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Protection of lung epithelial cells from protease-mediated injury by trappin-2 A62L, an engineered inhibitor of neutrophil serine proteases

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ABSTRACT

Neutrophil serine proteases (NSPs), including elastase, proteinase 3 and cathepsin G, play critical roles in the pathogenesis of chronic inflammatory lung diseases. The release of excess NSPs leads to the destruction of lung tissue and an overexuberant, sustained inflammatory response. Antiproteases could be valuable tools for controlling these NSP-mediated inflammatory events. We have examined the capacity of trappin-2 A62L, a potent engineered inhibitor of all three NSPs, to protect human lung A549 epithelial cells from the deleterious effects of NSPs. Trappin-2 A62L, significantly inhibited the detachment of A549 cells and the degradation of the tight-junction proteins, E-cadherin, β-catenin and ZO-1, induced by each individual NSP and by activated neutrophils. Trappin-2 A62L also decreased the release of the pro-inflammatory cytokines IL-6 and IL-8 from A549 cells that had been stimulated with elastase or LPS. Trappin-2 A62D/M63L, a trappin-2 variant that has no antiprotease activity, has similar properties, suggesting that the anti-inflammatory action of trappin-2 is independent of its antiprotease activity. Interestingly, we present evidence that trappin-2 A62L, as well as wild-type trappin-2, enter A549 cells and move rapidly to the cytoplasm and nucleus, where they are likely to exert their anti-inflammatory effects. We have also demonstrated that trappin-2 A62L inhibits the early apoptosis of A549 cells mediated by NSPs. Thus, our data indicate that trappin-2 A62L is a powerful anti-protease and anti-inflammatory agent that could be used to develop a treatment for patients with inflammatory lung diseases.

1. Introduction

There is now good evidence that neutrophil serine proteases play major roles in a variety of progressive inflammatory lung disorders such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, acute respiratory distress syndrome and pulmonary fibrosis. COPD, which is associated with tobacco smoking, is currently the fifth leading cause of death in the world and is predicted by the World Health Organization to reach the third place by 2030. Pioneering studies showed that a genetic α1-PI deficiency (also known as α1-antitrypsin), the major inhibitor of neutrophil elastase (HNE), was associated with the development of pulmonary emphysema [1]. These early studies led to the protease–antiprotease hypothesis of COPD pathogenesis, according to which the concentrations of proteases exceed those of the natural protease inhibitors in the lung. Since then, many other proteases and other neutrophil-derived serine proteases including proteinase 3 (Pr3) and cathepsin G (CG), have been shown to participate in destruction of the structural and extracellular matrix proteins of the lungs and to amplify inflammation (see Ref. [2] for review). Although other factors such as the oxidant–antioxidant imbalance [3] or enhanced apoptosis of structural lung cells [4] are also involved in the pathogenesis of COPD, there are no doubt that the excess proteolysis that results from an influx of neutrophils into the airways is pivotal in progressive destruction of the lung parenchyma. While neutrophil serine proteases (NSPs) take part in the homeostasis of healthy lungs by such processes as tissue regeneration and repair and killing of pathogens, an excess of them has deleterious effects due to their direct proteolytic actions on various substrates and their pro-inflammatory properties. The injurious effects of neutrophils and NSPs on the lung epithelium were recognized quite early [5,6], but our view of the role of NSP has evolved with the recent discovery that they are in fact key regulators of the inflammatory response [7,8]. NSPs not only upregulate the expression of other pulmonary proteases, they also activate the zymogens of several MMPs including MMP-2, MMP-7

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and MMP-9 (reviewed in Ref. [9]), thereby increasing the proteolytic potential within lungs. Also, active MMPs cleave and inactivate α1-PI [10], allowing NSPs and in particular HNE to remain active. Lastly, NSPs stimulate goblet cells, resulting in the hypersecretion of mucus [11,12] and airflow obstruction, a characteristic feature of COPD and other inflammatory chronic lung diseases. This reduces the mucociliary clearance of pathogens, a process which is amplified by the proteolytic degradation of innate defence proteins like the surfactant proteins SP-A [13] and SP-D [14] by all three NSPs. The massive influx of neutrophils into the lungs of patients with inflammatory disorders such as COPD or cystic fibrosis is maintained by both the HNE-stimulated increase in the synthesis of the potent chemoattractant interleukin-8 (IL-8) [15] and by the NSP-induced conversion of native IL-8 into more active truncated forms [16]. Excess NSPs also impair the removal of dying apoptotic neutrophils from the lungs by macrophages by cleaving receptors on the surface of macrophages such as the phosphatidylserine receptor [10]. This, plus their actions listed above helps perpetuate the inflammation caused by the decreased removal of apoptotic cells and increased release of pro-inflammatory factors like NSPs from post-apoptotic neutrophils.

One way of reducing the NSP burden and its unwanted deleterious effects in chronic inflammatory lung diseases is to boost the overwhelmed antiprotease defences. The therapeutic potential of elafin and its precursor trappin-2 (T2), two natural inhibitors found in the lung, have generated great interest in recent years, not just because they are potent inhibitors of HNE and Pr3 but because they also have pleiotropic activities. They have anti-inflammatory, anti-microbial and immuno-modulatory activities [17] that could help control exaggerated inflammatory responses. Our efforts to develop an antiprotease-based, aerosol-delivered therapy that targets NSPs directly into the lungs led to the design of a trappin-2 mutant, trappin-2 A62L (T2 A62L), that is a potent inhibitor of all three NSPs (HNE, Pr3 and CG) [18]. We have now evaluated the capacity of trappin-2 A62L to protect A549 lung epithelial cells from protease-mediated injury. We found that trappin-2 A62L can protect epithelial cells from both the proteolytic and the non-proteolytic deleterious effects of each of the NSPs. Interestingly, the anti-inflammatory activity of trappin-2 A62D/M63L, a trappin-2 variant devoid of inhibitory activity [19] was similar to that of trappin-2 A62L. This suggests that the anti-inflammatory activity of trappin-2 A62L is independent of its antiprotease function.

2. Materials and methods

2.1. Materials

Human neutrophil elastase (HNE) (EC 3.4.21.37) was obtained from Biocentrum (Krakow, Poland), human proteinase 3 (Pr3) (EC 3.4.21.76) was from Athens Research and Technology (Athens, USA) and cathepsin G (CG) (EC 3.4.21.20) was from MP Biomedicals (Vannes, France). Fluorogenic substrates specific for each neutrophil proteinase were custom-synthesized by Gencust Europe (Dudelange, Luxembourg). The protease inhibitors trappin-2, trappin-2 A62L and trappin-2 A62D/M63L were synthesized as recombinant proteins in the *Pichia pastoris* yeast expression system and purified as previously described [18,20]. All other reagents were of analytical grade.

2.2. Cell culture

A549 pulmonary epithelial cells derived from a human bronchioalveolar lung carcinoma and representative of type II alveolar cells was obtained from ATCC (American Type Culture Collection). The cells were grown at 37 °C in an atmosphere of 5% CO_2 in RPMI-1640 medium supplemented with 2 mM GlutamaxTM, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen SARL, Cergy Pontoise, France).

2.3. Measurement of the proteolytic activity of neutrophil serine proteases in alveolar epithelial cells supernatants

A549 cells were seeded at 10^5 cells per well in 24-well plates in complete medium and grown to confluence. They were then washed and cultured for 24 h in serum-free RPMI. The starved cells were washed with PBS and incubated with a neutrophil serine protease (HNE, Pr3, 15 nM; CG, 130 nM) for 15 min. Lastly, trappin-2 or trappin-2 A62L was added at an enzyme:inhibitor molar ratio of 1:10. Culture supernatants were collected at 0, 15, 60, 120, 180 min after adding inhibitor and the residual activity of each protease was determined using fluorogenic substrates (10 μM each) Abz-APEEIMRRQ-EDDnp for HNE, Abz-VADnVADYQNitroTyr (nV = norvaline) for Pr3 and Abz-TPFSGQEDDnp for CG where Abz (ortho-aminobenzoic acid) is the fluorescent group and EDDnp (*N*-(2,4-dinitrophenyl) ethylenediamine) the quencher group. The increase in fluorescence due to substrate hydrolysis was recorded at λ_{exc} = 320 nm and λ_{emi} = 420 nm in a SPECTRAmax Gemini microplate fluorescence reader (Molecular Devices).

2.4. Cell detachment assays

2.4.1. Exposure of alveolar epithelial cells to neutrophil serine proteinases

A neutrophil serine protease (HNE, Pr3, 15 nM; CG, 130 nM) and trappin-2 or trappin-2 A62L (E:I molar ratio = 1:10) were added simultaneously to A549 cells that had been grown as above and starved for 24 h. In a second set of experiments, proteases were incubated with inhibitors (E:I molar ratio = 1:10) for 10 min before adding the mixture to the cell culture. In a third series, cells were incubated with each protease for 15, 30 or 60 min prior to adding trappin-2 or trappin-2 A62L (E:I molar ratio = 1:1 and 1:10). In all three experiments, cells were incubated for 24 h with various protease–inhibitor combinations. To study the effects of inhibitors on protease-induced cell detachment, the concentration of each protease used in these experiments was chosen from a series of preliminary experiments (data not shown) so that a significant % of detachment occurred. The morphological changes induced by the serine neutrophil proteases were examined by phase contrast microscopy and cell detachment was evaluated by counting cells in a hematocytometer. The percentage of detached cells was calculated using the following formula:

$$\%\,\text{detachment} = \frac{\text{number of cells in supernatant}}{\text{number of total cells per well}} \times 100$$

2.4.2. Exposure of alveolar epithelial cells to activated neutrophils

Human neutrophils (PMNs) were purified from 15 mL samples of peripheral blood collected from healthy volunteers into EDTA-containing tubes, essentially as previously reported [18]. The purified PMNs were kept at room temperature with gentle shaking and washed with PBS just before use. Cell viability was checked by trypan blue exclusion. PMNs were activated by suspending 3×10^6 cells/mL in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and incubating them with the calcium ionophore A23187 (1 μM final) (Sigma–Aldrich, St. Quentin Fallavier, France) for 15 min at 37 °C. They were then centrifuged at 2000 × *g* for 5 min at 20 °C. The PMN pellet was suspended in PBS and kept at room temperature under gentle shaking until further use.

A549 cells in 24-well plates were labeled with 10 μM of CellTracker™ Orange CMTMR (5-(and-6)-(((4-chloromethyl)