

Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids

Meghan Cundall, MS,^a Yongchang Sun, MD,^b Christina Miranda, MS, PA,^a
John B. Trudeau, BS,^a Stephen Barnes, BS,^a and Sally E. Wenzel, MD^a Denver, Colo,
and Beijing, China

Background: Matrix metalloproteinase (MMP)-9 levels are increased in bronchoalveolar lavage (BAL) fluid from patients with severe asthma on high doses of glucocorticoids (GCs). **Objective:** We sought to identify neutrophils as the source of increased BAL fluid MMP-9 in severe asthma and to evaluate the effects of GCs on this MMP-9.

Methods: MMP-9 protein, activity, and mRNA were measured in BAL fluid and cells at baseline, and after in vitro GCs in patients with severe asthma and controls using enzyme immunoassays, zymography, Western blotting, and real-time PCR.

Results: The high molecular weight (HMW) form of MMP-9 was significantly increased in severe asthma ($P = .02$). Western blotting confirmed a heterodimer of MMP-9 and neutrophil gelatinase-associated lipocalin. The HMW MMP-9 correlated with BAL neutrophils ($r = .65$, $P < .0001$). BAL cell supernatant MMP-9 protein levels also tended to be higher in patients with severe asthma (overall, $P = .09$), whereas the HMW activity form was increased ($P = .03$). MMP-9 protein (and HMW activity) correlated with neutrophils in the cell pellet ($r = .75$, $P < .0001$). In contrast to protein and activity, BAL cell mRNA levels were marginally lower in patients with severe asthma than in control subjects (overall, $P = .06$).

Although GCs decreased BAL cell MMP-9 protein and mRNA in vitro, the effect was significantly smaller in severe asthma ($P < .01$ for both). GCs decreased the pro-MMP-9 activity in patients with severe asthma and normal control subjects, while having no effects on the HMW form ($P = .22$). Peripheral blood neutrophil MMP-9 protein was not affected by GCs.

Conclusions: BAL neutrophils contribute to BAL fluid MMP-9 protein and activity and are poorly inhibited by GCs. (J Allergy Clin Immunol 2003;112:1064-71.)

Key words: Matrix metalloproteinase-9, neutrophil gelatinase-associated lipocalin, glucocorticoids, asthma, bronchoalveolar lavage

Abbreviations used

BAL:	Bronchoalveolar lavage
DEX:	Dexamethasone
GC:	Glucocorticoid
HMW:	High molecular weight
MMP:	Matrix metalloproteinase
MW:	Molecular weight
NGAL:	Neutrophil gelatinase-associated lipocalin
TIMP:	Tissue inhibitors of metalloproteinase

Neutrophils are reported to be increased in tissue, bronchoalveolar lavage (BAL) fluid, and sputum in subjects with severe, often glucocorticoid (GC)-dependent asthma, but the mechanisms or implications of this are not clear.¹⁻³ Because GCs prevent apoptosis of neutrophils, the increase might be secondary to the high doses used by this group.⁴ Whether neutrophils function to worsen the disease or whether they are “bystanders” in the process of severe asthma is not known.

Matrix metalloproteinase 9 (MMP-9) is one of more than 20 zinc-dependent metalloproteinases with gelatinolytic and elastinolytic properties. It is made by cells that include neutrophils, macrophages, epithelial cells, and fibroblasts. MMP-9 exists in at least 4 different forms: a biologically inactive “pro” form (92 molecular weight [MW]), an “active” form (88 MW), a high molecular weight (HMW) form (125 MW), and as a homodimer at 220 MW. The HMW form is believed to be a heterodimer of MMP-9 and a neutrophil protein, neutrophil gelatinase-associated lipocalin (NGAL).⁵ This HMW form has been reported to be more easily activated than monomers or homodimers of MMP-9.⁶ Increased MMP-9 protein levels have been described in BAL and sputum of patients with asthma; however, the relationship of MMP-9 activity forms to asthma severity has not been reported.⁷⁻⁹

GCs remain the most potent anti-inflammatory agents for the treatment of asthma. However, their effect on airway remodeling and extracellular matrix modifying elements, such as MMP-9, remains controversial.^{10,11} GCs inhibit the expression of MMP-9 by alveolar macrophages in vitro, but effects on other cell types remain incompletely studied.¹²

We hypothesized that the increased BAL fluid MMP-9 levels previously reported in severe asthma derived pri-

From the ^aDepartment of Medicine, National Jewish Medical and Research Center and University of Colorado Health Sciences Center, Denver, and the ^bDepartment of Respiratory Medicine, Peking University Third Hospital, Beijing.

Supported by NIH grants HL-64087, AI-40600, RR-00051 and ALA of Colorado, Oklahoma, and Alaska.

The first 2 authors contributed equally to the development of this manuscript. Received for publication May 15, 2003; revised July 16, 2003; accepted for publication August 6, 2003.

Reprint requests: Sally Wenzel, MD, National Jewish Medical and Research Center, 1400 Jackson St, Denver, CO 80206.

© 2003 American Academy of Allergy, Asthma and Immunology
0091-6749/2003 \$30.00 + 0
doi:10.1016/j.jaci.2003.08.013

marily from neutrophils and would be poorly suppressed by GCs.⁹ After confirming the increase in BAL fluid MMP-9 protein levels, MMP-9 activity was qualified and quantified. The presence of NGAL/MMP-9 heterodimers was evaluated by Western blotting. MMP-9 protein, activity, and mRNA from BAL cells was also evaluated and compared with neutrophils in BAL fluid. Finally, the *in vitro* effects of dexamethasone (DEX) on these systems were evaluated.

METHODS

Subjects

Subjects with asthma were divided into severity categories by use of previous criteria.¹³ Patients with severe asthma were all referred for severe asthma. Despite treatment with oral and high-dose inhaled steroids, with at least 1 other controller, they had symptoms and used urgent health care. Normal control subjects had normal pulmonary function and no history of respiratory illness. The study was approved by the National Jewish Institutional Review Board, and all subjects gave informed consent.

BAL

BAL was performed as previously described,¹³ with 1-mL aliquots frozen for analysis of MMP-9. Twenty-milliliter aliquots were concentrated for zymography and Western analysis.

Isolation and culture of BAL cells

BAL fluid was filtered, centrifuged, washed, and the cells counted. BAL cells (10^6) (Table 1) were placed into Teflon-coated wells and cultured in Dulbecco's modified Eagle medium/0.1% BSA with and without DEX (1 μ mol/L) for 24 hours (37°C, 5% CO₂). RNA was extracted from the cultured BAL cells, while the supernatant was collected for MMP-9 protein level, zymography, and, in some cases, Western blotting for MMP-9 and NGAL.

Isolation and culture of peripheral blood neutrophils

Peripheral blood neutrophils were isolated using Ficoll-Paque separation and cultured in 24-well plates (4 $\times$ 10⁶ cells/well) for 24 hours with or without DEX (1 μ mol/L) in Dulbecco's modified Eagle medium/0.1% BSA.¹⁴ Supernatants were harvested and stored at -80°C for MMP-9 measurement and cells processed for RNA extraction and subsequent MMP-9 mRNA analysis.

Quantitative real-time PCR

Cellular RNA was extracted using TriZol reagent (Life Technologies, Rockville, Md). Reverse transcription was performed using random hexamers, 1 μ g of RNA and the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, Calif). MMP-9 primers and probe, labeled with 5'-reporter dye FAM and TAMRA, were designed using Primer Express software (Applied Biosystems). The following are sequences for MMP-9 primers and probe: forward primer, 5'-GGGAGACGCCCATTTTCG-3'; reverse primer, 5'-CGCGCCATCTGCGTTT-3'; probe, 5'-AACCAC GACGCCCTTGCCAG-3'. 18S ribosomal RNA labeled with VIC (Applied Biosystems) was evaluated in the same PCR reactions. Real-time PCR was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems). The threshold cycle (Ct) was recorded for MMP-9 and the internal control from each cDNA sample, and duplicates were averaged. Relative mRNA expression levels were calculated using the Δ Ct method ($[1/2^{(\text{gene of interest average Ct} - 18\text{S rRNA average Ct})}] \times 1000$).

Zymography

Concentrated BAL fluid (20:1, Amicon, 3000 MW filter, Beverly, Mass) and cell supernatant samples were loaded onto premade 10% zymogram gelatin gels (Bio-Rad, Hercules, Calif) under nonreducing conditions. After electrophoresis, the gels were incubated in renaturing enzyme activation buffer, rinsed, and incubated in TRIS-HCl developing buffer. Gels were stained, rinsed, dried (Novex Gel Dry, Ann Arbor, Mich), then scanned and analyzed with a Scion imaging analysis program (NIH, Bethesda, Md). Results were expressed as average pixel density $\times$ mm² for the bands of proteolysis, standardized to volume added (BAL fluid) or cell numbers (BAL cell and neutrophil supernatants). Minimum detection was in the 2-page range.^{15,16}

Enzyme immunoassays

MMP-9 levels in unconcentrated BAL fluid or cell supernatants were measured by enzyme immunoassay. The MMP-9 assay used reagents from R&D Systems (Minneapolis, Minn). Plates are coated with a mouse anti-MMP-9 (MAB936) and use the detection antibody from the Quantikine kit (DMP900). This assay detects both pro- and active MMP-9.

Western blotting for MMP-9 and NGAL

Concentrated BAL fluid samples (20:1, 3000 MW filter, Amicon) or BAL cell culture supernatants were electrophoresed on 4% to 15% gradient gels under nonreducing conditions and transferred to Hybond ECL nitrocellulose membranes (Amersham, Arlington Heights, Ill). Purified human MMP-9/NGAL complex (Calbiochem, San Diego, Calif) was used as a positive control. The blots were blocked with 5% nonfat milk/TRIS-buffered saline-Tween for 2 hours and incubated with NGAL antibody (1:200) (Antibody Shop, Copenhagen, Denmark) or MMP-9 (1:500) antibody (R&D) overnight at 4°C. The blots were washed, incubated with horseradish peroxidase-conjugated anti-mouse antibody, developed using ECL Western blot detection reagents (Amersham), and exposed to Hyperfilm (Amersham).

Statistical analysis

Because most continuous variables were right skewed, these data were log transformed. Because BAL MMP-9 levels and activity contained "zero" values, these data were modified as " $y = \ln(x + 1)$ ", where x = original data point and y = the modified data that is then log transformed. For presentation, log-transformed means and SEMs were reconverted to their original scale. Numbers in text or tables are the log-transformed means, with the upper and lower limits derived from the log-transformed SEMs reconverted back to the original scale. Means were compared by use of 2-way ANOVA for multiple comparisons. When a significant overall difference was found among the groups, a comparison of each individual group was performed by use of Tukey-Kramer testing. Correlations were performed using log-transformed data. A value of $P < .05$ was considered significant. All testing was done by use of a JMP program (SAS Institute, Cary, NC).

RESULTS

Subject characteristics

Thirty-nine patients with severe asthma, 9 patients with moderate asthma, 13 patients with mild asthma, and 17 normal control subjects underwent bronchoscopy with BAL for evaluation of cell counts, differentials, and MMP-9 levels (Table 1). Subgroups were involved in studies of MMP-9 production and activity as described in

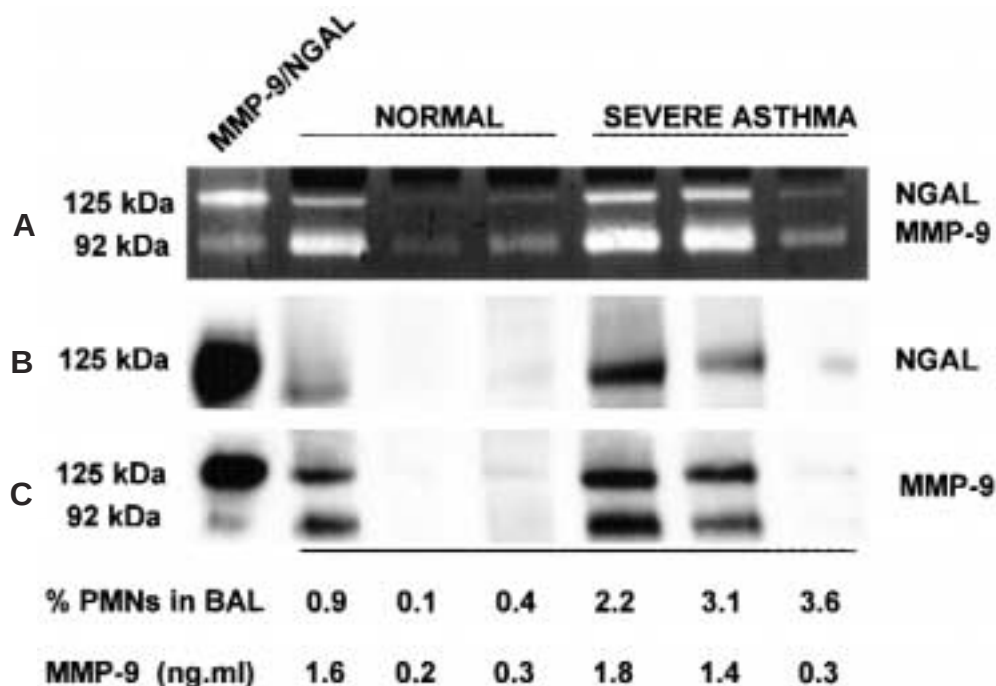


FIG 1. A, Zymograms of BAL fluid from 3 normal subjects and 3 severe asthmatics demonstrating the high molecular weight band. B, Corresponding Western blot for neutrophil-associated gelatinase lipocalin C, Corresponding Western blot for MMP-9. Accompanying neutrophil percentages and MMP-9 protein levels in lavage fluid are at the bottom.

TABLE I. Subject characteristics

	M/F	Age*	FEV ₁ (% predicted)†	BAL neutrophils (%)‡	BAL macrophages (%)‡	BAL eosinophils (%)‡	MMP-9 (ng/mL)‡	Oral steroids (mg/d)‡
Normal	8/9	29 ± 7	99 ± 3	0.6 (0.4-1.0)	86.6 (83.9-89.4)	0.15 (0.13-0.17)	0.18 (0.11-0.30)	—
Mild	8/5	35 ± 7	81 ± 2	0.7 (0.6-0.8)	85.1 (83.3-87.0)	0.31 (0.22-0.45)	0.15 (0.09-0.26)	—
Moderate	4/5	42 ± 12	67 ± 5	0.5 (0.4-0.6)	87.1 (84.3-90.0)	0.33 (0.20-0.53)	0.03 (0.02-0.06)	—
Severe	15/24	35 ± 11	45 ± 3	1.6 (1.3-2.0)	81.5 (79.6-83.3)	0.43 (0.33-0.55)	0.49 (0.37-0.65)	18 (13-25)
Overall P =	.44	.02	< .001	.009	.308	.102	.001	N/A

*Mean ± SD.

†Mean ± SEM.

‡Log-transformed mean and SEM converted back to original scale.

the individual sections. As reported previously, MMP-9 protein levels were highest in the severe asthmatics (overall, $P = .001$). Similarly, the group of patients with severe asthma had the highest percentages (and total numbers/milliliter) of neutrophils in BAL fluid compared with patients with milder asthma and normal control subjects ($P = .009$ and $P = .01$, respectively).

MMP-9 in BAL fluid

MMP-9 activity. Concentrated BAL fluid was evaluated for MMP-9 activity in 18 severe, 7 moderate, and 5 mild asthmatics and 8 normal controls by zymography. In most cases, 2 gelatinolytic bands were observed with

MWs of 92 and 125 kd, corresponding to the pro- (latent) and HMW forms (Fig 1, A).^{5,17} The active form was present in 3 of 18 patients with severe asthma but never in those with milder asthma or normal controls (band not shown). The pro-form of MMP-9 was detectable in all subjects, but differences between groups were not significant ($P = .18$) (Fig 2). The HMW form of MMP-9 was present in highest amounts in severe asthma and was significantly higher than the amount in moderate asthma (overall, $P = .02$) (Fig 2). Neither pro ($r = .31$, $P = .07$) nor HMW MMP-9 ($r = .32$, $P = .08$) activities correlated with BAL fluid MMP-9 protein level.

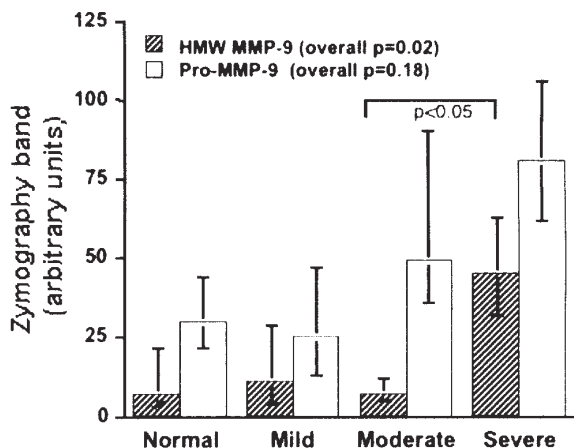


FIG 2. Pro- and HMW MMP-9 activity levels in BAL fluid among the 4 subject groups. There were no significant differences in the pro-form of MMP-9 among the groups (overall, $P = .18$). The HMW form was significantly different between groups ($P = .02$), and the severe asthmatics were significantly higher than those with moderate asthma.

NGAL/MMP-9 Western blotting. To confirm that the HMW form of MMP-9 was a heterodimer of MMP-9 and NGAL, sequential Western blots of concentrated BAL fluid were performed using antibodies to lipocalin and MMP-9 in 3 severe asthmatics and 3 normal controls. The 125-MW zymography band corresponded to a band that reacted with the NGAL and the MMP-9 antibody at the same MW shown on the Western blots in Fig 1, B and C. A strong association was found between the presence of the NGAL/MMP-9 band with neutrophils in BAL fluid (Fig 1).

Relationship of BAL MMP-9 levels to BAL cell types. MMP-9 levels and activity were compared with number and percentage of BAL cells. The MMP-9 protein level was positively correlated with both percentage ($r = .46$, $P < .0001$) and the number ($r = .38$, $P = .006$) of neutrophils, but not with percentage ($r = -.04$, $P = .77$) or number ($r = -.03$, $P = .66$) of macrophages in the BAL. Both the pro- and HMW activity form positively correlated with percentage of neutrophils ($r = .46$, $P = .005$ and $r = .65$, $P < .0001$, respectively), but correlation of neutrophils with the HMW form was higher/more significant (Fig 3). Similar correlations were observed when neutrophils per milliliter were compared with MMP-9 activity. In contrast, inverse relationships were seen with percentage macrophages in BAL and the pro- ($r = -.85$, $P = .007$) or HMW ($r = -.74$, $P = .03$) MMP-9 activity forms. Eosinophils did not correlate with MMP-9 levels.

MMP-9 protein/activity/mRNA from BAL cells

To further address the source of the elevated MMP-9 in severe asthma, studies of MMP-9 mRNA, protein, and activity were performed on isolated BAL cells from 18 severe asthmatics, 6 mild/moderate asthmatics, and 14 normal controls. BAL cell MMP-9 protein levels after 24

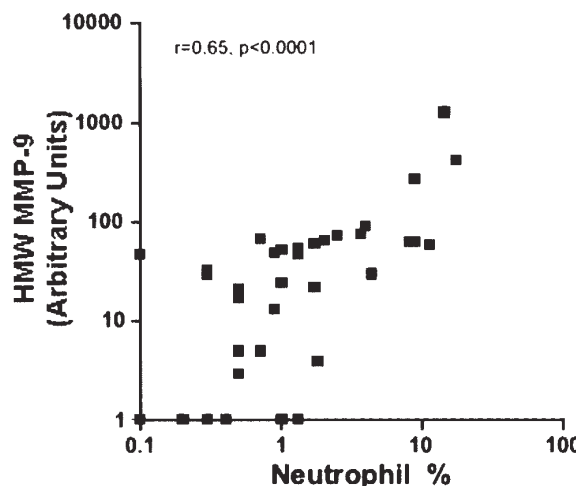


FIG 3. The percentage of neutrophils in the BAL cells was significantly correlated with the BAL fluid HMW MMP-9 level ($r = .65$, $P < .0001$).

hours in culture tended to be higher in those with severe (3.6 ng/mL, 2.8-4.4 ng/mL) asthma than in those with mild-moderate asthma (1.6 ng/mL, 1.2-2.2 ng/mL) and normal control subjects (1.9 ng/mL, 1.4-2.5 ng/mL) (overall, $P = .09$). The BAL cell MMP-9 protein concentration correlated with percentages of neutrophils in the BAL cell pellet ($r = .75$, $P < .0001$) and with BAL fluid MMP-9 protein level ($r = .45$, $P = .009$). Zymography was performed on BAL cell supernatants from 5 severe asthmatics and 5 normal controls. Activity was present at 92 MW in all samples. The HMW form was present in severe asthmatics and to a lesser degree in normal controls with neutrophils in the BAL cell pellet ($P = .03$) (Fig 4, A). In those with an HMW activity band, Western blotting for NGAL/MMP-9 demonstrated a HMW band positive for both (Fig 4, B and C).

BAL cells were also processed for MMP-9 mRNA. In contrast to protein, after 24 hours in culture, severe asthmatics had marginally lower amounts of MMP-9 mRNA than control subjects (overall, $P = .06$) (see Fig 5, B). BAL cell MMP-9 mRNA was not correlated with BAL fluid MMP-9 protein levels, activity, or neutrophil numbers.

Effect of GCs on MMP-9

In vitro effect of GCs on MMP-9 protein and mRNA in BAL cells. BAL cells from 17 severe asthmatics, 6 mild-moderate asthmatics, and 12 normal controls were cultured with and without dexamethasone for 24 hours. DEX had a significantly greater impact on BAL cell supernatant MMP-9 protein levels in normal controls than in severe asthmatics (overall, $P = .009$), despite marginally lower untreated MMP-9 protein levels in normal subjects (Fig 5, A). Pro-MMP-9 decreased after DEX in all groups ($P = .006$), but there was no significant change in HMW/NGAL protein/activity when compared with

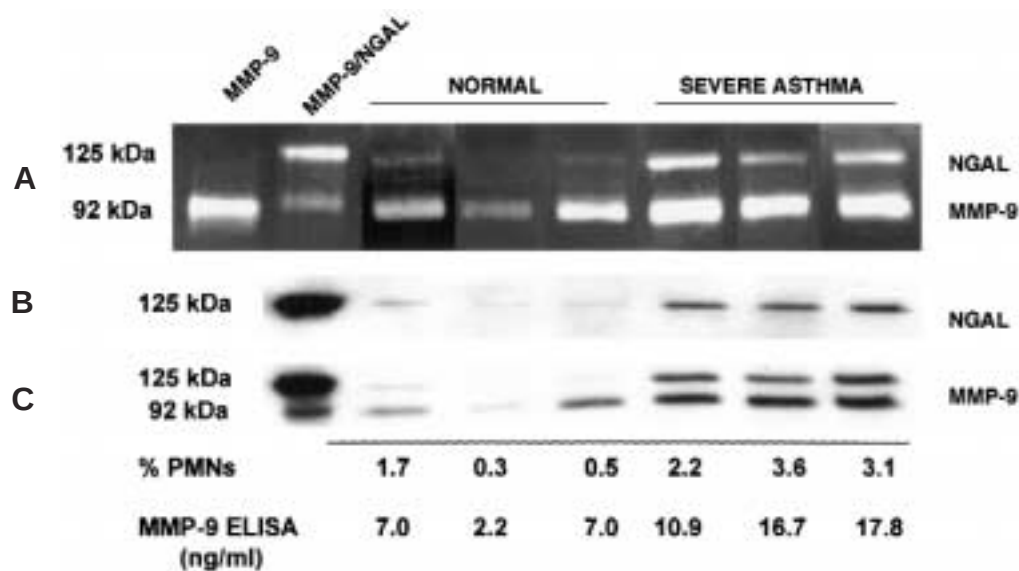


FIG 4. A, Zymograms from BAL cell supernatants from 3 normal controls and 3 severe asthmatics demonstrating high molecular weight and pro-form bands. B, Corresponding Western blot for neutrophil gelatinase associated-lipocalin. C, Corresponding Western blot for MMP-9. Neutrophil percentages in lavage fluid and MMP-9 protein levels in the BAL cell supernatants are at the bottom.

untreated cells in either asthmatics (-17 ± 8 units) or normal controls (-26 ± 9 units) ($P = .22$). DEX decreased MMP-9 mRNA in all groups. Similar to protein, the decrease was greater in normal controls than in severe asthmatics ($P = .005$) (Fig 5, B).

Effect of GCs on MMP-9 protein/mRNA in neutrophils. The effect of DEX on the protein and mRNA of MMP-9 in peripheral blood neutrophils from normal subjects ($n = 5$) and severe asthmatics ($n = 5$) was evaluated. MMP-9 mRNA and protein were detected in neutrophils and their supernatants in high amounts after 24 hours. MMP-9 protein levels were not decreased by DEX in either group (Fig 6, A). However, neutrophil MMP-9 mRNA was marginally inhibited in both groups ($P = .06$) without difference between the groups (Fig 6, B).

DISCUSSION

This study suggests that the increased MMP-9 protein in BAL fluid from patients with severe asthma is derived primarily from neutrophils, with minor contribution from monocyte/macrophages or other cells. Although it has been suggested that increases in MMP-9 after allergen challenge might be neutrophil derived, this is the first study to detail the relationship of BAL fluid MMP-9 to neutrophils and disease severity.^{18,19} In addition, in severe asthma, a significantly higher amount of the MMP-9 is in the HMW-NGAL complexed form, a form suggested to be a marker of neutrophil presence and activation.¹⁷ This HMW form of MMP-9 might be more proteolytically active or susceptible to activation than the pro-form. By inference, in areas where NGAL-associated MMP-9 is present, the tendency for proteolysis might be greater.⁶

The relationship of MMP-9 in BAL fluid to a neutrophil source is supported by the (1) significantly increased presence of the HMW MMP-9 in BAL fluid, (2) confirmation that the HMW form is the NGAL-MMP-9 heterodimer, (3) strong correlations between neutrophils in the BAL cell population with the HMW form of MMP-9 in the BAL fluid and cell supernatants, (4) low levels of BAL cell (predominantly macrophage) MMP-9 mRNA, and (6) poor inhibition of BAL fluid and cell MMP-9 protein, activity, and mRNA by *in vitro* GCs in subjects with severe asthma. In this study, correlations of macrophages or eosinophils with MMP-9 were either inverse (macrophages) or absent (eosinophils), suggesting that their contribution to MMP-9 is minor.

The implications for the increased release of NGAL/MMP-9 in the airways of patients with severe asthma are intriguing. Neutrophils are increased in severe asthma, but their activation state is not clear.^{1,2,13} Activated neutrophils release their granule components on stimulation.²⁰ NGAL is a unique protein released from activated neutrophil granules.⁵ Increased HMW MMP-9 activity has been described in patients with asthma, chronic obstructive pulmonary disease and subclinical emphysema, although the relationship to the NGAL complex has not been clear.^{17,21,22} In these diseases, the role of the neutrophil has been controversial, and it was suggested that in chronic obstructive pulmonary disease, the macrophage, not the neutrophil, was the primary source of the MMP-9.²³

In asthma, it has been unclear whether the increased BAL fluid MMP-9 is secondary to the increased neutrophils or a reflection of an increased activation state of the neutrophils. That question cannot be answered from

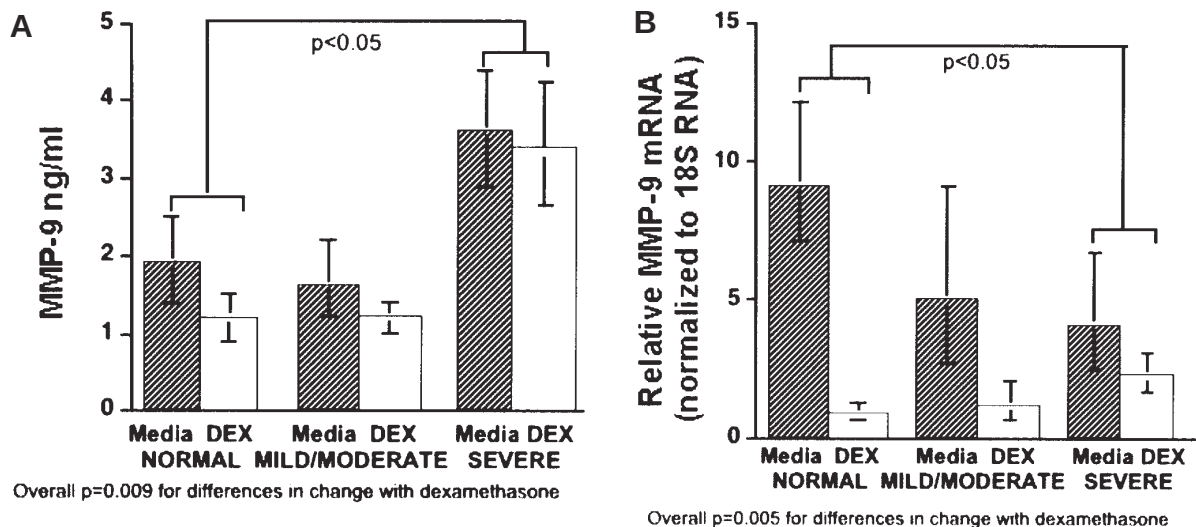


FIG 5. A, BAL cell supernatant MMP-9 protein levels (media versus dexamethasone). Dexamethasone had a greater effect on normal control than severe asthma MMP-9 protein (overall, $P = .009$). B, BAL cell MMP-9 mRNA (media versus dexamethasone). Dexamethasone had a significantly greater effect on normal control than severe asthma MMP-9 protein (overall, $P = .005$).

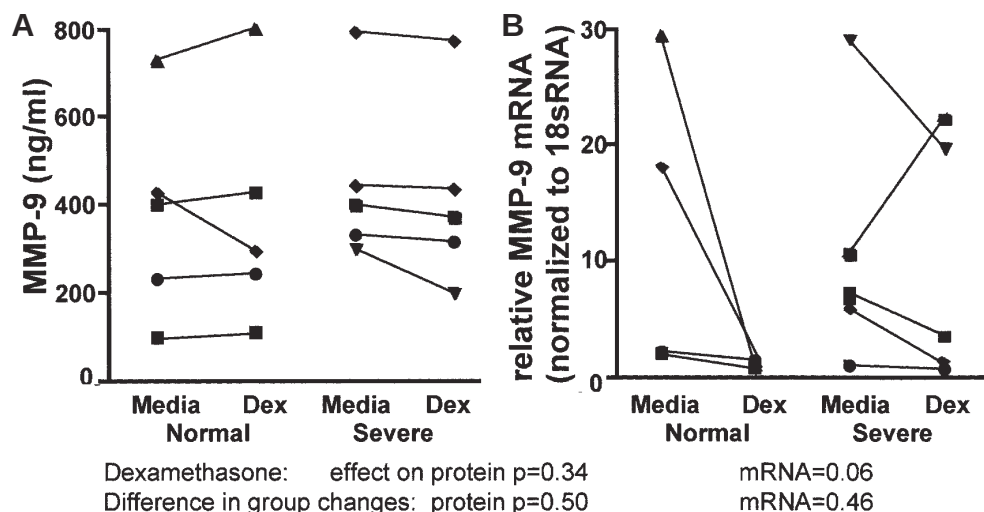


FIG 6. A, MMP-9 protein levels in peripheral blood neutrophil supernatants cultured for 24 hours in media versus dexamethasone (10^{-6} mol/L) from normal controls and severe asthmatics. B, MMP-9 mRNA levels from peripheral blood neutrophils cultured for 24 hours in the presence of media versus dexamethasone.

this study. However, because NGAL seems to protect MMP-9 from degradation, preserving MMP-9 enzymatic activity, the increased presence of the MMP-9/NGAL activity form in severe asthma could support an active role for neutrophils in proteolytic processes associated with this disease. In this regard, a recent report suggested that tissue MMP-9, when identified in the subepithelial basement membrane of patients with severe asthma, was associated with neutrophilic inflammation, worsened lung function, and potentially enhanced eosinophilic trafficking in tissue.⁹

These studies in toto suggest that evaluation of MMP-9 is complex. MMP-9 can exist in many forms, with dif-

fering degrees of activity. It can have several cell sources, differing regulatory elements, and be found in different compartments.^{9,22,23} Despite these complexities, many studies have attempted to develop MMP to tissue inhibitors of metalloproteinase (TIMP) ratios. It is unlikely that one simple ratio applied to complex biologic samples (lavage fluid or sputum) will provide a valid approximation of proteolytic activity within the lung. MMP protein assays generally measure total MMP, without regard for activation or binding to TIMP-1. TIMP-1 assays measure bound and free TIMP-1, and TIMP-1 inhibits other MMPs besides MMP-9. Finally, there is no apparent relationship between MMP-9 protein levels in BAL fluid and

MMP-9 (+) cells in the tissue ($r = -.01$) (data not shown). Description of ratios, if reported, should be precisely defined by cell source, compartment, activity/inhibition ratio, and additional presence of MMPs. Because of these complexities, although measured in all subjects, MMP-9/TIMP-1 data are not reported here.

GCs form the basis for our treatment of asthma. They influence both inflammatory and remodeling processes, although effects on extracellular matrix, MMPs, and TIMPs are less clear.^{24,25} It has been reported that moderate doses of inhaled GC (400 µg of beclomethasone twice a day) diminish MMP-9 (+) cells in the airways of patients with moderate asthma and decrease subbasement membrane thickening.²⁵ In this study, patients with moderate asthma receiving low-dose inhaled GCs had the lowest levels of BAL MMP-9 protein and activity. It is conceivable that the decrease in MMP-9 observed in the presence of low doses of GCs is predominantly through their profound inhibitory effect on macrophages.¹² However, in contrast to low-dose GCs, patients with severe asthma on high-dose GCs had the highest levels of MMP-9 protein and activity. This bimodal (dose) effect of GCs on MMP-9 in asthma might be due to their differing effects on macrophages and neutrophils.

High-dose GCs seem to have complex effects on neutrophil MMP-9. GCs produced a substantial decrease in peripheral blood neutrophil MMP-9 mRNA compared with untreated cells without difference between severe asthmatics and normal subjects. Because high-dose GCs prolong the survival of neutrophils, it is unlikely that the decrease in MMP-9 mRNA is due to diminished survival, but rather to diminished transcription or stabilization.^{4,26} Despite less mRNA, there was no effect on MMP-9 protein at 24 hours. Therefore, unlike macrophages, MMP-9 protein from neutrophils is not closely dependent on the amount of mRNA present. Similar to previous reports, peripheral blood neutrophils produced 100 to 1000 times greater MMP-9 protein than BAL cells, despite similar or even lower mRNA levels.²⁷ Therefore, even small neutrophil numbers (1% of total) could contribute disproportionately to BAL fluid/cell supernatant MMP-9 levels, especially in the presence of GC suppression of macrophage MMP-9.

Both MMP-9 mRNA and protein in BAL cells from severe asthmatics responded poorly to in vitro GCs. The effect on BAL cell protein is likely due to the higher percentage of neutrophils in the patients with severe asthma (Table I) and the limited effect of GCs on MMP-9 protein. However, the reasons for the differing effects on BAL cell mRNA are less clear, but they could be due to a slightly decreased response of neutrophil (compared with macrophage) mRNA to GCs, the marginally lower MMP-9 mRNA levels in severe asthmatics, or an example of "steroid resistance."²⁸

In conclusion, neutrophils contribute significantly to MMP-9 levels and activity in the luminal (BAL fluid) compartment of patients with severe asthma. This neutrophil-derived MMP-9 seems to be poorly suppressed by GCs in vitro and likely in vivo as well. Whether this

neutrophil-derived MMP-9 contributes to structural changes in asthma remains to be determined.

We thank Dr Niels Borregaard for the initial supply of NGAL antibody.

REFERENCES

- Wenzel SE, Szeffler SJ, Leung DYM, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma: Persistent inflammation associated with high dose glucocorticoids. *Am J Respir Crit Care Med* 1997;156:737-43.
- Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999;160:1532-9.
- Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care Med* 2000;161:9-16.
- Cox G. Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *J Immunol* 1995;154:4719-25.
- Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem* 1993;268:10425-32.
- Tschesche H, Zolzer V, Triebel S, Bartsch S. The human neutrophil lipocalin supports the allosteric activation of matrix metalloproteinases. *Eur J Biochem* 2001;268:1918-28.
- Vignola AM, Riccobono L, Mirabella A, Profita M, Chanez P, Bellia V, et al. Sputum metalloproteinase-9/tissue inhibitor of metalloproteinase-1 ratio correlates with airflow obstruction in asthma and chronic bronchitis. *Am J Respir Crit Care Med* 1998;158:1945-50.
- Mautino G, Oliver N, Chanez P, Bousquet J, Capony F. Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophages of asthmatics. *Am J Respir Cell Mol Biol* 1997;17:583-91.
- Wenzel SE, Balzar S, Cundall M, Chu HW. Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: association with asthma severity, neutrophilic inflammation, and wound repair. *J Allergy Clin Immunol* 2003;111:1345-52.
- Hoshino M, Nakamura Y, Sim JJ, Shimojo J, Isogai S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol* 1998;102:783-8.
- Bosse M, Chakir J, Rouabhi M, Boulet L-P, Audette M, Laviolette M. Serum matrix metalloproteinase-9: tissue inhibitor of metalloproteinase-1 ratio correlates with steroid responsiveness in moderate to severe asthma. *Am J Respir Crit Care Med* 1999;159:596-602.
- Shapiro SD, Campbell EJ, Kobayashi DK, Welgus HG. Dexamethasone selectively modulates basal and lipopolysaccharide-induced metalloproteinase and tissue inhibitor of metalloproteinase production by human alveolar macrophages. *J Immunol* 1991;146:2724-9.
- Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, et al. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med* 1999;160:1001-8.
- Chu HW, Trudeau JB, Balzar S, Wenzel S. Peripheral blood and airway tissue expression of transforming growth factor beta by neutrophils in asthmatic subjects and normal control subjects. *J Allergy Clin Immunol* 2000;106:1115-23.
- Zucker S, Mancuso P, DiMassimo B, Lysik RM, Conner C, Wu CL. Comparison of techniques for measurement of gelatinases/type IV collagenases: enzyme-linked immunoassays versus substrate degradation assays. *Clin Exp Metastasis* 1994;12:13-23.
- Kleiner DE, Stetler-Stevenson WG. Quantitative zymography: detection of picogram quantities of gelatinases. *Anal Biochem* 1994;218:325-9.
- Betsuyaku T, Nishimura M, Takeyabu K, Tanino M, Venge P, Xu S, et al. Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Respir Crit Care Med* 1999;159:1985-91.
- Kelly EAB, Busse WW, Jarjour NN. Increased matrix metalloproteinase-9 in the airway after allergen challenge. *Am J Respir Crit Care Med* 2000;162:1157-61.
- Cataldo DD, Bettioli J, Noel A, Bartsch P, Foidart JM, Louis R. Matrix

- metalloproteinase-9, but not tissue inhibitor of matrix metalloproteinase-1, increases in the sputum from allergic asthmatic patients after allergen challenge. *Chest* 2002;122:1553-9.
20. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* 1997;89:3503-21.
21. Keatings VM, Barnes PJ. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am J Respir Crit Care Med* 1997;155:449-53.
22. Cataldo D, Munaut C, Noel A, Frankenne F, Bartsch P, Foidart JM, et al. MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. *Int Arch Allergy Immunol* 2000;123:259-67.
23. Russell RE, Culpitt SV, DeMatos C, Donnelly L, Smith M, Wiggins J, et al. Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2002;26:602-9.
24. Jeffery PK, Godfrey RW, Adelroth E, Nelson F, Rogers A, Johansson S-A. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma: a quantitative light and electron microscopic study. *Am Rev Respir Dis* 1992;145:890-9.
25. Hoshino M, Takahashi M, Takai Y, Sim J. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immunol* 1999;104:356-63.
26. Meagher LC, Cousin JM, Seckl JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. *J Immunol* 1996;156:4422-8.
27. Fujisawa T, Kato Y, Terada A, Iguchi K, Kamiya H. Matrix metalloproteinase-9 in peripheral blood eosinophils. *Int Arch Allergy Immunol* 1999;120 Suppl 1:65-9.
28. Spahn JD, Szefer SJ, Surs W, Doherty DE, Nimmagadda SR, Leung DY. A novel action of IL-13: induction of diminished monocyte glucocorticoid receptor-binding affinity. *J Immunol* 1996;157:2654-9.