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Original Article

Enhancement of Pulmozyme activity in purulent sputum by combination with poly-aspartic acid or gelsolin☆

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Abstract

Background: DNase (Pulmozyme) effectiveness in cystic fibrosis treatment is in some cases limited by its inability to access DNA trapped within bundles in highly viscous fluids that also contain actin. Dissociating DNA-containing bundles using actin depolymerizing agents and polyanions has potential to increase DNase efficacy.
Methods: Fluorescence measurements of YOYO-1 and a rheological creep-recovery test quantified DNA content and viscoelasticity in 150 sputum samples from adult CF patients and their susceptibility to fluidization by DNase1, alone and in combination with gelsolin and poly-aspartate (p-Asp). Fluidization of sputum by these agents is compared to their capacity to increase antibacterial activity in sputum, measured using a luminescent *Pseudomonas aeruginosa* strain and a bacterial killing assay.
Results: The polyanion p-Asp (1–50 μg/g of sputum), the actin severing protein gelsolin (10-90 μg/g) and their combination enhance the ability of DNase 1 to increase the abnormally low mechanical compliance of CF sputum and to promote bacterial killing in sputum by colistin and tobramycin, two antibiotics commonly used to treat CF.
Conclusions: Addition of low concentrations of p-ASP or gelsolin can increase the therapeutic value of Pulmozyme (DNase 1).

Keywords: Cystic fibrosis; Extracellular DNA; F-actin; Gelsolin; Poly-aspartic acid

1. Background

In the respiratory tract of CF subjects, accumulation of biopolymers such as DNA [1] and F-actin [2], released mostly from neutrophils, is associated with dysfunction of ciliary transport, bronchial obstruction and chronic bacterial infection [3,4]. Previous rheologic analyses show that CF sputa have increased magnitudes of both elastic and viscous moduli compared to those of normal airway surface fluids. This thickening of sputum correlates with increased amounts of DNA and/or F-actin [1,2,5].

Inhaled recombinant human deoxyribonuclease 1 (Pulmozyme) has been used since the early 1990s in CF patients as a mucolytic drug, which cleaves DNA and reduces abnormal sputum viscosity, thereby improving cough clearance and reducing sputum adhesiveness [6]. Inhaled DNase can also cleave DNA in biofilms and sensitize bacteria within them to antibiotic agents [7]. Pulmozyme treatment significantly reduces the risk of infections requiring intravenous antibiotic treatment [8]. Positive response to DNase treatment indicated by improved lung function is not universal, and the reasons for a nonresponder rate of approximately 30% remain

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unclear [6]. Weak response to DNase in CF patients might be caused by the presence of large DNA/F-actin aggregates with limited drug penetration. These aggregates [9] are stabilized by small cationic molecules such as antimicrobial agents [10].

Both F-actin and DNA are strong polyelectrolytes, with sufficiently high surface charge density to form a condensed layer of counterions [11]. The attractive interactions between filaments in bundles can be mediated by multivalent cations even without specific actin- or DNA binding sites [12]. Cationic host antimicrobial agents such as cathelicidin LL-37 are at least part of the polyvalent counterions incorporated within actin/DNA bundles in CF [9]. Therefore, depolymerizing DNA with DNase I [6,13], severing actin filaments with gelsolin [2], sequestering actin monomers with thymosin beta 4 [14] or disrupting the filament bundles with soluble polyanions like p-Asp [15] fluidizes the sputum and releases antimicrobial peptides, leading to partial restoration of antibacterial function in CF sputum [9,10]. The hypothesis that addition of p-Asp or gelsolin to DNase I will enhance the effectiveness of sputum fluidization is tested here in samples obtained from patients with different infections to determine how the independent mechanisms of p-Asp, gelsolin, and DNase I act in concert to increase the rate or extent of sputum fluidization by Pulmozyme.

2. Materials and methods

2.1. Materials

Pulmozyme (recombinant human deoxyribonuclease I—rhDNase I) was from Genentech Inc. (South San Francisco, CA, USA). Poly-(D,L)-aspartic acid, colistin, tobramycin and fish sperm DNA were from Sigma (St Louis, MO, USA). A 10 amino acid long oligo D-Asp was from Peptide 2.0, Inc (Chantilly VA). Recombinant human plasma gelsolin was from Biogen-Idec, Inc. (Cambridge, MA, USA).

2.2. Sample collection

One hundred fifty sputum samples (Table 1) were collected by spontaneous expectoration in sterile containers from adult patients with cystic fibrosis attending the University of Pennsylvania Health System. Within about 2 h of collection the sample was divided into aliquots containing ~500–1000 mg, then aliquots were flash frozen in liquid nitrogen. The study was approved by University of Pennsylvania Office of Regulatory Affairs and written consent was obtained from all subjects (IRB 811197).

2.3. DNA content in CF sputum

DNA concentration was evaluated by comparing CF sputum fluorescence after addition of YOYO-1 with a series of known DNA concentration standards. Three plugs were taken at random from each sputum sample and homogenized 1:100 *w/v* in PBS. 10 μl of diluted sputum was added to 400 μl of 1 μM YOYO-1. DNA content was measured by fluorescence intensity (ex 491 em 509) and compared to a standard curve using purified DNA.

2.4. Evaluation of viscoelasticity

The viscoelastic properties of CF sputum were quantified by their shear compliance after imposition of 1 Pa shear stress to a sample confined between two parallel plates. Compliance is defined as the ratio of shear strain (deformation) to shear stress, and for viscoelastic materials, compliance is a time-dependent quantity as depicted in Fig. 1A. The shear compliance was determined from the relation between applied force and the resulting deformation using a Bohlin CVO Rheometer (Malvern, Inc). Samples were thawed at room temperature for ~25 min prior to treatment with PBS (50 μl/g of sputum), PBS containing DNase I (1 μg/g sputum), poly-aspartic acid (1–50 μg/g), gelsolin (1–90 μg/g) or their combination. Once treated, three different regions were taken at random from a 500–1000 mg sputum sample and mixed with a plastic pipette tip just enough to spread evenly over the bottom plate of the rheometer. The top plate was then lowered to the appropriate height (typically 0.8 mm) to hold 100 μl between the plates. Water-saturated filter paper surrounded the sample to prevent drying. Creep tests were performed by applying a constant stress of 1 Pa for 20 s, and recovery was measured for 30 s after the stress was removed (Fig. 1A).

2.5. Optical microscopy

DNA/F-actin aggregates in CF sputum were observed using a Leica microscope (Bannockburn, IL) with a 10× objective and a Cool SNAP(HQ) camera (Trenton, NJ). F-actin was labeled with 165 nM Alexa fluor 633 phalloidin (Sigma, St Louis, MO) and DNA was labeled by adding 500 nM YOYO-1 (Molecular Probes, Boulder CO).

2.6. Antimicrobial testing

Pseudomonas aeruginosa Xen 5 was grown overnight to reach a logarithmic phase of growth. Bacterial concentration was measured by spectrophotometry (OD_{600nm}). To assess antibacterial

Table 1
Demographic characterization of CF patients who provided CF sputum samples (n = 150).

Type of CF mutations	Number of samples	DNA (mg/g)	Sex		Age	
			M	F	M	F
CFTRΔF508—homozygous	69	3.1 ± 2.6	28	41	27.4 ± 7.1	29.1 ± 6.1
CFTRΔF508—heterozygous	55	3.8 ± 3.3	45	10	31.4 ± 8.7	31.1 ± 10.0
Others	26	3.4 ± 2.4	19	7	32.2 ± 6.4	34.9 ± 13.0