Rheology of Cystic Fibrosis Sputum after in vitro Treatment with Hypertonic Saline Alone and in Combination with Recombinant Human Deoxyribonuclease I

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Treatment with recombinant human deoxyribonuclease I (rhDNase) is currently used as therapy for cystic fibrosis (CF) lung disease. Hypertonic saline (HS) acts as an expectorant promoting mucus secretion and augmenting the volume of sputum. We evaluated the individual and combined effects of HS and rhDNase in vitro on the viscoelasticity of CF sputum. Sputum samples were collected from nine CF patients to use for in vitro testing. Aliquots of CF sputum (0.20 to 0.40 g) were subjected to the following protocols: (1) negative control sample without any treatment; (2) positive control sample, adding 10% volume of normal saline (0.9% NaCl); (3) application of hypertonic saline (HS-3% NaCl); (4) combining approximately 100 nM concentration of rhDNase with protocols 2 and 3. The samples in protocols 2 through 4 were incubated for 30 min at 37°C. For each protocol, CF sputum was analyzed at baseline and at 30 min for spinnability by filancemeter and viscoelasticity by magnetic microrheometry. Spinnability decreased for the sputum samples that were treated with rhDNase, in combination with either HS or normal saline. Treatment with HS alone and combined treatment with rhDNase and HS decreased log G* (the principal viscoelasticity index) to the same degree. Saline alone and rhDNase in normal saline both increased the predicted cough clearability of the sputum; however, the combined treatment with rhDNase and hypertonic saline had the best overall effect on cough clearability. The change in predicted mucociliary clearability, although greatest after HS, was not significant. These in vitro results suggest that combined treatment with rhDNase and HS should be evaluated further as a potential mucotropic approach to augment the clearance of purulent sputum in CF lung disease. King M, Dasgupta B, Tomkiewicz RP, Brown NE. Rheology of cystic fibrosis sputum after in vitro treatment with hypertonic saline alone and in combination with recombinant human deoxyribonuclease I.

Defects in the cystic fibrosis (CF) transmembrane regulator protein are responsible for abnormal ion transport in airway epithelial cells leading to complex pathophysiology of mucociliary function with impaired mucus clearance in CF lung disease (1). Impaired lung clearance in CF has been correlated with deteriorating lung function (2), and severely impaired lung function has been associated with poor short-term survival of patients with CF (3).

The rheological properties of the mucus, along with the efficiency of ciliary action, are both responsible for the effectiveness of mucociliary clearance (4). Two groups of macromolecules, DNA and mucous glycoproteins, have been reported to be the major contributors of the physical properties of CF airway secretions (5). Mucus rheology can be influenced by the concentration of ions within the mucous gel (6). One of the pathologic mechanisms in CF is thought to be due to decreased ionic content in respiratory mucus that is believed to increase repulsive interactions within the mucin macromolecules, increasing molecular dimensions and thereby augmenting the rigidity of the mucous gel network (7).

Since accumulation of thickened secretions plugging the airways is considered a major contributing factor to the decreased lung function, the current treatment of CF lung disease includes strategies aimed at changing the physical properties of respiratory mucus (1). One such strategy deals with another mechanism responsible for increased rigidity of mucus, namely the increased concentration of high molecular neutrophilic DNA found in inflammatory secretions. Secretions from patients with CF have been shown to have DNA contents up to 10.2% of the dry weight (8), and DNA has been reported to accumulate at an average concentration of 59 mg/ml (9). Treatment of CF sputum with rhDNase decreases the molecular size of the DNA, thus reducing its contribution to viscoelasticity (10). Clinical studies with rhDNase have shown it to improve lung function in patients with CF and decrease...
the number of exacerbations of infections necessitating the use of antibiotics (11).

A administration of hypertonic saline (HS) has been reported to increase whole lung clearance in CF patients (12), and treatment of CF sputum in vitro has been demonstrated to increase its clearability on ciliated epithelium (13). The mechanism for these effects is not fully understood. HS may be changing the physical and transport properties of CF secretions by modifying the hydrogen and ionic bonding between mucus macromolecules.

Therefore the purpose of this study was to examine the effects of administration of HS on the rheological properties of CF sputum that could provide a mechanism for the reported effects of administration of HS on the rheological properties of CF sputum. A iso the in vitro effect of combined treatment with HS and DNase was evaluated. The individual effects of hypertonic saline (HS 3% NaCl), as well as normotonic saline (NS 0.9% NaCl) were compared with the combined effects of DNase and HS or NS on the rheology and clearability of CF sputum.

METHODS

Subjects

Sputum samples were collected from nine patients with CF by voluntary expectoration during a routine clinical visit. The patients (18 to 37 yr, mean age 27 yr) were all infected with Pseudomonas aeruginosa and treated with steroids, antibiotics, and bronchodilators as required. None of the patients had been exposed to either rhDNase or HS aerosol up to the time of the sputum collection, and none were using any other type of mucolytic preparation. A approval to collect and use sputum for this in vitro analysis was obtained from the University of Alberta Research Ethics Board.

Study Design

A aliquots of each sputum sample (0.2 to 0.4 g) were subjected to three different treatment protocols: (1) baseline (negative control), without application of any treatment (e.g., rhDNase, HS); (2) positive control, to test the dilution effect, adding 10% (vol/wt) of 0.9% NaCl (NS); (3) incubation with ten percent of 3% NaCl (HS). The samples in protocols 2 and 3 were incubated at 37°C for 30 min. Ten percent volume of treatment solutions per weight of sputum (vol/wt) was chosen because by itself this vehicle treatment had no significant effect on viscoelasticity in previous in vitro experiments involving DNase (14, 16).

To observe the combined effects of HS or NS with rhDNase, protocols 2 and 3 were combined with rhDNase treatment (Pulmozyme®; Genentech Inc., South San Francisco, CA) to achieve a final concentration of 2.5 µg/ml or approximately 100 nM and then incubated at 37°C for 30 min. This concentration of DNase was chosen on the basis of previous experience in our laboratory (14–16), while 3% NaCl solution has been commonly used for clinical sputum induction.

For each treatment protocol, spinnability and viscoelasticity were measured where possible, and mucociliary clearability index (MCI) and cough clearability index (CCI) were calculated prior to any treatment (baseline), and then after 30 min of application of the treatment. Three spinnability readings per aliquot were taken, and the arithmetic mean of the three readings were calculated. V viscoelasticity measurements were performed in duplicate.

Rheological Measurements on CF Sputum

In this in vitro study, two techniques were used to measure the rheological properties of sputum: spinnability by filancemeter and viscoelasticity by magnetic rheometry.

Spinnability is the thread forming ability of mucus under the influence of low amplitude elastic deformation. The spinnability of CF sputum samples was measured using a Filancemeter (SE F Am N, Nancy, France) (17), in which a 20 to 30 µl mucus sample is stretched at a distraction velocity of 10 mm/s. A n electric signal conducted through the mucus sample is interrupted at the point where the mucus thread is broken. The length of this thread is known as the mucus spinnability (measured in mm).

**Viscoelasticity and Clearance Indices**

The magnetic microrheometer technique was used to measure the viscosity and elasticity of the sputum samples. A 100 µm steel ball was positioned in a 5–10 µl sample of sputum, and the motion of this sphere under the influence of an electromagnet was used to determine the rheological properties of the sputum. The image of the steel ball was projected via a microscope onto a pair of photocells, whose output was amplified and transmitted to an oscilloscope. By plotting the displacement of the ball against the magnetic driving force, the viscoelastic properties of the mucus were ascertained (18).

The parameters of mucus viscoelasticity determined were the rigidity index or mechanical impedance, i.e., G*+, reported here on a log scale, expressing the vector sum of “viscosity + elasticity”, and the loss tangent, i.e., tan δ, reflecting the ratio of “viscosity/elasticity”, at low (1 rad/s) and high (100 rad/s) frequency (18). Two derivative parameters—mucociliary clearability index (MCI) and cough clearability index (CCI)—were computed from in vitro relationships (19). These two indices predict mucus clearability by ciliary and cough mechanisms, respectively, based on the measured rheological properties and observations of clearance from model studies. The respective formulas are as follows (20):

\[
\text{MCI} = 1.62 - (0.22 \times \log G^+ - 0.77 \times \tan \delta 1) \tag{1}
\]

\[
\text{CCI} = 3.44 - (1.07 \times \log G^* 100 + (0.89 \times \tan \delta 100) \tag{2}
\]

**Statistical Analysis**

Data from each protocol are presented as mean ± SD of the mean. To analyze the significance of changes in spinnability, log G*+ at 1 rad/s, MCI, and CCCI after administration of NS, rhDNase, and HS, the spu- tum from each patient served as its own control. Equality of means was tested by analysis of variance (ANOVA), and post hoc analysis of changes from baseline was determined by the two-tailed, paired t-test. The paired t-test was also used to determine the differences between spinnability and viscoelasticity after different treatments. The Stat-View statistical package (Abacus Concepts, Palo Alto, CA) was used to carry out these analyses.

**RESULTS**

Complete spinnability measurements pre- and post-treatment were obtained on six samples of CF sputum; no spinnability data were obtained on three other samples because of inadequate sample size. Post-treatment viscoelasticity measurements by magnetic rheometry were successful on eight of nine samples; one sample was rejected because the sputum was too liquid for analysis by this rheological technique. Pretreatment viscoelastic data were only obtained for six samples in order to preserve sufficient sample for the spinnability measurements.

**Spinnability Measurements**

Compared with baseline, 30 min of single treatments with NS (0.9% NaCl) and HS (3% NaCl) resulted in a mean decrease in spinnability of CF sputum from baseline of 16% and 26%, respectively. The mean decrease in spinnability for the combined treatment of NS and DNase was 37%, while after combined treatment of HS and DNase the decrease was 40%. When compared with NS as a control (i.e., at 30 min), HS versus NS gave a 12% decrease in spinnability, and the combination of HS and DNase treatment versus NS gave a 25% decrease, whereas the combination of HS and DNase versus NS treatment resulted in a 29% decrease.

Individual effects of mucolysis. Incubation with HS over 30 min resulted in a small decrease in spinnability in comparison with incubation with NS (10.7 ± 0.61 versus 12.18 ± 0.79 mm, respectively; p = 0.0085). A dministration of NS itself resulted in a small but highly significant decrease in spinnability compared with the undiluted control, which had no treatment applied over 30 min (12.18 ± 0.79 versus 14.43 ± 1.17 mm, respectively; p = 0.0001) (Figure 1).
Combined effects of mucolysis. After a period of 30 min, the combination of DNase and HS treatments decreased spin-nability considerably more than the administration of HS alone over the same period of time (8.67 ± 1.03 versus 10.7 ± 0.61 mm, respectively; p = 0.0009). Combined treatment with DNase and NS also decreased spin-nability significantly more than administration of NS alone over a period of 30 min (9.10 ± 1.04 versus 12.18 ± 0.79 mm, respectively; p = 0.0011). The additional effect of HS in the presence of DNase, although very small, was consistent and statistically significant (8.67 ± 1.03 versus 9.10 ± 1.04 mm, respectively; p = 0.0071) (Figure 1).

**Viscoelasticity and Clearance Indices**

Incubation of CF sputum samples with HS demonstrated a decrease in the sputum rigidity index (log G* at 1 rad/s) compared with treatment with NS (1.41 ± 0.049 for HS versus 1.97 ± 0.35 for NS; p = 0.041). Combined treatment with HS and DNase showed a comparable, significant decrease in log G* 1 compared with CF sputum treated with NS and DNase (1.39 ± 0.46 for HS + DNase versus 1.91 ± 0.81 for NS + DNase; p = 0.049). However, singular treatment with DNase (in NS) resulted in only a small, nonsignificant decrease in rigidity compared with NS treatment itself. A diminution of NS itself resulted in no significant change in viscoelasticity compared with the undiluted control. These viscoelastic data (log G* 1) are presented in Figure 2.

The changes in rigidity determined at 100 rad/s were similar to those seen at 1 rad/s, i.e., a decrease for HS treatment, with or without DNase, but only a small, nonsignificant decrease in log G* 100 for DNase treatment itself. None of the changes in tan δ by treatment group were statistically significant.

As illustrated in Figure 3, HS treatment of CF sputum also demonstrated significant changes in the predicted cough clearability index (CCI) in comparison to treatment with NS (2.31 ± 0.80 for HS versus 1.57 ± 0.46 for NS; p = 0.046). The mean CCI obtained for DNase treatment was numerically similar (2.33 ± 2.17), but the difference from NS control was nonsignificant. The largest change in CCI was seen for the combination of DNase and HS treatment (2.90 ± 1.60 for HS + DNase versus 1.57 ± 0.46 for NS alone; p = 0.050). The differences in predicted mucociliary clearability index (MCI) were smaller than the CCI changes. The greatest MCI occurred for HS treatment, but the difference from NS control was nonsignificant (0.99 ± 0.14 versus 0.92 ± 0.09, respectively; p = 0.071).

**DISCUSSION**

Previous in vitro studies report HS to be more effective in reducing mucoid sputum viscosity in comparison to water (21, 22). A decrease in viscoelasticity and an increase in sputum clearability was also demonstrated in this study. These in vitro results provide support for the work done by Pavia and co-workers (23), who observed enhanced mucociliary clearance rates in patients with chronic bronchitis after inhalation with 1.21 M NaCl, and for Robinson and colleagues (12), who re...
cently found that inhalation of 7% NaCl (1.20 M) stimulated pulmonary mucus clearance in cystic fibrosis patients better than either aerosolized amiloride or coughing. A recent observation in the results of our study and work done by others, it would seem that HS solutions have the potential to stimulate airway mucus clearance in both chronic bronchitis and CF patients, by changing its ionic content and rheological properties.

A addition of H S to CF sputum demonstrated a small reduction in spinnability compared to treatment with NS. Treatment with rhDNase, as in our previous studies (14-16), resulted in a significant decrease in spinnability. A thorough, combined treatment of HS and DNase showed a greater reduction in spinnability than either treatment by itself, the total reduction was less than additive. In fact, in this study, the decrease in spinnability for combined in vitro treatment of rhDNase and HS was similar to the decrease demonstrated after treatment with rhDNase and NS. Thus, as opposed to the co-treatment with DNase and gelsolin (15) or DNase and N acystelyn (16), in this study, we saw no clear evidence for synergy of mucolytic action between DNase and HS.

The sputum samples in the study were treated with 3% NaCl (0.512 M) (1 part to 10 parts of sputum). The initial sputum NaCl content was not measured because of insufficient volume of sputum with four treatment protocols used; although this is one of the limitations of the study, we can calculate the amount of NaCl added by HS and estimate the final NaCl content in the sputum. Based on previous literature reports (5, 7), the initial NaCl concentration should have been about 88 mM (mean Na⁺ = 101 mM, mean Cl⁻ = 76 mM). Thus, the final concentration of NaCl in the sputum treated with 3% NaCl should have been about 126 mM, an increase of approximately 38 mM. These electrolyte concentrations are lower than serum levels and less than those reported for laryngectomized patients by Potter and coworkers (165 mM for Na⁺) (24).

Ten percent volume of solution treatments per weight of sputum (vol/wt) was chosen for this in vitro study because by itself this vehicle treatment had no significant effect on viscoelasticity in previous experiments involving DNase (15, 16). It is not known if the clinical use of HS or DNase results in delivery of 1:10 of total volume of secretions in the airways; this ratio will most likely vary depending on the distribution of aerosol deposition. Therefore it is difficult to make any predictions related to an optimal volume/dose of solutions delivered to the airways.

The 38 mM increase in sputum NaCl content by this in vitro treatment resulted in a modest decrease in spinnability compared with NS, as well as a major decrease in viscoelasticity by magnetic microrheometry. The increase in NaCl content was comparable to that reported by Tomkiewicz and coworkers (7) for long-term amiloride treatment of CF patients, where the mean Na⁺ content went from from 95 mM to 121 mM. (Sputum chloride content increased from 64 to 77 mM with chronic amiloride treatment; further acute treatment increased the sodium content to 143 mM, and 95 mM for Cl⁻). These changes in ion content were associated with a significant decrease in viscoelasticity despite the absence of detectable change in sputum water content. It was suggested at the time that CF sputum rheology might be particularly susceptible to small changes in ion content (25), and the results of the current study would seem to confirm this. The interpatient variation in rheological data for a log transformed mean ± SD for G* in the control group was 1.97 ± 0.35 (i.e., 124% coefficient of variation), while the intrapatient variation was approximately half of that; this is comparable to what has been reported previously (7, 20).

The combined treatments of DNase and HS and DNase and NS showed a greater reduction in spinnability in comparison to individual treatments of HS alone. The additive effects of both HS and DNase and DNase and NS in reducing the sputum spinnability is largely due to DNase. Treatment with rhDNase has been reported to cleave neutrophil-derived DNA present in CF infected lungs, reducing the adhesiveness and viscoelasticity of CF sputum (26). By separating the DNA molecules from the mucoprotein, it was expected that HS would make the DNA more susceptible to enzyme digestion (27). However, this was not the case, as indicated by the results. The combination of HS and DNase may have possibly had a less than additive effect on spinnability due to a reduction in activity of DNase at high salt concentrations, given the fact that DNase and HS were combined prior to incubating the sputum.

In the previous studies (15, 16), as well as in the present investigation, the change in molecular weight of the DNA present in the sputum samples after administration of rhDNase was reflected in the results obtained by the filancemeter, but not in the results from the magnetic microrheometer. The reasons why spinnability by filancemeter appears to be a more sensitive indicator for this type of mucolysis (i.e., molecular weight reduction) than viscoelasticity by magnetic rheometer are likely related to the molecular weight dependence of the two techniques. Viscosity (and probably viscoelasticity) classically exhibits a 3.4-power dependence on molecular weight (28). The power dependency for spinnability is less certain, but to the extent that it is related to the first normal stress difference, its power law dependency should be much higher, theoretically 6.8 power (29). This extra power dependency means that a mere 10% reduction in molecular weight would reduce spinnability to (0.9)⁶.⁸ or 49% of control, which is comparable to the reduction in spinnability observed for rhDNase treatment. At the same time, viscoelasticity should only decrease to (0.9)¹.⁴ or 70%. On the logarithmic scale used for viscoelasticity (log G*), this reduction would only amount to 0.16 log units, which is within the usual limit of detectability for magnetic rheometry (0.2 log units). With hypertonic saline treatment, it is assumed that there is no cleavage of intramolecular bonds and no reduction in molecular weight. However, the treatment (shielding of ionic charge) should produce substantial reductions in intermolecular interaction and macro-molecular conformation, both of which will reduce the degree of entanglement coupling. For this type of alteration, viscoelasticity and spinnability changes should become comparable (30).

In conclusion, this in vitro study demonstrated that treatment with HS alone, as well as combined treatment with HS and DNase, but not DNase alone, decreased log G* (the principal viscoelasticity index) to a similar degree, suggesting that this effect was mainly due to HS and that DNase had smaller effect on viscoelasticity in these samples than HS. The clinical relevance of our results is based mainly on established correlations between viscoelasticity and mucociliary clearance, whereas cough clearance also increases with decreased spinnability (31). Spinnability decreased for the sputum samples that were treated with rhDNase, in combination with either HS or normal saline. Hypertonic saline alone and rhDNase in normal saline both increased the predicted cough clearability of the sputum; however, the combined treatment with rhDNase and hypertonic saline had the best overall effect on cough clearability.

Our results of combined treatment with HS and rhDNase seem encouraging since they demonstrate a change in the physical property of CF sputum from a rigid gel to a lower elasticity fluid, more readily clearable by airflow interaction.
Because of the possibility that co-administration of HS with rhDNase might reduce the effectiveness of rhDNase, the potential for alternate or consecutive treatments with those two forms of mucotropic agents should be considered. Further studies are required to confirm these findings with a larger number of CF sputum samples and to carry out similar experiments with different ratios of HS and rhDNase.

References


