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A role for mitogen kinase kinase 3 in pulmonary inflammation validated from a proteomic approach

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ABSTRACT

Proteomics is a powerful tool to ascertain which proteins are differentially expressed in the context of disease. We have used this approach on inflammatory cells obtained from patients with asthma to ascertain whether novel drugs targets could be illuminated and to investigate the role of any such target in a range of in vitro and in vivo models of inflammation.

A proteomic study was undertaken using peripheral blood mononuclear cells from mild asthmatic subjects compared with healthy subjects. The analysis revealed an increased expression of the intracellular kinase, mitogen activated protein kinase (MKK3), and the function of this protein was investigated further in preclinical models of inflammation using MKK3 knockout mice.

We describe a 3.65 fold increase in the expression of MKK3 in $CD8^+$ T lymphocytes obtained from subjects with asthma compared with healthy subjects using a proteomic approach which we have confirmed in $CD8^+$, but not in $CD4^+$ T lymphocytes or human bronchial epithelial cells from asthmatic patients using a Western blot technique. In wild type mice, bacterial lipopolysaccharide (LPS) caused a significant increase in MKK3 expression and significantly reduced airway neutrophilia in $MKK3^{-/-}$ mice (median, 25, 75% percentile; wild/LPS; 5.3 (0.7–9.9) $\times$ 10^5 cells/mL vs $MKK3^{-/-}$/LPS; 0 (0–1.9) $\times$ 10^5 cells/mL, $P < 0.05$). In contrast, eosinophilia in sensitized wild type mice challenged with allergen (0.5 (0.16–0.65) $\times$ 10^5 cells/mL) was significantly increased in $MKK3^{-/-}$ mice (2.2 (0.9–3.5) $\times$ 10^5 cells/mL, $P < 0.05$).

Our results suggest that asthma is associated with MKK3 over-expression in $CD8^+$ cells. We have also demonstrated that MKK3 may be critical for airway neutrophilia, but not eosinophilia, suggesting that this may be a target worthy of further consideration in the context of diseases associated with neutrophil activation such as severe asthma and COPD.

1. Introduction

Asthma is an inflammatory disorder of the airways that is associated with an increase in airway hyperresponsiveness, leading to recurrent episodes of airflow obstruction. The incidence of asthma continues to increase in most countries, currently affecting over 300 million people worldwide [1,2]. Despite the availability of numerous therapies, a significant proportion of asthma sufferers are unable to control their disease through the use of the currently available drugs. These deficiencies in current therapy have led to considerable research effort to identify novel drug targets, particularly to help identify novel anti-inflammatory drugs to replace glucocorticosteroids or to be used in the treatment of patients with severe asthma who may be resistant to glucocorticosteroids [3]. High throughput proteomics has become an important research tool for the assessment of clinical samples in oncology [4,5], neurology [6], cardiovascular diseases [7], diabetes [8] and in

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Abbreviations

AU	absorbance unit
CD	cluster of designation
COPD	chronic obstructive pulmonary disease
DTT	dithiothreitol
HBEC	human bronchial epithelial cells
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MKK3	mitogen kinase kinase 3
OVA	ovalbumin
PBS	phosphate buffered saline
Tc	cytotoxic T cell
Th	T helper cell
TLR	toll like receptor
TNF	tumour necrosis factor

inflammation and asthma [9]. Such studies have uncovered the potential role of numerous genes and proteins in these various conditions, although enormous challenges remain to define the functional relevance of many of the proteins identified in such studies.

Mitogen activated protein kinase kinase 3 (MKK3) is a member of the p38 MAPK signalling pathway, which plays a pivotal role in inflammatory processes [10]. MKK3 is expressed in various cells and has been shown to cause a selective increase in p38 MAP kinase activity [11]. MKK3 is known to be activated following numerous stress stimuli in a wide range of cell types [12,13] and various studies have demonstrated that MKK3 is an important factor in the development of non-allergic inflammatory processes and Th1 responses, both in vivo and in vitro [14–16], although less is known about the role of MKK3 in the regulation of lung inflammation characteristic of diseases such as asthma and COPD.

The current study describes the identification of upregulated MKK3 expression in peripheral blood $CD8^+$ lymphocytes isolated from subjects with asthma compared to healthy control subjects and the characterization of this protein in the regulation of non-allergic and allergic lung inflammation.

2. Methods

2.1. Proteomic studies

2.1.1. Study subjects and sample collection

Peripheral blood (200 mL) was obtained by antecubital venepuncture into lithium heparin tubes from 22 healthy non-smoking subjects and 18 non-smoking subjects with well characterized mild asthma (Table 1). The latter were allowed treatment with a beta2-agonist prn, but refrained from taking any medication 24 h prior to venepuncture. This study was approved by the Ethics committee of King's College London. This was conducted according to the declaration of Helsinki principles and informed written consent was obtained from all participants. Lymphocytes were isolated using the MACS® Microbeads Cell Sorting system according to the manufacturer's instructions.

2.1.2. Proteomic analysis

Proteomic analysis was performed on 50 μL of supernatant from peripheral blood cells obtained from healthy and asthmatic subjects. Soluble proteins were separated by 2D gel electrophoresis: in the first dimension isoelectric focussing was performed using pH 3–10 immobilized non-linear gradient strips (GE Healthcare), followed by SDS-PAGE using 9–16% linear polyacrylamide gradient gels covalently attached to their glass support (using BindSilane, GE Healthcare). Gels were stained using Sypro Red (Molecular Probes, Inc.) and gels were scanned and processed using MELANIE7 II 2D PAGE analysis program. Features were detected using the following parameters: smooths = 2, Laplacian threshold = 2, partials threshold = 2, saturation = 25, peakedness = 100, minimum parameter = 7. Significantly upregulated spots were excised and tryptic in-gel digests were analysed by means of peptide mass fingerprinting and tandem mass spectrometry. Peptide mass fingerprinting was performed using MALDI-TOF-MS (PerSeptive Biosystems Voyager-DETM STR Matrix-Assisted Laser Desorption Ionization Time-of-Flight mass spectrometer) and resulting peptide masses were searched against a protein database using the MOWSE software [17]. Tandem MS was performed on a Quadrupole Time-of-Flight (Q-TOF) mass spectrometer (Micromass) and data analysis using the SEQUEST software [18].

Table 1
Demographics of the healthy and asthmatic volunteers.

	Non-asthmatic	Asthmatic
Number	22	18
Sex (male:female)	11:11	12:6
Age (yr) mean ± SEM	25.8 ± 1.3	28.7 ± 1.7
Atopic	No	Yes
Pulmonary function tests		
FVC (litre)	4.65 ± 0.16	4.53 ± 0.22
FVC (% predicted)	106.04 ± 1.88	97.22 ± 3.35
FEV1 (litre)	3.92 ± 0.11	2.86 ± 0.13
FEV1 (% predicted)	105.27 ± 2.16	72.47 ± 2.25
PEF (litre/second)	532.25 ± 22.36	443.5 ± 27.97
PEF (% predicted)	104.46 ± 2.79	81.30 ± 4.8
PC20 to methacholine (mg/mL)	>16	0.57 ± 0.23
Reversibility to salbutamol (200 μg), %	Not done	14.7 ± 2.76

Data expressed as mean ± SD.

2.2. Expression profiling of MKK3

2.2.1. Study subjects and sample collection

Human bronchial epithelial cells (HBECs) were cultured from epithelial tissue explants dissected from bronchial biopsies obtained from healthy and asthmatic volunteers (Additional file 1: Table S1 for demographics). Peripheral venous blood was taken from donors to isolate different inflammatory cells. Healthy non-smoking subjects with no history of asthma, allergic rhinitis or allergic dermatitis, as well as volunteers with a history of asthma were recruited and the study was approved by the Ethics Committee of King's College Hospital or King's College London. All subjects with asthma exhibited a positive skin response to at least one allergen, whilst none of the healthy subjects responded to any of the allergens tested.

2.2.2. Western blotting

Cells were lysed in lysis buffer, (50 mM Tris, 5 M urea, 55 mM DTT in PBS solution containing protease inhibitors) (approximately 100 μL buffer for 10^5 cells) and sonicated for maximum lysis (See Additional file 1; for further details). Tissues were lysed in lysis buffer (approximately 500 μL buffer for 1 tissue) and homogenized by three 30 s bursts using an Ultra Turrax T25 (IKA-Werke GmbH & Co). The protein solution was centrifuged at 13,000× *g* for 10 min at 4 °C and the supernatant was removed for analysis. Protein concentration was determined using the 2-D Quant Kit Protein Assay according to the manufacturer's instructions (Amersham). For