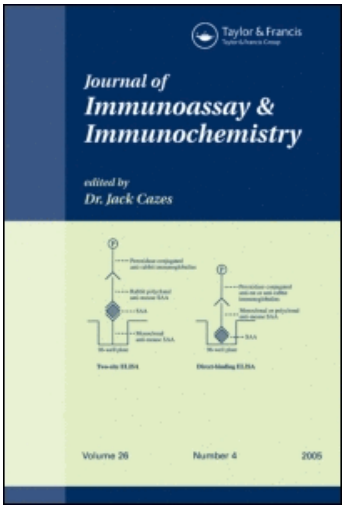

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Effects of Protease Inhibitors on Mediator Preservation in the Supernatant of Induced Sputum

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Abstract: Addition of protease inhibitors in induced sputum samples may help improve the recovery of mediators. The effect of protease inhibitors, in induced sputum, on the measurement of fibronectin, VEGF, IL-5, IL-6, and IL-8 was assessed. Protease inhibitors were added to sputum supernatant of atopic asthmatic subjects. Mediators were measured by ELISA or EIA. No differences were found in VEGF, IL-5, and IL-8 levels between protease inhibitors studied. Concentrations of IL-6 and fibronectin were higher when using, respectively, the commercial cocktail, and aprotinin. Protease inhibitors, if added, should be carefully chosen at the beginning of each study, to optimize the results.

Keywords: Sputum, Protease inhibitor, Standardization, ELISA

INTRODUCTION

Asthma is a chronic inflammatory disease characterized by lower airway obstruction and remodeling features such as subepithelial fibrosis, smooth muscle hyperplasia/hypertrophy, and glandular hyperplasia.[1] Influx of inflammatory cells is also observed in the asthmatic airways. These cells release various cytokines and mediators which are thought to play a major role in the pathophysiology of asthma.

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Induced sputum (IS) is a non-invasive method, increasingly used to study airway inflammation.[1,2] Analysis of induced sputum samples allows the quantification of a variety of proteins and mediators released by various airway inflammatory cells.[3,4] Different mediators have been successfully measured in IS supernatant, such as growth factors (vascular epidermal growth factor (VEGF), transforming growth factor-β (TGF-β)), cytokines (interleukin (IL)-5, IL-6, IL-8, IL-10, IL-12, endothelin (ET)-1, tumor necrosis factor (TNF)), and other mediators (fibronectin (FN)).[5]

Some of these mediators are commonly found in asthma. IL-5 is an important proinflammatory cytokine that plays a key-role in eosinophil recruitment and survival in asthma.[6] IL-6 is a pro-inflammatory cytokine involved in response to injury and infection.[7] IL-8 is a chemotactic factor for neutrophils.[8] VEGF is one of the most important growth factors for endothelium and is highly expressed in the airways of asthmatic subjects.[9] Finally, FN is an extracellular adhesion molecule involved in the structural changes of asthmatic airways.[10]

Mediators and cytokines may be subject to degradation by proteases *in vivo*[11–15] and the effects of proteases during sputum processing are not known. Adding protease inhibitors (PI) to sputum samples may help to preserve the various mediators, allowing their measurement. However, the methods used for IS analysis differ from one laboratory to another, one of these differences being the addition or not of PI to the samples.

Kelly et al. previously evaluated the addition of PI in IS supernatant for the measurement of IL-5. They demonstrated that IL-5 levels were increased by the addition of PI in the supernatant.[14] Moreover, Simpson et al. documented the same results with the addition of a mixture of PI.[16] However, to our knowledge, no comparison has been done between the different types of PI commonly used. The standardization of the method is important in order to obtain good quality samples and consistent analysis. Moreover, the addition of PI in IS supernatant should be evaluated in order to determine the importance of PI on the conservation of various cytokines.

The aim of this study was to determine the effects of adding different PI to IS samples on the measurement of IL-5, IL-6, IL-8, FN, and VEGF.

EXPERIMENTAL

Subjects and Study Design

Fifteen allergic subjects aged 18 to 40 years were recruited. PC_{20}, the provocative concentration of methacholine inducing a 20% fall in forced expiratory volume in one second (FEV_1), was <16 mg/mL. All subjects presented asthma symptoms that required treatment with only an inhaled short-acting β_2-agonist on demand and all were atopic, as shown by at least one positive allergy skin-prick test. Finally, IS were performed for all subjects. Informed

consent forms were signed by all subjects and the study was approved by the Hospital Ethics Committee.

Sputum Induction and Processing

Sputum was induced and processed as described by Pin et al.[2] and modified by Pizzichini et al.[17] Briefly, subjects were pretreated with 200 μg of salbutamol before inhaling concentration of hypertonic saline solution (3, 4, and 5%) for 7 min each with an electronic nebulizer (Medix, Catthorp, UK) without valve or nose clip. After each inhalation, subjects were asked to blow their nose and rinse their mouth with water to minimize post nasal drip and squamous epithelial cell contamination, respectively, and to expectorate into a sterile container.

Sputum was processed within 2 h following induction. Mucus was selected from saliva, and dithiothreitol (DTT) (Sputolysin; Calbiochem-Novabiochem, San Diego, CA, USA) was added. Sample was rocked for 15 min and the reaction was stopped by the addition of Dulbecco's phosphate buffered saline (D-PBS) (Invitrogen, Burlington, ON, Canada). Following filtration, total cell count and viability were determined using the trypan blue exclusion method. The cell suspension was centrifuged and the supernatant was divided into 4 equal volumes. PI was added to 3 of the aliquots. The fourth aliquot served as a negative control (no PI). Finally, supernatants were frozen at -80°C until mediator measurements.

Protease Inhibitors

The PI studied were aprotinin (Sigma, Oakville, ON, Canada) at a concentration of 1 μg/mL as suggested by the manufacturer and 10 μg/mL as often used in IS protocols, and the Complete Mini protease inhibitor cocktail 1X (Roche Diagnostics, Mannhein, Germany).

Mediator Measurements

ELISA measurements were performed for IL-5 (Amersham Biosciences, Buckinghamshire, UK), IL-6 and IL-8 (BD Biosciences, Mississauga, ON, Canada), VEGF (R&D systems, Minneapolis, MN, USA), and EIA was used for FN (Takara Biomedicals, Shiga, Japan) according to the manufacturer's instructions. Concentrations were determined using a microplate reader (Molecular Devices, Microplate Reader, Sunnyvale, CA, USA). The sensitivity of the assay was 9 pg/mL for VEGF, 2 pg/mL for IL-5, 4 pg/ml for IL-6, 2 pg/mL for IL-8, and 25 ng/mL for FN.

Statistical Analyses

Results were expressed in mean ± SEM. To analyse IL-5, IL-6, IL-8, FN, and VEGF data, the randomized complete block design was involved to the comparison among the different PI. A compound-symmetry covariance structure was involved to analyse data. For FN and IL-6 data, the graphical analyses of residuals have revealed a logarithm transformation and a gamma distribution. Statistical results from these variables were expressed with their transformed values. The quantile-quantile plot representation was used to compare residuals to the normal or gamma distributions, respectively. The results were considered significant with p-values ≤ 0.05. The data were analyzed using the statistical package program SAS v9.1.3 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Subject Characteristics

Subject characteristics are shown in Table 1. All subjects were atopic with mild asthma and they were aged (mean ± SEM) 25 ± 1 years and their geometric mean PC_{20} was 2.6 ± 1.8 mg/mL.

Sputum Analyses

Differential cell count was perfomed for all samples (Table 2). Macrophages were the most numerous cells found in IS samples, representing 63% of total cells. Levels of VEGF, IL-5, and IL-8 in IS samples are shown in Figure 1. The addition of PI to IS supernatants did not influence levels of these mediators.

The levels of FN and IL-6 are shown in Figure 2. The concentrations of FN in the supernatant containing aprotinin 1 μg/mL was significantly higher compared to control or to samples treated with the commercial cocktail (585.5 ± 347.9 vs 511.3 ± 285.5 ng/mL, $p < 0.05$; 585.5 ± 347.9 vs

Table 1. Subject characteristics

Number of subjects	15
Age (years)	25 ± 1
Atopy (y/n)	15/0
FEV_1 (% pred)	94.5 ± 2.5
PC_{20} (mg/mL)[a]	2.6 ± 1.8

Mean ± SEM.
[a]Geometric mean ± SEM.

Table 2. Differential cell count

Cell type	Absolute number ($\times 10^6$ cells)	Percentage (%)
Eosinophils	0.09 ± 0.05	3.8 ± 2.3
Neutrophils	1.2 ± 0.4	30.3 ± 4.7
Macrophages	2.7 ± 0.6	63.3 ± 5.5
Lymphocytes	0.05 ± 0.02	1.2 ± 0.3
Bronchial cells	0.05 ± 0.02	1.2 ± 0.4

Mean $\pm$ SE.

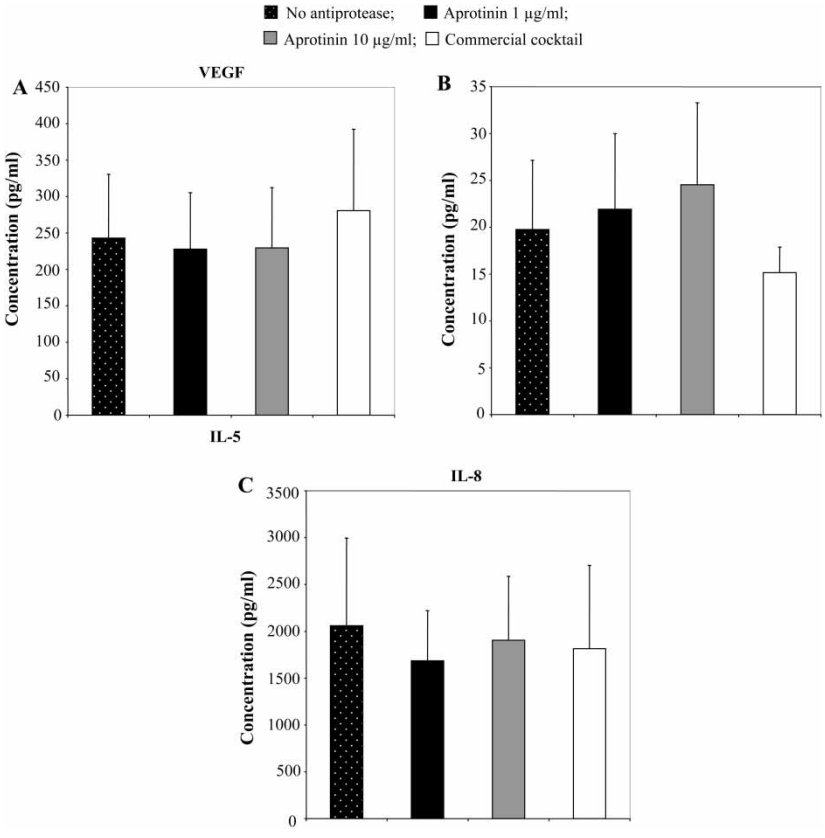

Figure 1. Levels of different mediators in IS supernatant treated with protease inhibitors: A) VEGF (n = 15); B) IL-5 (n = 8); C) IL-8 (n = 7). Mean $\pm$ SEM.

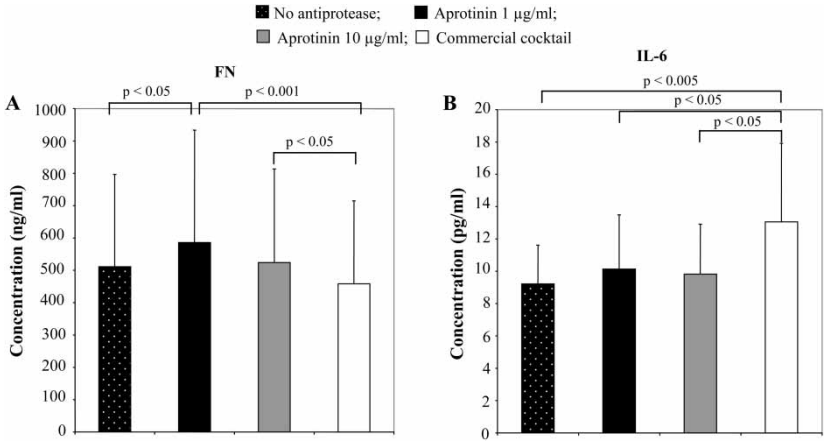

Figure 2. Levels of FN and IL-6 in supernatants treated with protease inhibitors. A) FN (n = 15); B) IL-6 (n = 7). Mean ± SEM.

458.3 ± 256.4 ng/mL, $p < 0.001$, respectively, n = 15). The addition of aprotinin, 10 μg/mL, resulted in higher concentrations of FN than with the commercial cocktail (524.0 ± 289.5 vs 511.3 ± 285.5 ng/mL, $p < 0.05$, n = 15). Moreover, no significant differences were observed between the concentrations of aprotinin 1 μg/mL and 10 μg/mL.

For the expression of IL-6, the presence of the commercial cocktail in IS supernatant significantly increased detectable levels of IL-6 compared with control (without PI), or aprotinin 1 μg/mL and 10 μg/mL (13.1 ± 4.9 vs 9.2 ± 2.4; 10.1 ± 3.4; 9.8 ± 3.1 pg/mL, $p < 0.05$, respectively, n = 7).

Since sputum induction can last as long as 30 min, we also added PI to the container before the induction, to verify if it would help to preserve the mediators from degradation in the first minutes of induction, compared to adding them later, during sputum processing. However, no significant differences were found whether the PI were added before or following IS treatment (data not shown). Moreover, we added the same PI (aprotinin or commercial cocktail) in the mucus plugs, before the addition of DTT, at the beginning of sputum treatment. Again, no differences were observed in the measurement of IL-5, IL-6, IL-8, VEGF, and FN between the PI used (data not shown).

DISCUSSION

We evaluated the role of PI in the measurement of IL-5, IL-6, IL-8, FN, and VEGF, when added in IS supernatant after treatment. Our results show that the commercial cocktail seems to be the best protease inhibitor studied for IL-6 measurement, while, aprotinin 1 μg/mL and 10 μg/mL are the most appropriate for the conservation of FN.

The aprotinin structure is quite stable, and can withstand exposure to high temperatures and acids.[18] It inhibits numerous serine proteases and all plasmin, kallikrein, elastase, trypsin, chymotrypsin, trypsinogen, and urokinase, but not carboxypeptidase A and B, papain, pepsin, subtilisin, thrombin, renin, and lysozyme.[18] Commercial aprotinin reduces or eliminates the biological activity of protein complex contaminants. The mechanism of action of aprotinin is to block the active site of enzymes or proteases by forming tight complexes with them.[18]

The commercial cocktail is a mixture of many protease inhibitors. According to the manufacturer, the commercial cocktail inhibits 87 to 99% of the proteolytic activity of chymotrypsin, thermolysin, papain, pronase, pancreatic extract, and trypsin. We hypothesized that it would give better results for the conservation of the mediators studied, since it had a larger spectrum of action than aprotinin alone. However, in our study, the commercial cocktail did not protect all mediators chosen.

The majority of the cells found in our IS samples were neutrophils and macrophages. These cells contain a large quantity of various proteases, such as serine proteases, that could degrade many mediators.[19–23] It is documented that all mediators involved in this study, IL-5, IL-6, IL-8, FN, and VEGF, were susceptible to the action of one or more of the proteases targeted by the protease inhibitors used.[11–15] However, protease inhibitors studied do not seem to influence the conservation of all mediators in IS supernatants.

Kelly et al.[14] evaluated the recovery of IL-5 when low and high concentrations of protease inhibitors were added to sputum supernatant. In this study, samples were spiked with IL-5 before freezing. A combination of 4-(2-aminoethyl)-benzensulphonyl fluoride (AEBSF), pepstain A, leupeptin, and disodium salt of ethylenediaminetetraacetic acid (EDTA-Na_2) was added to the samples. They found that the concentrations of IL-5 recovered were increased by the addition of protease inhibitors. In our study, we did not observe such an increase in levels of IL-5 when using 1 and 10 $\mu g/mL$ of aprotinin or the cocktail. However, these discrepancies between our results and the ones by Kelly et al. may be explained by the fact that we used different PI.[14]

IS treatment uses DTT, a mucolytic agent that cleaves and splices the glycoprotein disulfide bonds, thus liberating the cells from the mucus.[24] Disulfide cross-linkage maintains the three-dimensional molecular configurations and the biological activities of proteins. The reduction of disulfide bonds often leads to complete loss of catalytic activity for various enzymes.[24] Many proteases, such as plasmin, papain, chymotrypsin, and kallikrein, contain disulfide bonds.[25–27] It may be possible that DTT reduces the effectiveness of protease inhibitors in that way. DTT could also act similarly on the mediators themselves, and can interfere with their measurement. This could explain why we observed no differences in mediator levels between samples containing protease inhibitors or not. Some studies compared physical (ultrasound) and chemical (PBS and DTT) methods to disperse

sputum cells and some mediator concentrations were increased (eosinophil cationic protein (ECP)) or decreased (IL-8, IL-5) after DTT treatment.[28–30] We added DTT to some mediators, such that IL-6, IL-8, FN, and VEGF. For all mediators, except FN, the addition of DTT decreased the concentration of mediators by ELISA measurements (data not shown).

In conclusion, higher concentrations of IL-6 and FN were observed when protease inhibitors were added to the supernatant, suggesting that the addition of PI could be important for the preservation of some mediators. However, knowing the spectrum of protease inhibitors before to add them to the sputum is essential. Choosing the right PI is important before starting a study including mediator measurements in IS supernatants.

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