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Short Communication

Multi-analyte profiling of inflammatory mediators in COPD sputum – The effects of processing

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ABSTRACT

Prior to using a new multi-analyte platform for the detection of markers in sputum it is advisable to assess whether sputum processing, especially mucus homogenization by dithiothreitol (DTT), affects the analysis. In this study we tested a novel Human Inflammation Multi Analyte Profiling® Kit (v1.0 Luminex platform; xMAP®).

Induced sputum samples of 20 patients with stable COPD (mean FEV1, 59.2% pred.) were processed in parallel using standard processing (with DTT) and a more time consuming sputum dispersion method with phosphate buffered saline (PBS) only. A panel of 47 markers was analyzed in these sputum supernatants by the xMAP®.

Twenty-five of 47 analytes have been detected in COPD sputum. Interestingly, 7 markers have been detected in sputum processed with DTT only, or significantly higher levels were observed following DTT treatment (VDBP, α-2-Macroglobulin, haptoglobin, α-1-antitrypsin, VCAM-1, and fibrinogen). However, standard DTT-processing resulted in lower detectable concentrations of ferritin, TIMP-1, MCP-1, MIP-1β, ICAM-1, and complement C3. The correlation between processing methods for the different markers indicates that DTT processing does not introduce a bias by affecting individual sputum samples differently.

In conclusion, our data demonstrates that the Luminex-based xMAP® panel can be used for multi-analyte profiling of COPD sputum using the routinely applied method of sputum processing with DTT. However, researchers need to be aware that the absolute concentration of selected inflammatory markers can be affected by DTT.

1. Introduction

Induced sputum is a well established, non-invasive technique to investigate airway inflammation in various lung diseases such as chronic obstructive pulmonary disease (COPD), asthma or cystic fibrosis [1].

Dithiothreitol (DTT) is routinely used to process sputum samples in order to cleave the disulfide bonds of mucus proteins, which liquefies the sputum and enables the separation of the cellular and the fluid sputum phase, referred to as sputum supernatant [2]. This processing step is fast, simple to use and established in both observational and clinical studies, where high-quality differential cell counts are the primary variables of interest [3–5]. DTT, however, has a well-known effect on the analysis of a number of inflammatory markers in sputum supernatant either by directly affecting the analyte or by affecting the antibodies or processes within the analysis platform [6–8].

The novel multiplex bead array technology allows for simultaneous analysis of multiple inflammatory markers in small sample volumes [9]. However, this technology is designed to detect soluble cytokines and chemokines in serum, plasma, or cell culture supernatants, that usually do not require pre-processing. We previously used this technique to assess systemic inflammatory pathways in

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serum of patients with COPD [10]. Limited data are currently available regarding the feasibility of this technology for biomarkers analysis in sputum samples [11,12]. Therefore, it was the aim of our study to analyze sputum supernatant of patients with COPD using a Luminex platform and to directly compare two different sputum matrices processed with or without DTT which were derived from the same expectorate.

2. Methods

2.1. Patients

Induced sputum samples of stable patients with moderate to severe COPD were obtained at the Pulmonary Research Institute at LungClinic Grosshansdorf, Grosshansdorf, Germany. Patients did not receive any anti-inflammatory treatment and were free of a respiratory tract infection or exacerbation for four weeks before sputum collection. Previous publications of this cohort were dealing with the preservation of the sputum cell pellet following sputum processing [13] and the effects of DTT on the sputum transcriptome [14]. The study was approved by the local ethics committee of the Medical Chamber of Schleswig–Holstein (reference number: AZ 030/11(II)) and all patients gave written informed consent.

2.2. Sputum induction and processing

Sputum was induced and processed according to standardized procedures [13]. Patients were asked to inhale isotonic (0.9%; 2 × 5 min) and hypertonic (3%; 2 × 5 min) nebulized saline for a total inhalation time of 20 min as previously described [13]. In case lung function according to the forced expiratory volume in one second (FEV1) deteriorated by more than 10% the saline concentration was not increased; in case FEV1 deteriorated by more than 20% sputum induction procedure was prematurely stopped for safety reasons. Sputum plugs were selected directly after expectoration and stored at 4–8 °C until further processing. Selected sputum cell plugs of all induction periods were processed within one hour after collection. The sputum samples of all inhalation periods were pooled, weighed, and split into two portions. One portion was processed with four volumes of 6.5 mM dithiothreitol (DTT, Sputolysin®; Calbiochem, Bad Soden, Germany) for 15 min at room temperature and four volumes of phosphate buffered saline (PBS) [13]. The second portion was mixed with eight volumes of PBS only and divided into five aliquots. Each aliquot was homogenized by gentle passage (20 times) through an 18-gauge needle [15]. Therefore, the same dilution (dilution factor × 9) was applied to process the two sputum matrices.

Subsequently, the DTT and the needle processed cell suspensions were both filtered through a 53 μm nylon mesh and centrifuged (1800 rpm, 10 min, 4 °C). Supernatants were frozen at −80 °C. The remaining cell pellets were re-suspended in PBS. Cell counts were performed in a haemocytometer and viability was determined using trypan blue solution (Sigma, Deisenhofen, Germany). Cytospins were prepared and differential cell counts were performed as previously described [13].

2.3. Analysis of sputum supernatant

In pre-study experiments the matrix of COPD sputum supernatant (4 samples with DTT and 3 samples without DTT) was validated by determination of linearity, spike recovery, and the stability of the analytes (short term and freeze–thaw stability tests) using the Human Inflammation Multi Analyte Profile panel (xMAP®; Rules-Based Medicine, Inc., Austin, TX) according to established standards [16].

Thereafter, 47 inflammation-associated biomarkers were analyzed in the sputum supernatant samples of 20 COPD patients investigated in the present study.

2.4. Statistical analysis

Sputum cell concentrations were expressed as means ± SD. Protein concentrations were calculated as medians with interquartile ranges (IQR). Signed rank tests were performed using a statistical software package (SAS®, version 9.2, SAS Institute Inc.) for statistical analysis of differences between the two processing techniques. Statistical significance was defined as $p < 0.05$. Pearson correlation coefficients (r) were calculated to identify data correlation.

3. Results

3.1. Pre-validation of sputum supernatants

The results of spike recovery experiments for all detectable analytes are given in Supplementary Table 1. Overall, the short term (data not shown) as well as the freeze–thaw stability were within the acceptable range between 70 and 130% recovery for all detectable analytes (Supplementary Table 2). Stability of IL-18 and TNF-RII could not be assessed in samples processed without DTT (Supplementary Table 2).

3.2. Study subjects

Twenty patients with moderate to severe COPD (15 males, 5 females; 12 current smoker; 8 ex-smokers; mean age, 64.7 ± 7.1 years; mean FEV1 predicted, 59.2 ± 9.7%, smoking history, 53.1 ± 20.5 pack years) were recruited for this study. 17 out of 20 patients inhaled saline for a total inhalation time of 20 min. There were 3 patients, in whom saline inhalation had to be stopped prematurely for safety reasons (one patient after 5 min of inhalation, one patient after 10 min of inhalation, and one patient after 15 min of inhalation, respectively).

3.3. Cellular characteristics of induced sputum samples

The mean quantity of selected sputum cell plugs was 2141 ± 611 mg. The cellular characteristics of the sputum samples processed with and without DTT are summarized in Table 1. There were no significant differences in the number of inflammatory cells between both methods of processing. Furthermore, total cell count and cell viability were similar between the two processing methods. The average contamination of sputum samples with squamous cells was 9.89 ± 9.80% in samples processed with DTT and 8.91 ± 9.61% in samples processed without DTT.

Table 1
Comparison of the cellular characteristics of COPD sputum samples processed with and without DTT.

Cell type	With DTT	Without DTT	*P* value
SM	0.286 ± 0.260	0.326 ± 0.317	0.3885
NG	2.110 ± 2.860	1.710 ± 2.143	0.4477
EOS	0.016 ± 0.021	0.020 ± 0.037	0.7420
LY	0.021 ± 0.024	0.027 ± 0.026	0.1290
Viability	95.53 ± 3.16	92.71 ± 9.58	0.2057
TCC	2.458 ± 2.931	2.128 ± 2.219	0.7246

Abbreviations: SM: Sputum macrophages; NG: Neutrophil granulocytes; EOS: Eosinophil granulocytes; LY: Lymphocytes; TCC: total cell count. All concentrations are given in absolute numbers × 10E6/ml. Data are presented as mean ± SD; *P* values were determined by signed rank test; $n = 20$.