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Glutathione S-transferase M1 modulates allergen-induced NF- κ B activation in asthmatic airway epithelium

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Abstract

Background—Glutathione S-transferase M1 (GSTM1) is a Phase II enzyme and regulator of inflammatory signaling in airway epithelial cells. We have found upregulation of neutrophilic airway inflammation in atopic asthmatics expressing GSTM1 gene (GSTM1+) compared to GSTM1null asthmatics. We hypothesized that GSTM1 modulates NF- κ B activation in bronchial epithelium in atopic asthmatics. We determined regulation of allergen-induced NF- κ B activation in bronchial epithelium by GSTM1 in human atopic asthmatics *in vivo*.

Methods—Endobronchial biopsies and bronchoalveolar lavage fluid samples were collected from 13 GSTM1+ and 12 GSTM1null human atopic asthmatics at baseline and 24 h after segmental allergen challenge. A quantitative analysis of NF- κ B activation in airway epithelium was accomplished using a polyclonal antibody against the phosphorylated p65 component of NF- κ B. Elastase-positive neutrophils in the bronchial wall were quantified.

Results—Post-allergen neutrophilia in airway subepithelium and epithelial lining fluid was greater in GSTM1+ compared to GSTM1null asthmatics. Airway eosinophilia was similar in GSTM1+ and GSTM1null asthmatics. Allergen-provoked NF- κ B induction in bronchial epithelium was significantly greater in GSTM1+ compared to GSTM1null asthmatics. Activation of NF- κ B activation in airway epithelial cells correlated with interleukin-8 concentrations and absolute neutrophil numbers in bronchoalveolar lavage fluid in GSTM1+ but not GSTM1null asthmatics.

Conclusions—Allergen-induced neutrophilic airway inflammation in GSTM1+ asthmatics is associated with NF- κ B activation in airway epithelial cells *in vivo*. These novel data provide a potential mechanism of the genomic link between GSTM1 polymorphism and airway neutrophilia in atopic asthma.

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Keywords

Atopic asthma; GSTM1 polymorphism; NF- κ B; airway epithelium; neutrophilic airway inflammation

INTRODUCTION

GSTM1 is one of a family of cytosolic GSTs that act as multifunctional Phase II enzymes and regulators of stress-activated inflammatory signaling (1). The GSTM1 gene has 2 alleles: a wild-type allele *GSTM1*1* and a non-functional variant *null* allele *GSTM1*0* (1). Approximately 70% of European-Americans and nearly half of African-Americans have the homozygous *null* GSTM1 genotype and express no GSTM1 protein (2). In normal lungs, GSTM1 is predominantly expressed in airway epithelium and smooth muscle cells (3). GSTM1 conjugates endogenous and xenobiotic electrophiles with glutathione (1) and regulates stress-activated kinases (4–6) that participate in regulation of immune, inflammatory, and adaptive cellular responses to environmental changes (7). GSTM1 reduces airway inflammation and oxidative stress produced by ozone (8), tobacco smoke (9), diesel exhaust particles (10), and endotoxin (11). GSTM1 also mitigates nasal inflammation in response to allergen in atopic nonasthmatics exposed to diesel exhaust particles and second-hand smoking (9,10). Although epidemiologic studies suggest a modest reduction of asthma risk in GSTM1 carriers, these data are inconsistent (12). There are also studies suggesting that GSTM1 can aggravate responses to inflammatory stimuli and have pro-asthmatic role in humans (13,14,15).

Little is known about the regulation of airway inflammation by the GSTM1 genotype in human atopic asthmatics. Recently, we found greater neutrophilia and higher levels of pro-neutrophilic chemokines and cytokines in epithelial lining fluid following local allergen challenge in GSTM1+ compared to GSTM1*null* mild atopic asthmatics *in vivo* (16).

We hypothesized that GSTM1 modulates NF- κ B activation in bronchial epithelium in atopic asthmatics. The objective of the study was to examine allergen-induced NF- κ B activation in airway epithelial cells in human atopic asthmatics *in vivo*. We found greater expression of phosphorylated p65 (a surrogate of activated NF- κ B) in bronchial epithelium that positively correlated with airway neutrophilia in GSTM1+ but not GSTM1*null* asthmatics. Moreover, the percentage of p65-positive epithelial cells correlated with levels of neutrophil chemotactic protein, interleukin-8 (IL-8), in bronchoalveolar lavage fluid (BAL) in GSTM1+ patients.

METHODS

Subjects

Data was derived from volunteers with atopic asthma participating in a study on the effect of high doses of natural source d- α -tocopheryl on oxidant stress *in vivo* (17). Twenty five nonsmoking asthmatics with a physician diagnosis of mild atopic asthmatic according to National Asthma Education and Prevention Program (18) were recruited from Middle Tennessee. All participants had positive skin test with aeroallergens typical for this area,

methacholine test, and inhaled allergen challenge. The purpose of performing inhaled allergen challenge was to document that the specific allergen chosen for segmental allergen challenge was capable to provoke asthmatic response in each volunteer. The inhaled and bronchoscopic challenges were performed at least 3 weeks apart. Asthma drugs were stopped for at least 48 h prior to experimental procedures. Discontinuation of regular treatment was well tolerated by all volunteers. In addition, changes in asthma treatment were not permitted during the study. All women had negative urine HCG tests. The study was approved by the Vanderbilt University Committee for the Protection of Human Subjects and the use of instilled allergens by the U.S. Food and Drug Administration.

Protocol

Patients underwent fiberoptic bronchoscopy with baseline (before allergen challenge) bronchoalveolar lavage (BAL) and four endobronchial biopsies in the lingua of the left upper lobe followed by segmental allergen challenge (SAC) in a subsegment of the right middle lobe. The bronchoscopy procedure was repeated 24 h later to obtain BAL and four endobronchial biopsies from the allergen-challenged subsegment of the lung.

Experimental procedures

Prick skin test was done with diluent and histamine controls, and a set of standardized allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat hair, Bermuda grass, Kentucky bluegrass, fescue meadow grass, orchard grass, redtop grass, ryegrass, sweet vernal grass, timothy grass, and short ragweed (Greer Laboratories, Lenoir, NC) (19). The methacholine (Methapharm Inc., Brantford, Canada) challenge was accomplished using a Salter dosimeter (Salter Labs, Arvin, CA) and Flow Screen spirometer (VIASYS Healthcare GmbH, Hoechberg, Germany) (20). Provocations with inhaled allergen were carried out using the protocol described by Cockcroft and colleagues (21). Allergen aerosols were generated using a Wright nebulizer connected to a 2-way Hans Rudolph valve mouthpiece (Roxon, Montreal, Canada) attached to a wall oxygen source at 50 psi. Patients fasted overnight before the bronchoscopy studies. Intravenous midazolam 1–2 mg and fentanyl 50–100 mcg were administered for sedation and topical lidocaine (7 mg/kg) to anesthetize airways. BAL was performed using three 50 ml aliquots of sterile normal saline. Endobronchial biopsies were taken at bifurcations of segmental airways using disposable alligator forceps (Olympus FB-211D-A, Olympus America Inc.). SAC was performed with two incremental doses of the allergen to which the subject showed positive skin reactivity, each dose in a 5 ml aliquot of normal saline. The first dose of the allergen was 10-fold higher than the dilution that caused 5–8 mm wheal/20–30 mm erythema on intradermal test followed by the second dose of the same allergen at concentration 100-fold higher than the 2+ skin reaction, and the bronchoscope was then removed (16).

Laboratory methods

GSTM1 genotype was determined as previously described (16). Bronchial biopsies were fixed in 10% formalin and embedded in paraffin. One tissue section was routinely stained with hematoxylin and eosin for histological assessment and eosinophil count. Serial 5 µm sections were heated (60°C for 45 min), deparaffinized in xylenes, hydrated in graded

alcohol, and washed in PBS. Deparaffinized sections were heated in 10 mM Sodium Citrate Buffer (90°C for 20 min), cooled to room temperature, treated with 1% solution of Bovine Serum Albumin in PBS for 20 min, and incubated with primary and secondary antibodies. Analysis of NF- κ B was accomplished by employing rabbit polyclonal antibodies against phosphorylated p65 (phospho-p65, Ser276, Santa Cruz Biotechnology, Santa Cruz, CA), followed by secondary donkey antibodies labeled with Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were mounted using VECTASHIELD medium with DAPI (Vector Laboratories, Burlingame, CA). For neutrophil detection, sections were treated with rabbit polyclonal antibodies against neutrophil elastase (ab68672, Abcam, Cambridge, MA). Antigen retrieval was achieved by heating in citric acid pH 6 buffer for 15 minutes. A standard immunoperoxidase/avidin-biotin complex protocol (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) and biotinylated goat polyclonal antibodies were employed for rabbit anti-neutrophil elastase antibodies detection. Then, VECTASTAIN ABC reagent was utilized for detection of secondary antibodies. Peroxidase substrate solution (Vector Laboratories, Burlingame, CA) was used for visualization of the immunostaining. Quantitative assessment of NF- κ B activation in airway epithelial cells was accomplished by measuring fluorescent intensity for Cy3 (phospho-p65 expression) and normalizing it to the level of DAPI (nuclear content) fluorescence using Image-ProPlus 7.0 software (Media Cybernetics, Silver Springs, MD). Specifically, each nucleus was marked (Supplementary Figure 1A) and fluorescent intensities were measured for both Cy3 and DAPI separately. Then, phospho-p65 expression was calculated as a ratio of red fluorescent intensity (Supplementary Figure 1B) to the blue fluorescent intensity (Supplementary Figure 1C). Neutrophils and eosinophils were counted per 1 mm² of the epithelium or lamina propria, i.e. the space between reticular basement membrane and smooth muscles employing Image-Pro Express software (Media Cybernetics, Silver Springs, MD). IL-8 in BAL fluid was measured by human Plex Multi-Spot assay (Meso Scale Discovery, Gaithersburg, MD).

Statistical analysis

A two-tailed Mann Whitney test was used for comparison of GSTM1⁺ vs. GSTM1^{null} asthmatics, Wilcoxon signed rank test for comparisons within the groups, and Spearman's Rank Correlation Coefficient for correlation analyses. Data was expressed as mean $\pm$ standard deviation (SD). Significance was accepted when $p < 0.05$. Data were analyzed employing GraphPad Prism software version 5.0 (GraphPad Software Inc., La Jolla, CA).

RESULTS

The demographic and clinical characteristics of patients and allergens employed for segmental challenges are presented in Table 1. All asthmatics had normal baseline lung function. Although there was a trend toward greater spirometric values in asthmatics with the GSTM1^{null} versus GSTM1⁺ genotypes, the difference did not reach statistical significance (0.08 vs. 0.07, for FVC and FEV₁, respectively).

Prior to allergen challenge, the number of neutrophil elastase-positive cells (neutrophils) in airway walls on endobronchial biopsies (Figure 1) and in BAL fluid (Figure 2) was similar between GSTM1⁺ and GSTM1^{null} asthmatics. However, at 24 h after SAC, neutrophilic

influx into the airway walls and airspaces was observed only in GSTM1+ subjects. At this time point, neutrophil counts were significantly greater in the GSTM1+ compared to GSTM1null patients. In contrast, no differences in bronchial wall or BAL fluid eosinophil counts were detected between GSTM1+ and GSTM1null asthmatics before or after allergen challenge (Figures 1 and 2). SAC resulted in a similar increase in eosinophils in both groups. These data indicate a selective effect of the GSTM1 genotype on neutrophilic inflammatory responses to allergen challenge.

To test the hypothesis that GSTM1 modulates neutrophilic inflammation through activation of allergen-induced NF- κ B, we analysed phosphorylated Ser276 of the p65 component of NF- κ B (phospho-p65) as a measurement of activated NF- κ B in bronchial epithelial cells obtained by endobronchial biopsies from atopic asthmatics with GSTM1+ and GSTM1null genotypes at baseline and 24 h following SAC. Quantitative analysis of the intensity of the phospho-p65-specific fluorescence in airway epithelial cells from the GSTM1+ patients showed an increase from baseline in the percentage of bronchial epithelial cells with activated NF- κ B as well as an increase in relative NF- κ B activation in the bronchial epithelium (Figure 3). In contrast, no changes were observed after allergen challenge in the percentage of bronchial epithelial cells with activated NF- κ B or the relative NF- κ B activation in GSTM1null subjects. Consistent with these findings, the percentage of phospho-p65 positive airway epithelial cells on biopsies correlated with the concentration of the NF- κ B-dependent chemokine IL-8 in BAL fluid only in GSTM1+ asthmatics (Spearman $r=0.8$, $p=0.001$) (Figure 4). Moreover, the intensity of phospho-p65 fluorescence in bronchial epithelium positively correlated with the absolute neutrophil count in BAL fluid in GSTM1+ asthmatics (Spearman $r=0.42$, $p=0.04$). In contrast, no relationship was found between phospho-p65 and either airway neutrophilia or production of IL-8 in BAL fluid in subjects with the GSTM1null genotype. Together, these findings link the GSTM1+ genotype with allergen-induced NF- κ B activation, IL-8 production, and neutrophilic inflammation in the airways.

DISCUSSION

Our study demonstrates an association of neutrophilic airway inflammation with activation of NF- κ B in bronchial epithelial cells during the late response to allergen in human atopic asthmatics with the GSTM1+ genotype *in vivo*. These novel data provide a mechanistic insight into the relationship between GSTM1 genotype and neutrophilic airway inflammation in atopic asthma that we have recently reported (16).

Airway epithelium forms the first line of lung defense against aeroallergens and plays a central role in the onset and propagation of asthma (22). NF- κ B is an essential transcription factor regulating expression of inflammatory genes in airway epithelial cell in response to various environmental factors including allergens (23,24). IL-8 is a potent proneutrophilic chemokine produced by airway epithelial cells (as well as other cell types) under the control of NF- κ B (25). Thus, the correlation between production of IL-8 and percentages of phospho-p65-positive cells supports the notion that NF- κ B is mechanistically involved in the GSTM1-dependent regulation of airway neutrophilia in atopic asthmatics. Indeed, previous studies have shown upregulation of NF- κ B-dependent genes in asthmatics with

neutrophilic asthma phenotype (26). Our data are in a disagreement with studies showing protective role of GSTM1 on inflammatory response to ozone and endotoxin in healthy subjects (8,11). Wu and colleagues demonstrated an inhibitory role of GSTM1 on ozone-induced NF- κ B activation and IL-8 production in cultured human bronchial epithelial cells (BEAS-2B) *in vitro* (27). Nonetheless, presently it is unknown whether the airway epithelial cell response to various non-allergic environmental stimuli is differently regulated in asthmatics. Likewise, the mechanism modifying regulatory function(s) of GSTM1 on NF- κ B activation in atopic asthmatic airway epithelial cells is undetermined. Although our results are not unexpected given biological dysfunction of asthmatic airway epithelial cells as compared to normal bronchial epithelium (28–30), additional studies will need to be undertaken to explain these discrepancies.

Currently, the mechanism of the GSTM1-dependent allergen-induced NF- κ B activation in atopic asthmatic airways is speculative. Because asthma is associated with oxidative stress and depletion of glutathione (31), it is possible that the mechanism is redox-dependent (31–33). GSTM1 is a Phase II enzyme that depletes glutathione as it carries out its detoxifying actions (1). Paradoxically, this protective function of GSTM1 may result in reduction of intracellular glutathione and oxidative cell injury (32). Although it has been documented that activation of NF- κ B in bronchial cells involves redox-dependent mechanisms due to depletion of glutathione (33,34), additional studies are necessary to explain how GSTM1 modulates NF- κ B activation in atopic asthmatic airway cells.

In summary, we show here that activation of NF- κ B in bronchial airway epithelium and airway neutrophilia during the late airway response to allergen in human atopic asthmatics occurs predominantly in patients with the GSTM1+ genotype *in vivo*. These data provide a potential genomic linkage to the neutrophilic asthma phenotype in humans. The mechanism and pathophysiological role of the GSTM1-dependent NF- κ B-regulation in human asthma is yet to be explored. The genetic and molecular causes leading to non-eosinophilic asthma phenotypes are unknown, but uncovering them could identify molecular targets for new therapies. Understanding the molecular mechanism of the GSTM1-dependent neutrophilic inflammation may reveal new molecular targets for pharmacological treatment of asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The effect of GSTM1 genotype on bronchial wall neutrophils and eosinophils in atopic asthmatics *in vivo*

Exemplary airway biopsies stained with neutrophil elastase at baseline and after allergen challenge in GSTM1null (A–B) and GSTM1+ (C–D) asthmatics. Neutrophil (E) and eosinophil (F) counts in bronchial subepithelium at baseline (Baseline) and after allergen challenge (Allergen) in GSTM1null and GSTM1+ asthmatics.

Figure 2. The effect of GSTM1 genotype on neutrophils and eosinophils in BAL fluid in atopic asthmatics *in vivo*

Representative cytopsin slides stained with hematoxylin and eosin at baseline and after allergen challenge in GSTM1null (A–B) and GSTM1+ (C–D) asthmatics. Absolute neutrophil (E) and eosinophil (F) counts in BAL fluid at baseline (Baseline) and after allergen challenge (Allergen) in GSTM1null and GSTM1+ asthmatics.

Figure 3. Expression of p65 in airway epithelial cells in atopic asthmatics with GSTM1null and GSTM1+ genotypes *in vivo* (x200)

Typical images of airway biopsies stained with an antibody against phosphorylated p65 (ser276) at baseline and after allergen challenge in GSTM1null (A–B) and GSTM1+ (C–D) asthmatics. **E.** Percentages of phospho-p65-positive airway epithelial cells at baseline (Baseline) and post allergen (Allergen) in GSTM1null and GSTM1+ asthmatics. **F.** Intensity of phospho-p65-specific fluorescent signal in GSTM1null and GSTM1+ asthmatics at baseline (Baseline) and after allergen challenge (Allergen).

Figure 4. Relationship between expression of p65 in airway epithelium and production of IL-8 in airway space after SAC in human atopic asthmatics *in vivo*

The post-allergen percentages of p65 positive bronchial cells detected in airway biopsies positively correlate with levels of IL-8 in BAL fluid (* Spearman $r=0.8$, $p=0.001$).

Table 1

Clinical characterization of the volunteering asthmatics.

	GSTM1+	GSTM1null-
	Count or mean±SD, range	
Male/Female	3/10	4/8
Age	29.1±6.9 (22–42)	29.4±9.0 (20–46)
Race		
White	11	12
African American	2	0
Family h/o atopy	13	12
Baseline spirometry		
FVC (L)	4.26±0.6	4.83±0.99
FVC (%predicted)	98.7±9.9	107.7±10.5
FEV ₁ (L)	3.29±0.64	3.43±0.6
FEV ₁ (%predicted)	77.6±8.0	95.0±15.4
Treatment (regular or as needed)	77.6±0.9	71.6±6.5
Albuterol inhaler	13	12
Long-acting bronchodilator	1	0
Inhaled corticosteroids	2	0
Intranasal corticosteroids	1	0
Montelukast	3	1
Antihistamines	5	4
Allergen used for challenge		
Dust mite	4	3
Cat	4	6
Ragweed	1	1
Bermuda	1	1
Bluegrass	1	0
Rye grass	1	1
Timothy grass	1	0