German cockroach proteases regulate matrix metalloproteinase-9 in human bronchial epithelial cells

**Background:** Matrix metalloproteinases (MMPs) digest extracellular matrix proteins and may play a role in the pathogenesis of bronchial asthma. MMP-9 levels are increased in the bronchoalveolar lavage fluid and sputum of asthmatics compared with that of controls. As exposure to cockroaches is an environmental risk factor for asthma, we sought to investigate the role of German cockroach fecal remnants (frass) on MMP-9 expression.

**Methods:** Human bronchial epithelial cells (16HBE14o-) and primary normal human bronchial epithelial cells were treated with cockroach frass in the absence or presence of tumor necrosis factor (TNF)α. MMP-9 mRNA, protein levels and pro-MMP-9 activity were determined using real-time polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and zymogram assays. Pretreatment of frass with aprotinin abolished protease activity. PD98059, a chemical inhibitor of extracellular signal regulated kinase (ERK), and SLIGKV, an activator of protease-activated receptor (PAR)-2 were also used. AP-1 DNA binding was determined by electrophoretic mobility shift assay (EMSA) and ERK phosphorylation by Western blot analysis.

**Results:** Cockroach frass augmented TNFα-mediated MMP-9 mRNA and protein expression by a mechanism dependent on active serine proteases within frass and not on endogenous endotoxin. Frass increased ERK phosphorylation, and chemical inhibition of ERK attenuated cockroaches/C.213 effects on MMP-9. Serine proteases are known to activate the PAR-2 receptor. We found that selective activation of PAR-2 using the peptide SLIGKV augmented TNFα-induced MMP-9 protein levels and increased ERK phosphorylation. Frass and SLIGKV each increased AP-1 translocation and DNA binding.

**Conclusions:** These data suggest that German cockroach frass contains active serine proteases which augment TNFα-induced MMP-9 expression by a mechanism involving PAR-2, ERK and AP-1.

**Key words:** activator protein-1; Blattella germanica; extracellular regulated kinase; frass; serine protease.

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Materials and methods

Cockroach frass

The fecal remnants (frass) from one cage of German cockroaches were transferred to a sterile container and stored at 4°C. Frass was resuspended in endotoxin-free double-distilled water (2 h at 4°C while rocking), centrifuged to remove debris (13 000 g for 5 min at 4°C), supernatants harvested, and total protein was measured using the Bio-Rad Protein Assay Dye (Bio-Rad, Hercules, CA, USA). Protease activity was determined using the Azocoll method (17) as previously described (6). Endotoxin levels were determined by Charles River Laboratories using the Kinetic Chromogenic method (Charles River Laboratories, Charleston, SC, USA).

Cell culture

An SV40-transformed human bronchial epithelial cell line (16HBE14o-), provided by S. White (University of Chicago) was studied as described previously (18). Normal human bronchial epithelial cells (Clonetics Corp, Walkersville, MD, USA) were grown according to the manufacturers' specifications. Normal human bronchial epithelial cells (Clonetics Corp, Walkersville, MD, USA) were grown as described previously (18).

Cell supernatants were harvested and total protein was measured using the Bio-Rad Protein Assay Dye (Bio-Rad, Hercules, CA, USA). Protease activity was determined using the Azocoll method (17) as previously described (6). Endotoxin levels were determined by Charles River Laboratories using the Kinetic Chromogenic method (Charles River Laboratories, Charleston, SC, USA).

Real-time PCR

RNA was extracted using a standard TRIzol method of phenol extraction. Total RNA is converted to cDNA by reverse transcript-
isolated as previously described (11). Protein concentrations were
determined by Bradford assay (Bio-Rad) and stored at −70°C until
use. The AP-1 probe [sense 5'-GAT CCG AGT GTG ATG ACT
CAG GGT-3' and antisense 5'-AAC CTG AGT CAT CAC ACT
CGG ATC-3'] was synthesized from Invitrogen. The probe was
labeled with [γ-32P]ATP using T4 polynucleotide kinase (Gibco
BRL, Gaithersburg, MD, USA) and purified in Bio-Spin chroma-
tography columns. The gel was run using 4 μg of nuclear protein as
previously described (11). Cold-specific and nonspecific probes were
added at 5x the concentration of the radiolabeled probe. Gels were
transferred to Whatman 3M paper, dried under a vacuum at 80°C
for 1 h, and exposed to photographic film at 70°C with an inten-
sifying screen.

Statistical analysis
When applicable, statistical significance was assessed by one-way
analysis of variance (ANOVA). Differences identified by ANOVA were
pinpointed by Student–Newman–Keuls' multiple range test.

Results
German cockroach frass synergistically increased TNFα-induced
MMP-9 mRNA levels

We hypothesized that exposure to German cockroach 
fecal remnants (frass) may regulate MMP-9 expression in
human bronchial epithelial cells. To test this, we treated
SV40-transformed human bronchial epithelial cells (16HBE14o-)
with frass in the absence or presence of TNFα and performed quantitative real-time PCR. Treatment
with TNFα induced MMP-9 mRNA expression (Fig. 1A). Alone, frass had minimal effects on MMP-9
mRNA expression. When cells were treated with frass in
the presence of TNFα, a synergistic increase in MMP-9 
mRNA levels was detected compared with treatment with
TNFα alone.

To confirm that changes in MMP-9 mRNA resulted in
changes in protein abundance, we performed ELISA on
supernatants from cells treated with frass in the absence
or presence of TNFα, and found that frass synergistically
increased TNFα-induced MMP-9 protein abundance in
16HBE14o-cells (Fig. 1B) and in primary human bron-
chial epithelial cells (Fig. 1C). As tissue inhibitor of
metalloproteinase (TIMP)-1 acts to negatively regulate
MMP-9, we measured TIMP-1 protein levels. The con-
centration of TIMP-1 was not significantly altered by any
treatment. It is important to note that in human bronchial epithelial cells, the amount of TIMP-1 secretion
(control cells 202 ± 11 ng/ml; frass-treated cells 197.3 ±
13 ng/ml; TNFα-treated cells 222.8 ± 42 ng/ml; and
TNFα plus frass-treated cells 223 ± 42 ng/ml) greatly
exceeded the amount of MMP-9 secretion (control cells
0.42 ± 0.08 ng/ml; frass-treated cells 0.53 ± 0.07 ng/ml;

Figure 1. Cockroach frass regulates TNFα-induced MMP-9
levels. (A) 16HBE14o-cells were treated with cockroach frass,
TNFα or both for 4 h. Quantitative real-time PCR was per-
formed. MMP-9 normalized to SDHA and expressed as fold-
increase over control is shown. Data represent mean ± SEM
for four experiments (compared with control *P = 0.005;
compared with TNFα alone **P = 0.004; ANOVA). (B) 16HBE14o-cells were given either no pretreatment or pretreated
with polymyxin B (50 μg/ml for 1 h) prior to the addition of
frass, TNFα or both for 16 h. Supernatant was harvested and
analyzed by ELISA. Data represent mean ± SEM for four to
seven experiments (compared with control *P = 0.006,
#P = 0.001; compared with TNFα alone, **P = 0.002,
##P = 0.004; ANOVA). (C) Normal primary human bronchial
epithelial cells were treated as in (B). Data represent
mean ± SEM for four experiments (compared with TNFα
alone, *P < 0.001, ANOVA).
Endotoxin does not play a role in the synergistic effect

The endotoxin level in frass was measured by Charles River Laboratories and found to be 10.9 EU/mg protein (or 1.09 lg/mg protein). Incubation of cells with 100 ng/ml frass resulted in the addition of 109 pg/ml endotoxin. To rule out the possibility that endotoxin plays a role in the upregulation of MMP-9 expression by frass, we incubated 16HBE14o-cells with 100 pg/ml endotoxin in the absence or presence of TNFα and measured MMP-9 release by ELISA. Endotoxin had no effect on MMP-9 release, alone or in the presence of TNFα, suggesting that the effect of frass was not because of endotoxin contamination (data not shown). As a result of the complex composition of frass, we also wanted to determine if removal of endotoxin from frass would alter the synergistic increase in MMP-9 protein expression. To do this, we pretreated cells with polymyxin B for 1 h prior to addition of TNFα and/or frass. Polymyxin B treatment did not affect frass-induced synergistic increase in TNFα-mediated MMP-9 release (Fig. 1B).

German cockroach proteases modulate MMP-9 expression

Frass contained active serine proteases (19 μg/mg frass). Pretreatment of frass with the serine protease inhibitor aprotinin (10 μg/ml for 30 min at 37°C) inhibited 80% of protease activity as determined by the Azocoll assay. Treatment of cells with aprotinin-treated frass failed to augment TNFα-induced MMP-9 expression (Fig. 2). These data suggest the importance of active serine proteases in frass for modulating MMP-9 protein abundance.

The effect of TNFα and cockroach frass on pro-MMP-9 activity

Gelatin zymogram assays were used to detect the 92-kDa pro-MMP-9. Treatment with frass alone had no effect on pro-MMP-9 activity. Treatment with TNFα slightly increased pro-MMP9 activity; however, treatment with frass and TNFα resulted in a synergistic increase in active pro-MMP-9 (Fig. 3). Incubation of the control sample with an MMP-9 antibody shifted the band on the zymogram validating that the band detected on the zymogram is indeed pro-MMP-9 (data not shown).

Cockroach frass does not activate NF-κB

Nuclear factor (NF)-κB is a well-known activator of MMP-9 transcription. TNFα induced NF-κB translocation and DNA binding. However, frass had no effect alone or on TNFα-induced NF-κB translocation and DNA binding as determined by electrophoretic gel shift assay (data not shown).

Cockroach frass-induced activation of MMP-9 is due to activation of ERK

Treatment of primary normal human bronchial epithelial cells (Fig. 4A) or 16HBE14o-cells (Fig. 4B,C) with frass increased ERK phosphorylation. Both ERK1 (44 kDa) and ERK2 (42 kDa) are detected in the primary cells, while ERK2 is the primary form detected in 16HBE14o-cells. Pretreatment of cells with PD98059, a chemical inhibitor of MEK, abolished frass-induced synergy (Fig. 4D). Similar observations were obtained using another commercially available ERK inhibitor, U0126 (data not shown). We also investigated the possibility that frass increased phosphorylation of p38, another mitogen-activated protein kinase. TNFα increased phospho-p38 in a statistically significant manner, but the addition of frass to TNFα-treated cells did not result in a further phosphorylation of p38. Frass had no effect on phosphorylation of p38 either alone or in the presence of control frass aprotinin-frass

Figure 2. Protease activity in frass regulates MMP-9 protein abundance. 16HBE14o-cells were treated with frass, aprotinin-treated frass, or TNFα, alone or in combination for 16 h prior to harvest. Supernatant was collected and analyzed for MMP-9 protein by ELISA. Data represent mean ± SEM for five experiments (compared with control *P = 0.045; compared to TNFα alone **P < 0.001, ANOVA).

Figure 3. Frass increased TNFα-induced proMMP-9 activity. 16HBE14o-cells were treated with TNFα (T), frass (F), alone or in combination (T + F) for 16 h prior to harvest. Cell supernatants were subject to SDS-PAGE/gelatin electrophoresis under non-reducing conditions. Gelatinolytic activity is identified as clear zones on a blue background. The experiment was performed three times with similar results.
TNFα (data not shown). In addition, pretreatment with SB202190, a specific p38 inhibitor attenuated TNFα-induced MMP-9 expression, but did not affect synergy when frass was also added (data not shown). Furthermore, inhibition of protein kinase C (with calphostin C) or phosphatidylinositol 3-kinase (with wortmannin) did not affect frass-induced synergy of TNFα-mediated MMP-9 production (data not shown).

PAR-2 increases MMP-9 synthesis and activation of ERK

Activation of PAR-2 using the selective PAR-2-activating peptide SLIGKV resulted in the increased expression of MMP-9 (Fig. 5A). In addition, treatment of cells with SLIGKV increased ERK activation (Fig. 5B).

Cockroach frass and SLIGKV increase AP-1 translocation

To test for the involvement of AP-1, another transcription factor important in MMP-9 regulation, we performed an electrophoretic gel shift assay using an oligonucleotide probe designed against the AP-1 binding site on the IL-8 promoter. Treatment of cells with frass...
modulate TNFα-induced MMP-9 expression in a mechanism dependent on PAR-2, ERK, and AP-1 translocation and DNA binding. The human MMP-9 promoter contains NF-κB, AP-1, AP-2, SP-1 and Ets binding sites (20). An absolute requirement for NF-κB in MMP-9 transcription has been previously reported (21). That frass has no effect alone but augments TNFα-induced MMP-9 expression is likely because frass does not activate NF-κB. The activation of AP-1 may not be essential for induction but may be required for maximal stimulation (21). Frass-induced activation of ERK and AP-1 further increases translocation and DNA binding of AP-1 to the MMP-9 promoter to cause an upregulation of MMP-9. Frass-induced synergy was not altered by pretreatment with inhibitors to p38, phosphatidylinositol 3-kinase or PKC. As MMP-9 plays an important role in tissue remodeling, understanding the mechanism of regulation could lend insights into therapeutic interventions.

In this report, we used cockroach frass to determine the effects on MMP-9 expression. Frass is a likely source of allergen exposure as desiccated fecal remnants may easily be incorporated in house dust. It has been shown that after disturbance, dust particles containing cockroach proteins >10 μm in diameter may be deposited in the airways (22). Once in the airways, proteins elute from these particles and may reach high local concentrations. In addition, we have preliminary data to suggest that proteases remain active in dust collected from carpeting in homes (K. Page and B. Lanphear, unpubl. obs.). This suggests that the proteases may continue to be active even after lying dormant for a period of time.

MMP-9 protein levels were increased following treatment with TNFα and augmented with frass and TNFα treatment; however, these treatments did not significantly affect TIMP-1 levels. TIMP-1 is a natural inhibitor of MMP-9 and is thought to bind to MMP-9 in a 1:1 ratio. There was a significant excess of TIMP-1 compared with MMP-9 in these cells. This is likely why we were only able to detect the latent pro-MMP-9 (92 kDa) form of MMP-9 and not the active (88 kDa) form by zymogram. It is conceivable that the ratio of MMP-9 and TIMP-1 synthesized from different types of cells would be different. We have data to suggest that in neutrophils, which can degranulate and release MMP-9, the secretion of MMP-9 is in excess over TIMP-1 (K. Page and V.S. Hughes, unpublished data). The excess of TIMP-1 secretion from airway bronchial epithelial cells could be a mechanism to prevent disruption of the type IV collagen basement membrane. In a study comparing sputum samples from non-asthmatics, stable asthmatics and acute asthmatics, it was shown that pro-MMP-9 (92 kDa) activities were higher in asthmatic patients (1). They were unable to detect the active form of MMP-9 (88 kDa) in any patient, but rationalized that the concentration of active MMP-9 was small compared with the pro-MMP-9 concentration. Their data suggested that

Discussion

The airway epithelium plays a dynamic role in the asthmatic response with its ability to synthesize important metabolically active cytokines and other proteins. Airborne proteases may come in direct contact with airway cells and may play an important role in modulating inflammation and repair processes. In this report, we find that cockroach frass contains serine proteases which

![Figure 6. Frass and SLIGKV increased AP-1-DNA binding.](image)

(A) 16HBE14o-cells were untreated (C), treated with frass (F), TNFα (T), or TNFα and frass (T + F). (B) Cells were treated with the PAR-2 agonist SLIGKV (P) in the presence or absence of TNFα (T). Nuclear extracts were obtained, incubated with a 32P end-labeled double stranded AP-1 oligonucleotide, and resolved on a gel. Cold AP-1 probe (S) and cold NF-κB probe (NS) were used to show specificity. Each experiment was repeated twice with a representative experiment shown.
airway inflammation following an asthma exacerbation correlates with overproduction of MMP-9.

The consequence of increased MMP-9 expression following inhalation of cockroach proteases could be to aid in the migration of inflammatory cells. MMP-9 has been shown to be important for human polymorphonuclear neutrophil (PMN) and eosinophil migration across Matrigel-coated micropore membranes (23, 24). The transmigration of dendritic cells, an important antigen-presenting cell in asthma, was impaired in a MMP-9 knockout mouse. In addition, MMP-9 deficiency inhibited allergic airway inflammation characterized by a decrease in PMN and eosinophilic infiltration (25). In another study using MMP-9 knockout mice, lymphocyte and PMN infiltration was decreased following exposure to allergen (26). However, at least one study showed that neutrophil migration is not altered in MMP-9 null mice (27). Recent evidence suggests that pores exist within the basement membranes, and matrix degradation may not be as important as previously expected (28, 29). Additional stomachs are needed to clarify the importance of MMP-9 in cellular migration.

The MMP-9 could also play a role in the repair response in asthmatics. Some growth factors (such as platelet-derived growth factor, basic fibroblast growth factor and transforming growth factor) are stored in the extracellular matrix by being bound to proteoglycans (30, 31). It has been shown in the endobronchial biopsies of patients with asthma that MMP-9 immunoreactivity is localized in the extracellular matrix of the bronchial submucosa (32). In support of this, it has been shown that MMP-2, -3 and -7 have been shown to degrade decorin, a proteoglycan found in the extracellular matrix (33). It is possible that MMP-9-induced degradation of the collagen type IV in the basement membranes would release growth factors which could stimulate airway smooth muscle cell proliferation, a feature found in the asthmatic airway.

Our previous work has shown that serine proteases in cockroach whole body extract synergistically increased TNFα-induced IL-8 and IL-6 expression (6). IL-8 is a potent neutrophil chemoattractant, and can cause degranulation of recruited neutrophils. Van den Steen (20) has shown that MMP-9 derived from primary human neutrophils cleaves the amino terminus of IL-8 to increase its potency by more than 10-fold. It is conceivable that airway epithelial cells which encounter both cockroach proteases and a pro-inflammatory mediator such as TNFα. TNFα binds to a receptor and causes an intracellular signaling cascade which results in the increase of NF-kB activation. Cockroach proteases bind and cleave the PAR-2 receptor, which triggers its own intracellular signaling cascade resulting in activation of G-coupled protein activation, Ras, MEK (10), ERK and AP-1 (11). The end result is the increase in IL-8, IL-6 and MMP-9 secretion from human bronchial epithelial cells. Neutrophils will be attracted to the airways by the secretion of IL-8 and may also degranulate and release more MMP-9 into the airway. The amount of MMP-9 secretion from the human bronchial epithelial cell is minimal compared to what a neutrophil could secrete, but may play an important role in mediating the early stages of airway inflammation or repair.

Overall, our data suggest that the active serine proteases in cockroach frass play an important role modulating airway responses in the presence of an inflammatory stimulus, like TNFα. Inhalation of active proteases from cockroach frass, or from other sources, may elicit local inflammatory events in the airways, thus causing asthma exacerbations. Further understanding of the role of proteases in modulating airway remodeling and inflammation could result in novel therapeutic interventions for the treatment of asthma.

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References


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