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Alpha-defensin enhances expression of HSP47 and collagen-1 in human lung fibroblasts

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Abstract

Neutrophils and lung fibroblasts are thought to play a role in the pathogenesis of pulmonary fibrosis. We reported previously that heat shock protein 47 (HSP47), a collagen-specific molecular chaperon, and collagen-1 synthesis were involved in pulmonary fibrosis, and that plasma levels of α-defensins (HNP; human neutrophil peptide), cationic proteins with antimicrobial and cytotoxic activity in neutrophils, were significantly higher in patients with idiopathic pulmonary fibrosis than in control subjects. Here, we investigated the direct effect of HNP-1 *in vitro* on the expression of HSP47 and collagen-1 in human lung fibroblasts (NHLF). HNP-1 at 5 μg/ml induced fibroblast proliferation but at concentrations >50 μg/ml, HNP-1 reduced cell viability. Incubation of NHLF with 10 to 25 μg/ml of HNP-1 for 24-h increased the expression of HSP47 and collagen-1 mRNAs ($p<0.05$). The levels of HSP47 protein also increased significantly at 50 μg/ml, and those of collagen-1 protein increased at 10 to 50 μg/ml of HNP-1 ($p<0.05$). The mitogen-activated protein kinase (MAPK) signaling pathway in NHLF was activated by HNP-1 stimulation, but inhibitor of MEK (PD98059) did not block HNP-1-induced HSP47 protein production. Our results suggest that α-defensin is a fibrogenic mediator that promotes collagen synthesis through the upregulation of HSP47 and collagen-1 in lung fibroblasts and participates in the pathogenesis of neutrophil-induced pulmonary fibrosis.

Keywords: Human neutrophil peptide-1; HSP47; Collagen synthesis; Pulmonary fibrosis

Introduction

Neutrophils are considered to play a pathologic role in acute and chronic injurious diseases of the lung. A marginal increase of neutrophils in the respiratory tract can be associated with major damage and irreversible architectural changes in the lung (Sibille and Marchandise, 1993). Idiopathic pulmonary fibrosis (IPF) is a progressive and usually fatal lung disease characterized by patchy fibrotic areas with fibroblast proliferation and extracellular matrix remodeling, which result in irreversible distortion of the lung architecture. Increasing scientific evidence defines the importance of neutrophils in the pathogenesis of pulmonary fibrosis (Crystal et al., 1984; Hunninghake et al., 1981; Obayashi et al., 1997; Turner-Warwick and Haslam, 1987; Wells et al., 1998). For example, large numbers of neutrophils were found in the bronchoalveolar lavage (BAL) fluid (Crystal et al., 1984; Hunninghake et al., 1981) and lung tissue of patients with IPF (Hunninghake et al., 1981; Obayashi et al., 1997). Furthermore, BAL neutrophil counts in IPF patients correlated with the severity of the disease as determined by chest CT (Wells et al., 1998), poor treatment outcome (Turner-Warwick and Haslam, 1987) and disease activity (Crystal et al., 1984). However, other studies reported that the number and proportion of neutrophils in BAL fluid did not correlate with the activity of neutrophil alveolitis and that they had limited prognostic value (Boomars et al., 1995; Schwartz et al., 1994). Thus, the exact role of neutrophils in the pathogenesis of IPF remains unclear.

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α-Defensins are cationic proteins with antimicrobial activity, which constitute 5–7% of the total protein content of the human neutrophil and 30–50% of the total protein content of the azurophilic granules (van Wetering et al., 1999). They are also one of the products of activated and deteriorating neutrophils and released extracellularly during inflammatory reactions (Ashitani et al., 1998a,b). Six α-defensin peptides have so far been isolated and characterized in human. Since they are mainly present in neutrophil azurophilic granules, human α-defensin-1 to -4 are conventionally called human neutrophil peptides (HNP)-1 to -4 (van Wetering et al., 1999). High concentrations of HNP-1, -2 and -3 were observed in various inflammatory and fibrotic lung diseases including IPF (Ashitani et al., 1998a,b, 2004; Mukae et al., 2002), suggesting that α-defensins are related to the pathogenesis of neutrophil-related lung diseases. In these reports, the mean plasma levels of HNP-1 to 3 in IPF (768.2±422.4 ng/ml, ±SD) and acute respiratory distress syndrome (2010±1483 ng/ml) were significantly higher than in control subjects (200 to 300 ng/ml). In IPF, we have shown previously that the concentrations of HNP-1 to 3 in plasma correlated inversely with respiratory function tests and they reflected the clinical course with an acute exacerbation (Mukae et al., 2002). Immunohistochemically, positive staining for α-defensins was observed inside and outside neutrophils in the alveolar septa, especially in dense fibrotic areas (Mukae et al., 2002). These results suggest that neutrophils could be involved in the lung fibrotic process via the expression of α-defensins.

Heat shock protein 47 (HSP47), localized in the endoplasmic reticulum, is involved in the synthesis/assembly of various collagens as a collagen-specific molecular chaperone (Matsuoka et al., 2004; Nagai et al., 2000; Nakai et al., 1992). Expression of HSP47 has been reported to increase in parallel with the expression of collagens during the progression of various fibrosis models (Masuda et al., 1994; Pan and Halper, 2003). Indeed, strong immunostaining of both HSP47 and procollagen-1 was noted predominantly in interstitial spindle-shaped cells around active fibrotic areas in the lung of patients with IPF and murine bleomycin-induced pulmonary fibrosis (Ishii et al., 2003; Kakugawa et al., 2004, 2005). These findings suggest that lung fibroblasts and myofibroblasts play a role in fibrotic process through the upregulation of HSP47.

In this study, to assess the relationship between α-defensin and lung fibrotic process, we examined the expression of HSP47 and collagen-1 in primary cultures of human lung fibroblasts following stimulation with HNP-1 or transforming growth factor (TGF)-β1, which is a well-known chemotactic factor for fibroblasts and promotes the production of extracellular matrix proteins such as collagen.

Materials and methods

Cells and agents

Normal human lung fibroblasts (NHLF) obtained from three different donors were purchased from Clonetics-BioWhittaker (Walkersville, MD). Cells were grown in fibroblast basal medium (FBM; Clonetics-BioWhittaker) supplemented with 2% fetal bovine serum, human recombinant fibroblast growth factor (1.0 μg/ml), insulin (5 mg/ml), gentamicin and amphotericin-B in humidified incubator (5% CO_2 at 37 °C). The synthetic products of HNP-1 and TGF-β1 were purchased from Peptide Institute (Osaka, Japan) and R&D Systems (Minneapolis, MN), respectively.

Subculture and stimulation of NHLF

For experiments to evaluate the mRNA and protein expression levels of HSP47 and collagen-1, subcultures of NHLF were plated in 60-mm dishes (BD Falcon™, Franklin Lakes, NJ) at a density of 5×10^5 cells/dish. When the cells reached a confluence of approximately 70–80%, the medium was replaced by serum-free FBM. Cells were subsequently stimulated for 3, 6, 9, 12, 24 and 36 h with medium alone (control), synthetic HNP-1 5 to 100 μg/ml or TGF-β1 1 to 10 ng/ml. To examine the activation of the mitogen-activated protein kinase (MAPK) signaling pathway, NHLF were plated in 6-well dishes at a density of 5×10^4 cells per well and cultured to 70–80% confluence. After overnight starvation in serum-free FBM, the cells were incubated with 10 μg/ml HNP-1 in serum-free medium for 15 min to detect the phosphorylated ERK-1/2, or 24 h to confirm the production of HSP47 protein. In addition, the cells were incubated with PD98059 (50 μM; Alexis, Nottingham, UK) 1 h before addition of HNP-1.

Alamar Blue reduction assay

After 24-h incubation with HNP-1, the cells were treated for 3 h with 10-fold diluted Alamar Blue (Serotec, Oxford, UK) with FBM. Cell viability, the reducing environment of proliferating cells, was measured at 540 nm excitation and 620 nm emission. The results were compared with non-treated control cells.

Northern blot analysis

Human HSP47 and collagen-1 complementary DNAs (cDNAs) were generously provided by the Institute for Frontier Medical Science, Kyoto University, Japan. HSP47 mRNA was detected by using a 1.5 kilobase pair (kbp) *Eco*R-I fragment of full-length human HSP47 (CBP2) cDNA probe, whereas collagen-1 mRNA was detected by using a 0.6 kbp *Eco*R-V of full-length pCOLA1-I-CP cDNA probe. Total RNA was extracted from NHLF using RNeasy Mini Kit (Qiagen, Tokyo) using the method recommended by the supplier. Aliquots of total RNA (10 μg/lane) were size-fractionated in a 1.2% formaldehyde agarose gel and transferred to a positively charged nylon membrane (Amersham Biosciences Corp., Piscataway, NJ). The membrane was separately hybridized with ^{32}P-labeled HSP47 and collagen-1 cDNAs for 20 h at 42 °C. Autoradiographed membranes were analyzed using a BAS5000 bioimage analyzer (Fuji Photo Film). Relative transcription was normalized with GAPDH probe hybridization.

Western blotting

Cells were washed with phosphate-buffered saline (PBS) and solubilized in lysis buffer (20 mM Tris–HCl, 133 mM NaCl, 1%