Matrix metalloproteinase-8 in sera and from polymorphonuclear leucocytes in rheumatoid arthritis: *In vitro* characterization and correlation with disease activity

L. Rajasekhar, L.-B. Liou, C.Y. Chan, W.-P. Tsai, C.Y. Cheng

Division of Rheumatology, Allergy and Immunology, Department of Internal Medicine, Chang Gung Memorial Hospital, Tao-yuan County, Taiwan

Abstract

Objectives

To determine matrix metalloproteinase- 8 (MMP-8) secretion from rheumatoid arthritis (RA) peripheral blood polymorphonuclear leucocytes (PMNs), in response to immune complexes (IC), cytokines and their combinations, and to study correlation of serum MMP-8 with disease activity.

Methods

PMNs from RA patients and controls were stimulated in vitro *with interleukin -15 (IL-15), IL-18, adherent immune complexes, rabbit anti-human immunoglobulin G (anti-HIgG), human immunoglobulin G (HIgG), and their F (ab') 2 prongs, phorbol myristate acetate (PMA) or combinations of above. Supernatants from these experiments and sera from both groups were assayed for MMP-8 using ELISA and correlated with disease activity measures in patients.*

Results

MMP-8 secretion from stimulated PMNs was compared to unstimulated PMNs. Immune complexes elicited significant MMP-8 secretion (p=0.006 and 0.001, control and RA respectively). Unlike HIgG and its F (ab')2 fragment, very high secretion was elicited by anti-HIgG (242.37±10.85 ng/ml) and its F (ab')2 prong (195.85±28.67 ng/ml). IL-15 did not elicit any secretion. IL-18 with PMA increased secretion significantly only from RA PMNs (p=0.003). Serum MMP-8 correlated positively with serum CRP (p=0.017) and not with disease activity score (p=0.199).

Conclusions

We for the first time demonstrate that immune complexes elicit MMP-8 secretion from PMNs. Except for higher secretion from RA PMNs in response to combination of IL-18 and PMA, both control and RA PMNs respond similarly to various stimuli. Secretion by anti-HIgG occurs by a mechanism independent of Fc receptor. Correlation with CRP suggest that serum MMP-8 may be an indicator of acute inflammatory activity.

Present address of Liza Rajasekhar: Division of Rheumatology, Nizam's Institute of Medical Sciences, Hyderabad, India; supported by Chang Gung Memorial Hospital, Lin-kou.

Please address correspondence and reprint requests to: Lieh-Bang Liou, MD, PhD, Chief, Division of Rheumatology, Allergy and Immunology, Department of Internal Medicine, Chang Gung Memorial Hospital, 5, Fu-shin Street, Kwei-san Hsiang, Tao-yuan County, Taiwan 333. E-mail: b890121@adm.cgmh.org.tw

Introduction

Destruction of cartilage and periarticular bone, subsequent to hypertrophied synovium is the hallmark of pathophysiology of rheumatoid arthritis. Cumulative evidence suggests a complex interplay between an as yet undefined antigen, transformation of synoviocytes and collagenolytic systems involving matrix modifying enzymes and their inhibitors to be involved in the process of this destruction (1). The matrix metalloproteinases are a group of more than 20 enzymes, collectively having the ability, to degrade extracellular matrix in pathological conditions. Neutrophil collagenase, also known as matrix metalloproteinase-8 (MMP-8) or collagenase-2 is one of the three collagenases which cleave the triple helical structure of type I, II and III collagen, exposing the denatured collagen to proteolysis by other metalloproteinases. MMP-8 is co-localized with lactoferrin in the specific granules of neutrophils (2).

The inflammatory effusion of the rheumatoid joint has large number of neutrophils. Electron microscopic studies have demonstrated aggregates of immunoglobulins in damaged areas of cartilage in RA(3). Immune complexes (IC) can cause neutrophil degranulation by acting on surface Fc receptors (4, 5). There is evidence that IgG containing IC is elevated in the serum, synovial fluid and inside granulocytes in rheumatoid arthritis (4, 6). It has been shown that, by interacting with surface Fc receptors, IC is able to cause degranulation from neutrophil specific granules (5).

High concentrations of IL-15 have been demonstrated in the synovial lining and interstitium of rheumatoid joint but not in osteoarthritic joints (6). Moreover, increased levels of IL-15 mRNA and IL-18 protein have been demonstrated in the rheumatoid synovial lining (7, 8). IL-15 delays the apoptosis of neutrophils and enhances their phagocytic ability via cytoskeletal rearrangement (9). IL-15 also increases the secretion of IL-8 from neutrophils (10). In turn, IL-8 binds to surface CXCR2 receptors to cause neutrophil degranulation (11). Interleukin 18 (IL-18), a recently identified cytokine, has demonstrated pro- and anti-inflammatory effects. In the context of rheumatological conditions, IL-18 is believed to be predominantly proinflammatory (12). IL-18 can elicit lactoferrin secretion from peripheral blood neutrophils of RA (13). However, no one has ever examined the effects of IL-15, IL-18 or immune complexes on MMP-8 secretion from RA polymorphonuclear leucocytes (PMNs). With such a lot of recent data suggesting that IL-15 and IL-18 play important roles in the neutrophil inflammation of rheumatoid arthritis, these cytokines were thus chosen as stimuli for PMNs in our study.

We therefore decided to explore whether cytokines (IL-15, IL-18), immune complexes or PMA could cause secretion of MMP-8 from PMNs and whether the secretion was different in healthy people and patients with rheumatoid arthritis. In addition, the unanswered question of possible correlation between RAdisease activity and serum MMP-8 levels was investigated.

Materials and methods

Patients and serum collection

Forty consecutive patients, fulfilling at least four of the 1987 American College of Rheumatology (ACR) criteria for classification as rheumatoid arthritis (14) were enrolled. These patients were 59.10 ± 10.43 (mean ± S.D.) years old, with female/male ratio as 33/7; had a mean duration of arthritis of 24.67 ± 33.47 months, swollen joint count of 10.27 ± 5.13 and tender joint count of 12.82 ± 6.60; and were seropositive (RF > 40 IU/ml) in 18 out of 33. The controls (n = 25, mean age 49.72 ± 1.48 years, 21 female) were hospital staff or relatives of patients and had no diabetes, hypertension, arthritis or active viral infection. Except two, all patients were newly diagnosed and untreated with corticosteroids and disease modifying anti-rheumatic drugs (DMARDS). In four patients, *in vitro* experiments and sera collection was done before and after treatment with DMARDS. Disease activity was assessed by core set of clinical and laboratory measurements as suggested by the ACR (15). The swollen joint and tender joint count was recorded. Pain was assessed

on a visual analogue scale of 10. Physician's and patient's global assessment of disease severity was on a 5 point Likert scale (0-4), higher scores suggesting more severe disease. ESR was measured by the Westergren method. CRP and rheumatoid factor was measured by nephelometry, the results being reported in mg/L and IU/ml respectively. Unheparinised blood was collected from 27 patients and 25 healthy volunteers, allowed to stand at room temperature for 30 minutes, then centrifuged at 400g for 5 minutes and the supernatant stored at -20^0 C till assayed for MMP-8.

Reagents
Phorbol myristate acetate (PMA), HIgG and rabbit anti-HIgG were obtained from Sigma (St. Louis, MO, USA), F (ab')2 prong of HIgG, rabbit anti-HIgG, FITC-goat IgG F(ab')2, and FITC-goat F(ab')2 anti-human IgG from Jackson ImmunoResearch (West Grove, PA, USA), recombinant human IL-15 from R& D Systems Inc. (Minneapolis, MN, USA), IL-18 from Pepro Tech Inc. (Rocky Hill, NJ, USA). RPMI-1640, used as medium for all experiments and Hank's balanced salt solution (HBSS) was purchased from Gibco (Grand Island, NY, USA). Limulus Amebocyte lysate (LAL) gel clot assay was obtained from Cape Cod, Falmouth, MA, USA. Fetal calf serum (FCS) was obtained from Biological Industries (Israel) and treated at 56^0 C for 60 minutes before use.

Immune complex formation
Immune complexes were made as described (16) except that HIgG and rabbit anti-HIgG were used instead of human serum albumin and anti-human serum albumin. Briefly, 300 mg of HIgG in 500 µl of phosphate buffered saline (PBS) was incubated in 24 well flat bottom plates (Co-star, Corning Incorporated, Corning, NY, USA) at 37ºC for 2 hours. The wells were then washed three times with PBS and afterwards 300 mg of rabbit anti-HIgG in 500 µl of PBS was added to each well and stood at room temperature for 1.5 hour. The wells were washed again three times with PBS and the adherent IC so formed was used to stimulate PMNs. As controls for immune complexes, adherent HIgG, rabbit anti-HIgG and their F(ab')2 prongs were used at a concentration of 0.6 mg/ml.

Cell separation and in vitro *culture with various reagents*
Twenty five ml of heparinised blood was collected and run on Ficoll-Hypaque and processed as described (16). Briefly, 0.4 ml of 3% methylcellulose was aspirated into 5 ml syringes with 18 gauge needles. Then 5 ml of blood was aspirated into each prepared syringe and placed vertically at room temperature for 30-45 minutes. The upper layer of cells was then collected in a 50 ml tube and adjusted to 20 ml with PBS. Three ml of Ficoll-Hypaque (sp. gr. 1.077, Pharmacia, Uppsala, Sweden) was added to the bottom of 15 ml tubes, followed slowly by 10 ml of upper layer cell solution on top. This was then centrifuged at room temperature at 450g force for 30 minutes. PMNs were recovered from the lowest layer of precipitated cells. After being got rid of contaminating red blood cells, PMNs preparation purity was at least 95% by Wright stain.
$5x 10^5$ cells in RPMI-1640 media (containing 2 mM L-glutamate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10 mM HEPES with 10% FCS), 500 µl per well, in duplicate, were incubated with different concentrations of stimulating agents at 37ºC, 5% CO_2 for varying lengths of time as indicated. The supernatant was then recovered and stored at -20ºC till further assay. Cells in incubation were rated by trypan blue criteria as surviving (totally no blue dye inside cells), dying (with blue dye inside cells), dead (the rest of total number subtracting surviving and dying).

Determination of MMP-8 levels
MMP-8 levels in sera and from supernatants of PMNs stimulated with various reagents were estimated by two-site sandwich ELISA (Amersham Pharmacia Biotech UK Limited, England) using Emax precision microplate reader (Molecular Devices Corporation, CA, USA). The MMP-8 could be mea-

sured in the range of 0.25-4 ng/ml. The sensitivity of the assay was 0.032 ng/ml. The p value using Pearson correlation coefficient between serum rheumatoid factor and serum MMP-8 levels is 0.966, thus, no adjustment is done.

Statistical analysis
Values of supernatant and serum MMP-8 are reported as mean ± S.E.M, unless otherwise stated. All comparisons of stimulated groups were with supernatants from PMNs in medium only (unstimulated PMNs), unless otherwise stated. Two tailed $p<0.05$ by the student t test was considered significant except where indicated. Correlation between MMP-8 and clinical (disease activity score was used to encompass swollen joint count, tender joint count and ESR) and laboratory (CRP) variables was estimated using Spearman rank correlation coefficient.

Results
Cell survival and dying results are shown in Table I. Expectedly, after incubating with IL-15, there were more living PMNs from stimulated groups than corresponding groups of cells only (Table I), especially for RAPMNs.

Immune complexes elicit MMP-8 secretion in both groups and independent of Fc portion
Adherent IC was able to elicit a higher secretion of MMP-8 from PMNs of both groups when incubated for 12 and 24 hours (Fig.1). When compared to unstimulated PMNs, this increased secretion, was statistically significant ($p<0.05$) after 24 hours of incubation. Surprisingly, the antibody molecule as a control for the adherent IC, rabbit anti-HIgG produced significantly higher ($p < 0.001$) mean MMP-8 levels in controls (226.49 ± 9.00 ng/ml) and RA (253.49 ± 16.88 ng/ml) at 24 hours of incubation, when compared to medium only (Fig. 2). A similar trend was seen at 12 hours of incubation. This was much higher than the MMP-8 secreted in response to IC (Fig. 1). To decide whether this effect was due to the Fc portion of the molecule, the F (ab') 2 prong of the rabbit anti-HIgG was also used as a stimulant for 24 hours and

Table I. Percentages of cell survival and dying examined by trypan blue.

Subgroups		Surviving cells (%)					Dying cells (%)				
Culture duration (hours)		4	2	24	48	72	4	12	24	48	72
	RPMI	90	96	82	43	26	2	2	11	26	21
Control*	PMA	20	11	3+	ND#	ND	0	4	10+	ND	ND
(%)	IL-15	ND	ND	ND	44	45	ND	ND	ND	21	15
	RPMI	99	96	95	42	19	1	4	5	43	36
RA*	PMA	26	5	2+	ND	ND	4	2	11+	ND	ND
(%)	IL-15	ND	ND	ND	70	37	ND	ND	ND	27	30

*From two healthy controls or rheumatoid arthritis (RA) patients; data are expressed as mean values. # ND: not done. PMAwas used at 50 ng/ml (+: PMAplus 100 ng/ml of IL-18) and IL-15 at 100 ng/ml; dead cell percentages equal to 100% minus the sum of surviving and dying cell percentages.
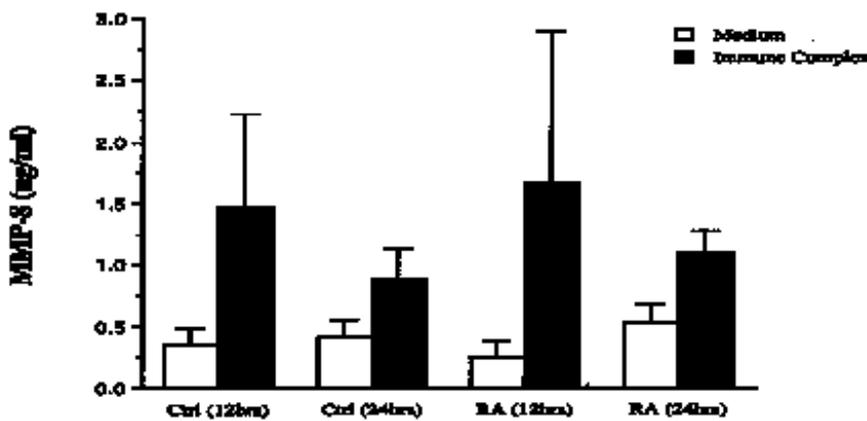
Fig. 1. Effect of stimulation with immune complexes. PMNs (5 x 10⁵ per well) stimulated for 12 hours (n = 4) and 24 hours (n = 5 and 9 for control and RA, respectively) either in medium only or with immune complexes as indicated. Data are represented as mean ± S.E.M. P< 0.05 when compared RA versus control PMNs after immune complex stimulation for 24 hours.
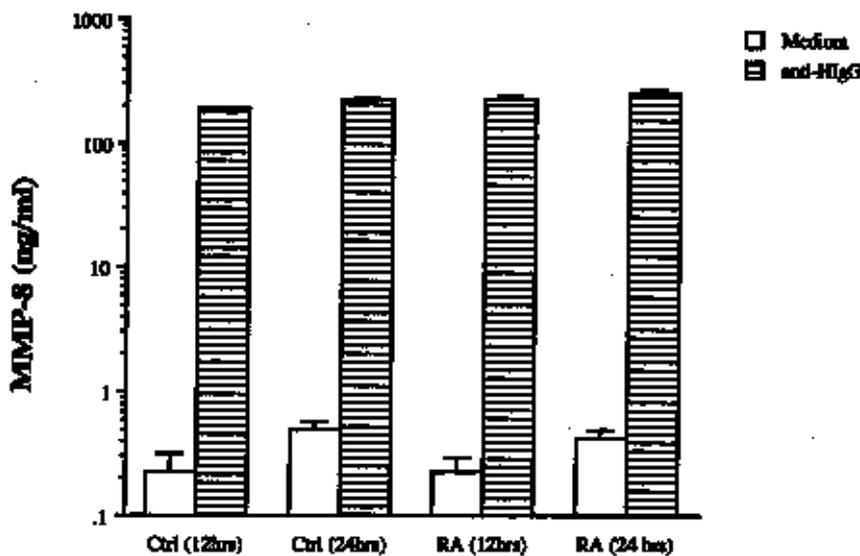
Fig. 2. MMP-8 levels in supernatants of PMNs treated with medium (n = 7, 9 for 12 hours, and 13, 19 for 24 hours, for control or RA, respectively) and rabbit anti-human IgG (anti-HIgG; n = 1, 2 for 12 hours and 7, 10 for 24 hours, for control or RArespectively). These supernatants were diluted, when necessary, to obtain readings within the range of measurement of the assay.

intriguingly it also produced similar results [212.9 ± 7.28 (mean ± S.D.) ng/ml and 225.9 ± 30.1 ng/ml in controls and RA respectively]. HIgG and its F (ab')2 fragment, however, were unable to induce any MMP-8 secretion from PMNs (data not shown).

We next investigated whether IL-15 plays an important role in the secretion of MMP-8 from neutrophils.

The change in MMP-8 levels after stimulation with IL-15 is partially different in RA and controls

Generally, *in vitro* production of MMP-8 from PMNs in both groups, when stimulated with different concentrations of IL-15, gradually increased from one hour in culture to a peak at 48 hours of culture (not shown). In both groups, there was no significant difference in the MMP-8 levels of unstimulated PMNs and those stimulated with different concentrations of IL-15 ranging from 30 to 150 ng/ml (mainly 30 or 100 ng/ml), for various incubation periods at 1 hour, 4 hours, 12 hours, 24 hours, 48 hours and 72 hours. The mean difference of MMP-8 levels between PMNs stimulated with 30 ng/ml IL-15 (0.79 ± 0.16 ng/ml with n = 8 or 0.72 ± 0.15 ng/ml with n = 12 in control and RA, respectively) and unstimulated PMNs (0.98 ± 0.20 with n = 8 or 0.67 ± 0.14 with n=13, respectively), was significantly different in the two groups at 48 hours (p = 0.01).

Moreover, both control and RA PMNs, stimulated with 100 ng/ml IL-15 in the presence of IC for 24 hours, produced no different MMP-8 amount from that by IC alone. Nevertheless, 50 ng/ml PMAwith 100 ng/ml IL-15 at 48 hours stimulated lower MMP-8 from RA PMNs (0.55 ± 0.15 ng/ml of MMP-8) than 100 ng/ml IL-15 alone (0.83 ± 0.29 ng/ml) (p = 0.031). This indicates that IL-15 really could rescue MMP-8 production.

IL-18 in the presence of immune complexes induces MMP-8 secretion from RAPMNs

Stimulation of PMNs with 100 ng/ml IL-18 did not change the mean MMP-8 secretion significantly in either group (Table II). However, when compared to

unstimulated PMN, the combined stimulus of 100 ng/ml IL-18 with 50 ng/ml PMA for 24 hours, increased mean MMP-8 levels more in RA PMNs than in control (p = 0.06, 0.32 respectively). When compared to IL-18 stimulation alone, the combined IL-18 and 50 ng/ml PMA stimulus produced higher MMP-8 secretion from both groups but more from controls (p = 0.003 compared to p = 0.10 in RA). The combination of immune complexes and IL-18 elicited higher MMP-8 than from unstimulated PMNs in both groups, however this was not different than that obtained by using IC alone (Table II).

In both groups, MMP-8 levels increased following stimulation with 50 ng/ml of PMA(Table III). This increase peaked at 12 hours in control and at 24 hours in the RA group. Further incubation led to a significant decline in MMP-8 levels.

Correlation of disease activity with serum MMP-8 levels

Mean serum MMP-8 in controls was 0.05 ± 0.04 ng/ml (n = 25) and in RA 0.08 ± 0.07 ng/ml (n = 19) with no significant difference between patients with and without DMARD therapy (data not shown). In all patients (untreated or those treated with corticosteroids and/or DMARD), measure of acute inflammatory activity like CRP correlated with serum MMP-8. Serum MMP-8 levels, however, did not correlate with disease activity score (Table IV).

Discussion

In Figure 1, whether there is an autodegradation of MMP-8 by RAand control PMNs to make lower mean MMP-8 levels of 24 hr culture when compared to 12 hour culture is not known at this time though decreased survival in the control group (Table I) may be a reason. Nevertheless, adherent immune complexes, adherent rabbit anti-HIgG molecules and their F (ab')2 prong were able to elicit secretion of MMP-8 from PMNs of both groups (Figs. 1 and 2). It is therefore possible that the immune complexes present in the rheumatoid cartilage and the synovial fluid may contribute to increased MMP-8

Table II. Effect of IL-18 on MMP-8 production compared to the effects of IL-18 combinations with PMAor IC.

Stimulus	Control	RA
None	0.49 ± 0.09 (13)	0.41 ± 0.07 (20)
IL-18	0.30 ± 0.01 (2)	0.36 ± 0.12 (3)
IL-18 + PMA	0.74 ± 0.03 (2)**	0.82 ± 0.18 (3)*
IL-18+IC	0.86 ± 0.22 (2)	1.17 ± 0.57 (4)
IC	1.04 ± 0.17 (8)	0.98 ± 0.17 (11)

Cell were cultured for 24 hours with different reagents or combinations; PMA: phorbol myristate acetate; IC: immune complexes. *p <0.005 compared to unstimulated PMNs (independent t test); **p < 0.005 compared to PMNs stimulated with IL-18.

Table III. Effect of PMAas a stimulant on MMP-8 production.

Time (hours)	No stimulus Control	RA	50 ng/ml PMA Control	RA
1	0.09 ± 0.00 (1)	0.09 ± 0.04 (3)	0.26 ± 0.02 (1)	0.05 ± 0.01 (1)
4	0.19 ± 0.08 (2)	0.15 ± 0.12 (2)	0.42 ± 0.04 (1)	0.18 ± 0.03 (1)
12	0.22 ± 0.10 (7)	0.22 ± 0.07 (9)	0.72 ± 0.16 (4)*	0.62 ± 0.24 (5)
24	0.46 ± 0.10 (11)	0.40 ± 0.08 (17)	0.52 ± 0.12 (11)	0.79 ± 0.25 (15)
48	0.98 ± 0.20 (8)	0.67 ± 0.14 (13)	0.53 ± 0.18 (8)	0.51 ± 0.10 (13)
72	0.15 (1)	0.62 ± 0.28 (2)	0.17 (1)	0.48 ± 0.25 (2)

*p < 0.05 between the values with and without PMA(50 ng/ml) as a stimulant. Numbers in parentheses indicate the number of independent experiments. Where there is only one experiment, the results are expressed as mean ± SD of two wells.

Table IV. Correlation of clinical and disease activity measures with serum MMP-8 levels.

Measure	R	p value	N
C-reactive protein	0.541	0.017	19
Disease activity score	0.308	0.199	19

R = rho, Spearman rank order correlation coefficient; N = number of patients for whom data on the measure and MMP-8 level are available. P< 0.025 was considered significant and used for correction of multiple statitical correlation analysis by the Bonferroni method, as described in reference 16.

found in arthritic lesions. Since we did not explore other sources of MMP-8 as described previously (17), it is not possible to be sure that *in vivo* neutrophils are the source of the raised MMP-8 as described in osteoarthritic lesions (18). In terms of the very high and almost similar secretion of MMP-8 by the whole molecule of rabbit anti-HIgG and its F(ab')2 prong, there exists, in all probability a mechanism of specific granule degranulation which is entirely independent of the use of Fc receptors on neutrophils. This finding in our study differs from that shown by Voice *et al.* that IgG containing immune complexes elicit degranulation from neutrophil specific granules by using surface Fc receptors (5). It was postulated that Fc RII mediated this release since

degranulation was inhibited in neutrophils treated with monoclonal antibodies to Fc RII (19). However, in our study, adherent human IgG molecules or their F (ab')2 was unable to generate any significant MMP-8 secretion. This may be explained partly by the lower concentration of human IgG used by us (0.6 mg/ml instead of 10 mg/ml used in reference 19).

Furthermore, we found that HIgG or rabbit IgG anti-HIgG contains lower than 50 or 160 EU/ml of endotoxin, that is, less than 5 ng/ml or 16 ng/ml in the respective solution. Hence, LPS does not seem to be able to explain this difference (Fig. 2). Whether some human immunoglobulins already attached on control and RA neutrophils confer high MMP-8 secretion by anti-human

IgG stimulation was also investigated. We found that, by using FITC-goat IgG F(ab')2 as a background staining, FITC-goat F(ab')2 anti-human IgG gave the same pattern as the background (not shown) for both kinds of PMNs. Therefore, what molecules on the surface of neutrophils eliciting such high MMP-8 production remain to be determined.

Though IL-18 has been shown to elicit secretion of lactoferrin from peripheral blood neutrophils and synovial fluid neutrophils of RA (13), RA peripheral blood PMNs could not secrete MMP-8 by IL-18 stimulation alone in our study (Table II). However, when PMNs were co-stimulated with IL-18 and PMA, higher MMP-8 secretion was achieved (Table II). This indicates that the effect of IL-18 on MMP-8 secretion may *in vivo*, require other stimuli, which could facilitate activation of signal transduction pathways like PMA does for protein kinase C.

Like previous studies (20), PMA could elicit degranulation of neutrophils and hence an increase in supernatant MMP-8 levels. These levels declined beyond 12 hours in controls and beyond 24 hours in RA suggesting that prolonged exposure of PMNs to PMAis toxic (see Table I and Table III). However in the presence of the cytokine IL-18, addition of PMAwas not so toxic, since this combination produced higher MMP-8 levels compared to unstimulated PMN even after 24 hours of incubation (Table II). Lastly, we found that high serum CRP levels, usually associated with active arthritis, correlated with serum MMP-8 (Table IV). It is therefore likely that MMP-8 in the serum reflects acute inflammatory activity of rheumatoid arthritis.

In summary, to our knowledge, this is the first report on characterization of MMP-8 secretion from RA PMNs. Since MMP-8 secretion from unstimulated PMNs in both groups is similar and much lower (approximately 0.40 ng/ml) than that produced by adherent anti-human IgG (approximately 240 ng/ml), it is likely that circulating PMNs of RA are not in an activated state, i.e. have not already degranulated. Regardless of the group, PMNs degranulate in response to immune complex stimulation. Hence IC, which is found in the sera, on the surface of cartilage and inside granulocytes of patients with RA(4, 6) may be one stimulus responsible for the secretion of MMP-8 *in vivo*, besides other possible mechanisms (for example, local activation of lytic enzymes). Since combinations of such stimuli are more likely to be found in the inflamed rheumatoid joint, these may be important stimuli for MMP-8 secretion. Consequently, serum MMP-8 levels may be used as a marker of acute inflammatory activity in rheumatoid arthritis, just like serum CRP.

Acknowledgement

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