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The concentration-dependent chemokine release and pro-apoptotic effects of neutrophil-derived α-defensin-1 on human bronchial and alveolar epithelial cells

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Abstract

Defensins play a pivotal role in antimicrobial reactions, inflammatory responses, wound repair, and specific immunity. In inflammatory and infectious lung diseases, α-defensins are released from recruited neutrophils, and modulate a variety of lung cell functions. We found that human bronchial and alveolar epithelial cells treated with low and moderate concentrations (5 and 10 μg/ml) of purified neutrophil-derived α-defensin-1 secreted more interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1 in a dose- and time-dependent manner. Under moderate and high concentrations (10 and 20 μg/ml) of α-defensin-1, we observed typical apoptotic changes in the lung epithelial cells after stimulation for 24 h. Furthermore, α-defensin-1 triggered lung cell detachment in a time- and dose-dependent manner at moderate and high concentrations. Prior to the detachment, caspase-3 activity significantly increased. On confocal laser microscopy, rapid translocation of α-defensin-1 to the endoplasmic reticulum (ER) was noted. These findings suggest that neutrophil-derived α-defensin-1 has pro-inflammatory and apoptotic effects in human bronchial and alveolar epithelial cells, which are concentration-dependent and may be associated with ER activity.

Keywords: Defensin; Interleukin (IL)-8; Monocyte chemoattractant protein (MCP)-1; Apoptosis; Caspase; Endoplasmic reticulum (ER)

Introduction

The defensins are part of the antimicrobial peptide families that comprise the host innate defensive systems, which permit multicellular organisms to live in harmony with microbes (Zasloff, 2002). In humans, there are two main defensin subfamilies, α- and β-defensins (Ganz et al., 1990; Goldman et al., 1997), which are widely distributed in phagocytes, epithelial cells, and tissues that are involved in human host defense against microbial infections (Lehrer et al., 1993; Ganz, 2003). The α-defensins are expressed in neutrophils and Paneth's cells of the intestinal tract, whereas the β-defensins are mainly produced by epithelial cells of various origins (Van Wetering et al., 1999). Human neutrophil defensins, also called human neutrophil peptides (HNP), are small (3.5–4 kDa), cationic polypeptides (Ganz et al., 1990). They are constitutively synthesized by the neutrophil precursors during specific differentiation stages of neutrophil development (Cowland and Borregaard, 1999; Harwig et al., 1994) and are packaged in the primary (azurophil) granules (Rice et al., 1987).

In addition to their antimicrobial activity (Selsted et al., 1984; Lehrer and Ganz, 2002), α-defensins have the capacity to be chemotactic for monocytes (Territo et al., 1989), naïve T cells (Chertov et al., 1996) and dendritic cells (Yang et al., 2000) by inducing chemokine/cytokine synthesis (Van Wetering et al., 1997; Zhang et al., 2004; Sakamoto et al., 2005). α-Defensins also have the capacity to enhance bacterial adherence to airway epithelial cells (Rice et al., 1987; Gorter et al., 1998), induce LTB4 and IL-8 release by alveolar macrophages (Van Wetering et al., 1997; Paone et al., 1999), and induce histamine release by mast cells (Befus et al., 1999). High α-defensin concentrations

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are present in inflammatory tissues (Van Wetering et al., 1997), as well as in the airway secretions of patients with chronic inflammatory lung disorders (Ashitani et al., 1998). Furthermore, high α-defensin concentrations have been reported to be cytotoxic to airway epithelial cells (Aarbiou et al., 2002; Sakamoto et al., 2005). However, most of the intracellular molecular mechanisms that are related to α-defensin-associated biological phenomena have not been fully elucidated.

In this study, we calibrated the cell survival to study the chemokine release from human lung bronchial and alveolar epithelial cells treated with neutrophil-derived α-defensin-1. We found that α-defensin-1 had diverse concentration-dependent biological effects on the cells, which appear to be associated with endoplasmic reticulum (ER) activity.

Materials and methods

Reagents

α-Defensin-1 was purchased from Peptide Institute, Inc. (Osaka, Japan). The broad-spectrum caspase inhibitor (zVAD-FMK) and fluorogenic caspase-3 substrate (Ac-DEVD-AMC; *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) were purchased from Calbiochem (La Jolla, CA, USA). Propidium iodide (PI) and *n*-octyl-*b*-D-glucopyranoside (OG), and Hoechst nuclear dye were obtained from Sigma–Aldrich (St. Louis, MO, USA). Goat anti-human α-defensin-1 antibody, rabbit anti-mouse IgG antibody conjugated with FITC, and swine anti-goat IgG antibody conjugated with Cy3 were obtained from Chemicon International (Temecula, CA, USA). Mouse anti-human ER-specific glycoprotein antibody was obtained from Molecular Probes (Eugene, OR, USA). Anti-fade mounting medium was purchased from Vector Laboratories (Burlingame, CA, USA). HRP-conjugated rabbit anti-goat IgG antibody and the SuperSignal substrate were obtained from Pierce (Rockford, IL, USA). CytoBusterProtein Extraction Reagent was obtained from Novagen (Madison, WI, USA). Bovine serum albumin, cell culture media, and supplements were purchased from Life Technologies (Grand Island, NY, USA). Chemokine ELISA kits for IL-8 and MCP-1 were obtained from R and D Systems Inc (Minneapolis, MN, USA). Reverse transcriptase and *Taq* DNA polymerase were purchased from Invitrogen (San Diego, CA, USA) and Promega (Madison, WI, USA), respectively.

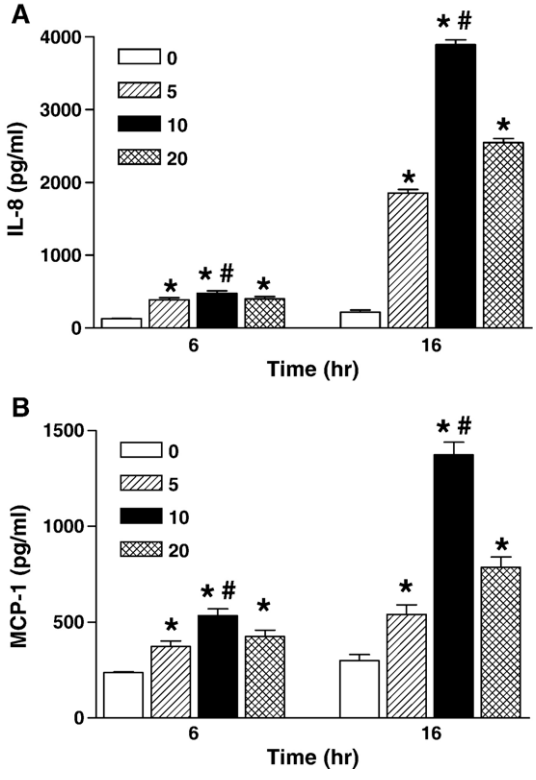

Fig. 1. Induction of IL-8 and MCP-1 secretion in lung A549 cells stimulated with neutrophil-derived α-defensin-1 at concentrations of 5, 10 and 20 μg/ml. (A) IL-8 and (B) MCP-1 protein secretion determined by ELISA; means±SEM of three independent assays are shown. *$p<0.01$, compared with the cells in the absence of defensin; #$p<0.01$, compared with the cells treated with α-defensin-1 at concentrations of 5 and 20 μg/ml.

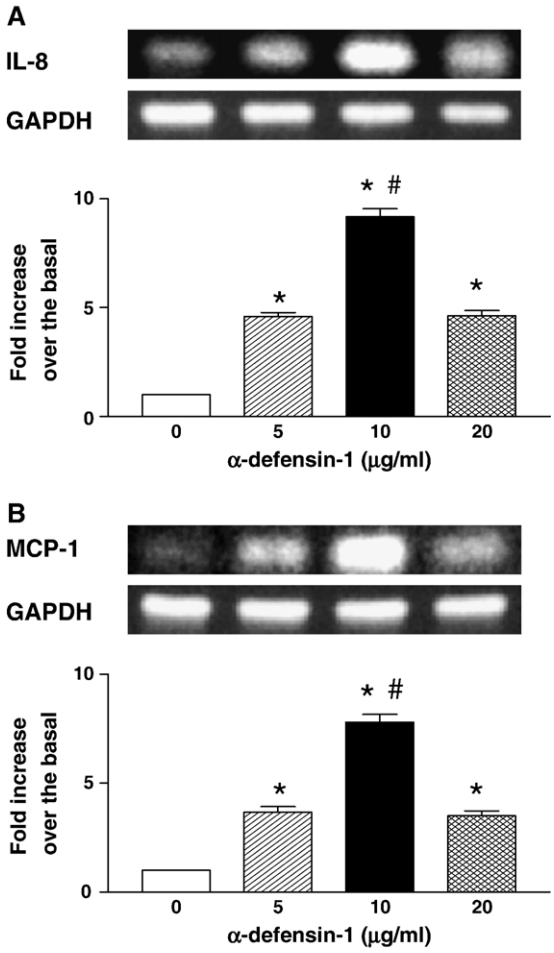

Fig. 2. Relative expression of (A) IL-8 and (B) MCP-1 genes determined by RT-PCR corrected for RNA density using GAPDH expression levels. Cells were treated with α-defensin-1 (5, 10 and 20 μg/ml) for 4 h. Fold change over expression in untreated control cells is demonstrated and represents the mean± SEM of three independent assays. *$p<0.01$, compared with the cells in the absence of defensin; #$p<0.01$, compared with the cells treated with α-defensin-1 at concentrations of 5 and 20 μg/ml.

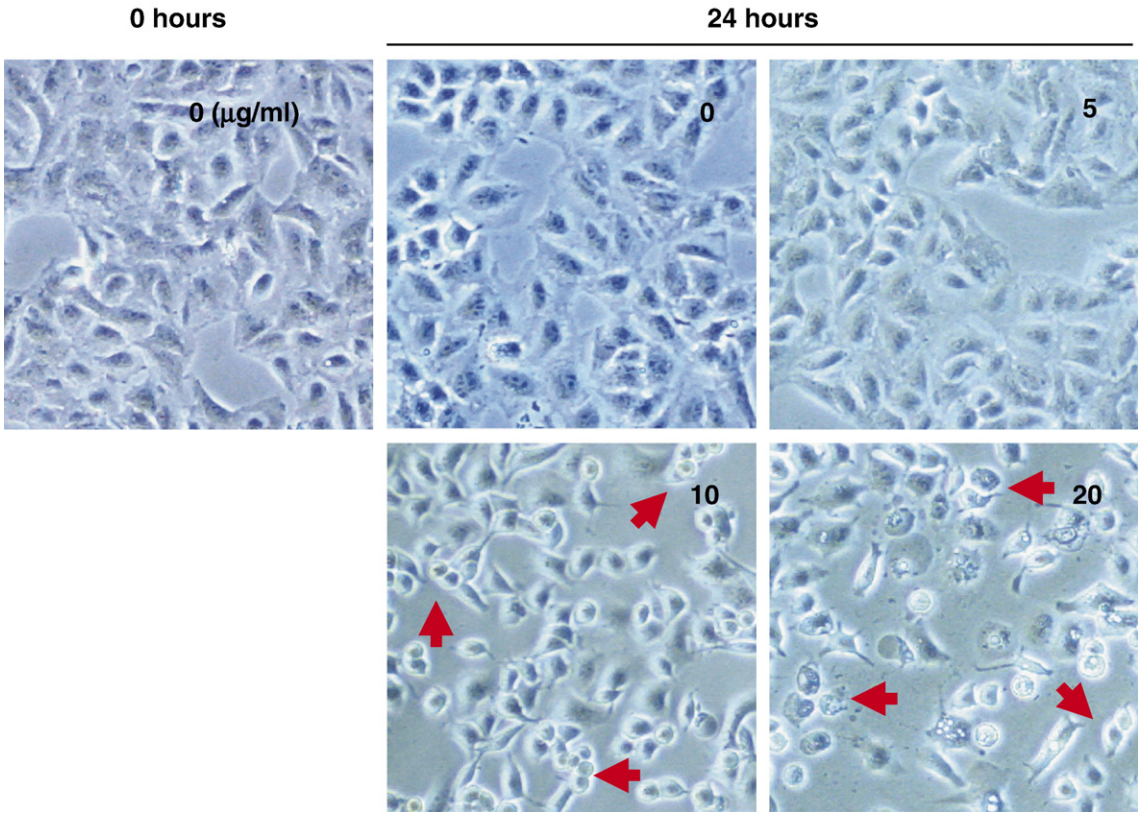

Fig. 3. Concentration-dependent effects of human neutrophil-derived α-defensin-1 on A549 cells. A549 cells were incubated with a variety of concentrations (0, 5, 10 and 20 μg/ml) of α-defensin-1 for 24 h, and observed using phase contrast microscopy. The monolayer confluence and the cell morphology showed no significant change in the cells treated with α-defensin-1 at the low concentration (5.0 μg/ml). When the concentrations of α-defensin-1 were increased to 10 and 20 μg/ml, the cells showed shrinkage (solid arrows) with decreased transparency and confluence. The results displayed are representative of five independent experiments (original magnification, 200×).

Cell culture

Human bronchial BEAS-2B and alveolar A549 epithelial cells (American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and passaged at 80% confluence. After the cells were over 98% confluent, they were further cultured in serum-free medium overnight and prepared for the experiments.

Enzyme-linked immunosorbent assay (ELISA)

The cells were cultured in 12-well plates at over 98% confluence, in the absence or presence of α-defensin-1 (5, 10 μg/ml, in 500 μl of medium) for 6 h and 16 h. The levels of IL-8 and MCP-1 were determined using a commercially available ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Cell-free supernatants were tested in duplicates. The IL-8 and MCP-1 ELISAs are sensitive to 10 and 5 pg/ml, respectively, and have an intra-assay coefficient of variation of <5% and an interassay coefficient of variation of <10%, according to the information provided by the manufacturer.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells were cultured in the absence or presence of α-defensin-1 (5 and 10 μg/ml) for 4 h. Total RNA was extracted using TRIzol reagent (Invitrogen) and quantified using RiboGreen™ quantification reagent (Molecular Probes Inc, Eugene, OR, USA). Readings were done on a Fluoroskan Ascent FL (Labsystems, Franklin, MA, USA). To synthesize cDNA, 1 μg of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase enzyme (Invitrogen) and a Minicycler™ (MJ Research, Waltham, MA, USA) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed using Promega *Taq* DNA polymerase, and the annealing temperature used for all primers was 55 °C. The primers used were (1) human IL-8 sense 5′-ATT TCT GCA GCT CTG TGT GAA-3′ and antisense 5′-TGA ATT CTC AGC CCT CTT CAA-3′ (255 bp); (2) human MCP-1 sense 5′-TCT CAG TGC AGA GGC T-3′ and antisense 5′-TGT GGA GTG AGT GTT CAA G-3′ (235 bp); and (3) human GAPDH sense 5′-ATG CAA CGG ATT TGG TCG TAT-3′ and antisense 5′-TCT CGC TCC TGG AAG ATG GTG-3′ (221 bp). PCR products were run on a 2% agarose gel and were stained with ethidium bromide (5 μg/ml).

Detachment assay

Cells plated in 48-well plates were labeled with 20 μM CellTracker Green CMFDA for 30 min at 37 °C in darkness. Cells were washed twice and incubated in 400 μl of fresh, phenol red-free medium. At this point, the baseline fluorescence intensity (FI)

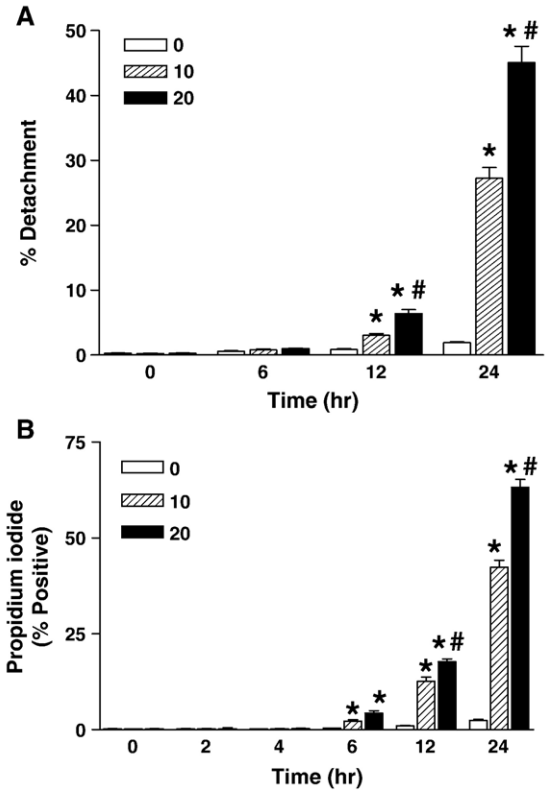

Fig. 4. Time- and dose-dependent α-defensin-1-induced A549 cell detachment. (A) A549 cells, plated in 48-well plates and labeled with 20 μM of CellTracker Green CMDFA for 30 min at 37 °C, were stimulated with α-defensin-1 (10 and 20 μg/ml). Detachment of A549 cells was measured using a CytoFluor plate reader. Results are expressed as mean±SEM of five experiments performed in triplicate. *$p<0.01$, compared with medium alone; #$p<0.01$, compared with the cells treated with 10 μg/ml of α-defensin-1. The cell membrane integrity of A549 cells was breached by α-defensin-1 in a time- and dose-dependent manner. (B) A549 cells were treated with α-defensin-1 and, at the predetermined time points, cells were stained with propidium iodide. The percentage of propidium iodide-stained adherent A549 cells was determined using flow cytometry. Data are expressed as mean±SEM of five experiments performed in triplicate. *$p<0.01$, compared with medium alone; #$p<0.01$, compared with the cells treated with 10 μg/ml of α-defensin-1.

in the wells was measured using CytoFluor (PerSeptive Biosystems, Framingham, MA, USA) with excitation at 485 nm and emission at 530 nm. Following treatment with α-defensin-1 at different concentrations for predetermined times, detached and loosely adherent cells were removed by gently washing the plate three times with phenol red-free medium. Next, the remaining fluorescence in the wells was measured. After subtracting background fluorescence (phenol red-free medium alone without cells), the percent of the detachment was calculated using the following formula (Liu et al., 2003):

$$\left[1-\frac{\text{FI in the test well at predetermined times}-\text{FI of background}}{\text{FI in the test well at time 0}-\text{FI of background}}\right]\times 100\%$$

Propidium iodide staining and flow cytometric analysis

The remaining adherent cells were washed twice with PBS and stained with propidium iodide (5 μg/ml in RPMI 1640) for 5 min at 37 °C. After two gentle washes, the cells were examined under fluorescence microscopy and then trypsinized for flow cytometric analysis (with excitation at 488 nm and emission at 585 nm) to quantify the damage to cell membrane integrity (Liu et al., 2003).

Caspase-3 activity assay

Cells that were cultured in 10-cm dishes at a predetermined time-point post-treatment were washed with PBS and lysed in 100 μl of buffer (10 mM potassium phosphate, 1 mM EDTA, 0.5% Triton X-100, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 mM DTT) for 10 min on ice. After centrifugation (15,000 ×g, 20 min, 4 °C), the protein concentration of the supernatant was determined with the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Subsequently, 40 μg of the sample was diluted to a final volume of 200 μl with the assay buffer (50 mM HEPES, 10% sucrose, 0.1% CHAPS, and 10 mM DTT) containing the fluorogenic caspase-3 substrate Ac-DEVD-AMC (100 μM) and incubated for 2 h at 30 °C in a 96-well plate. Fluorescence was determined (excitation 360 nm; emission 460 nm) with a CytoFluor series

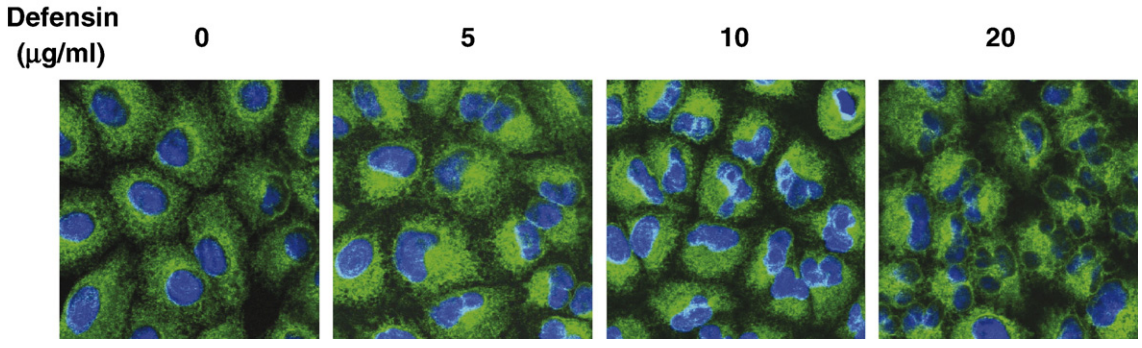

Fig. 5. α-Defensin-1 induced apoptotic-like morphologic changes in A549 cells. Immunofluorescent preparations of A549 cells 24 h after the addition of α-defensin-1 (10 or 20 μg/ml) or buffered saline were examined by confocal microscopy (cytoplasmic staining using mouse anti-human anti-ER-specific glycoprotein primary antibody and secondary rabbit anti-mouse antibody conjugated with FITC, nuclear staining using Hoechst dye (original magnification, 400×). α-defensin-1-stimulated A549 cells displayed cell shrinkage, nuclear fragmentation, and condensation similar to that observed in classic apoptosis. The apoptotic-like changes in morphology appeared more dramatic in the cells treated with 20 μg/ml of α-defensin than in cells treated with 10 μg/ml. The results displayed are representative of five independent experiments.

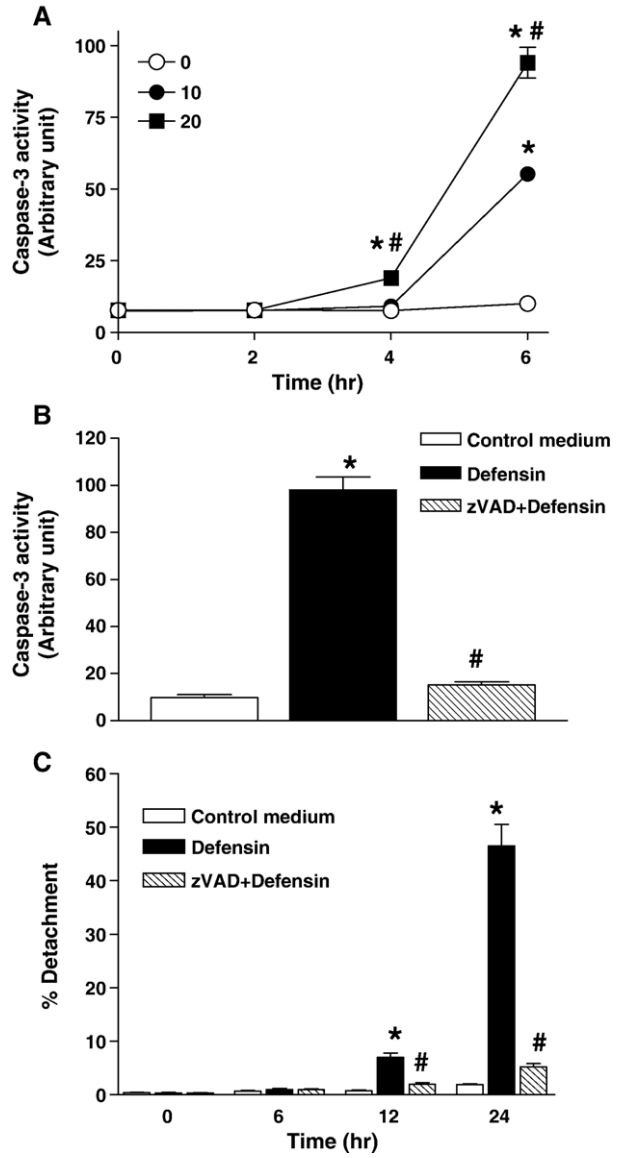

Fig. 6. The caspase-3 activity was significantly increased in the A549 cells treated with α-defensin-1. (A) Cleavage of the caspase-3-preferred fluorogenic substrate, Ac-DEVD-AMC, was measured in whole-cell lysates prepared from A549 cells stimulated with or without α-defensin-1 (10 or 20 μg/ml) for 6 h. Results shown represent mean±SEM of three experiments performed in triplicate. *$p<0.01$, compared with medium alone; #$p<0.01$, compared with the cells treated with 10 μg/ml of α-defensin-1. Broad-spectrum caspase inhibitor zVAD abrogated caspase-3 activity and further attenuated the detachment of A549 cells. (B) Cleavage of the caspase-3-preferred fluorogenic substrate, Ac-DEVD-AMC, was measured in whole-cell lysates prepared from A549 cells preincubated with or without zVAD (100 μM) for 1 h and then stimulated with or without α-defensin-1 (20 μg/ml) for an additional 6 h. Results shown represent mean±SEM of three experiments performed in triplicate. *$p<0.01$, compared with medium alone; #$p<0.01$, compared with the cells stimulated with α-defensin-1 alone. (C) A549 cells labeled with Celltracker Green were preincubated with zVAD (100 μM) for 1 h before the addition of α-defensin-1 (20 μg/ml) or an equivalent volume of buffered saline at time 0. The extent of cell detachment was subsequently assayed using a CytoFluor plate reader. Results are mean±SEM of three independent experiments performed in triplicate. *$p<0.01$, compared with medium alone; #$p<0.01$, compared with cells stimulated with α-defensin-1 alone.

4000 plate reader (Applied Biosystems, Foster City, CA, USA). Background fluorescence was determined in wells containing the assay buffer only.

Western blotting for α-defensin-1

Cells at the end of the treatment were harvested and washed with ice-cold PBS. Cells were resuspended in 500 μl of 0.05% Tween 20-TBS buffer containing 10 mM Tris–HCl (pH 7.5) and 150 mM NaCl, and, subsequently, they were homogenized using a Potter–Elvehjem homogenizer at 4 °C in the presence of Complete Protease Inhibitor Cocktail (Boehringer Mannheim, Indianapolis, IN, USA). The cell extracts were then sonicated, centrifuged at 12,000 ×*g* for 20 min, and the protein concentration was measured. Thirty micrograms of protein extract was subjected to gradient (4%–20%) SDS-PAGE and electrotransferred to nitrocellulose membranes. After blocking overnight with 5% dry milk in TBST (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), the membranes were incubated with primary goat anti-human α-defensin antibody. After washing with TBST, the membranes were incubated with secondary anti-goat antibody conjugated with HRP. Chemiluminescence of protein bands was detected using the SuperSignal Substrate Western Blotting Kit (Pierce).

Confocal laser microscopic analysis

At the end of treatment, the cells were fixed in 4% paraformaldehyde solution and permeabilized using 3.7% OG at room temperature for 5 min. Then the cells were incubated with primary goat anti-human α-defensin-1 and mouse anti-human ER-specific glycoprotein antibodies at room temperature for 1 h After washing, the cells were further incubated with secondary rabbit anti-mouse antibody conjugated with FITC and secondary swine anti-goat antibody conjugated with Cy3 sequentially, as well as Hoechst nuclear dye. After washing and air-drying, the cells were mounted on slides with anti-fade mounting medium. Images were acquired with a confocal laser-scanning microscope (Leica, Exton, PA, USA); they were then analyzed using Metamorph Image Analysis software (Universal Imaging, West Chester, PA, USA).

Statistical analyses

Data were expressed as the mean±SEM. For normally distributed data, Student's *t*-test was used to evaluate differences between sets. For non-normally distributed data, the Mann–Whitney *U* test was used. GraphPad Prism (version 3.01, GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. Statistical significance was defined as $p<0.05$.

Results

α-Defensin-1 induced IL-8 and MCP-1 release from human alveolar A549 epithelial cells

To investigate the inflammation-modulating effects of neutrophil-derived α-defensin-1 on lung epithelial cells, A549

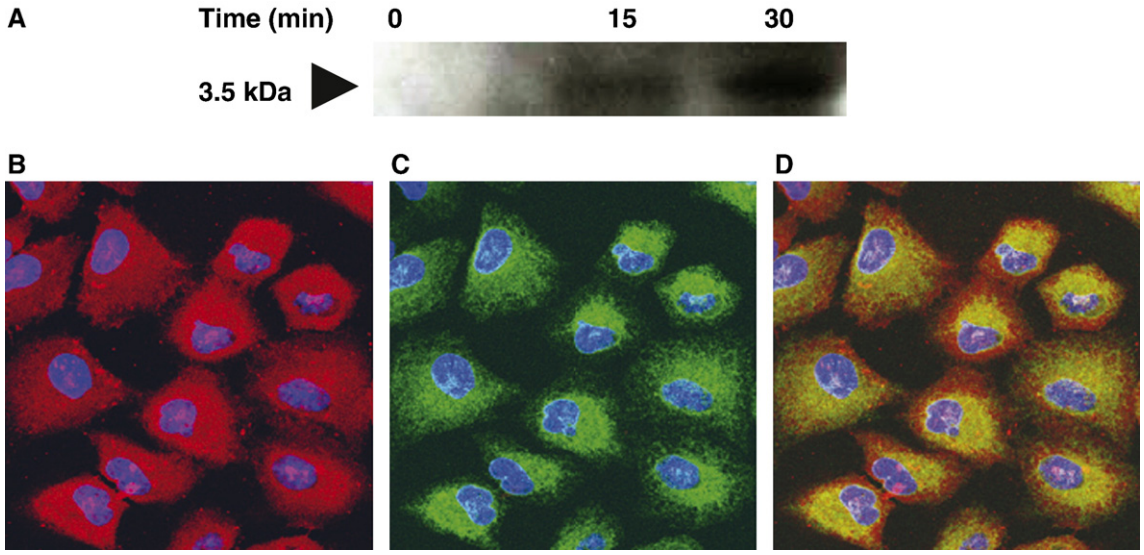

Fig. 7. Neutrophil-derived α-defensin-1 entered A549 cells within 15 min and was co-localized with the endoplasmic reticulum. (A) Immunoblot for α-defensin-1 (3.5 kDa) on the whole cell lysates prepared from A549 cells stimulated with or without α-defensin-1 (10 μg/ml). Using primary goat anti-human α-defensin-1 antibody and secondary swine anti-goat antibody conjugated with Cy3 for fluorescent immunostaining, the confocal image revealed that the intracellular distribution of α-defensin-1 had a branched reticulum pattern in A549 cells (B). Using primary mouse anti-human ER-specific glycoprotein antibody and secondary rabbit anti-mouse antibody conjugated with FITC for additional fluorescent immunostaining, confocal images showed that the fluorescence had turned from greenish (C) to yellowish (D) in color within 30 min (original magnification, 400×). The results displayed are representative of five independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cells were treated with α-defensin-1 at concentrations of 5, 10 and 20 μg/ml for 6 h and 16 h. Secretion of the chemokines IL-8 and MCP-1 was increased significantly in a dose- and time-dependent manner at concentrations of 5 and 10 μg/ml (Fig. 1A and B). Additionally, the expression of IL-8 and MCP-1 mRNA in the A549 cells that were treated with α-defensin-1 was increased significantly in a dose-dependent manner at concentrations of 5 and 10 μg/ml at 4 h (Fig. 2A and B). When the concentration of α-defensin-1 reached 20 μg/ml, the dose-dependent effect on the expression of IL-8 and MCP-1 mRNA and the secretion of chemokines (Figs. 1 and 2) disappeared.

Human α-defensin-1 induced cytotoxic changes in A549 cells

Under phase contrast light microscopy, human alveolar A549 epithelial cells treated with a low concentration of α-defensin-1 (5.0 μg/ml, for 24 h), retained more than 98% confluence, which was similar to the confluence before treatment (Fig. 3). In contrast, moderate and high concentrations of α-defensin-1 (10 and 20 μg/ml) were cytotoxic to lung epithelial cells; cell shrinkage and loss of transparency were seen (Fig. 3).

Furthermore, the detachment of the α-defensin-1-stimulated A549 cells was significant at 12 h and increased dramatically up to 24 h in a dose- and time-dependent manner (Fig. 4A). As shown by flow cytometric analysis, cell membrane permeability to propidium iodide, a DNA dye, was significantly increased before detachment after stimulation with α-defensin-1 for 6 h (Fig. 4B). This suggests that the integrity of A549 cells was significantly breached before cell detachment. On confocal laser microscopic examination, A549 cells that were treated with α-defensin-1 at concentrations of 10 and 20 μg/ml for 24 h showed apoptotic-like changes, including dense condensation of the nuclear chromatin, fragmentation of the nuclear lobes, extensive cytoplasmic fragmentation, and formation of many membrane-bound bodies (Fig. 5). On the other hand, cells treated with 5 μg/ml of α-defensin-1 appeared to be morphologically normal and were similar in appearance to the untreated cells (Fig. 5).

Caspase-3 activity implicated in α-defensin-induced apoptosis and cell detachment

Caspase-3 activity was significantly increased at 4 h in the cells treated with α-defensin-1 at a concentration of 20 μg/ml, and at 6 h in the cells treated with the reagent at concentrations of 10 and 20 μg/ml (Fig. 6A). The broad-spectrum caspase inhibitor zVAD was able to abrogate α-defensin-1-stimulated caspase-3 activity (Fig. 6B). zVAD also attenuated α-defensin-1-induced cell detachment (Fig. 6C). These results implicate caspase-3 in α-defensin-1-triggered cell apoptosis and detachment.

α-Defensin-1 trafficked to the ER without cell surface accumulation

The localization of α-defensin-1 was further explored to elucidate the intracellular mechanisms involved in the effects of α-defensin-1. Western blotting analysis detected α-defensin-1 intracellularly within 15 min (Fig. 7A). Under confocal laser microscopy, a branched reticulum pattern of exogenous α-defensin-1 was noted, similar to ER architecture (Fig. 7B). With this novel finding, an anti-ER glycopeptide antibody conjugated with FITC was used to label the ER (Fig. 7C). When the α-defensin-1 was labeled with anti-α-defensin antibody conjugated with Cy3 (red fluorescence), we could not detect any red

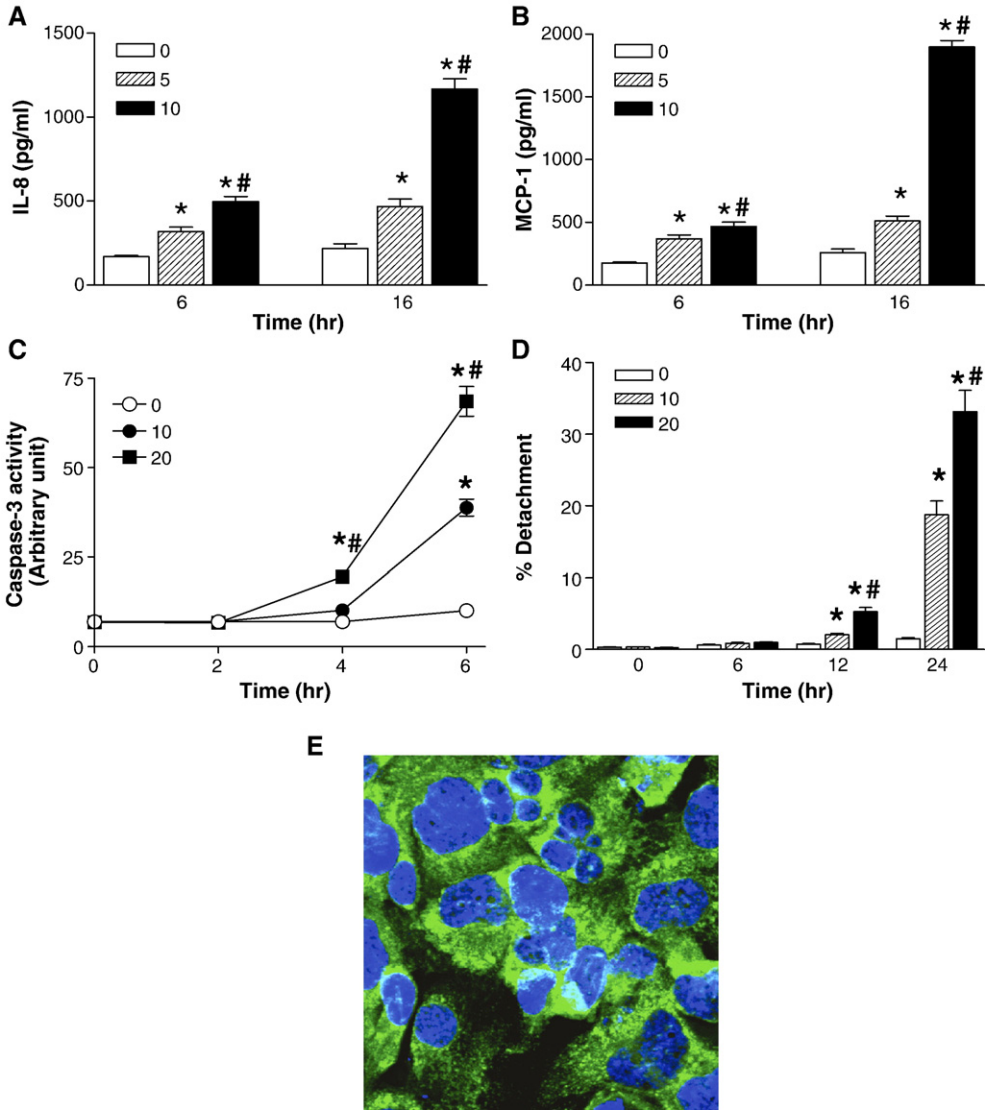

Fig. 8. In bronchial epithelial BEAS-2B cells, neutrophil-derived α-defensin-1 induced IL-8 and MCP-1 secretion and apoptosis in a concentration- and time-dependent manner. (A) IL-8 and (B) MCP-1 protein secretion determined by ELISA; mean ± SEM of three independent assays are shown. *$p<0.01$, compared with the cells in the absence of defensin; #$p<0.01$, compared with the cells treated with α-defensin-1 at the concentration of 5 μg/ml. (C) Cleavage of the caspase-3-preferred fluorogenic substrate, Ac-DEVD-AMC, was measured in whole-cell lysates prepared from BEAS-2B cells stimulated with or without α-defensin-1 (10 or 20 μg/ml) for 6 h. Results shown represent the mean ± SEM of three experiments performed in triplicate. *$p<0.01$, compared with medium alone; #$p<0.01$, compared with the cells treated with 10 μg/ml of α-defensin-1. (D) BEAS-2B cells, plated on 48-well plates and labeled with 20 μM of CellTracker Green CMDFA for 30 min at 37 °C, were stimulated with α-defensin-1 (10 and 20 μg/ml). Detachment of cells was measured using a CytoFluor plate reader. Results are expressed as mean ± SEM of five experiments performed in triplicate. *$p<0.01$, compared with medium alone; #$p<0.01$, compared with the cells treated with 10 μg/ml of α-defensin-1. (E) Immunofluorescent preparations of BEAS-2B cells after the addition of α-defensin-1 (20 μg/ml) for 24 h were examined by confocal microscopy (cytoplasmic staining using mouse anti-human anti-ER specific glycoprotein primary antibody and secondary rabbit anti-mouse antibody conjugated with FITC, nuclear staining using Hoechst dye (original magnification, 400×). α-defensin-1-stimulated cells displayed cell shrinkage, nuclear fragmentation, and condensation similar to that seen in classic apoptosis. The results displayed are representative of five independent experiments.

fluorescence staining on the cell membrane (Fig. 7D). Within 30 min, the green fluorescence representing the ER turned to yellow fluorescence representing co-localization with α-defensin-1 (Fig. 7D).

Effects of α-defensin-1 on bronchial epithelial cells

The human immortalized bronchial epithelial BEAS-2B cell line was used to further explore the effect of human neutrophil-derived α-defensin-1 on lung cells. Our data showed that the secretion of IL-8 and MCP-1 was upregulated in the cells treated with α-defensin-1 at concentrations of 5 and 10 μg/ml (Fig. 8A and B). Additionally, an increase in the caspase-3 activity and detachment were observed in the BEAS-2B cells treated with α-defensin-1 at concentrations of 10 and 20 μg/ml (Fig. 8C and D). On confocal microscopy, cell shrinkage and segregation of nuclei were seen in the cells that were treated with moderate and high concentrations of α-defensin-1 (Fig. 8E).

Discussion

In the present study, we have demonstrated that human alveolar A549 and bronchial BEAS-2B epithelial cells, when treated with low and moderate concentrations of neutrophil-derived α-defensin-1 (5 and 10 μg/ml), release the chemokines IL-8 and MCP-1 in a dose-dependent manner. In contrast, when treated with moderate and high concentrations of α-defensin-1 (10 and 20 μg/ml), the lung cells undergo apoptotic cell death. This apoptotic process is caspase-3-dependent, as shown by the caspase-3 assay and the pharmacologic inhibition study. Furthermore, our results also indicate that the ER might be implicated in α-defensin-1-related chemokine secretion and its pro-apoptotic effect, as demonstrated by the rapid translocation of the molecules to the ER observed on confocal microscopy. Our observations are compatible with previous reports showing that the modulation of airway epithelial cell function by neutrophil defensins is concentration-dependent (Okrent et al., 1990; Aarbiou et al., 2002).

Chemokines, which are chemotactic cytokines, not only recruit inflammatory cells but also have effects that are related to angiogenesis, hematopoiesis, and the activation of immunocompetent cells (Yoshie et al., 2001). Interleukin-8 (IL-8), a member of the CXC chemokine family, has an important role in the continuous accumulation of neutrophils in the airways of patients with chronic inflammatory and infectious lung diseases (Oishi et al., 1994). This chemokine is secreted by several types of cells, including lung epithelial cells (Asokananthan et al., 2002). In lung diseases, it is believed to play a role in the pathogenesis of bronchiolitis (DiGiovine et al., 1996). IL-8 is also associated with asthma and acute respiratory distress syndrome (ARDS) (Aggarwal et al., 2000). On the other hand, human monocyte chemoattractant protein-1 (MCP-1), a member of the CC chemokine family, is produced by a variety of stimulated cell types, including lung epithelial cells (Olszewska-Pazdrak et al., 1998). MCP-1 is a potent chemoattractant for monocytes and it also activates lymphocytes, basophils and NK cells (Gu et al., 1999). MCP-1 is produced in chronic inflammatory conditions, such as atherosclerosis (Gu et al., 1998), HIV replication (Garzino-Demo et al., 2000), glomerular nephritis (Tang et al., 1995), allergic and chronic inflammatory diseases (Alam et al., 1996), and in malignant tumors (Luciani et al., 1998). MCP-1 is thought to have an important role in the regional exudation of monocytes and macrophages (Lu et al., 1998). Accumulating evidence indicates that MCP-1 may be involved in Th subset differentiation and has immunoregulatory functions (Karpus and Kennedy, 1997).

Human neutrophil-derived α-defensins are contained in the azurophilic granules of neutrophils and contribute to the intracellular killing of ingested microorganisms (Lehrer and Ganz, 2002). α-Defensins can also be released into the extracellular space, where they not only contribute to the extracellular killing of microorganisms, but also affect the function of other tissue cells (Van Wetering et al., 1999, 2005). In this study, we demonstrated that α-defensin-1 upregulated the expression of the chemokines IL-8 and MCP-1 in the lung epithelial cells at low and moderate concentrations, suggesting that neutrophil-derived α-defensin-1 has pro-inflammatory and immune modulating effects on lung epithelial cells.

Apoptosis, or programmed cell death, is a pivotal physiological process that is required for the normal development and maintenance of tissue homeostasis in multicellular organisms (Glucksmann, 1965; Wyllie, 1997). It is known to be involved in a wide range of pathologic conditions (Thompson, 1995). In the present study, α-defensin-1 displayed cytotoxic activity at moderate and high concentrations. α-Defensin-1 given at concentrations of 10 and 20 μg/ml caused lung A549 and BEAS-2B cells to exhibit morphological changes typical of apoptosis. The morphological changes of apoptosis and an increase in cell membrane permeability to propidium iodide were followed by extensive cell detachment in the α-defensin-1-stimulated lung cells. This suggests an important function of α-defensin-1 in preventing cell necrosis and attenuating the inflammatory responses that are induced by lung epithelial cells (Van Wetering et al., 2005).

Several signaling pathways have been reported to be involved in apoptosis. Proteins of the B cell lymphoma (Bcl)-2 family, as well as caspases, mitochondria, cytochrome *C*, and other nucleus-targeting proteins, such as apoptosis-inducing factor (AIF), DNA fragmentation factor (DFF) and endonuclease, have been identified as essential components of the intracellular apoptotic signaling pathways (Newton and Strasser, 1998). Among them, the caspases are a central component of the apoptotic machinery and are typically activated in the early stages of apoptosis (Grutter, 2000). In the caspase cascade, caspase-3 is a downstream effector that is responsible for the activation of the executing proteins that lead to the typical morphological changes observed in cells undergoing apoptosis (Grutter, 2000). In the present study, we observed that caspase-3 activity is elevated in A549 and BEAS-2B cells stimulated with α-defensin-1 at moderate and high concentrations, confirming that α-defensin-1 has a pro-apoptotic effect on lung epithelial cells. We also observed that cell detachment is attenuated by the general caspase inhibitor zVAD, which indicates that cell detachment can be attributed to the prior cell apoptosis.

Confocal microscopic observations showed that α-defensin-1 rapidly enters A549 cells without accumulating on the cell membrane; this suggests that a cell membrane receptor-mediated mechanism is not likely. Furthermore, the distribution of α-defensin-1 was not random; it had a branched reticulum pattern, which was similar to the architecture of the ER. On confocal microscopy, α-defensin-1 was translocated to and co-localized with ER within 30 min; this occurred before caspase-3 activation was detected, which suggests that the α-defensin-1 accumulation at the ER might be related to subsequent lung epithelial cell apoptosis.

The ER plays an important role in the maintenance of intracellular calcium homeostasis, protein synthesis, post-translational modifications, proper protein folding and the sorting and trafficking of proteins. The ER is also an important subcellular compartment that has been implicated in apoptotic execution (Mehmet, 2000; Nakagawa et al., 2004). A disruption of calcium homeostasis and the accumulation of excess proteins in the ER have been reported to induce stress signals that trigger cell apoptosis (Trump and Berezesky, 1995; Mehmet, 2000;

Nakagawa and Yuan, 2000). In fact, the signaling pathway that initiates ER stress-induced apoptosis has been defined; it involves the activation of cysteine proteases that are distinct from those that trigger mitochondrial and death receptor apoptotic pathways. ER stress-mediated apoptosis is dependent on an ER-resident cysteine protease, caspase-12 (Mehmet, 2000; Nakagawa et al., 2004), whose activation is regulated by processing of the procaspase-12 zymogen by cysteine protease calpain (Nakagawa and Yuan, 2000) and by TRAF2-mediated zymogen clustering (Yoneda et al., 2001). Considering the evidence obtained in our study and previous reports suggesting that proinflammatory and immunomodulating cytokines were increased in the α-defensin-stimulated lung epithelial cells, high defensin concentration-dependent pro-apoptotic mechanisms may indicate the presence of a modulating effect on the resolution of inflammation mediated by α-defensin (O'Sullivan et al., 2003; Van Wetering et al., 2005).

In conclusion, our results demonstrate that, at low and moderate concentrations, neutrophil-derived α-defensin-1 upregulates the secretion of chemokines IL-8 and MCP-1 in human alveolar A549 and bronchial BEAS-2B epithelial cells. In contrast, at moderate and high concentrations, α-defensin-1 has a pro-apoptotic function in lung cells, which may be associated with ER activity. Our findings suggest that small molecules of pro-inflammatory mediators, such as α-defensin, may directly translocate to and accumulate at the ER to exert their various concentration-dependent biological activities in order to maintain homeostasis in the tissues. The molecular mechanisms by which α-defensin-1 induces chemokine expression and cell apoptosis and the role of ER activity in the α-defensin-1-related cellular responses deserves future study. It is important to clarify the mechanisms that are involved to advance our knowledge of the neutrophil-mediated pathophysiological functions of lung epithelial cells.

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