DNA Quantification and Fragmentation in Sputum after Inhalation of Recombinant Human Deoxyribonuclease

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Key Words

Cystic fibrosis • Deoxyribonucleic acid • Recombinant human deoxyribonuclease • Fragmentation • Sputum

Abstract

Background: Inhaled rhDNase may improve sputum viscosity and mucociliary clearance by cleavage of extracellular DNA derived for instance from dead leukocytes in purulent, highly viscous patient sputum. **Methods:** Here we established a method to quantify rhDNase-mediated DNA fragmentation in sputum using gel electrophoresis. Sputum of *Pseudomonas aeruginosa* colonized cystic fibrosis (CF) patients with (CF+) or without (CF-) rhDNase treatment or mechanically ventilated non-CF patients receiving rhDNase (non-CF+) or not (non-CF-) was analyzed. DNA measurements from T-lymphocytes served as controls. Absolute DNA content and the relative quantity within eight molecular mass ranges (12000 to 200 bp) was determined by gel electrophoresis and densitometric analysis. **Results:** Geometric mean sputum DNA concentrations were 0.41 mg/dl for CF- (n=54), 0.78 mg/dl for CF+ (n=60), 0.053 mg/dl for non-CF- (n=41) and 0.049 mg/dl for non-CF+ (n=28). Treatment with rhDNase resulted in fragmentation of

DNA that was quantified by separation and densitometric analysis of the DNA on agarose gels. The new analysis method permits analysis of DNA cleavage with high accuracy. **Conclusion:** This new monitoring method facilitates DNA quantification and *in vitro* monitoring of rhDNase in sputum.

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Introduction

Inhaled recombinant human deoxyribonuclease (rhDNase; Pulmozyme®; Roche AG, Grenzach, Germany) has been successfully applied since the early 90s in patients with cystic fibrosis (CF) [1-4]. The compound is a mucolytic drug, which cleaves extracellular DNA in CF patients airways [5-8] leading to a reduction in the proportion of high molecular weight DNA which is supposed to be responsible for abnormal high sputum viscosity [9, 10] in CF. An additional direct anti-inflammatory effect of rhDNase is suspected [11, 12] and this topic has been addressed in a large multicenter study (BEAT Study) [13]. In general, after treatment for a few days, a proportion of CF patients show improved lung function, increased workload and oxygen uptake [14], but the reason for a considerably high nonresponder rate

of approximately 30% remains unclear [15]. A few articles report beneficial effects of rhDNase even in intubated children [16, 17] or adult patients with atelectasis or asthma, respectively, independent of cystic fibrosis [18–20]. However, all these reports rely on clinical observations as lung function, subjective quality of life or dyspnea [21], while a method for measuring rhDNase effects on sputum DNA has not been established at present.

DNA quantification in sputum of CF and non CF children was first reported by Kirchner et al [6] using a Hoechst dye-binding assay. A proposed quantification method by measurements of the extent of unrecoiled solids compression when a complex biopolymer is subjected to a centrifugal force (“compaction assay”) [22] did not find its way into clinical routine. Shah and coworkers [7] studied posttreatment DNA fragmentation and sputum rheology and found improved lung function and improved rheological characteristics of the sputum after rhDNase treatment. Successful DNA fragmentation by rhDNase was determined colorimetrically in terms of four classes of DNA size (12000–2000, 2000–750, 750–150, and < 150 kD). Probably due to a small study population ($n = 20$), the authors did not find a correlation between clinical and the *in vitro* effects of rhDNase (rheology plus DNA fragmentation). They did not discriminate between *Pseudomonas aeruginosa* positive and negative patients. Brandt and coworkers [23] reported similar *in vitro* findings in a larger population of 135 CF patients. DNA concentration was determined by colorimetry and DNA fragment lengths were measured by gel electrophoresis, which showed a decrease of overall DNA content and a fragmentation down to median 1.3 kbp pieces. The study did not correlate these laboratory findings with clinical data.

Here, we report a novel and easy-to use method to quantify DNA fragmentation in the sputum upon rhDNase treatment to facilitate comparisons of clinical effects with *in vitro* measurements of sputum DNA in CF and non CF patients.

Materials and Methods

Material

Sputum samples were induced with 1 M NaCl. Samples were obtained from 54 *Pseudomonas aeruginosa* colonized cystic fibrosis (CF) patients without pre-study rhDNase treatment (CF-), 60 CF patients with prior rhDNase treatment (CF+). Sputa were also obtained by bronchoalveolar lavage in 41 mechanically ventilated pediatric non-CF patients receiving no rhDNase (nonCF-) and 28 mechanically ventilated pediatric non-CF patients receiving rhDNase (nonCF+). DNA from

T-lymphocytes (Jurkat cells) served as a reference (Ref) [24, 25]. For CF patients and non CF patients the ethics committee approved the study.

Electrophoresis

Sputum sample aliquots were weighed, diluted 1:1 with dithiothreitol (DTT) and incubated for 30 min at room temperature. This was followed by incubation with gentle shaking for 90 min at 37°C with addition of 10 μ l rhDNase to completely digest free DNA (serving as negative control) or buffer consisting of 132 mM NaCl, 20 mM HEPES (pH 7.4), 5 mM KCl, 1 mM CaCl₂, 0.7 mM MgCl₂, and 0.8 mM MgSO₄. 15 μ l of each sample was added to 5 μ l 1x loading buffer and separated on a 1% agarose gel using ethidium bromide to label the DNA and its relative quantity within eight molecular mass ranges (12000, 4000, 3000, 2000, 1600, 1000, 500 and 200 kD). Lambda-BstEII digest or 1 Kb DNA ladder (peqlab: Erlangen, Germany) was used as size marker. After separation for approximately 30 min at 300 volts (650 mA), the gel was photographed and analyzed by densitometry. The gels were photographed with raytest®-equipment (Straubenhardt, Germany) and densitometric analyses were performed with the enclosed AIDA image analyser software. The area of interest was defined by the two-dimensions of the loading slot and the lane in the agarose gels. This area was used to quantify DNA amounts in the gel. The line in Figure 3 was employed to calculate the slope of DNA-fragmentation.

DNA fragmentation analysis (molecular mass of DNA fragments)

DNA eluted from human T lymphocytes (Jurkat cells, $n = 35$) was separated with and without rhDNase by electrophoresis and used as a reference value for comparing rhDNase activity in patient samples *in vitro*. Densitometry of DNA gel electrophoresis scans prior to and after the *in vitro* addition of rhDNase (reference value: the T lymphocyte value) were used for measurement (analysis Doku®, Soft Imaging System GmbH, Leinfelden, Germany), selecting grayscale values at eight labeled DNA lengths (12000, 4000, 3000, 2000, 1600, 1000, 500 and 200 bp) (Figures 2, 3). The relative quantities (as percentage of total DNA content) representing the respective DNA lengths/grayscale peaks were calculated and plotted. Percentages were converted to logits to equalize variances. The slope of the regression on log DNA length (relative quantity as percentage; eight point measurement) for each patient was calculated by a computer program. Decreasing slopes of the interpolation line between the percentages reflect a successful, rhDNase mediated cleavage of high- to low-molecular DNA in contrast to distinctly positive slopes in native sputum with a high percentage of high molecular, unfragmented DNA. Thus, the *in vitro* effect of rhDNase on T lymphocytes was extrapolated into the *in vivo* measurements of individual patients. The difference between the slopes of the two T lymphocyte lines was interpreted as the treatment effect.

DNA quantification (total DNA concentration)

Figure 1 describes the DNA quantification. An aliquot of T-lymphocyte DNA extract containing 0.155 mg/ml DNA was

Fig. 1. DNA quantification in sputum electrophoresis. Size marker (first line from left), *in vitro* addition of rhDNase to sputum sample (second line), sputum sample (third line) of a CF patient and as a reference denaturated T lymphocytes (fifth line). All densities are measured in the white rectangles.

Fig. 3. Original electrophoresis with untreated sputum sample (first lane, sputum of a CF patient), sputum sample after incubation with 10 µl of rhDNase (second lane, aliquot from the same sputum as in the first lane) and kb-ladder (third lane) as a size marker for length of DNA. The white line marks the overlay of computer based grayscale analysis curve. This curve was employed to calculate the slopes of DNA fragmentation.

measured photometrically several times and used as reference for the densitometric comparison of all samples. DNA concentration in the probes was calculated using the following formula:

$$x = (b - c) / a \times 0.155 \text{ mg/ml}$$

where "x" is the concentration of sputum DNA, "b" is the density (pixel intensity) of the probe (line 3), "c" is the density of the sample after incubation with 10 µl rhDNase (line 2) *in vitro* (for complete digestion of free DNA with remaining DNA in intact cells) and "a" is the density of the reference (line 5). All densities were measured within the rectangles, which were set in each electrophoresis exact equally with the same length and width from the mid of the loading slot.

Fig. 2. Schematic drawing: Fragmented DNA in diluted sputum sample aliquots were separated on a 1% agarose gel using ethidium bromide, after separation photographed and analyzed by densitometry. The relative quantities representing the respective DNA lengths (grayscale peaks) were calculated and plotted. The slope of the regression from logits on the -log DNA length (eight point measurement) for each patient was calculated. by the computer programme. Positive slope is the result of a high percentage of small fragments as a result of strong DNase effect. A negative slope represents minor DNase cleaving effect with large amounts of large fragments.

Statistical analysis

Statistical analysis was performed using JMP 7.0.1 (SAS Institute Inc., Cary NC, 2007). A paired t-test was used to describe the treatment effect in the reference samples and two samples t-tests to describe differences between patient groups. The corresponding 95%-confidence intervals (CI) were computed. DNA concentrations were lognormally distributed, so that geometric means, their ratios, and coefficients of variation (CV) are reported. *P* values less than 0.05 were considered statistically significant. CI for sensitivity and specificity were computed by the exact Clopper-Pearson formula.

Tables. Results of DNA quantification in comparison with (+) and without (-) rhDNase treatment and calculated slopes of the interpolation line between the fragment size peaks.
^aabsolute; ^bdifferent populations; * p<0.05, ‡ p<0.00001

Population	rhDNase	Number	Sputum DNA (mg/ml): geometric mean (coefficient of variation)	ratio of geometric means (CI)
Reference samples		36	0.155 ^a mg/ml (%)	
CF patients	-	54	0.411 mg/ml (190 %)	1.89
	+	60	0.779 mg/ml (178 %)	(1.25 to 2.86)*
NonCF patients	-	41	0.0527 mg/ml (141 %)	1.07
	+	28	0.0494 mg/ml (83 %)	(0.62 to 1.84)

Population	rhDNase	Number	Slope: mean (SD)	difference (CI)
Reference samples	-	36	0.93 (0.54)	1.34
	+		-0.41 (0.44)	(1.10 to 1.58)‡
CF patients	-	49	0.77 (0.48)	0.74
	+	57	0.03 (0.35)	(0.57 to 0.90)‡
NonCF patients	-	41	1.57 (0.53)	1.54
	+	28	0.03 (0.31)	(1.34 to 1.75)‡

Fig. 4. Slope (computed after logit-transformation of percentages) by material and treatment with rhDNase as dotplot and parametric boxplot with 2.5%-, 25%-, 50%-, 75%-, 97.5%-quantiles of the respective normal distributions. The reference line at -0.3 shows the suggested threshold between native and cleaved DNA.

Results

Quantitative analysis

As expected, we detected approximately ten-fold higher DNA concentrations in CF patient sputum (*Pseudomonas* positive) compared to non-CF patients under mechanical ventilation. A significant reduction of sputum DNA upon treatment with rhDNase was detected in the non-CF group. In the CF group we studied different populations excluding a meaningful comparison of DNA concentrations. Independent of a rhDNase treatment, the DNA concentrations in sputum varied much more in CF patients than in non CF patients (Tables).

Fragmentation

RhDNase was highly effective on DNA fragmentation from reference cells, CF and non-CF patients (Tables). The maximum effect was reached on DNA from reference cells with even a conversion of the slope towards negativity (Tables). The slope significantly

Fig. 5. ROC curves (areas under them) for (from the top left corner) reference samples (0.999), nonCF patients (0.980) and CF patients (0.947). The tangents were used to find the threshold of -0.3.

flattened in non-CF as well as in CF patients, but did not reach negativity. This represents a very strong enzyme effect on DNA of reference cells in vitro and in non-CF patients (with overall low amounts of sputum DNA) in comparison to CF patients. RhDNase showed a highly

significant cleaving activity as well, but not as strong as in non CF patients. The variance between the individual slopes is similar in the reference cells and both patient groups.

Sensitivity, specificity

We computed ROC-curves (Figure 5) for the discrimination of treated and untreated CF patients, non-CF patients and T lymphocyte cell cultures, and suggest a slope = -0.3 as a threshold, as it is near the points with maximal sum of sensitivity and specificity in both, CF and nonCF patients. Sensitivities were 0.88 % (CI 76 % to 95 %) in CF patients, 89 % (CI 72 % to 98 %) in non-CF, 100 % (CI 90 % to 100 %) in T lymphocyte cell culture. Specificities were 86 % (CI 73 % to 94 %) in CF patients, 95 % (CI 84 % to 99 %) in non-CF patients, 94 % (CI 81 % to 99 %) in T-lymphocyte cell culture when this threshold was used.

Discussion

Here we present a reliable and convenient novel method to analyze the effects of rhDNase on the DNA in sputum. We analyzed sputum samples of CF patients receiving rhDNase, CF patients not receiving rhDNase and non-CF patients on mechanically ventilation receiving rhDNase for DNA content and DNA fragmentation as an *in vitro* diagnostic measurement of the rhDNase effect. We established a DNA gel electrophoresis method for densitometric DNA quantification. Analysis of grayscale values at eight labeled DNA lengths for using a densitometric approach after gel electrophoresis served to determine digestion of the DNA. Calculation of the slopes of these eight DNA length-measurements permitted us to report not only ratios [7] between high and low DNA levels, but also to determine the amount of DNA shift between from high to low molecular weight DNA.

Brandt and coworkers [23] determined DNA in CF patients and detected up to 9.5 mg/ml, prior to rhDNase treatment, which dropped to 0.6 mg/ml to 0.3 mg/ml after treatment. These pre-treatment values are approximately 20-fold higher than in our patients, which is probably explained by the analysis of different patient populations. Similar to our method, Brandt *et al.* [23] used human blood cell DNA as a standard, but only compared the overall median DNA length prior and after rhDNase treatment. Therefore, our results are difficult to compare with these data.

We have shown that the slopes of the DNA size curve prior and after rhDNase therapy are a more accurate monitor than the ratio determination of an endpoint measurement, as performed by Shah *et al.* [7].

At present, the role of the chronic microbial colonization of the lungs (all patients of the present study were infected with *P. aeruginosa*) for accumulation of DNA in CF patients requires definition, despite its critical impact on pulmonary inflammation and hence on pulmonary DNA content. Suri *et al.* [27] described a direct correlation between elevated mucociliary clearance on rhDNase therapy and decreased proinflammatory parameters. Unfortunately this study did not consider the chronic microbial colonization or the circadian change in tracheal secretion on rhDNase therapy. We have detected marked qualitative differences in tracheal secretion in 15 patients to date depending on the timing of sputum collection (2 vs 8 hours after rhDNase inhalation), although this needs to be confirmed in a larger population.

Quantitative DNA fraction analysis in the tracheal secretions of CF patients to date [7, 23] has been performed exclusively with spontaneously expectorated sputum resulting in a high fluctuation of the quality and reproducibility [23]. Induced sputum (e.g. with 1 M NaCl) is currently considered to be the best method for sample collection [26]. Thus, we used this method in the present study to collect the samples from our CF population treated with rhDNase. Both Shah *et al.* [7] and Brandt *et al.* [23] performed DNA quantification predominantly in frozen sputum samples, which may result in a mechanical fragmentation of the DNA. We, therefore, performed all measurements in fresh native tracheal secretions to achieve more reproducible results. Our own observations show that samples stored for more than three days undergo fragmentation and yield falsely low values.

In summary, we describe a novel technique that permits a reproducible measurement of the *in vivo* effects of rhDNase on DNA in the lung.

Conclusion

The presented refinement of the electrophoresis sputum DNA fragmentation methods based on the pioneer work of Shah [7], Brandt and coworkers [23] markedly increases the sensitivity for monitoring the direct rhDNase effect *in vivo*. This offers the possibility for detailed longitudinal and cross sectional treatment studies in the future to gain insight into the processes of DNA cleavage in sputum and its role in chronic pulmonary inflammation.

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