel, Switzerland]) on ice. Eight to fifteen micrograms total protein of each sample was separated on a 10% glycine-based gel using the Mini-protean 3 (Biorad, Hercules, CA) SDS-PAGE system. Separated proteins were transferred to polyvinylidene difluoride membranes using the Mini-transblot system (Biorad) and used for detection of native and activated MAPKs. Therefore, the membranes were pre-incubated with 0.05% Tween-20 in PBS (PBST) containing 0.5% (wt/vol) casein or 5% (wt/vol) nonfat dry milk (ELK; Campina, Zoetermeer, The Netherlands) for at least 1 h, followed by incubation with anti-phospho-ERK1/2, -p38, or -JNK rabbit polyclonal antibodies (all from New England Biolabs, Beverly, MA) overnight at 4°C. After incubation with horseradish peroxidase–conjugated goat anti-rabbit (for ERK1/2; BD Transduction Laboratories, Franklin
EFG Receptor Immunoprecipitation Assays

For the detection of phosphorylated EGF receptor, NCI-H292 cells were cultured to near confluence, serum-deprived overnight, and subsequently incubated with medium alone or supplemented with 8 μg/ml HNP1–3 or 20 ng/ml TGF-α for 15 min or 10 h. After washing in ice-cold buffer (1 mM CaCl₂, 1 mM MgCl₂ in PBS), the cells were lysed in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.8, 1% [vol/vol] Triton-X 100, 50 mM NaF, 5 mM PMSF, 1 mM Na₃VO₄, complete protease inhibitor cocktail) for 10 min. The EGF receptor was immunoprecipitated by incubating the cell lysates with anti-EGF receptor monoclonal antibodies (BD Transduction Laboratories) and subsequent binding to protein A Sepharose 4 fast flow beads (Pharmacia Amersham) overnight at 4°C. Immunoprecipitated native and phosphorylated EGF receptor were detected by Western blot analysis, using anti-EGF receptor monoclonal antibodies (BD Transduction Laboratories), or horseradish peroxidase–conjugated anti-phosphotyrosine monoclonal antibodies (PY99; Santa Cruz Biotechnologies, Santa Cruz, CA), respectively.

Cell Proliferation Assay

Cell proliferation was assessed by 5-bromo-2-deoxyuridin (BrdU) incorporation. Confluent NCI-H292 cell monolayers were wounded as described and incubated with various stimuli. Following addition of 10 mM BrdU (Sigma Aldrich), the cells were incubated for a further 24 h and fixed in 70% ethanol (vol/vol) for at least 1 h. BrdU incorporation was assessed by immunocytochemistry as previously described (7). BrdU-positive nuclei were enumerated within a distance of 10 cells from the wound edge and in intact areas. At least 300 nuclei were counted for each condition.

Reverse Transcriptase–Polymerase Chain Reaction for MUC5B and MUC5AC

For analysis of mucin mRNA expression, PBEC and NCI-H292 cells were cultured and starved as described for Western blot experiments. Following growth factor deprivation, cells were cultured and starved as described for Western blot experiments. Following growth factor deprivation, cells were cultured to near confluence, serum-deprived overnight, and subsequently incubated with medium alone or supplemented with 8 μg/ml HNP1–3 or 20 ng/ml TGF-α, or 8 or 50 μg/ml HNP1–3. RNA was extracted using TRIzol Reagent (Gibco) and reverse transcribed using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (RT; Gibco) as recommended by manufacturers. Equal loading of cDNA was evaluated using the housekeeping gene β-actin. Amplification by polymerase chain reaction (PCR) was performed using primers for β-actin: 5’-CTA CAA TGA TGA GCT GCC TGT GG and 5’-AAG GAA GGC TGG TGG AAG ATG GC; MUC5AC: 5’-ATT TTT TCC CCA CTC CTG ATG and 5’-AAG ACA ACC CAC CCT TCC AAC CC; MUC5B: 5’-CAC ATC CAC CCT TTC AAC and 5’-GCC TCA TTT CGT CTC CTG. PCR amplification was performed in a final volume of 25 μl PCR buffer (Eurogentech, Seraing, Belgium) containing MgCl₂ (2 mM for β-actin, 2.5 mM for MUC5B and MUC5AC), 1 μM of each primer, 0.2 mM dNTP (Invitrogen, Carlbad, CA) and 0.04 U/μl Taq polymerase (Eurogentech). Amplified products were loaded on a 1% agarose gel.

Statistical Analysis

Results are expressed as mean ± SEM. Data obtained from three to four separate experiments were analyzed for statistical difference by the Student’s t test for paired samples and differences were considered significant when P < 0.05.

Results

Defensin-Enhanced Epithelial Wound Closure

To investigate the effects of neutrophil defensins on epithelial wound closure, mechanically wounded NCI-H292 cell monolayers were incubated with medium alone (negative control) or with HNP1–3, TGF-α, or FCS, and the wound area was measured at various time points. Wounded monolayers incubated with 8 μg/ml HNP1–3 showed a slight delay in wound closure in the first 18 h of incubation, followed by a subsequent enhancement in wound closure resulting in a significant increase after 48 and 72 h as compared with monolayers incubated with medium alone (Figure 1A).
TGF-α– and FCS-treated monolayers, 80–85% of the wound area was closed after 48 h and 95–100% after 72 h. To study the effect of HNP1–3 on wound closure, NCI-H292 cell monolayers were incubated with various HNP1–3 concentrations and residual wound area was determined at various time points (Figure 1B). Maximal increase of wound closure was observed at concentrations of 4–10 μg/ml after incubation for 48 or 72 h, whereas at a concentration of 50 μg/ml wound closure was delayed.

Defensin-Induced MAPK Activation
To assess the involvement of MAPK signaling pathways in defensin-enhanced epithelial repair, cell lysates prepared from stimulated NCI-H292 cells were analyzed for activated ERK1/2, p38, and JNK by Western blot analysis. In cell lysates obtained from cells stimulated for 15 min with 8 μg/ml HNP1–3, 20 ng/ml TGF-α, or 25 ng/ml TNF-α, a marked increase in activation of ERK1/2, p38, and JNK was observed (Figure 2A). To study the kinetics of defensin-induced ERK1/2 activation, NCI-H292 cells were incubated with 8 or 50 μg/ml HNP1–3 or 20 ng/ml TGF-α for various time periods (Figure 2B). Like TGF-α, HNP1–3 showed a biphasic activation of ERK1/2. Both HNP1–3 concentrations resulted in activation of ERK1/2 after 5 min which persisted up to 1 h, whereas TGF-α already resulted in activation after 1 min that was maintained for 2 h. The second activation phase was observed after 10 h of incubation with HNP1–3 and TGF-α.

Activation of ERK1/2 by HNP1–3 was also analyzed in PBEC (Figure 2C). The results showed that HNP1–3 and TGFα also induced activation of ERK1/2 in PBEC after 15 min.

Involvement of MAPK Signaling in Defensin-Induced ERK1/2 Activation
To further delineate the involvement of the EGF receptor signaling pathway, NCI-H292 cells were incubated with inhibitors of EGF receptor tyrosine kinase (AG1478), MEK (PD98059 and U0126), PI-3K (LY294002), and Src (PP1) 1 h before addition of HNP1–3 or TGF-α for 15 min (Figure 3A). Western blot analysis of activated ERK1/2 demonstrated that MEK inhibitors completely (U0126) or partially (PD98059) blocked neutrophil defensin–induced ERK1/2 activation. Neutrophil defensin–induced ERK1/2 activation was not blocked by AG1478, in contrast to activation induced by TGF-α. The PI-3K and Src inhibitors did not affect neutrophil defensin– and TGF-α–induced ERK1/2 activation, indicating that these kinases are not involved in the signaling pathway leading to ERK1/2 activation.

Involvement of the EGF receptor in defensin-induced ERK1/2 activation was also assessed by studying the effect of blocking antibodies directed against the EGF receptor on the activation of ERK1/2 (Figure 3B). These antibodies did not affect the early phase (after 15 min) phosphorylation of ERK1/2 induced by defensins, whereas they did block the effect of TGF-α at 2 ng/ml. These results are in line with the results observed when using AG1478. In contrast to what was observed at 15 min, late phase activation (after 10 h) by both defensins and TGF-α was blocked by anti-EGF receptor antibodies. In addition, we studied the effect of neutrophil defensins on EGF receptor activation (Figure 4). No phosphorylation of the EGF receptor could be detected after incubation with 8 μg/ml HNP1–3 for 15 min, whereas after 10 h of incubation a weak but reproducible phosphorylation was observed.
phosphorylation was observed. Incubation with TGF-α resulted in a clear activation of the EGF receptor at both time points.

Involvement of EGF Receptor and MAPK Signaling in Defensin-Enhanced Wound Closure

To investigate whether the observed induction of epithelial wound closure by neutrophil defensins is mediated via the EGF receptor signaling pathway, the effect of the previously described inhibitors on neutrophil defensin- and TGF-α-enhanced wound closure was assessed (Figure 5). All individual inhibitors were shown to block the basal wound closure. In line with the results on ERK1/2 activation, PD98059 and U0126 significantly blocked both neutrophil defensin- and TGF-α-enhanced wound closure. Interestingly, AG1478 completely blocked neutrophil defensin–enhanced wound closure, although it did not affect ERK1/2 activation (Figure 3). PI-3K and Src have been implicated in mediating cell migration and thereby may also contribute to wound repair (18, 19). Inhibitors of these kinases significantly blocked wound closure in neutrophil defensin– and TGF-α–stimulated cultures, suggesting that PI-3K and Src are required for neutrophil defensin–enhanced wound closure.

Effect of Neutrophil Defensins on NCI-H292 Cell Migration

To examine the involvement of cell migration in defensin–enhanced epithelial wound repair, the effect of neutrophil defensins on cell migration of NCI-H292 cells was assessed (Table 1). Neutrophil defensins at 8 μg/ml caused a significant increase in cell migration as compared with medium alone (34.1 ± 2.8 versus 11.9 ± 0.9 cells per high-power field, respectively). Control stimulation with FCS also resulted in a significant increase (27.9 ± 2.6) in cell migration. When these stimuli were added to the upper compartment, or to both compartments, no increase in migration was observed. These results indicate that the activity of HNP1–3 is chemotactic rather than chemokinetic.

BrdU Incorporation

Previous studies have demonstrated that neutrophil defensins may induce proliferation of various cell types, including human airway epithelial cells. We therefore used the wound repair model in NCI-H292 cells to investigate the effect of neutrophil defensins on proliferation of cells located within the wounded area after (partial) wound closure, and of cells in intact epithelial layers (Table 2). In all cultures, cell proliferation was higher in epithelial cells present in the original wound area as compared with cells in noninjured areas of the same culture. HNP1–3, FCS, and TGF-α stimulated proliferation of epithelial cells; however, this was not observed in the first 24 h in the wound area, and also not in the last 24 h (48–72 h) in intact areas.

Mucin mRNA Expression

Effects of neutrophil defensins on mucin mRNA expression were examined in PBEC and NCI-H292 cells (Figure 6). In NCI-H292 cells, increased MUCSB and MUC5AC mRNA expression was observed with 8 μg/ml HNP1–3 already after 5 h, that was maintained up to 24 h (MUC5AC) or 48 h (MUCSB). Higher HNP1–3 concentrations (50 μg/ml) did not affect MUCSB in NCI-H292 cells, whereas TGF-α increased expression of MUCSB only after 24 h. PBEC incubated with 8 or 50 μg/ml HNP1–3 showed an increase in MUCSB and MUC5AC mRNA expression already after 5 h, and this was maintained up to 48 h. Cells that were incubated with TGF-α showed an increase in MUCSB mRNA expression after 24 h, but not after 5 or 48 h. Elevated MUC5AC mRNA in PBEC with TGF-α was observed after stimulation for 5 or 24 h.

Figure 3. (A) Effect of inhibitors of the EGF receptor signaling pathway on neutrophil defensin–induced ERK1/2 activation. NCI-H292 cells were preincubated with AG1478 (AG; 1 μM), PD98059 (PD; 50 μM), U0126 (U; 25 μM), LY294002 (LY; 10 μM), or PP1 (PP; 25 μM) for 1 h before addition of 8 μg/ml HNP1–3 (HNP) or 20 ng/ml TGF-α (TGF 20) for 15 min. (B) Effect of EGF receptor blocking antibodies on early and late phase ERK1/2 activation. NCI-H292 cells were preincubated with AG1478 (AG; 1 μM), PD98059 (PD; 50 μM), U0126 (U; 25 μM), LY294002 (LY; 10 μM), or PP1 (PP; 25 μM) for 1 h before addition of 8 μg/ml HNP1–3 (HNP) or 20 ng/ml TGF-α (TGF 20) for 15 min or 10 h. Phosphorylated (pERK1/2) and total ERK1/2 was determined by Western blot. Data in A and B are from one experiment (similar results were obtained in another separate experiment).

Figure 4. Effect of neutrophil defensins on EGF receptor activation. NCI-H292 cells were incubated with medium alone or supplemented with 20 ng/ml TGF-α, 8 μg/ml HNP1–3 (HNP 8), or 50 μg/ml HNP1–3 (HNP 50) for 15 min or 10 h. After immunoprecipitation of the EGF receptor, phosphorylated (pEGFR) and total EGF receptor was detected by Western blot. Data are from one experiment (similar results were obtained in another separate experiment).
Figure 5. Effect of inhibitors of the EGF receptor signaling pathway on neutrophil defensin–induced wound closure of NCI-H292 monolayers. Mechanically wounded NCI-H292 cell monolayers were preincubated with AG1478 (1 μM), PD98059 (50 μM), U0126 (25 μM), LY294002 (10 μM), or PP1 (25 μM) 1 h before addition of 8 μg/ml HNP1–3 or 20 ng/ml TGF-α. Data are mean ± SEM of three separate experiments. Closed triangles, medium; closed circles, TGF-α; closed squares, HNP1–3; open triangles, inhibitor; open circles, inhibitor + TGF-α; open squares, inhibitor + HNP1–3.

Discussion

The results demonstrate that neutrophil defensins induce a dose- and time-dependent enhancement of airway epithelial wound closure. In addition, neutrophil defensins were shown to cause activation of ERK1/2, p38, JNK, and the EGF receptor. Inhibitors of the MAPK kinase MEK blocked defensin-induced ERK1/2 activation, whereas EGF receptor–blocking antibodies only inhibited late phase activation of ERK1/2. The involvement of these signaling molecules in defensin-enhanced wound closure was demonstrated by the blocking effect of inhibitors of MEK and EGF receptor tyrosine kinase on this process. The enhancement of wound closure by neutrophil defensins involved cell migration and cell proliferation. Finally, neutrophil defensins increase airway epithelial mucin expression, as shown by mRNA expression of the mucins MUC5B and MUC5AC.

In previous studies, we and others demonstrated that neutrophil defensins induce proliferation of human airway epithelial (7) and renal carcinoma cells (12) and murine fibroblasts and retinal epithelial cells (6), possibly allowing neutrophil defensins to play a role in tissue repair. The tissue repair process is complex and requires migration of cells surrounding the injury before cell proliferation and differentiation (13). Using mechanically wounded NCI-H292 cell monolayers, we demonstrated that neutrophil defensins enhance wound closure at the same range of concentrations that were described to enhance airway epithelial cell proliferation (7). Addition of higher concentrations (> 50 μg/ml) resulted in a delay of wound closure. This delay is most likely due to cytotoxic effects of neutrophil defensins and is in line with previous studies showing that neutrophil defensins induce cytotoxicity in airway epithelial cells at these concentrations (20). The effects of neutrophil defensins on wound closure were time-dependent, and maximal closure rate was observed in the time period between 24 and 72 h. Interestingly, the rate of wound closure in the early phase (first 18 h) after addition of neutrophil defensins was lower than that observed with TGF-α. This may be due to an indirect effect of defensins on EGF receptor activation, and is in line with the observed activation of the EGF receptor at a later phase (10 h), but not in the early phase (15 min).

Various studies have shown that growth factors and their receptors play a pivotal role in airway epithelial repair processes. Amongst these growth factor receptors, the EGF receptor has been shown to modulate epithelial cell migration and proliferation (13). Upon activation of this receptor by its ligands (e.g., EGF, TGF-α), various signaling pathways are activated, such as the MAPK ERK1/2, p38, and JNK signaling pathways. Although the latter two MAPKs may be

Table 1

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<th>Lower Compartments</th>
<th>Upper Compartment</th>
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<tr>
<td></td>
<td>Medium</td>
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<tr>
<td>Medium</td>
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<td>HNP1–3</td>
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<td>FCS</td>
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Definition of abbreviations: FCS, fetal calf serum; HNP, human neutrophil peptide.

NCI-H292 cell migration was assessed by modified Boyden chamber assays after addition of medium alone or supplemented with 8 μg/ml HNP1–3 or 10% FCS to the lower, upper, or both compartments. Data are expressed as mean number of migrated cells per high-power field ± SEM of three independent experiments.

\(^a\) P < 0.05 versus serum-free medium in both compartments.

\(^b\) P < 0.01 versus serum-free medium in upper and HNP1–3 in lower compartment.
activated by growth factors, in general they are activated by stress-induced signals. The functional consequence of their activation may vary depending on the stimulus and the cell type, and may comprise induction of inflammatory cytokines (21), growth arrest, and apoptosis (22). The ERK1/2 signaling pathway is considered to be the most important in the induction of epithelial cell growth (23, 24). This pathway involves the subsequent activation of Ras, Raf, MEK1/2, and ERK1/2. Activated ERK1/2 are then translocated to the nucleus, where they activate various transcription factors, resulting in transcription of various genes involved in cell growth. The importance of this pathway in epithelial cell growth, and the observation that the MEK inhibitor U0126 blocked defensin-induced airway epithelial cell proliferation (7), suggests a role for ERK1/2 signaling in defensin-induced airway epithelial wound closure. Time-kinetics experiments showed a biphasic activation of ERK1/2 in NCI-H292 cells: an early activation occurred already after 5 min and persisted for approximately 1 h, and a late activation phase after 10 h. This biphasic activation of ERK1/2 is comparable to that observed with TGF-α and has also been described in vascular smooth muscle cells (25) and hepatocytes (26) stimulated with cytokines or growth factors. In previous studies performed in hamster fibroblasts it was demonstrated that thrombin, which caused an early- and a late-phase activation of ERK1/2, was able to induce cell proliferation, whereas substances (e.g., serotonin, carbachol) that only induced an early phase activation did not (27, 28). These studies suggest that the observed late phase activation of ERK1/2 may be involved in the enhancement of wound closure by neutrophil defensins. This is further supported by the observation that neutrophil defensins fail to enhance EGF receptor activation in the early phase although they do induce late phase activation. Specific inhibitors of the ERK1/2 signaling pathway blocked defensin-induced ERK1/2 activation and wound closure, confirming the requirement of this pathway for neutrophil defensin–enhanced wound closure. These inhibitors also blocked basal wound closure of NCI-H292 monolayers incubated with medium alone. This is probably the result of release of endogenous EGF receptor ligands by the epithelial cells surrounding the wound area, as previously demonstrated in a scrape wounding model (14). Interestingly, the EGF receptor tyrosine kinase inhibitor AG1478 did not affect defensin-induced ERK1/2 activation, although it did block defensin-enhanced wound closure. A possible explanation for this discrepancy may be that neutrophil defensins induce EGF receptor–independent ERK1/2 activation, which may subsequently lead to EGF receptor activation and wound closure. This is possibly mediated by defensin-induced shedding of growth factors, because shedding of the EGF receptor ligands TGF-α and HB-EGF has been shown to be regulated via ERK1/2 and other MAPKs (29, 30). The possible indirect activation of the EGF receptor via ERK1/2 activation is in line with our observation that early ERK1/2 activation is not inhibited by anti-EGF receptor antibodies, whereas late-phase activation is. Our studies on EGF receptor and MAPK phosphorylation were performed using near-confluent cultures, in which spontaneous release of EGF receptor ligands is lower than in the wounded cultures (14). Therefore, in

### Table 2

**Induction of cell proliferation in wounded NCI-H292 cell monolayers**

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>% BrdU-Positive Nuclei*</th>
<th>0–24 h</th>
<th>24–48 h</th>
<th>48–72 h</th>
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<tr>
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<td>HNP1–3</td>
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<td>TGF-α</td>
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<td>54.4 ± 0.8</td>
<td>32.6 ± 1.1</td>
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</table>

Definition of abbreviations: FCS, fetal calf serum; HNP, human neutrophil peptide; TGF-α, transforming growth factor-α.

* Percentage BrdU-positive nuclei within a distance of 10 cells from the wound edge (i.e., within the original wound area) and in intact areas. Data are mean ± SEM of three independent experiments.

P < 0.05 versus serum-free medium-treated cells.

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**Figure 6.** Induction of MUC5B and MUC5AC mRNA expression in NCI-H292 cells and PBEC by neutrophil defensins. Cells were cultured in medium alone, or supplemented with 20 ng/ml TGF-α, 8 μg/ml HNP1–3 (HNP 8), or 50 μg/ml HNP1–3 (HNP 50) for 5, 24, or 48 h. At these time points, RNA was isolated and mRNA expression of MUC5B, MUC5AC, and β-actin was determined by RT-PCR. Data are from one experiment (similar results were obtained in another separate experiment).
the wound closure assays defensins may act by increasing wounding-induced EGF receptor phosphorylation. Despite this difference, it should be noted that defensins increase cell proliferation both in near-confluent cultures (7) and in cells near the wound area (this study), indicating a similar response to defensins.

Our data suggest that neutrophil defensin–enhanced wound closure may require activation of the EGF receptor, whereas the immediate ERK1/2 activation following addition of defensins (this study) and defensin-induced cell proliferation (7) may be EGF receptor–independent. How neutrophil defensins activate epithelial cells is not known. Human β-defensins have been shown to interact with dendritic cells through the chemokine receptor CCR6 (31) and Toll-like receptor-4 (32), whereas as yet unidentified G-protein–coupled receptors may be involved in the chemotactic activity of neutrophil defensins on T- and dendritic cells (33). The potential involvement of G-protein–coupled receptors in defensin-induced signaling as observed in the present study is of particular interest, as these receptors have been shown to transactivate the EGF receptor via metalloproteinase-mediated shedding of growth factors (34). A recent study demonstrated the involvement of another membrane protein in the effects of defensins on cellular behavior. In this study, neutrophil defensins were found to inhibit smooth muscle contraction via binding to the low-density lipoprotein receptor–related protein/α2-macroglobulin receptor (35). At present it is unknown whether defensins act as classical ligands for cellular receptors, or as recently suggested (36) may mediate their effects by a mechanism that is independent of a ligand–receptor interaction. The nature of the membrane structures involved in mediating the effects of neutrophil defensins on epithelial cell activity, as observed in the present study, remains to be established.

We have shown that neutrophil defensins induce a localized increase in cell proliferation in wounded areas, confirming the importance of cell proliferation in the defensin-enhanced wound closure. Interestingly, cell proliferation was not increased in the wounds in the first 24 h after stimulation with HNP1–3, FCS, or TGF-α, suggesting that migration of epithelial cells is the predominant mechanism of wound closure in the first phase of the process preceding cell proliferation. The involvement of cell migration in defensin-enhanced wound closure is further supported by the observation that neutrophil defensins increase NCI-H292 cell migration by acting as a chemoattractant for these cells. Two EGF receptor–mediated signaling pathways, the ERK1/2 and PI-3K pathways, have been shown to be implicated in cell migration (18). We assessed a possible involvement of the PI-3K signaling pathway in defensin-enhanced wound closure using specific PI-3K and Src inhibitors, and demonstrated that these inhibitors block defensin-enhanced wound closure. These results suggest that the ability of neutrophil defensins to increase epithelial cell migration and proliferation contributes to the observed increase in airway epithelial wound closure, and that this requires activation of PI-3K and ERK1/2 signaling pathways.

The final step in epithelial repair is cell differentiation. A characteristic of differentiated airway epithelium is that a subpopulation of the cells produce and secrete mucins such as the glycoproteins MUC5B and MUC5AC. Our studies show increased mRNA expression of MUC5B and MUC5AC in neutrophil defensin–treated airway epithelial cells. Whether this indeed indicates that defensins reconstitute an area of epithelial injury with fully differentiated epithelial cells remains to be determined, because demonstrating an increase in mucin mRNA does not suffice to conclude that neutrophil defensins cause goblet cell differentiation. Other neutrophil products have been previously shown to increase epithelial mucin expression. Takeyama and coworkers (4, 37) demonstrated that MUC5AC expression induced by oxidants or neutrophil supernatants in NCI-H292 cells is EGF receptor–dependent and antioxidant-sensitive. In addition, Fisher and colleagues (38) demonstrated that neutrophil elastase, a serine protease also stored in azurophilic granules, induces MUC5AC gene expression in A549 cells via an oxidant-dependent pathway. Although enhancement of mucin expression by defensins may contribute to a more efficient clearance of microorganisms, mucus hypersecretion may also lead to airway obstruction. Therefore, excessive stimulation of mucin expression by defensins may lead to mucus hypersecretion, a feature of neutrophil-dominated inflammatory diseases such as chronic bronchitis (39).

In summary, we demonstrated that neutrophil defensins enhance airway epithelial wound closure in vitro, possibly by inducing cell migration and proliferation. Furthermore, we have shown that this enhancement requires both EGF receptor and ERK1/2 activation. Neutrophil defensins were also found to increase mucin gene expression. These data suggest a pivotal role for neutrophil defensins in airway epithelial wound repair. Whether defensins enhance wound repair in vivo remains to be determined.

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References


