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Short paper

DNA concentration and length in sputum of patients with cystic fibrosis during inhalation with recombinant human DNase

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Abstract

***Background* – The clinical benefit of the administration of aerosolised recombinant human DNase (rhDNase) on pulmonary function in patients with cystic fibrosis has already been demonstrated but the biochemical action of rhDNase on DNA in bronchial secretions in vivo has not yet been investigated.**

***Methods* – Sputum was collected from 135 patients with cystic fibrosis before and during treatment with aerosolised rhDNase and examined to ascertain DNA concentration and length by colorimetric assay and densitometry of gel separated DNA.**

***Results* – Treatment with rhDNase reduced the concentration and the size of extracellular DNA in the sputum. The median interquartile range of DNA length decreased from 0·5–2·6 kbp before treatment to 0·3–1·0 kbp during treatment.**

***Conclusions* – rhDNase was delivered to the secretions and was enzymatically active in vivo.**

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Keywords: DNase, cystic fibrosis, sputum analysis.

Respiratory tract disease characterised by the accumulation of mucopurulent secretions and chronic endobronchial infection is responsible for more than 90% of the morbidity and mortality in cystic fibrosis.[1] On the basis of the hypothesis that an increased concentration of high molecular weight DNA contributes to the abnormal viscoelastic properties of sputum and airway obstruction, recombinant human deoxyribonuclease I (rhDNase) has been delivered as an aerosol to the airways of patients with cystic fibrosis.[2–7] The administration of rhDNase reduced but did not eliminate exacerbations of respiratory symptoms[7] and resulted in a slight improvement in pulmonary function.[3–7] Although the clinical benefit of aerosolised rhDNase has been demonstrated in several trials,[3–7] the biochemical action of rhDNase on its substrate in vivo has not yet been investigated. We measured the concentration and length of DNA in sputum samples from patients with cystic fibrosis collected before and during treatment with aerosolised rhDNase.

Methods

One hundred and thirty five patients with cystic fibrosis from seven German cystic fibrosis outpatient clinics participated in a 12 week phase III trial on the inhalation of 2·5 mg rhDNase (Pulmozyme, Genentech Inc, San Francisco, USA) once daily. Criteria for study entry were no indications for hospital admission and a forced vital capacity of 40–70% predicted.

Sputum expectorated during physiotherapy was collected on three consecutive days directly before the first inhalation of rhDNase and again on three consecutive days after six weeks of inhalation of rhDNase. Sputum containers were stored in the refrigerator and at the end of each sampling period the three sputum containers were brought to the Hannover laboratory, frozen to −20°C upon arrival, and stored for up to six weeks. Thawed sputum was liquified for 3–5 hours at room temperature under stirring with a twofold volume of 2% (v/v) aqueous mercaptoethanol. Cells and debris were spun down by centrifugation at 1200 *g* for 10 minutes. Supernatants from each patient's three day collection period were pooled and lyophilised, with the exception of the 63 pretreatment and 72 treatment samples from the 25 patients of the Hannover cystic fibrosis clinic which were analysed on a day-to-day basis. The freeze dried sample was reconstituted in distilled water and the DNA concentration was then determined by colorimetric assay with diphenylamine according to the method of Burton[8] modified by Croft and Lubran[9] using human blood cell DNA as standard. To determine the DNA length, DNA was isolated[10] from lyophilised samples by digestion with proteinase K, extractions with phenol/chloroform, precipitation with ethanol, and dissolution in 10 mM Tris/HCl, 1 mM EDTA, pH 8·0. The purified DNA was separated by 1% agarose gel electrophoresis using a λ-*BstE*II digest as a size marker. The size distribution was measured by laser densitometry of 8·5 × 10·8 cm Polaroid negative films of the ethidium bromide-stained agarose gel.

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Median (interquartiles and range) concentration and length of extracellular DNA in sputum samples from patients with cystic fibrosis

	No.	*No treatment with DNase*	*No.*	*Inhalation with DNase*
All sputum samples:				
DNA concentration (mg/ml sputum)	167	0·6 (0·3–1·2; 0·0–9·5)	177	0·3 (0·1–0·9; 0·0–4·8)
Median DNA length (kbp)[1]	159	1·3 (0·7–1·5; 0·3–3·0)	164	0·4 (0·3–0·6; 0·1–14·1)
Day-to-day variation during 3-day collection periods (Hannover)				
Mean DNA concentration (mg/ml sputum)	25	0·7 (0·2–1·0; 0·0–2·3)	25	0·4 (0·2–1·0; 0·0–2·2)
Variance of DNA concentration (mg/ml sputum)[2]	20	0·5 (0·4–1·6; 0·1–3·3)	24	0·5 (0·2–1·2; 0·1–3·0)

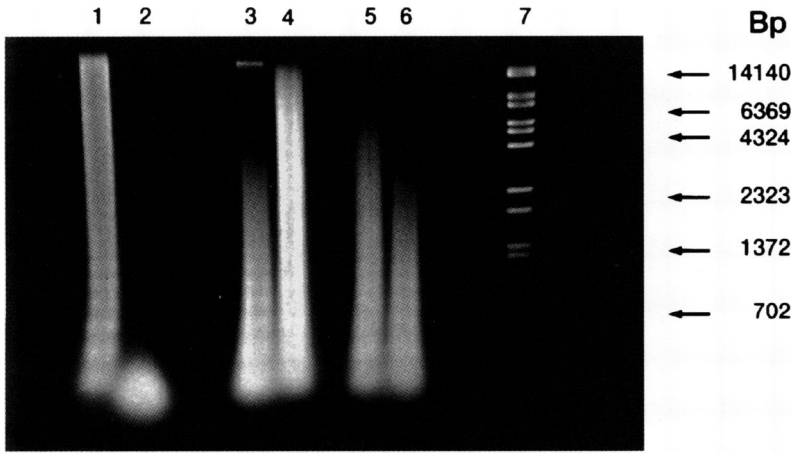
[1] Yield of purified DNA was too low in eight pretreatment and 13 treatment samples to perform densitometry of gel separated DNA.
[2] Variance was only calculated if patients delivered two (2, 1 patients) or three (18, 23 patients) sputum containers.

The statistical significance of the impact of rhDNase inhalation on DNA length and concentration was evaluated with the Mann-Whitney rank test. Aliquots of cystic fibrosis sputum controls which were either immediately processed after collection or intermittently stored at −20°C for up to eight weeks gave the same results within experimental error.

Results

The DNA contents in sputum samples from 132 patients were analysed before the first inhalation of rhDNase and from 129 patients after six weeks of inhalation of rhDNase. The extracellular DNA content in the sputum varied from 0 to 9·5 mg/ml (table). During inhalation with rhDNase the distribution of DNA concentration was significantly reduced ($p<0·005$), the median concentration falling from 0·6 to 0·3 mg extracellular DNA/ml sputum (table). Samples collected from the same patient on consecutive days exhibited a broad variation of DNA content which is reflected by the fact that the mean and variance of specimens from individual patients covered a similar concentration range (table).

Extracellular DNA in the sputum was degraded to molecules of a few kilobase pairs (kbp) or less. Lanes 1 and 2 of the figure exhibit a typical example of the distribution of DNA length in the absence (lane 1) and presence (lane 2) of inhaled rhDNase. The small DNA size was not an experimental artefact due to shearing because the isolation of DNA from peripheral blood cells processed in parallel by the same protocol yielded high molecular weight DNA with a median size of about 50 kbp. The median DNA length decreased significantly during inhalation with rhDNase ($p<10^{-9}$; table). The median interquartile range of DNA length decreased from 0·5 to 2·6 kbp before treatment with DNase to a smaller and narrower range of 0·3–1·0 kbp during inhalation with rhDNase ($p<10^{-9}$). No reduction of DNA size during treatment was seen in samples from 18 patients (13% of study population) of which two examples are shown in the figure (lanes 4 and 6).



Ethidium bromide stain of gel separated extracellular DNA isolated from sputum before (left lanes) and during (right lanes) administration of aerosolised rhDNase from three patients with cystic fibrosis. Lanes 1 and 2 represent a typical example, while the samples separated in lanes 3/4 and 5/6 belong to the 18 non-responders. Lane 7 = λ-BstEII digest as size marker.

Discussion

rhDNase was developed to reduce the contribution of high molecular weight DNA to the abnormal viscoelastic properties of bronchial secretions in patients with cystic fibrosis. We measured the length and content of extracellular DNA which should be the target of aerosolised rhDNase. To our knowledge, all data in the literature refer to the total amount of intracellular and extracellular DNA in cystic fibrosis secretions. Warren *et al*[11] detected a mean DNA content of 10% of dry weight in bronchial secretions, whereas 20 years later Picot and colleagues[12] found values of 1·1% in mucoid, 1·9% in mucopurulent, and 3·6% in purulent cystic fibrosis sputum samples. The lower values in this latter report may reflect improvements in the treatment of the airways disease in patients with cystic fibrosis and, indeed, changes of the DNA content of sputum from average values of 5·9 to 2·6 mg/ml were observed in association with clinical manifestations and treatment.[13] A comparison of the data in the literature with those in this study indicates that the extracellular DNA contributes about 10–20% to the DNA content in the sputum of patients with cystic fibrosis. The large day-to-day variation of the extracellular DNA content of the sputum of these patients is probably the result of the spatially uneven composition and mobilisation of secretions drained during physiotherapy.

Extracellular DNA in the sputum of patients with cystic fibrosis was regularly degraded, probably by nuclease activities released from shedded human cells into the secretions. DNA molecules of up to 1·5 kbp in size are quasirod-like in solution, but the hydrodynamic behaviour of larger molecules is more like that of a coil.[14] Since we observed a median DNA

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length of 1·3 kbp, rod-like and coil-like molecules were present in comparable quantities. Upon inhalation with DNase the extracellular DNA was further degraded to smaller polynucleotides whereby, on average, >90% of molecules fell into the category of rod-like chains of low intrinsic viscosity. The reduction in molecular weight was observed in all but 18 patients, indicating that rhDNase was enzymatically active in vivo and adequately aerosolised and delivered to the secretions. A second encouraging finding is the twofold decrease of median DNA content during inhalation with rhDNase. A reduced load of extracellular DNA is a measure of endobronchiolar cell debris and suggests that rhDNase affects local inflammation which could contribute to the reduced risk of respiratory exacerbations.[7]

This study was performed in collaboration with the members of the "German Clinical Study Group on rhDNase" at the Roche company (Dr Deuber) and the cystic fibrosis clinics in Berlin (Dr Tacke), Essen (Dr Stephan), Frankfurt (Dr Posselt), Gießen (Dr Lindemann), Halle (Dr Brömme), Hannover (Dr Ballmann) and München (Drs Harms).

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