

# Human neutrophil defensins induce lung epithelial cell proliferation in vitro

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**Abstract:** Repair of injured airway epithelium is often accompanied by an influx of leukocytes, and these cells have been suggested to contribute to the repair process. The aim of the present study was to investigate the effect of neutrophil defensins—antimicrobial peptides present in large amounts in the neutrophil—on proliferation of cultured lung epithelial cells. Neutrophil defensins at 4–10 µg/ml enhanced proliferation of the A549 lung epithelial cell line as assessed using cell counting, BrdU incorporation, and the tetrazolium salt MTT assay. Higher, cytotoxic concentrations of defensins decreased cell proliferation. Whereas defensin-induced cell proliferation was not inhibited by the EGF receptor tyrosine kinase inhibitor AG1478, it was completely inhibited by the mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor U0126, suggesting that defensins mediate cell proliferation via an EGF receptor-independent, MAP kinase signaling pathway. Although the cytotoxic effect of defensins was inhibited by α1-proteinase inhibitor, the defensin-induced cell proliferation was not affected. These data suggest that neutrophil defensins may possibly be involved in epithelial repair in the airways by inducing lung epithelial cell proliferation. *J. Leukoc. Biol.* 72: 167–174; 2002.

**Key Words:** antimicrobial peptides · wound repair · epidermal growth factors · α1-proteinase inhibitor

## INTRODUCTION

The airway epithelium forms a continuous barrier against potentially harmful inhaled agents. In response to epithelial injury, it is essential that a repair process is initiated that comprises subsequent epithelial cell migration, proliferation, and differentiation [1]. In inflammatory lung diseases such as asthma and chronic bronchitis, epithelial cell injury is observed [2]. Various studies have shown that activation of the epidermal growth factor (EGF) receptor following ligand binding may play an important role in epithelial repair processes by inducing cell migration, proliferation, and differentiation [3].

Repair of injured epithelium is often accompanied by an influx of inflammatory cells such as neutrophils and macrophages. Neutrophils contain and produce several products, including elastase, cathepsin G, defensins, and reactive oxygen species, which are released upon stimulation. These products are not only involved in defense against a broad spectrum of microorganisms, but upon release may also cause epithelial cell injury [4]. Besides their injurious potential, neutrophil products may also be involved in the subsequent repair process [5–9]. In vivo studies in rats demonstrated that neutrophil depletion resulted in decreased epithelial proliferation and repair following ozone-induced epithelial injury [5]. Data from in vitro studies also support a role for neutrophils in epithelial repair, as the neutrophil products lactoferrin and H<sub>2</sub>O<sub>2</sub> increase epithelial cell proliferation [6, 7] and differentiation [8]. In addition, neutrophil defensins have been shown to increase proliferation of mouse retinal epithelial cells and fibroblasts in vitro [9]. This is of potential importance in view of the abundance of neutrophil defensins in the neutrophil [10] and their presence in airway secretions [11].

Human neutrophil defensins, also called human neutrophil peptides (HNP), are small (3.5–4 kDa), cationic polypeptides that belong to the α-defensin subfamily. They are present in large amounts in azurophilic granules and are released upon neutrophil activation [10]. Four homologous members of the neutrophil defensins (HNP1–4) have been identified so far. Neutrophil defensins were originally identified as broad-spectrum antimicrobial peptides. Subsequent studies have shown that neutrophil defensins also display cytotoxic activity to eukaryotic cells and a variety of other pro- and anti-inflammatory activities (reviewed in ref. [12]). Defensins have now been shown to be active in regulating a variety of processes, including complement activation [13], chemotaxis of human immature dendritic cells, CD4<sup>+</sup>/CD45RA<sup>+</sup> and CD8<sup>+</sup> human T cells [14], and monocytes [15]. In vitro studies also revealed marked effects of defensins on epithelial cells, as defensins induce cytotoxicity [16, 17], interleukin (IL)-8 [18], and SLPI expression [19] and increase adhesion of *Haemophilus influenzae* [20]. Some of the proinflammatory activities of defensins

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appear to be inhibited by defensin-inhibitory components, such as the serpin serine proteinase inhibitors  $\alpha$ 1-proteinase inhibitor ( $\alpha$ 1-PI) and  $\alpha$ 1-antichymotrypsin [18, 20, 21].

Based on the possible involvement of neutrophils in epithelial repair processes and the observation that human neutrophil defensins increase proliferation of murine fibroblasts and retinal epithelial cells [9], we hypothesized that neutrophil defensins increase proliferation of human lung epithelial cells. Therefore, the aim of the present study was to examine the effect of neutrophil defensins on proliferation of cultured epithelial cells, to explore the possible involvement of EGF receptor activation and the downstream mitogen-activated protein (MAP) kinase signaling pathway in this process, and to determine the effect of  $\alpha$ 1-PI on defensin-induced cell proliferation.

## MATERIALS AND METHODS

### Defensin isolation

Human neutrophil defensins were isolated from neutrophil granules as a mixture of HNP-1, -2, and -3 using the procedure described previously [18, 22]. HNP-1 was further isolated from the mixture by reverse-phase high-pressure liquid chromatography. Purity of the isolates was determined by tricine-based sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; see Fig. 1A), acid urea (AU) PAGE, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using the Voyager-DE PRO Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA; see Fig. 1B).

In selected experiments, disulfide bond reduction and cysteine alkylation of HNP-1 were performed as described [23]. Briefly, 1 mg HNP-1 was dissolved in 1 ml 6 M guanidine hydrochloride, 0.1 M tris(hydroxymethyl)aminomethane, and 2 mM ethylenedinitrilo tetraacetic acid, pH 8.5 (reaction buffer). Reduction of disulfide bonds was performed by incubating 1 ml HNP-1 solution with 2 ml 1.5 mg/ml dithiothreitol in reaction buffer for 4 h at 50°C under saturated N<sub>2</sub> conditions to prevent spontaneous reoxidation. Free cysteine thiols were alkylated by incubation with 3 ml 0.1 mg/ml iodoacetamide in reaction buffer for 20 min at room temperature in the dark. Reduced HNP-1 was dialysed against a 0.01% (v/v) acetic acid solution and concentrated on Sep-Pac C18 cartridges (Waters, Milford, MA).

### Generation of anti-HNP mouse monoclonal antibodies (mAb)

Anti-HNP mAb were generated using conventional hybridoma technology. Briefly, female Balb/c mice were immunized subcutaneously with a mixture of native and glutaraldehyde-cross-linked HNP-1 in Freund's complete adjuvant and boosted with HNP-1 in Freund's incomplete adjuvant. Four days following

an intrasplenic injection with HNP-1, splenocytes were harvested and fused with SP2/0 myeloma cells. Hybridomas producing antibodies specific for HNP1-3 were subcloned by limiting dilution, tested for positivity by enzyme-linked immunosorbent assay using purified HNP1-3 as antigen, and screened for specificity by Western blot analysis of neutrophil granule extracts and immunohistochemistry on formalin-fixed bronchial tissue (data not shown). Antibodies from clone HNP-E3 (immunoglobulin G class) were purified from culture supernatant by protein G affinity chromatography using the ÅKTAprime system (Amersham Pharmacia Biotech, Upsala, Sweden).

### Cell culture

Cells from the A549 human lung carcinoma cell line (a cell line with type II alveolar epithelial cell characteristics) and the NCI-H292 human mucocpidermoid tumor cell line were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in tissue culture flasks at 37°C in a 5% CO<sub>2</sub>-humidified atmosphere in RPMI 1640 (Gibco, Grand Island, NY), supplemented with 2 mM L-glutamine, 200 U/ml penicillin, 200 µg/ml streptomycin (all BioWhittaker, Walkersville, MD), and 10% heat-inactivated fetal calf serum (FCS; Gibco).

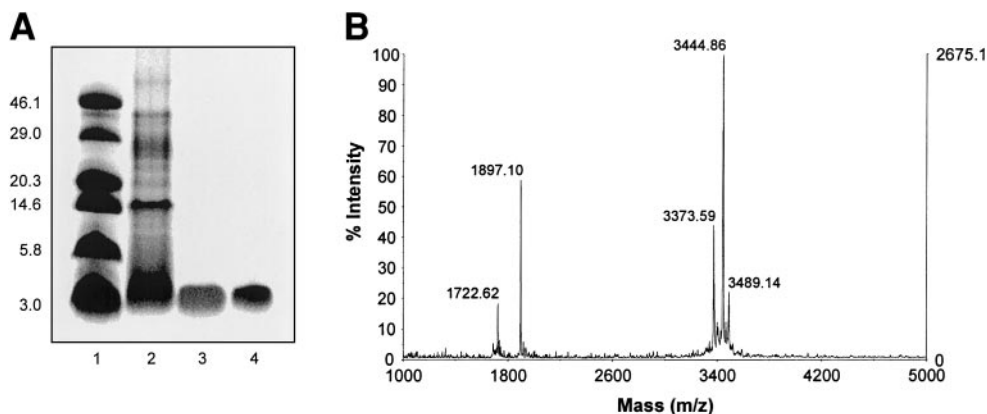
### Cell proliferation

Cell proliferation was assessed using automated cell counting, 5-bromo-2-deoxyuridine (BrdU) incorporation or by measuring mitochondrial activity using (4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT).

### Cell counting

A549 and NCI-H292 cell cultures were seeded at a concentration of 30,000 cells/cm<sup>2</sup> and were cultured overnight in 12- or 24-well plates (Costar, Cambridge, MA) in complete RPMI medium. After washing with prewarmed phosphate-buffered saline (PBS), the cells were starved for growth factors by overnight incubation in serum-free medium followed by incubation for 24 or 48 h in the same medium alone (negative control) or supplemented with HNP1-3, HNP-1, reduced and alkylated HNP-1 (red-HNP-1), transforming growth factor  $\alpha$  (TGF- $\alpha$ ; Sigma Chemical Co., St. Louis, MO), or FCS. Cell counting was performed after harvesting the cells using a cell scraper (Greiner, Alphen a/d Rijn, The Netherlands), and cell numbers were determined using an automated cell counter (Casy-1, Schärve System, Germany). To study the role of EGF receptor activation and the downstream MAP kinase signaling pathway, cells were incubated with 10 µM of the EGF receptor tyrosine kinase inhibitor tyrphostin AG1478 (Calbiochem, La Jolla, CA) or 25 µM of the MAP kinase kinase (MEK) inhibitor U0126 (Promega, Madison, WI) for 1 h, followed by incubation with HNP1-3 or TGF- $\alpha$  in the presence of AG1478 or U0126. Cytotoxic effects of AG1478 and U0126 were excluded by trypan blue exclusion assays. In other experiments, HNP1-3 was preincubated with 2 µg/ml polymixin-B (PB; Sigma Chemical Co.) or equimolar concentrations of  $\alpha$ 1-PI (Laboratoire Français de Fractionnement et des Biotechnologies, Lille, France) 1 h before the addition to cells. The effect of HNP-specific mAb on HNP1-3-induced cell proliferation was assessed by preincubation of HNP1-3 (58 µg/ml) with anti-HNP mAb (2.2 mg/ml) for 1 h at 37°C, followed by

**Fig. 1.** (A) Analysis of purified HNP-1 and HNP1-3 by Coomassie-stained tricine-based SDS-PAGE. Lane 1, Low  $M_r$  protein marker (expressed as kDa); lane 2, 10 µg acetic acid neutrophil granule extract; lane 3, 5 µg HNP1-3; lane 4, 5 µg HNP-1. (B) MALDI-TOF-MS spectrum of HNP1-3. The spectrum was calibrated internally on a synthetic peptide with an average mass of 1897.10 Da (MH<sup>+</sup>). Three analytes can be discerned at  $m/z$  (mass-to-charge ratio) 3373.59, 3444.86, and 3489.14, corresponding to HNP-2, -1, and -3, respectively. The peak at  $m/z$  1722.62 corresponds to the double-charged analyte at 3444.86 Da (HNP-1).



dilution to a final concentration of 8  $\mu\text{g/ml}$  HNP1-3 in medium and removal of anti-HNP/HNP1-3 aggregates by centrifugation prior to addition to cells.

## BrdU incorporation

For assessment of A549 cell proliferation by BrdU incorporation, A549 cells were seeded and starved for growth factors, as described above, on 12-well tissue-culture plates (Costar). Following incubation of the cells with various stimuli for the indicated time periods, 10 mM BrdU (Sigma Chemical Co.) was added, and cells were incubated for 1 h. Next, the cells were washed twice in PBS and fixed in 70% ethanol (v/v) for at least 1 h. BrdU incorporation was assessed using immunocytochemistry [24]. Briefly, cells were permeabilized with 1 M hydrochloric acid followed by subsequent washes with 0.1 M sodium tetraborate and PBS. BrdU incorporation was demonstrated by incubation with a mouse anti-BrdU mAb (kindly provided by Prof. Dr. F. C. S. Ramaekers, Dept. of Molecular Cell Biology, University of Maastricht, The Netherlands), followed by incubation with a peroxidase-labeled rabbit anti-mouse polyclonal antibody (Dako, Glostrup, Denmark). BrdU incorporation was visualized using Nova RED (Vector Laboratories, Burlingame, CA), and the percentage BrdU-positive nuclei was calculated.

## MTT assay

A549 cells were incubated, as described above, in 96-well plates (Costar). After a 24-h incubation of A549 cells in 100  $\mu\text{l}$  RPMI medium containing the stimulus, 50  $\mu\text{l}$  of a 5 mg/ml MTT (Sigma Chemical Co.) solution in PBS was added, and cells were incubated at 37°C for 2 h [25]. The cells were then lysed by addition of 100  $\mu\text{l}$  per well extraction buffer [20% (w/v) SDS, 50% (v/v) *N,N*-dimethyl formamide, 2% (v/v) acetic acid, pH 4.7]. After overnight incubation with extraction buffer, the optical density (OD) at 562 nm was measured.

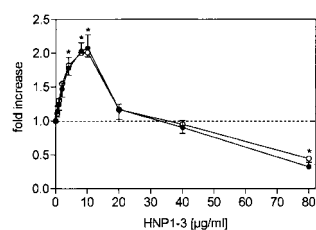
## Statistical analysis

Results are expressed as mean  $\pm$  SEM. Data obtained from three separate experiments were analyzed for statistical difference by the Student's *t*-test for paired samples. For individual experiments performed in triplicate, the unpaired *t*-test was used. Differences were considered significant when  $P < 0.05$ .

## RESULTS

### Defensin-induced A549 cell proliferation

The effect of neutrophil defensins on lung epithelial cell proliferation was examined in A549 cells using HNP1-3 isolated from neutrophil granules. The purity of the preparation used was demonstrated by acid urea PAGE (data not shown), SDS-PAGE, and mass spectrometry analysis (Fig. 1). This analysis of HNP1-3 demonstrated the presence of peaks with a molecular weight ( $M_r$ ) of 3445, 3374, and 3489 corresponding to the  $M_r$  of HNP-1, -2, and -3, respectively [22]. Automated cell counting revealed that HNP1-3 induced A549 cell proliferation in a dose-dependent manner, reaching a maximal 2.1-fold increase in cell numbers at HNP1-3 concentrations of 4–10  $\mu\text{g/ml}$  as compared with cells incubated in serum-free medium after 48 h (Fig. 2). At concentrations above 40  $\mu\text{g/ml}$ , cell numbers decreased. A549 cells treated with medium containing 10% FCS served as a positive control and showed a 4.0-fold increase in cell numbers. After 48 h of serum-free medium treatment, cells were tightly packed as adherent clumps of a few cells, and cells treated with FCS formed a confluent monolayer with tight cell-cell contacts (Fig. 3). Cells treated with low (proliferation-inducing) concentrations (8  $\mu\text{g/ml}$ ) of HNP1-3 formed monolayers resembling those observed



**Fig. 2.** Dose-dependent induction of A549 cell proliferation by neutrophil defensins. Proliferation was assessed by automated cell counting and calculated as fold increase compared with cells cultured in serum-free medium alone (dotted line). A549 cells were incubated with HNP1-3 at various concentrations in serum-free medium for 24 (○) or 48 h (●). For comparison, cells treated with medium containing 10% FCS showed a 3.2- and 4.0-fold increase in cell numbers at 24 and 48 h, respectively (data not shown). Data represent mean  $\pm$  SEM of three separate experiments. \*,  $P < 0.05$  versus serum-free medium-treated cells.

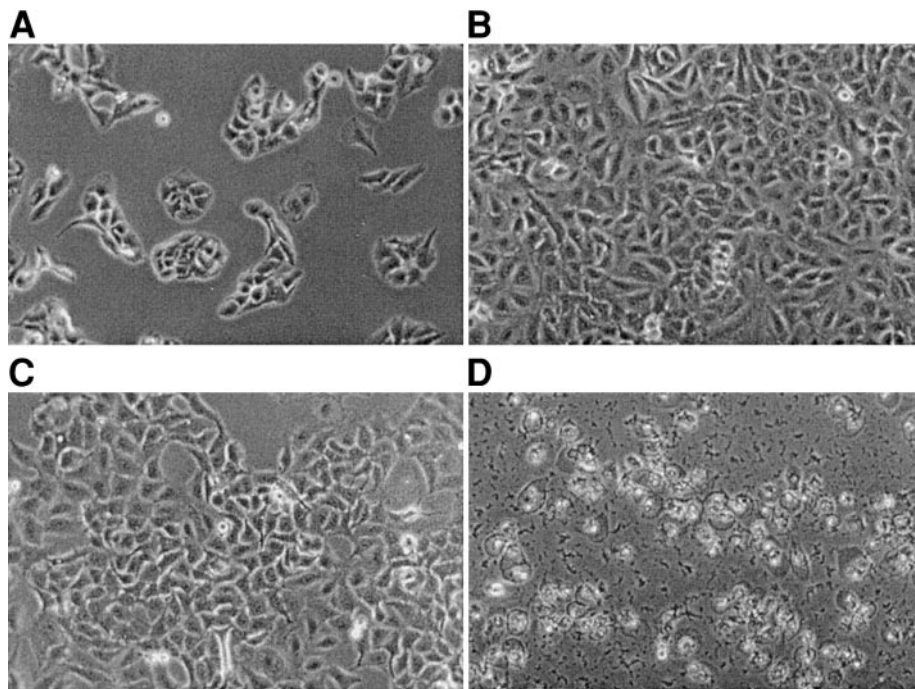
in FCS-treated cells. At 50  $\mu\text{g/ml}$ , cells lost cell-cell contacts and became rounded after 24 h. After 48 h, cellular debris was observed at these concentrations. In line with previous observations [17], round and “fragmented” cells did not detach from the plate and were resistant to trypsin treatment.

Next, the effect of HNP1-3 on the proliferation of the muco-epidermoid lung tumor cell line NCI-H292 was investigated (Table 1). After a 48-h incubation of NCI-H292 cells with 8  $\mu\text{g/ml}$  HNP1-3, a twofold increase in cell numbers was observed, whereas incubation with 50  $\mu\text{g/ml}$  resulted in a decrease as compared with cells incubated in serum-free medium.

To exclude the possibility that the observed effects of defensins are the result of trace contamination of our defensin preparations with lipopolysaccharide (LPS), HNP1-3 was preincubated with PB 1 h prior to addition to A549 cells (Fig. 4A). PB did not affect defensin-induced A549 cell proliferation. As neutrophil defensins are highly cationic, we considered the possibility that induction of proliferation is a nonspecific property of the cationic charge of defensins. Therefore, the effect of protamine (a peptide of similar size and charge as defensins) on A549 cell proliferation was assessed (Fig. 4A). Morphology and cell numbers of A549 cells incubated with protamine did not differ from untreated A549 cells. The effect of defensins was not a result of a defensin-induced change in the charge of the tissue-culture plates, as preincubation of these plates with HNP1-3 or medium alone followed by washing did not affect cell proliferation. The effect of conformational changes of defensins on defensin-induced cell proliferation was assessed by reduction and alkylation of HNP-1 disulfide bonds. The purity of the HNP-1 preparation used was demonstrated by AU PAGE (data not shown) and SDS-PAGE analysis. Mass spectrometry analysis of HNP-1 demonstrated a  $M_r$  3445, corresponding to that of HNP-1. Incubation of A549 cells with reduced and alkylated HNP-1 did not affect cell proliferation, whereas native HNP-1 increased A549 cell proliferation in a similar manner as HNP1-3 (Fig. 4A). Finally, the specificity of the effect of defensins on A549 cell numbers was also assessed by preincubation with a HNP1-3-specific mouse mAb (Fig. 4B). Preincubation of HNP1-3 with the anti-HNP antibody completely blocked the defensin-induced increase in A549 cell numbers after 48 h.

Using the MTT assay, a dose-dependent effect of defensins on the viable cell counts, as assessed by determining mitochondrial activity, was observed (Fig. 5). A maximal 1.4-fold

**Fig. 3.** Cell morphology of A549 cells shown by phase contrast microscopy following incubation with proliferation-inducing or cytotoxic concentrations of HNP1-3. A549 cells were incubated with serum-free medium (A), 10% FCS (B), 8  $\mu\text{g/ml}$  HNP1-3 (C), or 50  $\mu\text{g/ml}$  HNP1-3 (D) for 48 h. Original magnification, 100 $\times$ .



increase in OD562 nm was measured using 8–20  $\mu\text{g/ml}$  HNP1-3, whereas at 40  $\mu\text{g/ml}$  and higher concentrations, a marked (at least 1.8-fold) decrease was measured. It is interesting that at 20  $\mu\text{g/ml}$  HNP1-3, an increase in mitochondrial activity was observed, as assessed by the MTT assay, whereas this concentration did not increase cell counts (Fig. 2).

BrdU incorporation studies revealed a time-dependent increase in the percentage of BrdU-positive nuclei in A549 cells that were incubated with HNP1-3 as compared with cells incubated with serum-free medium (Table 2). Maximal BrdU incorporation was observed after 24 h of incubation with HNP1-3, whereas after 4 h, no differences in BrdU incorporation were observed between control and HNP1-3-treated cells. BrdU incorporation was increased in A549 cells incubated with 20 ng/ml TGF- $\alpha$ ; however, no differences were observed between the different time periods.

### Effect of $\alpha$ 1-PI on defensin-induced A549 cell proliferation

The serpin  $\alpha$ 1-PI has been shown to modulate selected defensin-induced activities. To assess the effect of  $\alpha$ 1-PI on defensin-induced cell proliferation and cytotoxicity, HNP1-3 was

incubated at proliferation-inducing (8  $\mu\text{g/ml}$ ) and cytotoxic (50  $\mu\text{g/ml}$ ) concentrations with equimolar concentrations of  $\alpha$ 1-PI before addition to A549 cells (Fig. 6).  $\alpha$ 1-PI alone at low concentrations (equimolar to 8  $\mu\text{g/ml}$  HNP1-3) did not affect A549 cell proliferation; high concentrations (equimolar to 50  $\mu\text{g/ml}$  HNP1-3) resulted in a small but significant increase in A549 cell proliferation. A549 cell proliferation induced by 8  $\mu\text{g/ml}$  HNP1-3 was not significantly affected by preincubation with equimolar concentrations of  $\alpha$ 1-PI. Incubation with concentrations of  $\alpha$ 1-PI equimolar to 50  $\mu\text{g/ml}$  HNP1-3 not only inhibited defensin-induced A549 cell cytotoxicity (ref. [21] and this study; data not shown), but also resulted in a 2.9-fold increase of A549 cell numbers.

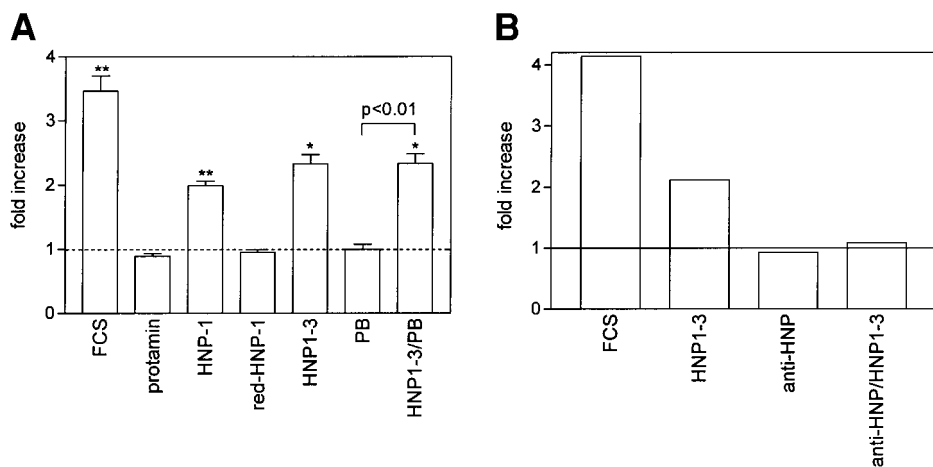
### Role of the EGF receptor and MAP kinase signaling in defensin-induced cell proliferation

As the EGF receptor exerts an important role in epithelial cell proliferation, we investigated a possible involvement of the EGF receptor in defensin-induced cell proliferation. A549 cells were preincubated with the EGF receptor tyrosine kinase inhibitor (tyrphostin) AG1478 1 h before addition of HNP1-3, using the EGF receptor ligand TGF- $\alpha$  as a control (Fig. 7A). AG1478 completely blocked TGF- $\alpha$ -induced A549 cell proliferation, and defensin-induced A549 cell proliferation was not affected. To assess a possible involvement of the MAP kinase signaling pathway downstream of the EGF receptor in defensin-induced cell proliferation, A549 cells were preincubated with the MEK inhibitor U0126 for 1 h prior to addition of HNP1-3 or TGF- $\alpha$  (Fig. 7B). Unlike AG1478, U0126 completely inhibited defensin-induced A549 cell proliferation. These findings indicate that although defensin-induced cell proliferation is not EGF receptor-dependent, this induction may be mediated via the downstream MAP kinase signaling pathway.

TABLE 1. Effect of Neutrophil Defensins on NCI-H292 Cell Proliferation Assessed by Automated Cell Counting

Stimuli	Fold-increase $\pm$ SEM
FCS	1.99 $\pm$ 0.21 <sup>a</sup>
EGF (5 ng/ml)	1.71 $\pm$ 0.03 <sup>b</sup>
HNP1-3 (8 $\mu\text{g/ml}$ )	2.00 $\pm$ 0.05 <sup>b</sup>
HNP1-3 (50 $\mu\text{g/ml}$ )	0.53 $\pm$ 0.10 <sup>a</sup>

Data represent the relative increase in NCI-H292 cell numbers after 48 h as compared with cells incubated in serum-free RPMI medium. <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.01$  versus serum-free medium-treated cells.



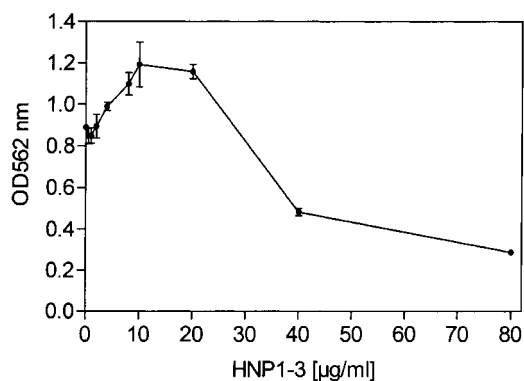
**Fig. 4.** A549 cell proliferation following incubation with HNP1-3, HNP-1, or red-HNP-1 (A) and after preincubation with a HNP1-3-specific mAb (B). Proliferation was assessed by automated cell counting and calculated as fold increase as compared with cells cultured in serum-free medium alone (dotted line). A549 cells were incubated with HNP1-3, HNP-1, red-HNP-1, protamine (all 8  $\mu\text{g/ml}$ ), or 10% FCS in serum-free medium for 48 h. Possible effects of LPS contamination were examined by preincubating HNP1-3 with 2  $\mu\text{g/ml}$  PB. Effects of a HNP1-3-specific mAb on defensin-induced cell proliferation were assessed by preincubating HNP1-3 with anti-HNP (clone HNP-E3). Data represent mean  $\pm$  SEM of three separate (A) or one experiment [similar results were obtained in two independent experiments (B)]. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus serum-free, medium-treated cells.

## DISCUSSION

The results from the present study show that neutrophil defensins enhance proliferation of cells from the airway epithelial cell lines A549 and NCI-H292. Enhanced proliferation was demonstrated by cell counting, assessment of BrdU incorporation, and enhanced mitochondrial activity as determined by MTT assays. The effect of defensins was dose- and time-dependent, with a maximum increase in cell proliferation at defensin concentrations ranging from 4 to 10  $\mu\text{g/ml}$  after 48 h of incubation. Higher concentrations of defensins decreased cell proliferation, presumably as a result of cytotoxicity. The involvement of the EGF receptor pathway was evaluated in experiments using the EGF receptor tyrosine kinase inhibitor tyrphostin AG1478. Involvement of EGF receptor is unlikely, as AG1478 did not affect defensin-induced epithelial cell proliferation. However, the MAP kinase signaling pathway downstream from the EGF receptor appears to be involved, as the MEK inhibitor U0126 completely blocked defensin-induced cell proliferation. Effects of  $\alpha 1$ -PI on defensin-induced cell proliferation were assessed to investigate whether this specific property of defensins can be modulated. While defen-

sin-induced cytotoxicity was inhibited by  $\alpha 1$ -PI, the defensin-induced cell proliferation was not.

Previous studies have shown that neutrophil defensins enhance proliferation of murine fibroblasts and retinal epithelial cells [9]. The present study extends these findings by showing that neutrophil defensins also enhance proliferation of human lung epithelial cells. We observed that this effect is dependent on structural integrity of the defensins and is not affected by  $\alpha 1$ -PI. Various studies have demonstrated the involvement of the EGF receptor and the MAP kinase signaling pathway in (epithelial) cell proliferation [26]. MEK-mediated activation of extracellular regulated kinase-1/2 was found to be especially important for epithelial cell proliferation. Our results show that defensin-induced epithelial cell proliferation is not dependent on EGF receptor activation but appears to be mediated via the MAP kinase signaling pathway downstream of the EGF receptor. Other studies have also provided evidence for a role of neutrophil products in epithelial cell proliferation. Hagiwara et al. [6] have demonstrated that lactoferrin and lactoferrin-derived peptides show growth-promoting activities toward the rat intestinal epithelial cell line IEC-18. It has been suggested that another neutrophil-derived antimicrobial protein, the por-

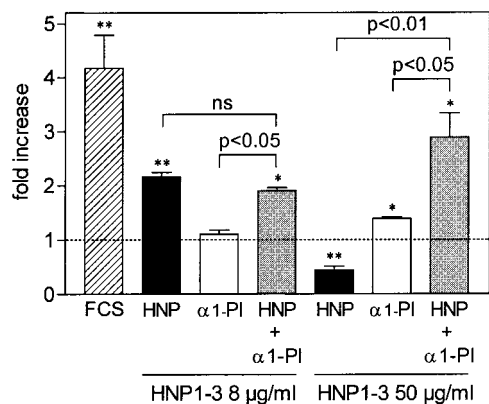


**Fig. 5.** Defensin-induced A549 cell proliferation assessed by MTT colometric OD562 nm measurement. A549 cells were incubated with HNP1-3 in serum-free medium at various concentrations for 24 h. Data represent mean  $\pm$  SEM of two experiments performed in triplicate.

**TABLE 2.** Effect of Neutrophil Defensins on A549 Cell Proliferation Assessed by BrdU Incorporation

Stimuli	% BrdU-positive nuclei		
	Incubation time		
	4 h	24 h	48 h
Medium	36.5 $\pm$ 1.5	33.1 $\pm$ 1.6	31.5 $\pm$ 0.7
FCS	36.9 $\pm$ 2.0	52.1 $\pm$ 2.9 <sup>a</sup>	44.5 $\pm$ 0.9 <sup>a</sup>
HNP-1-3	37.7 $\pm$ 1.8	49.5 $\pm$ 1.6 <sup>a</sup>	45.4 $\pm$ 2.9 <sup>a</sup>
TGF- $\alpha$	37.8 $\pm$ 2.0	39.8 $\pm$ 1.9 <sup>b</sup>	42.3 $\pm$ 3.6 <sup>a</sup>

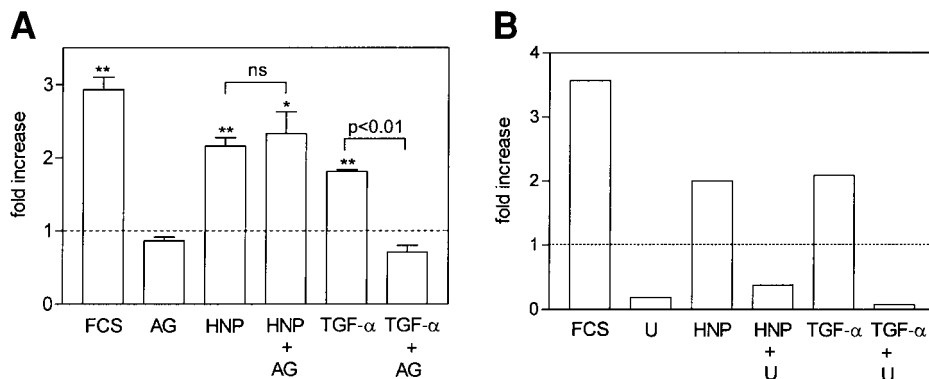
A549 cells were incubated with serum-free medium, 10% FCS, HNP-1-3 (8  $\mu\text{g/ml}$ ), or TGF- $\alpha$  (20 ng/ml) for the indicated time period and subsequently incubated with BrdU for 1 h. Data represent mean  $\pm$  SEM of nuclear cell counts made in at least six different high-power fields. Another separate experiment showed comparable results. <sup>a</sup> $P < 0.01$ , <sup>b</sup> $P < 0.05$  versus serum-free medium-treated cells.



**Fig. 6.** Effect of  $\alpha$ 1-PI on neutrophil defensin-induced A549 cell proliferation. Proliferation was assessed by automated cell counting and calculated as fold increase compared with cells cultured in serum-free medium alone (dotted line). A549 cells were incubated with HNP1-3 (8 or 50  $\mu$ g/ml; solid bars), HNP1-3 preincubated for 1 h with an equimolar concentration of  $\alpha$ 1-PI (shaded bars), or  $\alpha$ 1-PI alone (123 or 770  $\mu$ g/ml; open bars) in serum-free medium for 48 h. For comparison, data from cells cultured in FCS-containing medium are shown (hatched bar). Data represent mean  $\pm$  SEM of three separate experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus serum-free medium-treated cells. ns, not significant.

cine cathelicidin PR-39, is involved in wound repair, as it induced mesenchymal cell expression of syndecans, which are heparan-sulfate proteoglycans that show growth-promoting activities [27]. A third neutrophil product that has been implicated in cell proliferation is  $H_2O_2$ . In an in vitro study using a rabbit lens epithelial cell line, Ohguro et al. [7] demonstrated that  $H_2O_2$  induces cell proliferation. Similar to neutrophil defensins, the nature of the  $H_2O_2$  effects on epithelial cells is concentration dependent. At high concentrations (1 mM),  $H_2O_2$  is cytotoxic, whereas at low concentrations (1 nM–1  $\mu$ M), it induces cell proliferation. Furthermore, Goldkorn et al. [28] have shown that  $H_2O_2$  activates the EGF receptor, suggesting a role for EGF receptor signaling in  $H_2O_2$ -induced cell proliferation. However, we found that the EGF receptor is not involved in neutrophil defensin-induced cell proliferation. Taken together, these data illustrate that stimulation of neutrophils may lead to the release of potentially cytotoxic mediators that at low concentrations, may contribute to restoration of epithelial integrity.

**Fig. 7.** Effect of EGF receptor tyrosine kinase and MEK inhibition on defensin-induced A549 cell proliferation. Proliferation was assessed by automated cell counting and calculated as fold increase as compared with cells cultured in serum-free medium alone (dotted line). Cells were preincubated with EGF receptor kinase inhibitor tyrphostin AG1478 (AG; 10  $\mu$ M; A) or MEK inhibitor U0126 (U; 25  $\mu$ M; B) 1 h prior to incubation with HNP1-3 (HNP; 8  $\mu$ g/ml) or TGF- $\alpha$  (20 ng/ml) in serum-free medium for 48 h. Cells incubated with 10% FCS served as a positive control. Data represent mean  $\pm$  SEM of three separate (A) or one experiment [similar results were obtained in two independent experiments (B)]. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  serum-free medium-treated cells.



The present results were obtained using highly purified preparations of neutrophil defensins that were isolated from granules derived from peripheral blood human neutrophils. Therefore, it is unlikely that contaminants in the defensin preparations are responsible for the observed effects on cell proliferation. Contribution of LPS as a contaminating factor to defensin-induced cell proliferation was excluded by demonstrating that PB did not affect the defensin activity. Furthermore, we have demonstrated that LPS from *Escherichia coli* and *Pseudomonas aeruginosa* did not affect A549 cell proliferation, which makes a role of LPS in defensin-induced cell proliferation unlikely (data not shown). Effects of possible minor mitogenic contaminants could also be excluded, as the observed increase in cell numbers was fully blocked by HNP-specific mAb. The activity of defensins appeared to depend on an intact structure of defensins, as reduction and alkylation of defensins resulted in complete loss of the growth-promoting activity. Finally, defensin-induced cell proliferation is not likely to be a mere consequence of the cationic character of the peptide, as the cationic peptide protamine was devoid of growth-promoting activity. It is unknown whether neutrophil products that are released by stimulated neutrophils concomitant with defensins affect defensin-induced cell proliferation.

Based on structural differences, human defensins can be divided into two subfamilies: the  $\alpha$ - and  $\beta$ -defensins [10]. Neutrophil defensins belong to the subfamily of  $\alpha$ -defensins, whereas epithelial cells secrete  $\beta$ -defensins. Understanding the mechanism that underlies neutrophil defensin-induced epithelial cell proliferation is hampered by the fact that no receptor has been identified for these defensins.  $\beta$ -Defensins have been shown to use the chemokine receptor CCR6 to cause chemoattraction of CCR6-bearing memory T cells and immature dendritic cells [29]. It has recently been shown that neutrophil defensin-induced chemotaxis of T and dendritic cells is pertussis toxin sensitive, suggesting the involvement of G-protein-mediated signaling [14]. Therefore, it is possible that neutrophil defensins also use G-protein-coupled chemokine receptors to exert their various actions. The chemokine IL-8 has been implicated in the induction of cell proliferation and migration of epidermal and colon epithelial cells [30–32], suggesting a role for chemokine receptors in epithelial cell proliferation. Further experiments are required to delineate the

signal transduction pathways involved in defensin-induced epithelial cell proliferation.

What are the implications of the present findings? Neutrophilic inflammation is generally considered a beneficial response to potential, harmful intruders such as microorganisms. Neutrophils are involved in eradicating microorganisms, cause tissue injury, and may aid in restoration of epithelial integrity after injury. Neutrophilic inflammation may be harmful because of the tissue injury associated with sustained and extensive neutrophilic inflammation. Indeed, neutrophilic inflammation in the lung has been shown to be associated with chronic bronchitis [33], chronic obstructive pulmonary disease [2, 34], cystic fibrosis [35], and various other inflammatory lung diseases. We [17] and others [16] have previously shown that defensins at high concentrations cause epithelial injury and expression of proinflammatory genes in airway epithelial cells. In contrast, the present results indicate that at low concentrations, defensins may contribute to epithelial reconstitution by enhancing cell proliferation, considered as one of the crucial phases in the repair process. Therefore, the effect of neutrophil defensins is dependent on local defensin concentrations that may be determined by the extent of neutrophil inflammation, clearance mechanisms, and defensin-inhibitory substances. Whereas in healthy individuals neutrophil defensin concentrations in the epithelial lining fluid are low, these concentrations may markedly increase through neutrophil-dominated, inflammatory processes. Indeed, high (up to 1.6 mg/ml) concentrations of neutrophil defensins have been shown to be present in purulent secretions of patients with chronic bronchitis [21] and cystic fibrosis [36]. In addition, bronchoalveolar lavage fluid collected from patients with active pulmonary tuberculosis contains high levels of HNP1–3 ( $1.25 \pm 0.31 \mu\text{g/ml}$ ) [37], indicating that the concentration in the epithelial lining fluid is ten- to 100-fold higher. Defensin-binding substances that are present in the epithelial lining fluid may differentially affect the various activities of defensins, as shown in the present study by the ability of  $\alpha 1$ -PI to selectively block defensin-induced epithelial cell death, allowing high, potentially cytotoxic concentrations of defensins to induce proliferation. In line with this observation, Murphy et al. [9] have demonstrated that serum albumin (bovine) is also capable of enabling cell proliferation by defensin concentrations that are otherwise cytotoxic. Therefore, the range of defensin concentrations that may induce cell proliferation in vivo may be higher than predicted based on studies using purified defensins alone.

One previous report, which has not yet been confirmed further, indicated that rabbit neutrophil defensins increase wound healing in the skin [38]. This observation is in line with our results and suggests that defensins also enhance airway epithelial wound repair. Although defensin-induced cell proliferation may be beneficial to the host defense, it is possible that when the local defensin concentration is inadequately controlled, excessive and prolonged stimulation of cell proliferation may also have detrimental effects. An association between neutrophil influx and epithelial cell proliferation in human lung disease is suggested by the observation that bronchial biopsies obtained from chronic bronchitis patients showed an increased proportion of proliferating epithelial cells as compared with normal individuals and asthmatic patients

[39]. As other studies showed that airway neutrophilia is more prominent in patients with chronic bronchitis compared with patients with asthma or healthy controls [33, 40], epithelial cell proliferation and neutrophil influx may be associated features in chronic bronchitis. It is tempting to speculate that defensins are involved in the occurrence of epithelial abnormalities in chronic bronchitis, such as squamous and mucous cell metaplasia, which are often associated with neutrophilic inflammation [2].

## CONCLUSIONS

The results from the present study show that neutrophil defensins induce lung epithelial cell proliferation and thereby may possibly be involved in epithelial repair in the airways. Unlike other defensin-mediated activities such as cytotoxicity, defensin-induced cell proliferation is not affected by  $\alpha 1$ -PI. The defensin-induced cell proliferation appears to be mediated via an EGF receptor-independent activation of the MAP kinase signaling pathway.

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