Differential effects of human neutrophil peptide-1 on growth factor and IL-8 production by human lung fibroblasts and epithelial cells

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Short title: Effects of HNP-1 on human lung cells

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Abstract

α-Defensins, antimicrobial peptides produced mainly by neutrophils, have been reported to be associated with a wide variety of lung diseases, including idiopathic pulmonary fibrosis (IPF), cystic fibrosis (CF), and diffuse panbronchiolitis (DPB). In each disease, α-defensins are located in different areas, such as around the alveolar septa in IPF and around the airways in CF and DPB, suggesting that α-defensins play different roles. Meanwhile, growth factors are known to contribute to IPF, CF, and DPB. α-Defensins are known to induce IL-8 in airway epithelial cells, but the effects of α-defensins on the release of growth factors from various components in the lung have not been sufficiently investigated. In the present study, the *in vitro* effects of human neutrophil peptide (HNP)-1 (a subtype of α-defensin) on the expressions of IL-8 and growth factors in lung fibroblasts, bronchial epithelial cells, and alveolar epithelial cells were examined. HNP-1 mainly enhanced the expression of IL-8 in epithelial cells, while it enhanced transforming growth factor-β and vascular endothelial growth factor expressions in lung fibroblasts. These results suggest that α-defensins play different roles in the pathogenesis of IPF, CF, and DPB according to the location in the lung where the α-defensins are mainly produced.

Introduction

Neutrophils play a pathologic role in neutrophil-related lung diseases through α-defensins as well as neutrophil elastase [1-7]. α-Defensins are cationic peptides with antimicrobial activity. Of these, human neutrophil peptides (HNP)-1, -2, -3, and -4 are mainly present in neutrophils [8]. HNPs, especially HNP-1, also have the capacity to induce synthesis of mucin and chemokines/cytokines, including interleukin (IL)-8, IL-1β, monocyte chemoattractant protein-1, and epithelial neutrophil-activating protein 78, by lung epithelial cells [9-13] and collagen type I and heat shock protein (HSP) 47 by lung fibroblasts [14]. HNPs also enhance the interaction between lung epithelial cells and CD4+ lymphocytes by increasing cell adhesion and release of IL-8, indicating that HNPs link innate and adaptive immunity [15]. Voglis et al. reported that high concentrations of HNPs induce a defect in the antimicrobial function of neutrophils [16]. These reports suggest that HNPs are able to modulate inflammatory responses and that IL-8, a neutrophilic chemotactic factor, plays an important role in HNP-induced lung inflammation. Thus, α-defensins appear to be strongly associated with lung inflammation and fibrosis by activating lung epithelial cells and fibroblasts [3-5, 11, 14]; however, the exact mechanisms remain unclear.

Accumulating evidence has suggested that growth factors, including transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), and connective tissue growth factor (CTGF), also contribute to lung inflammation and fibrosis [17, 18]. The role of these growth factors has been studied in idiopathic pulmonary fibrosis (IPF) and cystic fibrosis (CF), diseases in which neutrophils are known to play an important pathogenic role [17, 19, 20]. However, the association between α-defensins and these growth factors has not been

clarified. We and other groups have demonstrated high concentrations of α-defensins in IPF, CF, and diffuse panbronchiolitis (DPB), suggesting that α-defensins are related to the pathogenesis of these chronic neutrophil-related lung diseases [3-5, 21]. DPB and CF are chronic inflammatory airway diseases, while IPF is an interstitial fibrotic lung disease. Immunohistochemistry of surgical lung biopsy specimens has shown that the main localization of α-defensins was in neutrophils and mucinous exudate in the airways, on the surface of bronchial epithelial cells in DPB [3], and inside and outside neutrophils in the alveolar septa, especially in dense fibrotic areas in IPF [4]. This information led us to the hypothesis that α-defensins may act differently in neutrophil-related lung diseases by inducing different mediators in different cell types, such as lung epithelial cells and mesenchymal cells, and this might clarify the role of α-defensins in the pathogenesis of IPF and DPB.

Thus, in the present study, the differences in the production of IL-8 and growth factors by HNP-1-stimulated, cultured human lung fibroblasts and human bronchial epithelial cells were investigated.

Materials and Methods

Cell culture

Normal human lung fibroblasts (NHLF; CC-2512, Lonza, Walkersville, VA, USA), human bronchial epithelial cells (HBEC; CC-2541, Lonza), and A549 cells (American Type Culture Collection, Rockville, MD, USA) were maintained in fibroblast growth medium (FGM; Lonza), bronchial epithelial growth medium (BEGM; Lonza), and Dulbecco's

modified minimal essential medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen). The cells were incubated in a humidified incubator (5% CO_2 at 37°C), and the experiments were performed at either the third or the fourth passage.

Defensin

Synthetic products of HNP-1 were purchased from the Peptide Institute (Osaka, Japan). HNP-1 solution was prepared in fibroblast basal medium (FBM; Lonza) for NHLF, in bronchial epithelial basal medium (BEBM; Lonza) for HBEC, and in DMEM for A549 cells.

Stimulation of cells with HNP-1

For the analysis of mRNA and protein expressions of IL-8 and growth factors, subcultures of NHLF, HBEC, and A549 cells were plated in 60-mm dishes at a density of 5×10^5 cells per dish. When the cells reached a confluence of approximately 70-80%, the medium was replaced with serum-free medium. The cells were subsequently stimulated for 3 and 24 h with medium alone (control) or 1, 5, 10, and 25 µg/ml of HNP-1. HNP-1 concentration was determined according to the previous study by Yoshioka et al., which demonstrated that an HNP-1 concentration >25 µg/ml is cytotoxic [14].

RNA extraction and reverse-transcriptase PCR

Expression levels of IL-8, TGF-β1, VEGF, and CTGF were investigated. Each cell was stimulated with HNP-1 for 3 h, and total RNA was isolated from the cells using an RNeasy Plus Mini Kit (Qiagen, Mississauga, Canada) according to the manufacturer's instructions. A volume of 2 to 4 µl of pooled RNA was reverse-transcribed using the SuperScript III

First-Strand Synthesis System for the real-time polymerase chain reaction (RT-PCR) kit (Invitrogen) in a total volume of 21 µl with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) at 65°C for 5 min, followed by 50 min at 50°C, 85 min at 85°C, and 20 min at 37°C. The resulting cDNA was subjected to quantitative RT-PCR. The RT-PCR amplification was performed using the TaqMan Gene Expression Assays set (Applied Biosystems) in combination with the TaqMan Universal PCR Master Mix (Applied Biosystems). Cycle-to-cycle fluorescence emission readings were monitored and analyzed by an ABI PRISM 7500 Sequence Detector System (Applied Biosystems). Human β-actin was used as an endogenous control. TaqMan primer probe sets of human IL-8, TGF-β1, VEGF, CTGF, and β-actin were purchased from Applied Biosystems. The comparative C_T method was used to quantify mRNA expression, and the ratio of the selected gene vs. β-actin was calculated.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected 24 h after addition of HNP-1, centrifuged, and stored at -80°C. IL-8, TGF-β1, and VEGF protein levels in supernatants from NHLF, HBEC, and A549 cells were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA). All measurements were performed in duplicate, and the values reported are the means of at least three measurements.

Immunocytochemistry

After 24 h incubation with HNP-1, cultured cells were fixed with acetone for 10 min, and immunocytochemistry was performed using the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) with mouse anti-human CTGF antibody (ab 51704,

dilution 1:100 for NHLF and A549 cells, 1:1000 for HBEC, Abcam, Tokyo, Japan).

Irrelevant mouse IgG primary antibodies were used as controls for nonspecific staining

(Santa Cruz Biotechnology, Santa Cruz, CA, USA). The staining intensity of CTGF in each

cell was scored semiquantitatively using a scale from 0 to 2 (0 = no staining; 1 = weak

staining; 2 = strong staining). Staining intensity was examined in more than five separate

fields at ×1000 magnification. Total cell count was converted to 100 so that the maximum

number was 200 in this scoring system. The results were reproducible based on

interobserver and intraobserver variability. Statistical analysis was applied to the

representative results of one observer.

Immunohistochemistry

Immunohistochemical staining of the surgical lung specimens obtained from patients with

IPF (n = 4) and DPB (n = 3) was performed using anti-HNPs-1, 2, and 3 antiserum as

described previously [3]. As a control, normal-looking lung tissue specimens resected from

patients with lung cancer were also studied. The Human Ethics Review Committee of

Nagasaki University School of Medicine approved the study protocol, and all patients

provided their written, informed consent.

Statistical analysis

All values are expressed as means ± SEM. Differences among various groups were

compared by one-way ANOVA. Statistical significance was defined as a p value <0.05. All

data were analyzed using StatView software (ver 5.0; SAS Institute Inc, Cary, NC, USA).

Results

Effects of HNP-1 on IL-8 RNA and growth factor expressions

Figure 1 shows the mRNA expressions of IL-8, TGF-β1, VEGF, and CTGF in NHLF,

HBEC, and A549 cells after 3 h incubation with medium alone (control) or 1, 5, 10, and 25

μg/ml of HNP-1. IL-8 mRNA expression in NHLF showed a significant increase at 5 μg/ml

of HNP-1. In HBEC and A549 cells, mRNA expression levels of IL-8 increased in a

dose-dependent manner, and the range of increase was much greater than that observed in

NHLF (Figure 1A). TGF-β1 mRNA expression in NHLF treated with HNP-1 at 25 μg/ml

was significantly increased, whereas TGF-β1 mRNA expression in HBEC did not show a

significant change (Figure 1B). In addition, HNP-1 at 1, 5, 10, and 25 μg/ml significantly

decreased TGF-β1 mRNA expression in A549 cells. In NHLF, VEGF mRNA expression

showed a significant increase with HNP-1 at 5, 10, and 25 μg/ml. In HBEC, it decreased

significantly at 5 μg/ml HNP-1, while it increased significantly at 25 μg/ml in A549 cells

(Figure 1C). CTGF mRNA expression in NHLF showed a significant increase at 5 μg/ml

HNP-1. In HBEC and A549 cells, the mRNA expression of CTGF also increased

significantly with HNP-1 at higher concentrations (Figure 1D).

Effects of HNP-1 on production of IL-8 protein and growth factors

Figure 2 shows IL-8, TGF-β1, and VEGF protein levels of supernatant analyzed by ELISA

in NHLF, HBEC, and A549 cells after 24 h incubation with medium alone (control) or 1, 5,

10, and 25 μg/ml of HNP-1. IL-8 production from NHLF and A549 cells showed a

significant increase at 5 μg/ml and at 5, 10, and 25 μg/ml of HNP-1, respectively. In HBEC,

protein production of IL-8 increased significantly at 25 μg/ml of HNP-1, and the range of

increase was much greater than that observed in NHLF (Figure 2A). In NHLF, TGF-β1

production significantly increased with 5 and 10 μg/ml of HNP-1. In HBEC, TGF-β1

production was under the detectable level. In A549 cells, production of TGF-β1 decreased

significantly with 25 μg/ml HNP-1 compared with control (Figure 2B). In NHLF, VEGF

production increased significantly in a dose-dependent manner. In HBEC and A549 cells,

there was no significant change in VEGF protein production (Figure 2C).

Immunocytochemical studies

Treatment with HNP-1 increased both the number and the intensity of immunopositive

cells for CTGF in a dose-dependent manner in NHLF, HBEC, and A549 cells. The average

grading score for CTGF also increased significantly in HBEC and A549 cells (p = 0.013

and p = 0.0027, respectively; Figure 3).

Immunohistochemical analysis

Figure 4 shows immunohistochemical staining of α-defensins using surgical lung biopsy

specimens from patients with IPF and DPB. In the lungs of IPF patients, strong positive

immunoreactivity for α-defensins was seen in infiltrated neutrophils. Thickened fibrous

stroma tissue composed of lung fibroblasts also showed mild positive immunoreactivity

that seemed to reflect exudation of α-defensins. In the lungs of DPB patients, neutrophils

rich in α-defensins were recognized in the terminal respiratory tract. The cilia of the

bronchial epithelial cells were slightly positive, which seemed to reflect exposure to

α-defensins.

Discussion

The present study demonstrates that HNP-1 induces the release from lung cells of growth factors, including TGF-β1, VEGF, and CTGF, as well as IL-8, and regulates the production of these mediators differently in NHLF, HBEC, and A549 cells. In IPF patients, HNP-1 was chiefly located in fibrotic interstitial lung tissue and consisted mainly of proliferated lung fibroblasts, but it was not present in airways. In contrast, a number of neutrophils highly positive for α-defensins accumulated in the airway spaces of DPB lung tissue. Bronchial epithelial cells did not show positive immunoreactivity to α-defensins, but the cilia of the bronchial epithelial cells showed positive immunoreactivity, which suggests that bronchial epithelial cells are strongly exposed to α-defensins in DPB. Thus, α-defensins in human lung show different local effects in different lung diseases. In fact, the bronchoalveolar lavage fluid (BALF) levels of α-defensins were markedly higher in DPB patients than in IPF patients [3].

IL-8 is a neutrophilic chemotactic factor that is involved in host inflammatory responses and is synthesized by many different cell types, including fibroblasts and epithelial cells [22, 23]. IL-8 is elevated in the BALF of patients with IPF and DPB, and it is considered to play a key role in the development of both diseases [4, 24-26]. We have previously demonstrated elevated IL-8 levels in the BALF of patients with IPF and DPB and a positive correlation between IL-8 and α-defensin levels in the BALF of these patients [3, 4]. However, the BALF concentration of IL-8 was also markedly higher in patients with DPB than in patients with IPF [3]. HNP-1 is known to stimulate IL-8 synthesis by airway epithelial cells [10, 11]. The present study demonstrated that HNP-1 also stimulates IL-8

production from NHLF. However, the degree of increase from HBEC was 10 times higher than that from NHLF. This might explain, at least in part, the difference in the degree of neutrophilic inflammation in the airways between DPB and IPF.

TGF-β1 is a 25-kDa peptide dimer secreted by several pulmonary cell types, including epithelial cells and fibroblasts [27]. TGF-β1 has been implicated in the pathogenesis of pulmonary fibrosis; increased levels of TGF-β1 are found in the fibrotic lung tissue of IPF patients [28], and TGF-β1 antibodies attenuate the fibrotic response in the bleomycin mouse model [29]. In this regard, HNP-1, as well as TGF-β1, directly enhanced the expression of collagen type I and HSP47, a collagen-specific molecular chaperon, on NHLF [14, 30]. In the present study, mRNA synthesis and protein production of TGF-β1 in NHLF was increased by HNP-1, whereas HBEC did not show a significant change, and a suppressive effect was seen in A549 cells. In addition, α-defensin expression was found in fibrotic interstitial lung tissues mainly associated with proliferative lung fibroblasts in IPF. These findings suggest that human lung fibroblasts stimulated by α-defensin promote fibrogenesis by promoting not only collagen production but also TGF-β1 production in IPF. In contrast, little effect of HNP-1 on TGF-β1 expression has been found in epithelial cells. TGF-β1 has a number of anti-inflammatory properties. For example, TGF-β1 acts as a negative regulator of NF-κB activity and inhibits IL-8 expression by airway epithelial cells [31, 32]. Therefore, α-defensin in the airway may enhance neutrophilic inflammation by inducing IL-8 production by epithelial cells and having little effect on TGF-β1, which suppresses IL-8 production [32].

VEGF is relatively specific for endothelial cells and is a major mediator for angiogenesis

and vascular permeability [33]. In patients with idiopathic interstitial pneumonias (IIPs),

Simler et al. demonstrated that patients who developed progressive diseases had

significantly higher baseline plasma levels of VEGF, as well as IL-8, and a rising VEGF

concentration was associated with an adverse physiological outcome, as suggested by lung

function and high resolution CT parameters [34]. McKeown et al. also demonstrated that

BALF levels of VEGF in IPF were higher in those with who died early during follow-up

and with rapidly declining lung function over 1 year [35]. These findings suggest that

angiogenic cytokines, including VEGF, are also involved in IPF. In the present study, the

levels of VEGF protein in NHLF tended to increase dose-dependently with HNP-1

stimulation, suggesting that the elevation of VEGF in IPF is, at least in part, associated

directly with α-defensins. In CF patients, serum VEGF levels are also generally elevated,

and they correlate with disease severity as measured by FEV_1, and VEGF-mediated

angiogenesis is considered to play an important role in the progression of CF [20, 36]. In

the present study, no direct effects of HNP-1 were observed on the production of VEGF by

bronchial or alveolar epithelial cells. Therefore, other factors might contribute to an

elevated VEGF level in the serum of CF patients; VEGF is also expressed by neutrophils,

which are increased in the airways of CF patients [37]. A recent report demonstrated that

α-defensins inhibit neovascularization by inhibiting endothelial cell adhesion and migration

to fibronectin, which is regulated by VEGF [38]. Further studies are needed to clarify the

association between VEGF and α-defensins in these diseases.

  CTGF belongs to the CCN family of multifunctional growth factors, with major roles in

angiogenesis and fibrosis [39-41]. CTGF is released from various cell types, such as

fibroblasts [42] and endothelial cells [43]. In IPF tissue, high levels of CTGF mRNA are found in proliferating type II alveolar cells and activated fibroblasts [44]. CTGF is induced exclusively by TGF-β and is thought to mediate the latter's profibrotic effects by modulating fibroblast cell growth and ECM protein synthesis, such as collagen and fibronectin [41, 45]. In the present study, mRNA expression of CTGF increased both in NHLF and HBEC with HNP-1 stimulation. Therefore, α-defensins may induce CTGF expression and play a role in the pathogenesis of these diseases, while few reports have demonstrated an association between CTGF and chronic respiratory tract infection, including CF and DPB.

In conclusion, these data indicate that α-defensins induce production of growth factors, as well as IL-8, in human lung cells. Furthermore, there is a considerable difference in the reaction to HNP-1 stimulation among cell types. Although the interaction between a large number of mediators and cells in lung inflammation and fibrosis is intricate, these results suggest that α-defensins play different roles in the pathogenesis of neutrophil-related lung diseases.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content

and writing of the paper.

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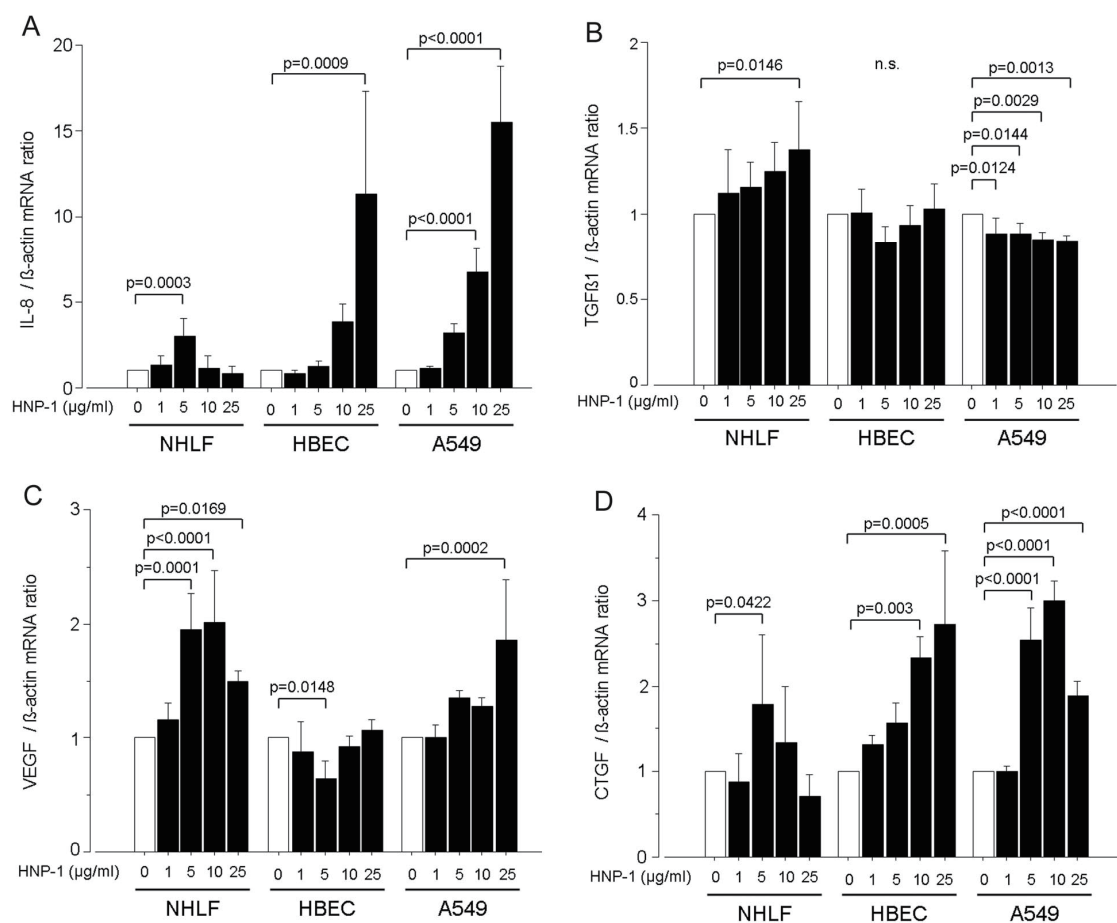
Figure Legends

Figure 1. Effects of HNP-1 on mediator mRNA expression

The mRNA expressions of IL-8 (A), TGF-β1 (B), VEGF (C), and CTGF (D) in NHLF,

HBEC, and A549 cells after 3-h incubation with medium alone (control, open bars), and 1,

5, 10, and 25 µg/ml of HNP-1 (black bars). Values are means ± SE of four independent
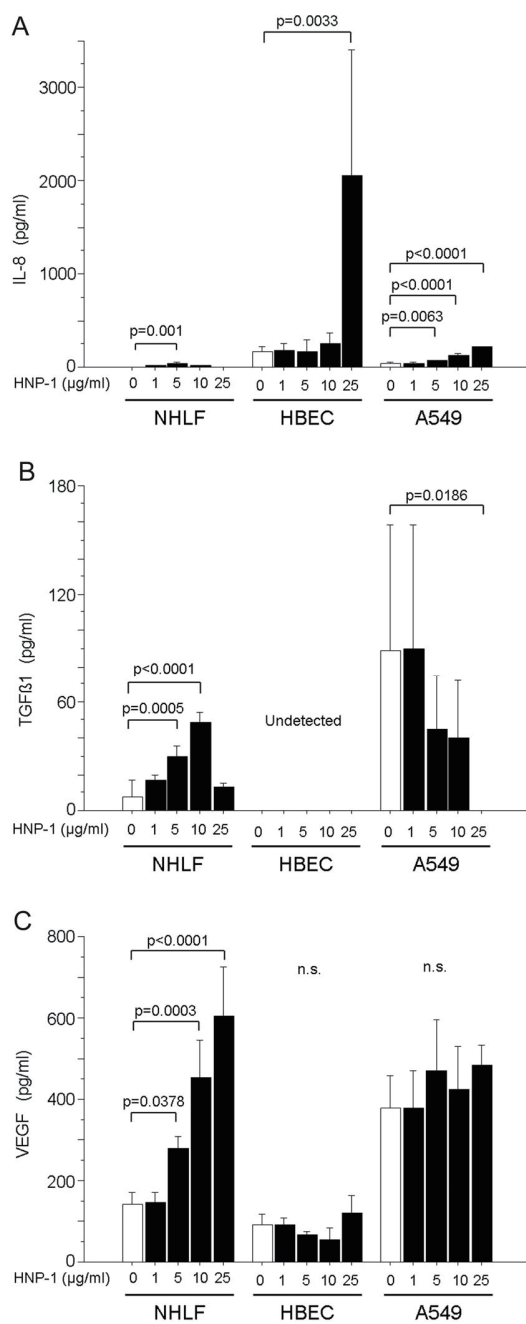
experiments. n.s. = not significant

Figure 2. Effects of HNP-1 on protein production

  The protein levels of IL-8 (A), TGF-β1 (B), and VEGF (C) in the supernatant of NHLF, HBEC, and A549 cells after 24-h incubation with medium alone (control, open bars), 1, 5, 10, and 25 μg/ml of HNP-1 (black bars). Values are means ± SE of four independent experiments.
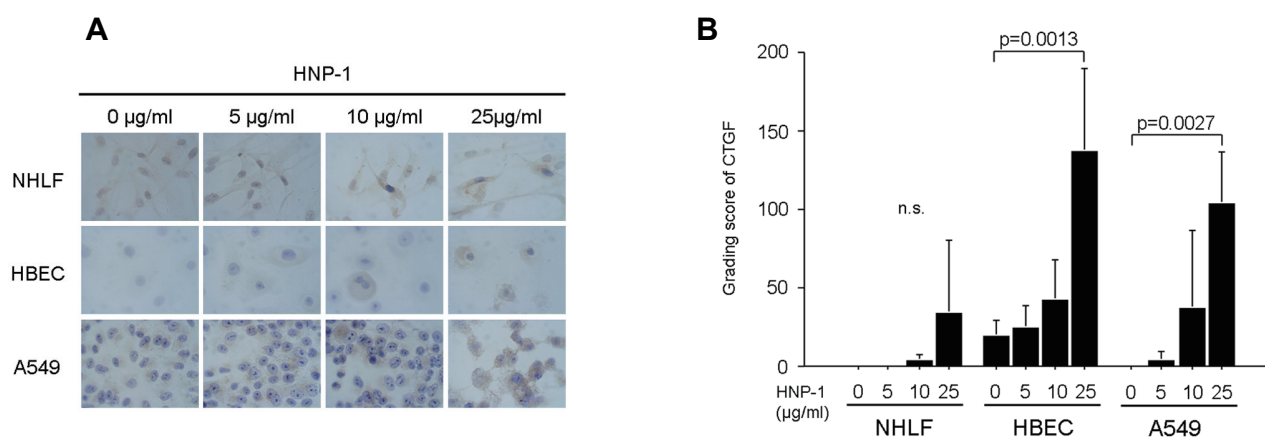
Figure 3.

(A) Representative immunocytochemical staining for CTGF in NHLF, HBEC, and A549 cells

(B) Grading score for CTGF immunocytochemical staining

Grading score for CTGF immunocytochemical staining after 24-h incubation with medium alone (control, open bars), 5, 10, and 25 µg/ml of HNP-1 (black bars). The staining intensity of CTGF in each cell was scored semiquantitatively using a scale from 0 to 2 (0 = no staining; 1 = weak staining; 2 = strong staining). Staining intensity was examined in more than five separate fields at ×1000 magnification. Values are means ± SE of four independent experiments. n.s. = not significant.
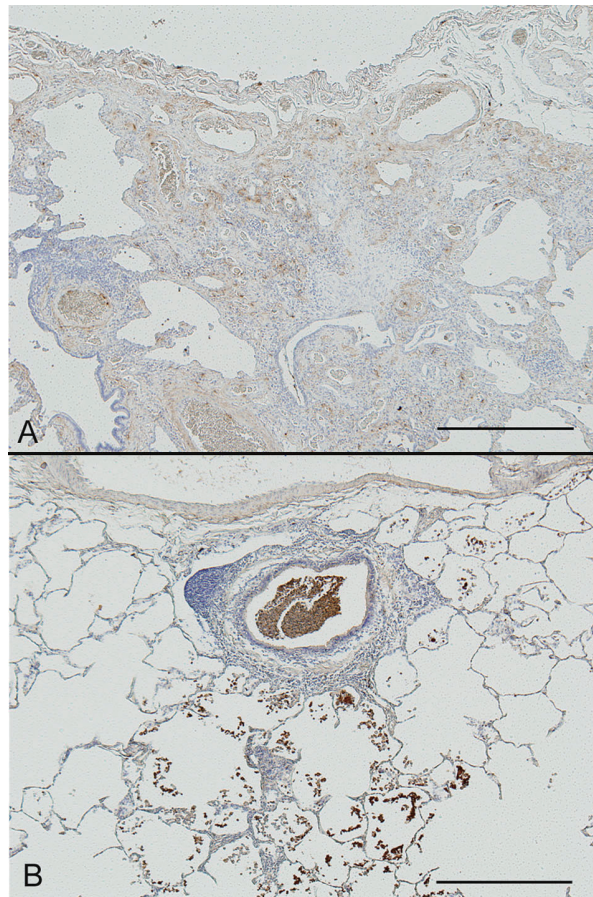
Figure 4. Immunohistochemical staining for α-defensins

(A) A surgical lung biopsy specimen obtained from a patient with IPF showing positive immunoreactivity for α-defensins to the thickened fibrous stroma tissue composed of lung fibroblasts. Infiltrated neutrophils with strong immunoreactivity are seen sparsely.

(B) A surgical lung biopsy specimen obtained from a patient with DPB showing a number of neutrophils rich in α-defensins in the airspace. The cilia of the bronchial epithelial cells are moderately immunopositive. Scale bar = 500 μm.