

Human eosinophils release matrix metalloproteinase-9 on stimulation with TNF- α

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Background: The eosinophil is a prominent cell in allergic lung inflammation and is exposed to a range of cytokines, including TNF- α , at the site of allergen challenge. Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) produced by inflammatory cells are thought to play a crucial role in interstitial matrix turnover and tissue remodeling in acute and chronic lung diseases. In addition, protein kinase C is known to be important in MMP-9 expression and secretion.

Objective: We investigated the regulation of eosinophil-derived MMP-9 and TIMP proteins by TNF- α .

Methods: Using RT-PCR and gelatin zymography, we investigated the ability of human eosinophils to produce and secrete active MMP-9 on stimulation with TNF- α . We also studied the production of TIMP-1 and TIMP-2 in eosinophils by using Western blotting.

Results: The gelatinolytic activity of MMP-9 in unstimulated eosinophils was low, but it increased by 95% after TNF- α stimulation. This increase was regulated at both the transcriptional and translational levels. The transcription inhibitor actinomycin D, the nuclear factor κ B (NF κ B) inhibitor N-CBZ-Leu-Leu-Leu-AL, the protein synthesis inhibitor cycloheximide, and the protein kinase C inhibitor H7 significantly decreased MMP-9 activity in TNF- α -treated cells. TIMP-1 and TIMP-2 gene expression and protein production varied significantly among different cell donors.

Conclusion: Eosinophils, on stimulation with TNF- α , may play a major role in asthmatic airway remodeling through increased MMP-9 production at the inflammatory site. (*J Allergy Clin Immunol* 1999;104:983-90.)

Key words: Eosinophil, asthma, matrix metalloproteinase, tissue inhibitor of matrix metalloproteinases, tumor necrosis factor α

A precise role for the eosinophil in inflammatory diseases has remained elusive.¹ Eosinophils are thought to exert a beneficial function in protecting the host from parasitic infection,² while at the same time they are considered

Abbreviations used

GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
MMP:	Matrix metalloproteinase
NF κ B:	Nuclear factor κ B
PKC:	Protein kinase C
PMA:	Phorbol-12-myristate-13-acetate
TIMP:	Tissue inhibitor of metalloproteinase

to play a detrimental role in a variety of acute and chronic airway disorders. In bronchial asthma strong evidence has been provided to indicate that eosinophils may be responsible for damage to the bronchial epithelium by releasing cytotoxic granule proteins³ and other proinflammatory mediators.^{1,4} The number of eosinophils is also increased in both peripheral blood and airways of atopic and nonatopic asthmatic subjects.⁵ Furthermore, the concentration of eosinophil products has been shown to correlate with disease severity.⁶ Nevertheless, evidence for an effector function of the eosinophil remains circumstantial. This fact is complicated by the presence of a mixed inflammatory reaction involving neutrophils,⁷ macrophages,⁸ lymphocytes,⁹ mast cells,¹⁰ and eosinophils.¹¹

At the histopathological level, one characteristic of asthmatic tissue is sub-basal membrane thickening and hypertrophy, as well as hyperplasia of smooth muscle cells and subepithelial glands.¹² These features are thought to correlate directly with the activity of matrix metalloproteinases (MMPs) in the airway interstitial tissue, including MMP-9.¹³ Abundant expression of MMP-9 messenger (m)RNA has been detected in airway biopsy specimens obtained from asthmatic subjects. Interestingly, the vast majority of cells expressing MMP-9 mRNA were eosinophils.¹⁴ Asthmatic tissue associated with the expression of MMP-9 was characterized by remarkable inflammation, including mucosal edema, cellular infiltration, and expression of adhesion molecules.¹² These findings suggest the overexpression of MMP-9 by eosinophils in the bronchial tissue of asthmatic individuals and the participation of MMP-9 in the maintenance of airway inflammation in asthma.

Among the cytokines involved in MMP regulation,¹³ there is evidence to suggest that TNF- α may be crucial, particularly with regard to MMP-9. In fact, TNF- α has been demonstrated to markedly upregulate MMP-9 production in human monocytes.⁸ These data have been confirmed in endothelial cells,¹⁵ fibroblasts,¹⁶ and leukemia cells.¹⁷ TNF- α effects in human inflammatory cells are

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regulated by means of specific receptors, TNF- α RI or TNF- α RII. Eosinophils express at least one of the 2 receptors.¹⁸

It is well known that not only cytokines, such as TNF- α , regulate MMP-9 activity but also the physiologic tissue inhibitors of matrix metalloproteinases (TIMP) 1 and 2.¹⁹ TIMP-1 seems to be closely associated with MMP-9 activity,¹³ although this has not yet been confirmed in eosinophils. Interestingly, in human monocytes TIMP-1, but not TIMP-2, expression was shown to be induced by TNF- α .⁸ Although the stimulatory effect of TNF- α on MMP-9 appears to be consistent, its effect on TIMPs needs further investigation.

In this study we hypothesized that in eosinophils TNF- α is a major activator of MMP-9 activity, and TNF- α influences the physiologic equilibrium between MMP-9 and TIMPs. To understand the equilibrium between MMP-9 and TIMPs on stimulation with TNF- α , we examined the expression of MMP-9 and TIMPs and the amounts of MMP-9 and TIMP proteins produced by peripheral blood eosinophils. Our data suggest that TNF- α markedly increases MMP-9 release from human eosinophils. Furthermore, the production of inhibitors of MMPs appears inconsistent among different atopic subjects. These results have important implications on the potential role of the eosinophil in airway remodeling in asthma.

METHODS

Isolation of peripheral blood eosinophils

Peripheral blood eosinophils were isolated from atopic asthmatic volunteers who had given their informed consent, as described previously.^{20,21} Briefly, heparinized blood (100 mL) was mixed with 20 mL of dextran (6%; Fluka, Buchs, Switzerland) in RPMI-1640 medium (Bio Whittaker, Walkersville, Md). Erythrocytes were then sedimented for 30 minutes at room temperature. The plasma was layered on a Ficoll (Pharmacia, Quebec, Canada) cushion (15 mL) and centrifuged for 25 minutes at 1000g at room temperature. Contaminating erythrocytes in the resultant pellet were lysed in 2 mL of sterile water for 5 seconds, and the cell suspension was then washed in RPMI-1640 medium. The pellet, containing approximately 5×10^7 cells, was resuspended in 600 μ L of RPMI-1640 medium containing 2% FCS/0.5 mmol/L EDTA (Sigma, St Louis, Mo) and incubated for 45 minutes with anti-CD16 (60 μ L), anti-CD14 (20 μ L), and anti-CD3 (20 μ L) immunomagnetic beads (MACS). These cells were passed through a magnetic column, and the eluant contained an eosinophil population of greater than 97% purity. Eosinophil counts were performed by using the Kimura staining technique.²²

Cell culture

Eosinophils were resuspended in RPMI-1640 medium (2×10^6 cells/mL) and incubated at 37°C in the presence or absence of 100 ng/mL TNF- α . In pilot experiments we optimized the conditions for MMP-9 release from eosinophils after stimulation by TNF- α . A generally accepted range for TNF- α concentration is between 1 and 100 ng/mL. We have therefore tested 5 different concentrations of TNF- α (0, 0.1, 1, 10, and 100 ng/mL). A dose of 100 ng/mL showed the highest increase in MMP-9 secretion, and this concentration was used throughout the current study. Similarly, time-course experiments indicated that 24 hours of incubation of eosinophils with or without TNF- α was optimal because, especially in unstimulated cells, at earlier time points (0, 1, and 8 hours) MMP-9 mRNA expression and protein release were low. To study the regulation of MMP-9 synthesis and

release, cells were incubated with either actinomycin D (10^{-6} mol/L), cycloheximide (10^{-6} mol/L), the protein kinase C (PKC) inhibitor H7 (500 μ mol/L), the nuclear factor κ B (NF κ B) inhibitor N-CBZ-Leu-Leu-Leu-AL (10 μ mol/L), or a neutralizing anti-human TNF- α antibody (0.04 μ g/mL) for 24 hours in the presence or absence of TNF- α . Except for the anti-TNF- α antibody, which was purchased from R&D Systems (Minneapolis, Minn), all reagents were obtained from Sigma.

Because transcription and translation inhibitors are known to be cytotoxic, cell viability was assessed after 24 hours of incubation by using trypan blue dye exclusion and an Annexin V-FITC Apoptosis Detection kit (PharMingen, Ontario, Canada) to detect dead cells. In all experiments cell viability was greater than 90% at all time points examined. These findings exclude the possibility that the observed increase in MMP-9 activity was caused by unspecific protein leakage caused by cell death.

RT-PCR

Total RNA was isolated from 2×10^6 eosinophils by using the Qiagen RNeasy kit (Ontario, Canada). The average amount of RNA obtained from 2×10^6 eosinophils was 300 ng. One third of the RNA was reverse transcribed by using superscript II reverse transcriptase (Gibco BRL, Ontario, Canada) and random hexamers (50A₂₆₀ U; Boehringer Mannheim GmbH, Mannheim, Germany) as primers. Thereafter, PCR was performed in 20- μ L reactions by using the following primers (25 μ mol/L) for MMP-9 (5'-CGTGGAGAGTCGAAATCTCTG-3', 5'-CCAAACTGGATGACGATGTCT-3'), TIMP-1 (5'-GGGGACACCAGAAGTCAACCAGA-3', 5'-CTTTTCAGAGCCTTGGAGGAGCT-3'), and TIMP-2 (5'-GATGCACATCACCTCTGTG-3', 5'-CTCGATGTCGAGAACTCCTG-3'). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-CCACCATGGCAAATTCATGGCA-3', 5'-TCAAGACGGCAGGTCA-GGTCCACC-3') was used as a housekeeping gene internal standard. One tenth of the complementary (c)DNA was used in PCR experiments. DNA amplification was obtained by annealing at 54°C for MMP-9 and TIMP-2, 50°C for TIMP-1, and 67°C for GAPDH for 30 seconds each, followed by an elongation step at 72°C for 1 minute. All sequences were amplified over 30 cycles, except for TIMP-1 sequences, which needed 40 amplification cycles to be detected. The amplified fragment size for GAPDH, MMP-9, TIMP-1, and TIMP-2 was 598 bp, 331 bp, 400 bp, and 292 bp, respectively.

Gelatin zymography

SDS-PAGE gelatin zymography (7% polyacrylamide gel containing 2% gelatin) was performed, as previously described.^{23,24} Serum-free culture supernatants were assayed to study MMP-9 release, whereas intracellular MMP-9 protein was studied in lysed cell pellets. Supernatants were concentrated (5 $\times$ concentration) in Centricon 10 concentrators (Amicon, Mass). Cell pellets were lysed by 3 $\times$ 1 minute freeze-thaw cycles on dry ice and a 37°C water bath. The resulting homogenate was resuspended in RPMI-1640 medium in a volume equal to that of the supernatants. Phorbol-12-myristate-13-acetate (PMA)-treated (100 nmol/L, 48 hours at 37°C) HT-1080 fibroblast supernatants were used as a positive control for gelatinolytic activity and to distinguish MMP-9 activity from that of MMP-2. After electrophoresis at 4°C, the gels were washed 3 times in 2.5% Triton X-100 at room temperature. The gels were then incubated in 50 mmol/L Tris-HCl buffer (pH 7.6) supplemented with 0.15 mol/L NaCl, 5 mmol/L CaCl₂, and 0.05% NaN₃ at 37°C for 24 hours. After incubation, the gels were stained for 1 hour with 0.05% Coomassie Brilliant Blue G-250 stain. Thereafter the gels were destained overnight in 20% isopropanol/10% acetic acid. All reagents were obtained from Sigma (Ontario, Canada), and the electrophoresis unit (Mini-Protein II Gel) was obtained from Bio-Rad (Hercules, Calif). Proteolytic activity was identified as clear bands on a blue background.

SDS-PAGE and Western blotting

Human eosinophils (2×10^6 cells/mL) were incubated with or without TNF- α (100 ng/mL) in RPMI-1640 medium for 24 hours at 37°C. After 5 minutes of centrifugation at 100g, supernatants were concentrated (10 $\times$ concentration), and the cells were lysed by repeated freeze-thaw cycles (3 $\times$ 1 minute) in 100 μ L of homogenization buffer containing 0.5 mmol/L EDTA (pH 7.4), 10 mg/mL poly-methylsulfonyl fluoride, and a mixture of 5 mg/mL tosyl-L-arginine methyl ester hydrochloride, aprotinin, and leupeptin. Untreated HT-1080 fibroblast supernatant was concentrated (20 $\times$ concentration) and used as a positive control for TIMP-2. PMA-treated (100 nmol/L, 48 hours at 37°C) HT-1080 fibroblast supernatant (10 $\times$ concentration) was used as a positive control for TIMP-1. Equal concentrations of eosinophil supernatant and cell homogenates (80–100 μ g of protein) were loaded onto a 12% SDS-polyacrylamide gel and transferred electrophoretically (25 V, 35 minutes) onto a polyvinylidene difluoride membrane (Bio-Rad) by using the Semi-Dry Trans Blot System according to the manufacturer's instructions. The membrane was then blocked overnight in 5% milk powder (Bio-Rad) in Tris-buffered saline containing 0.02% Tween at 4°C. To avoid nonspecific background staining caused by high concentrations of cationic proteins contained in the eosinophil preparations, the membrane was then blocked in 5% milk powder in Tris-buffered saline containing 5% goat serum (Gibco, Ontario, Canada) for 1 hour. Thereafter the membrane was probed with a mouse monoclonal anti-human TIMP-1 (1 μ g/mL) or anti-human TIMP-2 antibody (5 μ g/mL) in 5% milk powder containing 5% goat serum for 1 hour at room temperature. Both antibodies were obtained from Cedarlane Laboratories Ltd (Ontario, Canada). The membrane was then incubated with a peroxidase-conjugated polyclonal anti-mouse antibody (1:5000; Amersham, Ontario, Canada). Protein bands were detected by enhanced chemoluminescence (Amersham).

Statistical analysis and OD measurements

ODs of PCR gels and zymograms were measured by using the Sigma Gel Analysis program (Jandel Scientific, version 1.0.5.0, 1995). Band intensities of PCR products were expressed as a percentage of a GAPDH standard $\pm$ SEM. In pilot experiments GAPDH expression was shown to be stable on stimulation with IL-3, IL-5, and TNF- α . Band intensities of zymograms were expressed as the percentage of treated versus untreated cells $\pm$ SEM.

Densitometry is a quick and very convenient method to compare different band intensities on RT-PCR or gelatin-zymography gels. Advantages of this computerized technique are high sensitivity and rapidity in directly measuring large numbers of band intensities. Potential disadvantages include the possible subjective interpretations of band intensities during the measuring process, especially if performed by different investigators. To avoid these possible pitfalls in this study, only one investigator performed OD measurements. We were, however, aware of the fact that this method is neither helpful in detecting absolute copy numbers or protein concentrations nor able to detect subtle differences in gene expression or protein production.

For statistical analysis, the unpaired Student *t* test was used (InStat, Graph Pad Software Inc). A *P* value of less than .05 was considered significant.

RESULTS

MMP-9 and TIMP expression in human eosinophils

Transcriptional expression of MMP-9, as well as that of TIMP-1 and TIMP-2, was studied in human peripheral blood eosinophils by means of RT-PCR. MMP-9 and TIMP-2 gene expression could be detected in both rest-

ing and TNF- α -stimulated eosinophils (Fig 1, A), whereas TIMP-1 gene expression appeared to vary between different subjects (data not shown). Stimulation of eosinophils for 24 hours with TNF- α resulted in upregulation of MMP-9 gene expression (Fig 1, B; 29.5 ± 4.5 and 40.5 ± 3.5 , respectively), although the increase did not achieve statistical significance (*P* = .09, *n* = 4). In contrast, TIMP-2 gene expression (Fig 1, B) in eosinophils appeared to decrease after TNF- α treatment (46.8 ± 5 and 32.6 ± 5 , respectively), but the difference again did not achieve statistical significance (*P* = .08, *n* = 5). Throughout these preparations, GAPDH was equally and consistently expressed in unstimulated, as well as in TNF- α -stimulated, eosinophils.

TNF- α increases MMP-9 activity in human eosinophils

We examined the potential of TNF- α to increase MMP-9 release from human eosinophils by using gelatin zymography (Fig 2, A). Addition of TNF- α (100 ng/mL) to human peripheral blood eosinophils significantly increased MMP-9 activity after 24 hours of incubation by $95\% \pm 6\%$ (*P* < .01, *n* = 13) compared with untreated eosinophils (Fig 2, B). To ensure that this effect was specific to TNF- α and not caused by some nonspecific protein interactions, a neutralizing anti-TNF- α antibody was added to the cell cultures for 24 hours. In the presence of TNF- α and anti-TNF- α antibody, MMP-9 activity remained at baseline values (*n* = 3; Fig 2, A and B). Other cytokines, including IL-3 and IL-5, caused only a weak increase in MMP-9 activity (data not shown).

We also studied the effect of TNF- α on the ratio of released versus intracellularly stored MMP-9. Therefore, supernatants and cell lysates of both resting and stimulated cells were analyzed by using gelatin zymography (*n* = 4) after a 24-hour incubation period (Fig 3, A). A clear shift of MMP-9 activity from the lysed cell pellet into the supernatant could be observed on TNF- α stimulation (Fig 3, B). In resting eosinophils MMP-9 activity could be detected in both cell lysates (34%) and supernatants (66%). In TNF- α -treated eosinophils, however, most of the MMP-9 protein (92%) was released into the supernatant (*P* < .01), with only 8% retained in the cells (*P* < .01).

The increase in MMP-9 activity is regulated at different levels

To gain insights into MMP-9 activation, eosinophils were incubated with TNF- α in the presence or absence of transcription and translation inhibitors, as well as with a PKC inhibitor. In RT-PCR experiments, the combination of TNF- α with transcription and translation inhibitors, as well as the PKC inhibitor H7, decreased MMP-9 mRNA levels compared with TNF- α -stimulated cells (Fig 4, A). These results were also confirmed at the protein level (Fig 4, B). The transcription inhibitor actinomycin D decreased MMP-9 activity by $61\% \pm 7\%$ (*P* < .01, *n* = 7) compared with TNF- α -treated cells. To investigate whether NF κ B was involved in TNF- α -induced upregulation of MMP-9 transcription, an NF κ B inhibitor, N-

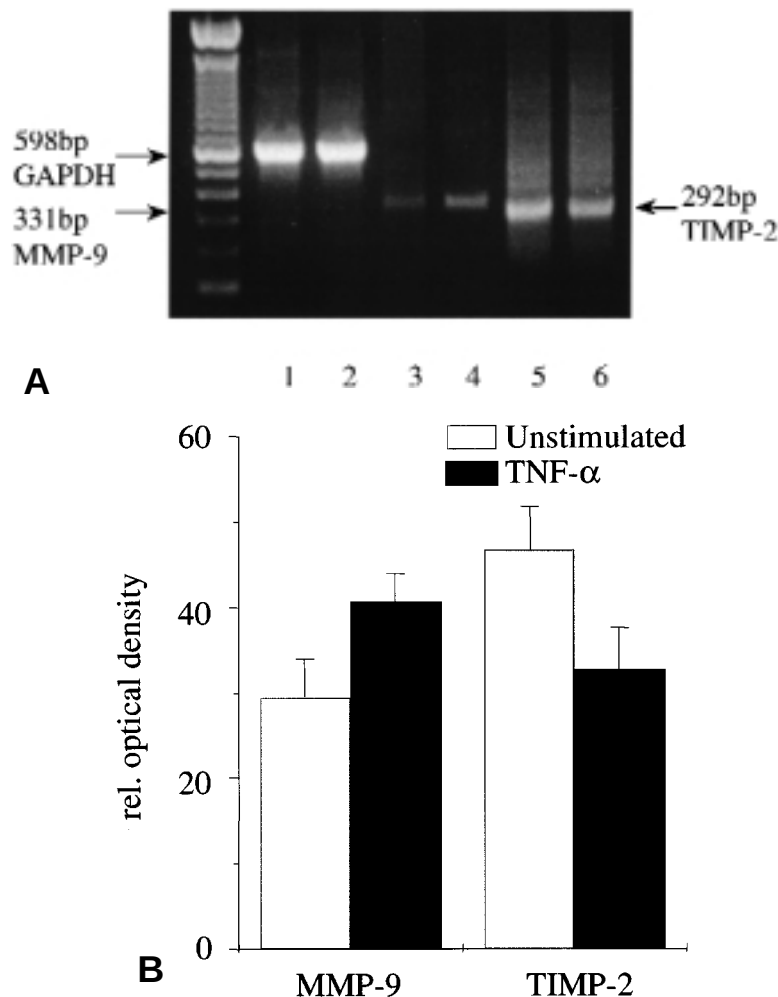


FIG 1. A, A representative RT-PCR experiment of resting and TNF- α -stimulated eosinophils. Cells were incubated for 24 hours at 37°C in the presence or absence of TNF- α (100 ng/mL). Sequences for GAPDH (lanes 1 and 2), MMP-9 (lanes 3 and 4), and TIMP-2 (lanes 5 and 6) were amplified over 30 cycles. MMP-9 expression in eosinophils was increased on TNF- α stimulation (lane 4) compared with unstimulated cells (lane 3). In contrast, TIMP-2 expression was weaker in TNF- α -treated (lane 6) than in untreated cells (lane 5). No difference could be observed in the expression of GAPDH between untreated (lane 1) and TNF- α -treated (lane 2) eosinophils. **B,** Densitometric analysis data of MMP-9 and TIMP-2 expression in unstimulated and TNF- α -stimulated eosinophils determined by using RT-PCR. Cells (2×10^6) were incubated for 24 hours in the presence or absence of TNF- α . MMP-9 and TIMP-2 band intensities are expressed as percentages (mean $\pm$ SEM) of a GAPDH internal standard. After TNF- α stimulation, MMP-9 expression increased by 11% compared with that found in unstimulated eosinophils. In contrast, TIMP-2 expression decreased by 14% on TNF- α stimulation.

CBZ-Leu-Leu-Leu-AL, was used. Inhibition of NF κ B resulted in a significant reduction of MMP-9 gelatinolytic activity by $61\% \pm 12\%$ ($P < .01$, $n = 5$) compared with TNF- α -stimulated eosinophils. These results indicate that on TNF- α stimulation MMP-9 protein was newly synthesized. In addition, we found that the TNF- α -stimulated MMP-9 activity profile was also regulated at the translational level. Eosinophils were incubated with TNF- α in the presence or absence of the protein synthesis inhibitor cycloheximide. The latter decreased TNF- α -induced MMP-9 activity by $60\% \pm 11\%$ ($P < .01$, $n = 7$) compared with TNF- α -treated cells. To determine whether PKC was involved in the TNF- α -induced effects, eosinophils were incubated in the presence of

this cytokine with or without the PKC inhibitor H7. Addition of H7 to TNF- α -stimulated eosinophils resulted in inhibition of MMP-9 activity by $57\% \pm 5\%$ ($P < .01$, $n = 8$) compared with TNF- α -stimulated cells. The presence of these inhibitors alone did not affect constitutive MMP-9 secretion as determined by using gelatin zymography ($n = 3$; Fig 4, B).

TIMP protein expression in human eosinophils

We investigated the ability of human eosinophils to produce TIMP-1 and TIMP-2 proteins by using Western blotting. Eosinophil supernatants and cell lysates were assayed to detect released and intracellular TIMP pro-

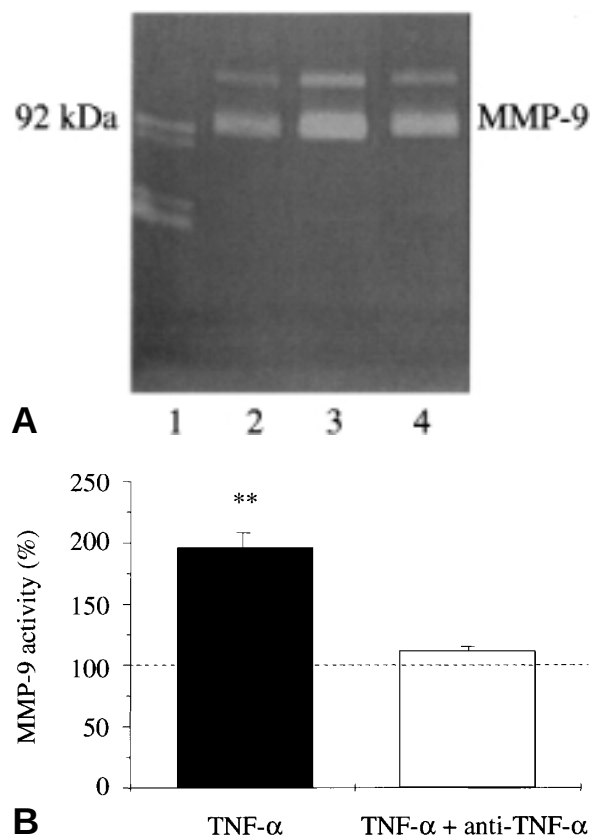


FIG 2. A, MMP-9 activity of unstimulated and TNF- α -stimulated (100 ng/mL) eosinophil supernatants determined by using gelatin zymography. After a 24-hour incubation period, supernatants obtained from 2×10^6 cells were $10\times$ concentrated and analyzed by gelatin zymography. PMA-stimulated HT-1080 fibroblast supernatant (lane 1) was used as a positive control for active MMP-9 (molecular weight, 92 kd). Unstimulated eosinophils constitutively showed MMP-9 gelatinolytic activity (lane 2). On TNF- α stimulation, MMP-9 gelatinolytic activity was significantly increased (lane 3). The TNF- α specificity of this effect was shown by using a neutralizing anti-TNF- α antibody, which clearly decreased MMP-9 activity (lane 4). **B**, Densitometric analysis of cell-free eosinophil supernatants determined by using gelatin zymography. Eosinophils were incubated for 24 hours with or without TNF- α or TNF- α and anti-TNF- α . Results are expressed as percentage of activity of TNF- α treatment to resting cells (mean $\pm$ SEM). MMP-9 gelatinolytic activity of resting cells is expressed as 100%. TNF- α significantly increased MMP-9 gelatin-degrading activity by 95% ($P < .01$) compared with that found in unstimulated cells. This effect could be reversed by the addition of neutralizing TNF- α antibody.

teins. We detected TIMP-1 protein in only 2 of 4 subjects studied (data not shown). The pattern of TIMP-1 release from cells of these 2 subjects also appeared to vary between different individuals. In one subject TIMP-1 localized primarily intracellularly, whereas in the other the majority of TIMP-1 protein was secreted. In both cases TNF- α stimulation increased the levels of TIMP-1 protein compared with that found in resting cells. In the other 2 subjects, no TIMP-1 protein could be detected in either supernatant or cell lysate.

We further investigated the ability of human eosinophils to produce TIMP-2. Eosinophils obtained from 4 subjects

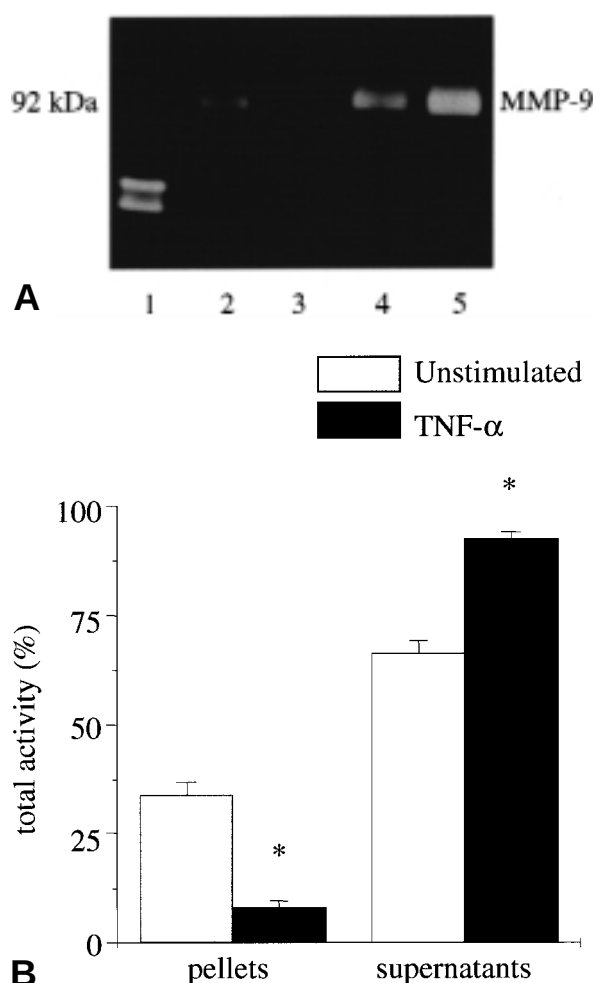


FIG 3. A, Intracellular and secreted MMP-9 protein of resting and TNF- α -stimulated (100 ng/mL) eosinophils after 24 hours of incubation in a representative gelatin zymography experiment. PMA-stimulated HT-1080 fibroblasts (lane 1) were used as a positive control for active MMP-9 (molecular weight, 92 kd). Resting eosinophils constitutively showed MMP-9 gelatinolytic activity in both lysed cell pellets (lane 2) and supernatants (lane 4). After TNF- α stimulation, the pellet was almost completely depleted of MMP-9 (lane 3), and the majority of active MMP-9 protein was released into the supernatant (lane 5). **B**, Densitometric analysis of intracellular and released MMP-9 in lysed eosinophil pellets and supernatants performed by using gelatin zymography. Results are expressed as percentage of total MMP-9 activity (mean $\pm$ SEM). After 24 hours of incubation, resting eosinophils showed constitutive MMP-9 activity in pellets (39%) and supernatants (61%). On TNF- α stimulation, MMP-9 activity in the pellets decreased (8%), whereas active MMP-9 levels were increased in the supernatants (91%).

showed TIMP-2 protein expression (Fig 5). However, like TIMP-1, the pattern of protein release was inconsistent, and the amounts of protein varied among subjects, with only 2 of the 4 subjects showing similar intracellular levels of TIMP-2. In both cases TIMP-2 protein was released at an increased rate after TNF- α stimulation (Fig 5). Eosinophils from the other 2 subjects showed TIMP-2 protein exclusively intracellularly.

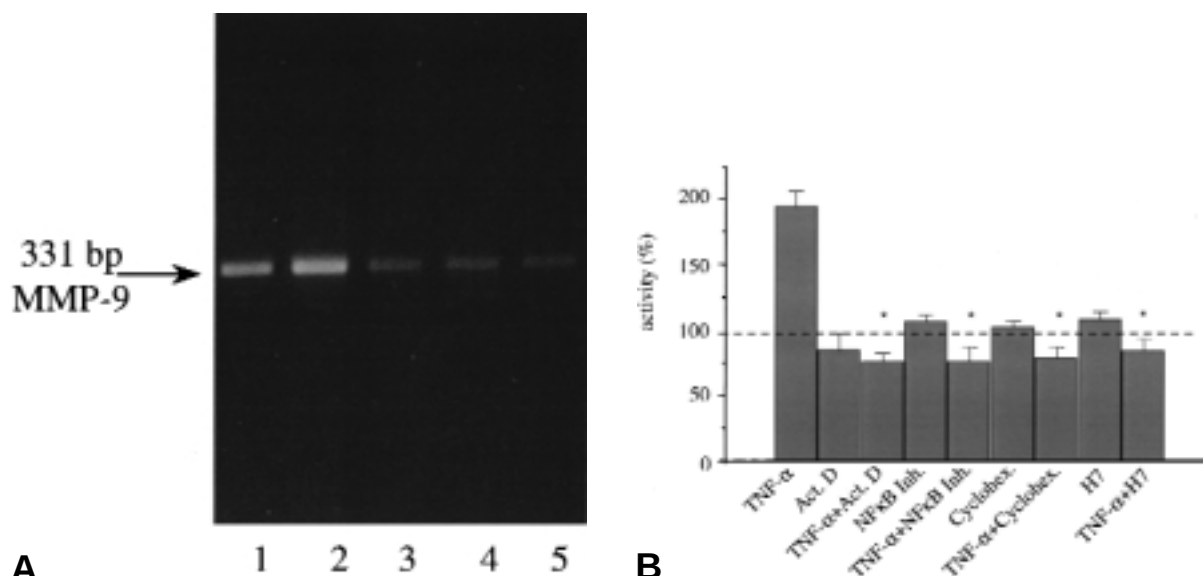


FIG 4. A, Regulation of TNF- α -induced increase in MMP-9 expression by using RT-PCR. Eosinophils were incubated for 24 hours in the presence or absence of TNF- α and with actinomycin D, the NF κ B inhibitor N-CBZ-Leu-Leu-Leu-AL, or the PKC inhibitor H7. Resting eosinophils showed constitutive MMP-9 (product size, 331 bp) expression (lane 1). On TNF- α stimulation, MMP-9 expression was markedly upregulated (lane 2). The addition of actinomycin D (lane 3), the NF κ B inhibitor (lane 4), and H7 (lane 5) to TNF- α -stimulated cells clearly decreased MMP-9 expression. **B,** Densitometric analyses of MMP-9 regulation in gelatin zymography. Eosinophils were incubated with or without TNF- α and actinomycin D (Act. D), the NF κ B inhibitor (Inh.) N-CBZ-Leu-Leu-Leu-AL, cycloheximide (Cyclohex.), or the PKC inhibitor H7. Results are expressed as percentage of activity of TNF- α -treated to untreated cells (mean $\pm$ SEM). MMP-9 gelatinolytic activity of untreated cells is expressed as 100%. As shown in Fig 2, B, TNF- α significantly upregulated MMP-9 activity by 95% $\pm$ 6%. The combination of TNF- α and actinomycin D or the NF κ B inhibitor decreased MMP-9 activity significantly by 61% $\pm$ 7% and 61% $\pm$ 12%, respectively, compared with TNF- α -stimulated cells. Similarly, cycloheximide decreased MMP-9 activity by 60% $\pm$ 11% and H7 by 57% $\pm$ 5% compared with TNF- α -treated cells. The inhibitors alone did not affect constitutive MMP-9 release. In all experiments the *P* value was less than .01.

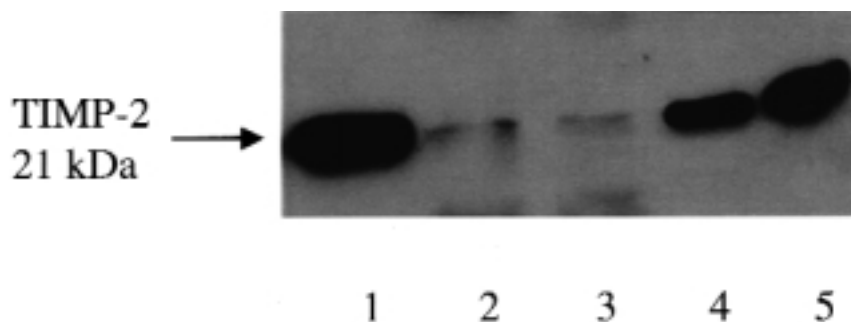


FIG 5. Western blot experiment for TIMP-2. As a positive control for TIMP-2, unstimulated HT-1080 fibroblast supernatant was used (lane 1). Lanes 2 and 3 show similar expression of TIMP-2 protein in cell pellets of resting (lane 2) and TNF- α -stimulated (100 ng/mL) eosinophils (lane 3) from this patient. In the supernatant increased amounts of released TIMP-2 protein were found after TNF- α stimulation (lane 5) compared with that found in the supernatant of resting cells (lane 4).

DISCUSSION

The recent description of the capacity of eosinophils to release MMP-9 has opened new vistas toward our understanding of the process of airway remodeling in asthma.²⁵ Our novel data revealed that human eosinophil-derived MMP-9 expression and secretion is upregulated on TNF- α stimulation. Gelatin zymography provided an excellent

method to show MMP-9 activity in these cells. NF κ B was previously shown to be involved in MMP-9 activation in fibroblasts,²⁶ transformed cells,^{27,28} and renal cells.²⁹ Our findings also confirm the validity of the hypothesis that TNF- α -induced binding of NF κ B to the MMP-9 promoter is a requirement for transcription activation.²⁶ However, other transcriptional factors, such as activator protein 1 are also likely to be involved.²⁸ Our data shed new important

light on the possible involvement of PKC in MMP-9 transcription and translation. However, this aspect requires further investigation. Studies in uterine fibroblasts have suggested that MMP-9 production is PKC independent.¹⁶ In addition, PMA, a PKC activator, increased MMP-9 secretion in alveolar macrophages of healthy control subjects but not asthmatic subjects.³⁰ However, PKC appears to be one of the intracellular signaling mediators of TNF- α effects. In bronchial epithelial cells TNF- α has been shown to increase PKC activity by 3- to 5-fold.³¹ Our data underscore the importance of PKC in MMP-9 expression and secretion and confirm this in eosinophils.

TNF- α is only one of many cytokines released at the inflammatory site. In preliminary experiments we investigated the effects of 2 other important cytokines in eosinophilic inflammation, namely IL-3 and IL-5.^{32,33} Both cytokines were able to elicit only a weak increase in MMP-9 activity (data not shown). Similar results have been reported by Fujisawa et al,³⁴ who could not induce a significant increase in MMP-9 release from eosinophils on IL-5 stimulation. These data emphasize the potential of TNF- α as a major regulatory molecule for MMP-9 regulation in eosinophils. We cannot, however, exclude the possibility that in vivo interactions between different combinations and concentrations of cytokines may have additional synergistic and/or antagonistic effects on MMP-9 regulation.

In addition to different types and concentrations of cytokines present at the inflammatory site, the equilibrium between MMPs and their specific tissue inhibitors (TIMPs) may also determine the degree of tissue degradation. It was therefore tempting to speculate that the production and secretion of both MMP-9 and TIMPs might be altered in an inflammatory environment. Other investigators who described TIMP-1 as an inducible protein in contrast to TIMP-2⁸ have reported similar observations. The inconsistent pattern of release, however, puts into question the physiologic relevance of TIMP production by eosinophils. More importantly, other inflammatory and tissue structural cells in the airways, including monocytes and macrophages,⁸ epithelial cells,³⁵ and myofibroblasts,³⁶ are known to produce considerable amounts of TIMP proteins. It is therefore likely that, in addition to limited autocrine TIMP production, eosinophil MMP-9 activity is primarily regulated by TIMP proteins secreted from other cells at the inflammatory site.

Increased production of MMP-9 at the inflammatory site has been shown to be associated with airway tissue remodeling.³⁷ We suggest that in a TNF- α -rich inflammatory milieu, MMP-9 activity might be increased. This does not exclude the possibility that other MMPs, such as the gelatinase MMP-2, together with collagenases and stromelysins may participate in increased interstitial matrix turnover in asthmatic airways.³⁸ The magnitude of contribution of these other MMPs, in addition to MMP-9, to overall tissue destruction in hyperreactive airways remains to be elucidated.

Our study relied on peripheral blood eosinophils

obtained from atopic asthmatic subjects. It would be ideal to compare these observations with cells obtained from bronchoalveolar lavage fluid,³⁹ segmental bronchoprovocation,⁴⁰ mucosal biopsy specimens,⁴¹ or induced sputum samples⁴² from asthmatic patients. There remains a paucity of data on possible differences between blood and tissue eosinophils. However, it is now generally accepted that peripheral blood eosinophils are a useful model of their tissue counterparts. Eosinophil numbers in the peripheral blood of asthmatic subjects appear to correlate significantly with tissue cell counts⁴³ and the degree of airflow obstruction.⁴⁴

In conclusion, our data propose a major role for TNF- α in MMP-9 regulation in asthmatic airways and an important function for the eosinophil in interstitial matrix turnover and airway tissue remodeling. These findings may have significant implications for future antieosinophil therapies in asthma and related conditions.

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