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Matrix metalloproteinases and TIMP-1 production by peripheral blood granulocytes from COPD patients and asthmatics

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Abstract

Both asthmatic and COPD patients were found to have increased amounts of granulocytes and matrix metalloproteinase-9 (MMP-9) in their sputum. The present study was conducted to investigate whether the elevated amounts of MMP-9 and TIMP-1 found in such patients' airways may be linked to an enhanced secretion by granulocytes. Blood granulocytes from asthmatics (n = 10), COPD patients (n = 11), and healthy controls (n = 11) were isolated and cultured under basal conditions or after stimulation by phorbol 12-myristate 13-acetate (PMA) or *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). MMP-9 activity was detected by zymography while MMP-8 and TIMP-1 levels were measured by ELISA. In zymography, pro- and activated forms of MMP-9 were present in each group (healthy subjects, asthmatics, and COPD patients). Spontaneous release was not different between the three groups. Stimulation by fMLP and PMA increased to a similar extent the release of MMP-9 by granulocytes in all the three groups. TIMP-1 levels were also increased after stimulation by PMA and fMLP only in healthy subjects and COPD patients. MMP-8 levels were barely detectable. We conclude that circulating granulocytes from COPD patients and asthmatics do not display an abnormal secretion of MMP-9, and that granulocytes from asthmatics have an impaired ability to release TIMP-1 upon stimulation.

Keywords: asthma; COPD; matrix metalloproteinases; MMP-8; MMP-9; TIMP-1.

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium dependent enzymes widely distributed and involved in many physiologic and pathologic processes (1). Most of MMPs are secreted from the cells as inactive zymogens requiring the proteolytic cleavage of an amino-terminal ~10-kDa domain for activation. Among the MMPs, MMP-9 (gelatinase B) is thought to play a key role in tissue remodeling and repair through the degradation of type IV collagen, the major component of basal membranes. This enzyme is expressed, produced and secreted by a large array of human cells including eosinophils and neutrophils (2, 3) In neutrophils, MMP-9 is thought to be synthesized during the maturation of the precursor in the bone marrow and stored in specific granules until needed (4). MMPs are selectively inhibited by tissue inhibitors of metalloproteinases (TIMPs). Four different TIMPs have been identified so far, and TIMP-1 has been shown to bind both the active and pro-form of MMP-9 and to be synthesized and released by human neutrophils (5, 6).

Asthma and chronic obstructive pulmonary diseases (COPD) are chronic inflammatory diseases of the airways associated with higher rates of decrease in FEV_1 during the life than in a healthy population (7, 8). The progressive function loss is accompanied by several pathologic changes of the airways, including extracellular matrix fibrosis of the airway wall (9, 10). It has been recently found that MMPs (mainly MMP-9) and TIMP-1 were increased in the sputum from asthmatics and COPD patients (11, 12). In these studies, the levels of MMP-9 were correlated with the number of granulocytes in the airways, suggesting that the latter might be implicated in the local production of MMPs and TIMPs.

The purpose of the present study was to evaluate the secretion of MMPs and TIMP-1 by peripheral blood granulocytes collected from asthmatics and COPD patients under both basal and stimulated conditions.

Material and methods

Granulocyte isolation and culture

Granulocytes were isolated from peripheral blood of asthmatics, COPD patients, and healthy controls without history of lung disease. Clinical characteristics are shown in Table 1. The patients were classified as having asthma or COPD according to the American Thoracic Society (ATS) guidelines (13). Granulocytes were isolated

from heparinized peripheral blood obtained by venous puncture in the cephalic vein. After sedimentation on hydroxyethyl starch 10% (Haes Steril, Fresenius, Schelle, Belgium), leukocytes were separated with a Percoll density gradient (Sigma Chemicals Co, St Louis, MO, USA). The granulocytes collected after centrifugation (10 min at 1500 *g*) were cultured in triplicate in 96-wheel plates (Nunc, Roskilde, Denmark) in RPMI 1640 (Bio-Whittaker Europe, Verviers, Belgium) at 37°C with 5% CO_2 for 2 and 24 h. PMA 5 µg/ml (phorbol 12-myristate 13-acetate, Sigma chemical Co, St Louis, MO, USA), fMLP 2.5 10^{-5} M (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine, Sigma Chemical Co, St Louis, MO, USA), and cycloheximide 50 µg/ml (Sigma Chemical Co, St Louis, MO, USA) were added to the culture medium as indicated.

The differential cell count of each granulocyte fraction was evaluated by counting 500 cells on a cytospin stained with Diff Quick (Dade Behring, Marburg, Germany). In all samples, mean counts were 89% neutrophils and 11% eosinophils in asthmatics; 92% neutrophils, 7% eosinophils, and 1% other cells in COPD patients and 95% neutrophils, 4% eosinophils, and 1% other cell types in controls.

Values were expressed as mean ± SEM unless otherwise stated. Comparisons between two groups were performed by an impaired Student's *t*-test, and the differences between different experimental conditions in the same group were sought by paired Student's *t*-test. *P* values of <5% were statistically significant.

***Table 1**: Patient characteristics*

	Controls	**Asthma**	**COPD**
N	11	10	11
Mean age (years)	32 (24-44)	37 (18-65)	61 (50-68)
Sex ratio (M/F)	5/6	6/4	11/0
Tobacco	11 NS	4 PS, 6 NS	11 CS (mean pack year = 40)
FEV_1 (%pred)	ND	70 (32-104)	56 (21-95)
FEV_1 (ml)	ND	2433 (741-3968)	1724 (582-3228)
FEV_1/VC	ND	75 (45-99)	56 (22-95)
Therapy	11 NT	6 NT, 4 IS	7 NT, 4 IS

Values are expressed as mean (range).
IS: inhaled steroids; NT: not treated (salbutamol as needed); PS: past smoker; CS: current smoker; NS: never smoked; ND: not done.
Statistical analysis

Assessment of gelatinolytic activity

Gelatin zymography was performed, as previously described (14). Gelatinase activity was detected as white lysis zones against a blue background. Quantitative evaluation of the gelatinolytic activity was performed by scanning the gel with a Bio-Rad GS 700 imaging densitometer (Bio-Rad, Hercules, CA, USA). On each gel, dilutions of culture medium conditioned by HT 1080 cells were used as an internal standard. Gelatinolytic activity of MMP-9 was determined by scanning the lysis bands in the 92- and 85-kDa areas. Results were expressed as arbitrary units/ml of culture supernatant.

Measurement of TIMP-1 and MMP-8

TIMP-1 and MMP-8 were measured by commercially available ELISA (Quantikine human TIMP-1 immunoassay, R&D Systems, Minneapolis, MN, USA; Biotrak MMP-8 human Elisa system, Amersham Pharmacia Biotech, Buckinghamshire, UK). The lower limits of detection were 0.08 ng/ml and 0.032 ng/ml for TIMP-1 and MMP-8, respectively. Measurements of TIMP-1 were performed after sixfold concentration of the sample by ethanol precipitation.

Quantitative RT-PCR for MMP-8, MMP-9, and TIMP-1 transcripts

Total RNA from granulocytes was purified by cesium chloride ultracentrifugation (15). 28S rRNA, MMP-9, MMP-8, and TIMP-1 mRNA were measured in 10-ng aliquots of total RNA by RT-PCR. An external control RNA template (synthetic RNA) was introduced in each sample to monitor the RT-PCR reaction and to allow the quantitation of each endogenous mRNA (100 copies for MMP-8, 100 copies for MMP-9, 10^5 copies for TIMP-1, and 5×10^5 copies for 28S) (16). RT-PCR was performed with the GeneAmp Thermostable rTth reverse

transcriptase RNA PCR kit (Perkin Elmer, Branchburg, NJ, USA) and two pairs of primers (Gibco BRL-Life Technologies) (Table 2). Reverse transcription was performed at 70°C for 15 min followed by 2-min incubation at 95°C for denaturation of RNA-DNA heteroduplexes. Amplification started at 94°C for 15 s, 20 s at 68°C, and 10 s. at 72°C (36 cycles for MMP-8, 35 cycles for MMP-9, 36 cycles for TIMP-1, and 19 cycles for 28S) and terminated by 2 min at 72°C. RT-PCR products were resolved on 10% acrylamide gels and analyzed with a Fluor-S Multilmager (BioRad, Hercules, CA, USA) after staining with Gelstar (FMC BioProducts, Rockland, ME, USA) dye. The expected sizes of the synthetic control RNA and the endogenous mRNA are 267 and 200 bp for MMP-8, 266 and 208 bp for MMP-9, 265 and 176 bp for TIMP-1, and 269and 212 bp for 28S rRNA, respectively.

***Table 2**: Primers used for quantitative RT PCR*

	Oligonucleotide sense	Oligonucleotide antisense
MMP-8	5'-CCAAGTGGGAACGCACTAACTTGA-3'	5'-TGGAGAATTGTCACCGTGATCTCTT-3'
MMP-9	5'-GCGGAGATTGGGAACCAGCTGTA-3'	5'-GACGCGCCTGTGTACACCCACA-3'
TIMP-1	5'-CATCCTGTTGTTGCTGTGGCTGAT-3'	5'-GTCATCTTGATCTCATAACGCTGG-3 '
28S	5'-GTTCACCCACTAATAGGGAACGTGA-3'	5'-GATTCTGACTTAGAGGCGTTCAGT-3'

Results

Zymography

MMP-9 was spontaneously produced in all samples, both in the pro-form (92 kDa) and in the activated form (85 kDa). Other bands of proteolytic activity were detected in the 130-kDa area. In addition, a 200-kDa gelatinolytic form corresponding to a dimer of pro-MMP-9 was found in each sample (representative example of zymography in Fig. 1).

The bands corresponding to pro-MMP-9 and activated MMP-9 were further quantified by densitometric scanning for their gelatinolytic activity (Fig. 2). Neither the spontaneous nor the stimulated production of MMP-9 was different between the groups in comparison of the granulocytes of healthy subjects, asthmatics, and COPD patients. We then compared the efficacy of PMA and fMLP to amplify spontaneous production. After 2 h of granulocyte culture, we observed that the gelatinolytic activity linked to MMP-9 was increased after incubation with PMA ($P<0.05$ in the three groups) and to a lesser extent after incubation with fMLP ($P<0.05$ in controls only). In all groups, protein synthesis inhibition by cycloheximide (CHX) failed to modulate significantly the gelatinolytic activity after 2 h. This was further confirmed by additional experiments of 24-h incubation of granulocytes from each patient with CHX (data not shown). MMP-9-derived gelatinolytic activity was similar whether a smoking history or an inhaled steroid therapy was reported or not.

TIMP-1

There were no significant differences between the groups in the spontaneous release of TIMP-1. TIMP-1 levels were increased after stimulation with PMA ($P<0.05$ in healthy subjects and COPD patients) and fMLP ($P<0.05$ in COPD patients only) in cultures of granulocytes derived from healthy subjects or COPD patients. However, no stimulation of TIMP-1 release by fMLP and PMA was observed with the granulocytes of asthmatics (Fig. 2).

MMP-8

MMP-8 (neutrophil collagenase) was barely detectable in the supernatant of cultured granulocytes in four controls, three asthmatics, and one COPD patient without any differences between the three groups. It was undetectable in the supernatants of all other subjects.

Expression of MMP-8, MMP-9, and TIMP-1 mRNA

Basal and stimulated expression of MMP-9, MMP-8, and TIMP-1 was investigated by RT-PCR in blood granulocytes from healthy subjects ($n = 4$). In order to standardize the method, results were expressed as number of RNA copies/10^5 copies of 28S RNA.

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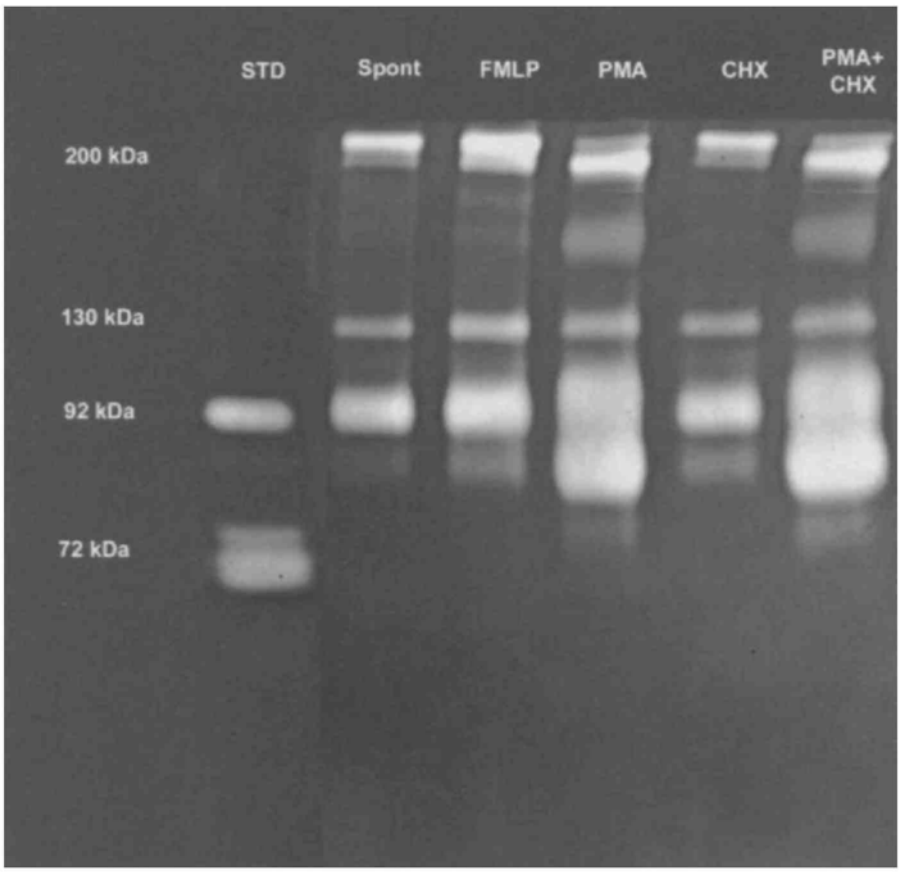
MMP-8, MMP-9, and TIMP-1 mRNA were expressed under basal conditions. The expression of MMP-9 mRNA and TIMP-1 mRNA was not modulated after stimulation by PMA or fMLP. By contrast, stimulation by PMA produced a significant enhancement of MMP-8 mRNA expression ($P<0.05$) (Fig. 3). Stimulation by PMA and fMLP failed to modulate the MMP-9 mRNA/TIMP-1 mRNA ratio.

***Figure 1**: Representative example of gelatin zymography of granulocyte culture supernatant of asthmatic. First lane corresponds to medium conditioned by HT 1080 cells. Granulocytes were untreated (spont) or treated for 2 h with PMA, fMLP, cycloheximide (CHX), or PMA and cycloheximide (PMA+CHX). Pro-form (92 kDa) and activated form (85 kDa) and additional gelatinolytic activities in 200- and 130-kDa area are visible in zymography.*



Discussion

We have demonstrated that circulating granulocytes from healthy subjects, asthmatics, and COPD patients produced spontaneously MMP-9 in both the pro-form and activated form. An additional release of MMP-9 was detected after stimulation by fMLP and PMA. The secretion of MMP-9 was not inhibited by the protein synthesis inhibitor cycloheximide, indicating that this protein was mostly secreted from the preformed gelatinase present in the gelatinase granules. In line with a very recent report investigating serum levels of MMP-9 in asthmatics and controls (17), no significant differences were detectable between the groups regarding the basal or stimulated MMP-9 production. TIMP-1 was secreted spontaneously by granulocytes in the three groups without differences. PMA enhanced TIMP-1 release in controls and COPD patients but failed to do so in asthmatics. The MMP-8 protein was barely detectable in our experimental conditions. In quantitative RT-PCR, we also showed that the expression of MMP-9 and TIMP-1 mRNA was present under basal conditions but was not enhanced by PMA and fMLP, indicating that the release of these two proteins originated from preformed stocks contained in the granules. By contrast, we demonstrated that the expression of MMP-8 was increased after stimulation by phorbol esters.

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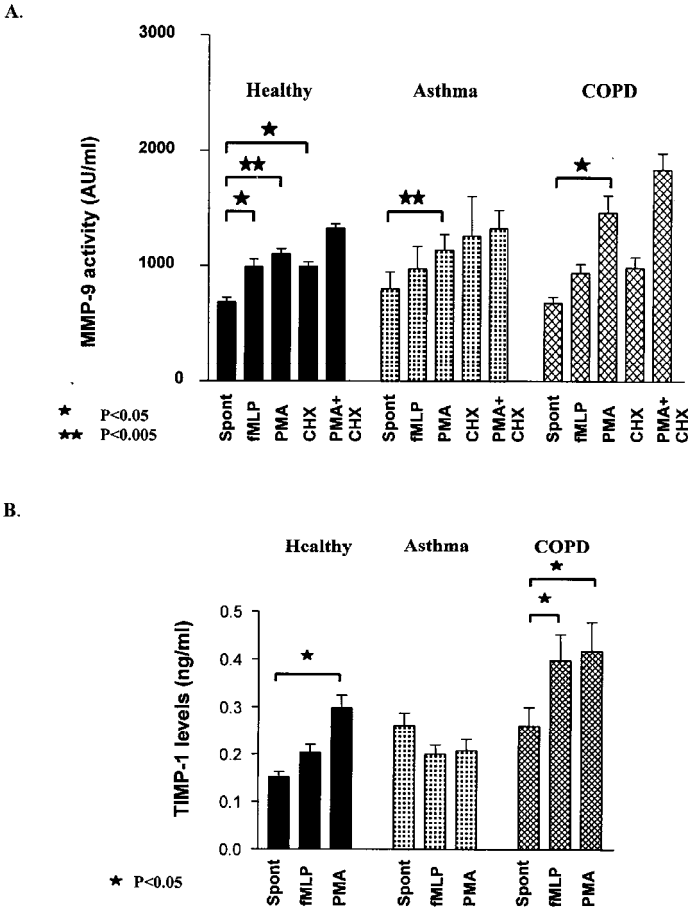
Although MMP9 levels were found to be increased in the airways of asthmatics and COPD patients in association with a local influx of granulocytes (11, 12), we found here their blood granulocytes to be of a similar potency in secreting this protease either spontaneously or after stimulation. This suggests that the raised MMP-9 activity found in the airways of patients with asthma and COPD reflects only the local increased number of granulocytes rather than a basic overproduction of this protease at the cell level. However, we acknowledge that other cell types, such as macrophages and epithelial cells, express the MMP-9 in the airways, and these cells might significantly contribute to the increased MMP-9 levels found in the airway secretions (18, 19). Moreover, the behavior of granulocytes in terms of production of MMPs might be somewhat different in the lung microenvironment as compared to the peripheral granulocytes. To the best of our knowledge, our study is the first to investigate the secretion of MMP-9 from isolated peripheral granulocytes in asthmatics and COPD patients. In a previous study, granulocytes from COPD were shown to respond with an enhanced migration and proteolytic activity against fibronectin when stimulated with fMLP (20, 21). As MMP-9 might play a critical role in cell migration by degrading extracellular matrix, it was worth comparing the production of this protease by granulocytes from COPD patients and control subjects. Our results do not support the hypothesis that excessive release of MMP-9 might contribute to the increased proteolytic activity described in COPD.

Figure 2*: MMP-9 activity detected by zymography and TIMP-1 levels. A) Gelatinolytic activity corresponding to pro-MMP-9 and activated MMP-9 was measured by densitometric scanning in supernatant of cultured granulocytes from healthy subjects, asthmatics, and COPD patients. B) TIMP-1 levels measured by ELISA in supernatants of cultured granulocytes.*



The failure of cycloheximide to modulate the release of MMP-9, combined with the inability of PMA and fMLP to upregulate the MMP-9 mRNA levels, indicates that MMP-9 was released from preformed stocks located in a specific granules subtype called "gelatinases granules" (2, 22). Interestingly, we found not only the pro-form (92kDa) but also the activated form (88 kDa) of MMP-9 in the supernatant of cultured granulocytes. As neutrophils have been described as potent activators of MMP-9 (23), several hypotheses can explain our findings (24). First, neutrophils are potent secretors of serine proteases (mainly elastase) that might, *in vitro*, activate the

MMP-9 by proteolytic cleavage of the aminoterminal prodomain. These enzymes are located in azurophil granules of mature neutrophils, which are partially released after PMA stimulation (2). *In vitro,* it has been shown that serine protease inhibitors strongly inhibit the activation of neutrophil-derived MMP-9, suggesting an important contribution of the neutrophil elastase in this process (25). Secondly, neutrophils are also known as producers of activated oxygen products that might lead to activation of MMPs *in vitro.* In neutrophils, inhibition of the H_2O_2/myeloperoxydase/ chloride system blunts the ability of these cells to activate pro-MMP-9 (23). Delclaux et al. have recently compared the relative contribution of elastase and activated oxygen products in activating the MMP-9, and found elastase to be the main contributor (26).

Two additional gelatinolytic species were detected in the 130- and 200-kDa area, corresponding probably to a complex between MMP-9 and lipocalin (27), and a dimer of MMP-9, respectively.

We also assessed the production of TIMP-1, the physiologic inhibitor of MMP-9. This protein was found to be produced by human neutrophils (5, 6). Our results show that TIMP-1 is spontaneously secreted by human granulocytes in both healthy subjects and patients with asthma and COPD without significant differences between the groups. In addition, PMA and fMLP clearly amplify the secretion in healthy subjects and COPD patients. It is noteworthy that this upregulation by PMA and fMLP was not observed in asthmatics. This lack of stimulation regarding TIMP-1 secretion combined with an enhanced stimulated MMP-9 release could lead to an imbalance favoring excessive proteolytic activity in asthma. This observation is in agreement with a recent report of Hoshino et al. (28) showing that, when compared with healthy subjects, asthmatics had an expression of MMP-9 not sufficiently counterbalanced by a corresponding expression of TIMP-1 in their bronchial biopsies. The enhancement of TIMP-1 secretion after fMLP and PMA, combined with the lack of TIMP-1 mRNA overexpression under the same conditions, suggests that these stimuli release the protein directly from an intracellular storage place, as has been shown for MMP-9. This is further supported by the fact that 10% of the secreted MMP-9 from human neutrophils is complexed with TIMP-1 (5).

Although amounts of secreted MMP-8 were almost undetectable by ELISA in our experimental conditions, we report here that MMP-8 mRNA was expressed by the circulating granulocytes. Furthermore, this expression was significantly upregulated by PMA, as recently described in other cell types, such as endothelial cells and human rheumatoid synovial fibroblasts (29).

We conclude that, in circulating granulocytes from patients with asthma and COPD, there is no upregulation of the production or extracellular secretion of MMP-9. These data strongly suggest that the increased MMP-9 activity observed in the airways of asthmatics and COPD patients is related to an increased number of granulocytes in the airways rather than to an enhancement of MMP-9 secretion by the granulocytes. TIMP-1 was secreted extracellu-larly in lesser amounts by the granulocytes of asthmatics after stimulation, an effect which might cause an imbalance between proteolytic and anti-proteolytic activities in the airways of these patients.

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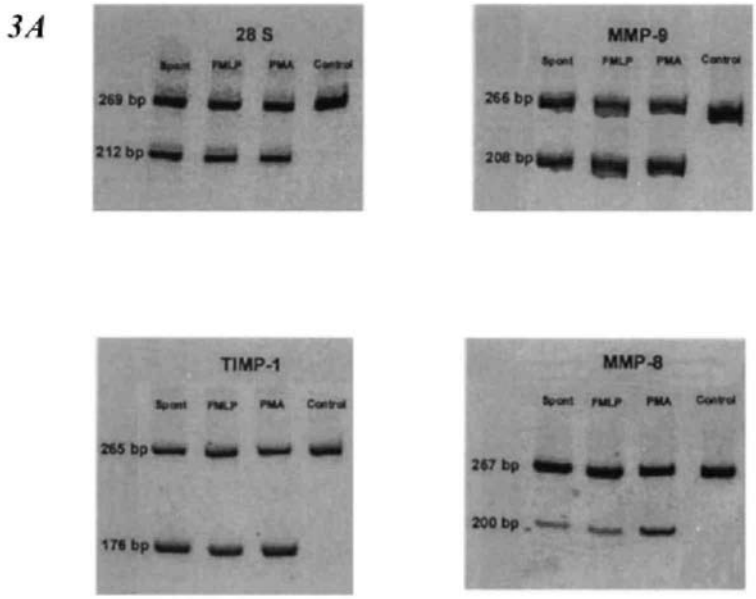
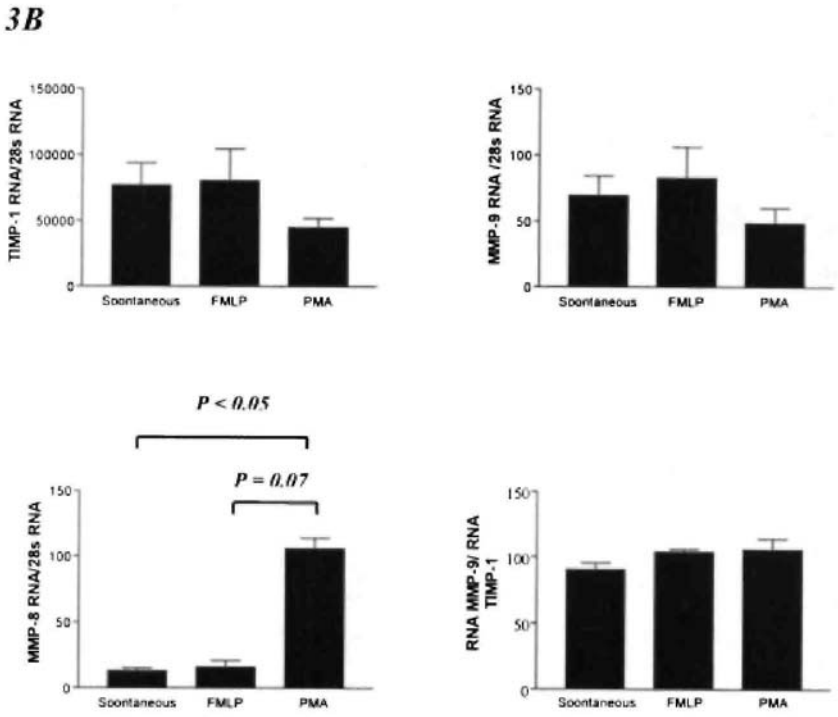
Figure 3: *mRNA expression of MMPs and TIMP-1 by blood granulocytes of healthy subjects. A) Representative example of acrylamide migration of RT-PCR products. On each gel, upper band corresponds to detection of internal standard and lower one corresponds to endogenous mRNA. Granulocytes were either untreated (spont) or treated with fMLP or PMA. In each experiment, internal standard was amplified in absence of sample (control). Intensity of bands detected for 28S, MMP-9, and TIMP-1 RNA was not modulated by either fMLP or PMA, while intensity of bands corresponding to MMP-8 RNA was clearly increased after incubation of cells with PMA. B) RT-PCR products are expressed as ratio of intensity of band detected in acrylamide gels to band of synthetic standard RNA, and normalized by same ratio calculated for 28S RNA (n= 4). Results are expressed as a number of RNA copies.*





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