Active MMPs captured by alpha$_2$Macroglobulin as a marker of disease activity in rheumatoid arthritis

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Abstract

Objective

The aim of the present study was to analyze α_2Macroglobulin/MMP (α_2M/MMP) complex formation and to investigate whether MMP activity in α_2M/MMP complexes in serum can be used as a disease marker in rheumatoid arthritis (RA).

Methods

High and low molecular weight (H/LMW) substrates and inhibitors and size exclusion were used to analyze α_2M/MMP complex formation. LMW fluorogenic substrates were used to quantify the level of MMPs in α_2M/MMP complexes in the serum of RA patients and healthy controls.

Results

Active MMPs were fully inhibited by LMW inhibitor BB94 in the presence of α_2M, whereas no inhibition was achieved by HMW inhibitor TIMP-1. Size exclusion analysis showed α_2M/MMP complex formation in buffer and in normal plasma spiked with activated MMPs, which indicated α_2M/MMP complex formation in the systemic circulation. MMP activity in α_2M/MMP complexes in the serum of RA patients was significantly higher than in the serum of healthy controls ($P < 0.001$). MMP activity levels in the serum of RA patients were correlated with ESR ($r = 0.72$, $P < 0.001$).

Conclusion

In the systemic circulation of RA patients, active MMPs form complexes with α_2M and can be detected using LMW fluorogenic substrates. MMP activity measurements in serum allow discrimination between RA patients and healthy controls and provide a new tool for the assessment of the disease process in RA.

Key words

Matrix metalloproteases, rheumatoid arthritis, α_2Macroglobulin.

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Abbreviations used:

ProMMPs: Pro-matrix metalloproteinases;
MMPs: Matrix metalloproteinases;
TIMPs: Tissue inhibitors of metalloproteinases;
α_2M: alpha$_2$Macroglobulin;
HMW: High molecular weight;
LMW: Low molecular weight;
RA: Rheumatoid arthritis;
CRP: C-reactive protein;
ESR: Erythrocyte sedimentation rate.

Introduction

Matrix Metalloproteinases (MMPs) are Zn^{2+} dependent extra-cellular enzymes that play a key role in normal and pathological remodeling of connective tissues. In rheumatoid arthritis (RA), a chronic disease characterized by polyarticular inflammation leading to loss of cartilage and bone, proMMPs1 are synthesized and released (1) by synovial fibroblasts, chondrocytes, macrophages, neutrophils and endothelial cells (2). Based on domain structure and substrate specificity, MMPs can be divided into subclasses, e.g. collagenases, gelatinases, stromelysins and membrane-type MMPs. Most of the proMMPs are activated extracellularly and they have the combined ability to degrade all components of articular cartilage (3). Stromelysins (MMP-3, –10 and –11) are believed to play an important role in this enzyme system due to their wide substrate specificity and ability to activate other MMPs. Collagenases (MMP-1, -8 and -13) are capable of degrading intact collagen (one of the main components of articular cartilage), which can be further degraded by gelatinases (MMP-2 and -9). Gelatinases can also degrade other components of the joint tissues such as aggrecan, fibronectin and elastin. Membrane-type matrix metalloproteinases (MMP-14, -15, -16, -17, -24 and -25) have also been shown to degrade various components of joint tissue and to be involved in activation of other MMPs (4).

MMP subclasses have been shown to be increased at the tissue level in inflammatory joint diseases (5). Also in the systemic circulation, antigen levels of proMMPs are increased, indicating their involvement in the disease process (4). Recent research on the use of serum proMMP antigen levels as a marker for disease activity or as a prognostic tool in RA indicates that serum proMMP levels reflect not only the inflammation but also the degradation of articular cartilage (6, 7). Although serum proMMP levels correlate with disease progression, they mainly reflect the potential of the proteolytic system to degrade cartilage. Other factors, such as the activation status (conversion of proMMPs into active MMPs) and the inhibitory capacity of the proteolytic system (presence of endogenous inhibitors) co-determine the eventual tissue degradation. Analysis of MMPs and their Tissue Inhibitors (TIMPs) shows a surplus of active MMPs (due to insufficient levels of TIMPs) at the tissue level, which supports the role of the MMP/TIMP imbalance in joint diseases (8-11). Quantification of this MMP surplus may provide a useful tool for the evaluation of the clinical course of the disease inasmuch as this surplus of active MMPs reflects the actual end-status of the system: i.e., the proteolytic capacity after production, activation and inhibition.

It has been shown that active, not-TIMP-inhibited MMPs can be entrapped by α_2Macroglobulin (α_2M), which results in α_2M/MMP complex formation in biological fluids (12-15). We hypothesized that in RA the surplus of active MMPs, i.e. the excess of active MMPs over TIMP, will result in an increased level of α_2M/MMP complexes in the systemic circulation which can be quantified using Low Molecular Weight (LMW) fluorogenic substrates (16).

In order to provide evidence of α_2M/MMP complex formation in the systemic circulation of RA patients, the MMP/TIMP imbalance as it exists in the inflammatory joint disease was mimicked and MMP activity was measured after size exclusion analysis. Furthermore, the use of LMW MMP-specific fluorogenic substrates for detection of α_2M/MMP complexes in the systemic circulation was investigated. To explore the feasibility of MMP activity measurements as a marker of disease activity, MMP activity levels in serum of RA patients were determined and compared to an inflammatory marker, ESR.

Patients and methods

Matrix metalloproteinases

ProMMP-13 was kindly provided by Dr. P. Mitchell (Pfizer Central Research, Groton, CT, USA) and was activated by incubation with 2 mM APMA for 2 h at 37°C in MMP buffer (50 mM Tris, 5 mM $CaCl_2$, 250 mM

NaCl, 1 μM $ZnCl_2$, 0.02% NaN3 and 0.01% Brij-35, pH 7.5). The amounts of active enzyme were calibrated by active-site titration with TIMP-1 (Oncogene Research Products, Cambridge, MA, USA) as described by others (18).

Fluorogenic MMP substrate

The internally quenched fluorogenic peptide substrate Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys(Fluorescein)-Ala-Lys-NH2 (TNO211-F) was synthesized according to the method described by Drijfhout *et al.* (17). TNO211-F is converted by MMP [mainly MMP-2, -8, -9 and -13; and also at lower rate by MMP-1 and -3 (15)] and not by other metalloproteinases such as ADAMs or ADAM-TS.

MMP activity measurements

A. Using fluorogenic MMP substrate. MMP activity was measured using 6.25 μM (all concentrations are final) fluorogenic substrate TNO211-F in the presence or absence of 5 μM BB94 (a general MMP inhibitor). TNO211-F is mainly converted by MMP-2, -3, -7, -9, -12 and -13. It is also converted, although at lower rate, by MMP-1 and -9 (15), whereas other metalloproteinases, such as ADAMs, do not cleave TNO211-F (19). Serum samples were diluted (final dilution 1/50) in MMP buffer and EDTA-free Complete™ serine and cysteine proteases inhibitor, Roche, Mannheim, Germany; 1 tablet in 50 ml) was added to all series. The difference in the initial rate of substrate conversion (linear increase in fluorescence in time) between samples with or without BB94 addition was used as a measure of MMP activity. Fluorescence was measured for 6 hrs at 30°C using a Cytofluor 4000 (Applied Biosystems, Foster City, CA, USA).

B. Using High Molecular Weight MMP substrate UKcol. UKcol is a modified pro-urokinase, which has a general MMP cleavage site. MMP activity was measured using UKcol (final concentration 50 mg/ml) and chromogenic substrate S-2444 (Chromogenix, Mölndal) (20). Color development was recorded in a Multiskan® MCC/340 (LabSystems, Helsinki, Finland) at 405 nm.

C. Collagen type I degradation by MMP-13. MMP-13 solutions were prepared (constant enzyme concentration of 0.5 nM) in buffer containing α_2M, of which the concentration varied from 0 up to 1.7 nM. MMP-13 was incubated for 2 hrs at room temperature with various concentrations of α_2M (MMP-13/α_2M ratios ranging from 0.01 through 100). An aliquot of each ratio (200 (μl) was incubated with collagen type I (100 μg/ml). Cleavage of collagen into characteristic TC_A and TC_B fragments after overnight incubation at 30°C was visualized by non-reducing SDS-PAGE (10% polyacrylamide gel) and analyzed using TINA software (Isotopenmeßgeräte, GmbH, Germany).

α_2M sandwich ELISA

To determine human α_2M levels a two-step ELISA was used. First, high binding ELISA plates (EIA/RIA Stripwell™ Plate, Corning Incorporated, NY, USA) were coated with sheep anti-mouse F(ab)' fragments (1.3 μg/ml, Jackson Laboratory) for 48 h at 4-8°C. The plates were washed 3 times with PBS/0.5% Tween-20 and blocked with PBS/0.5% Tween-20/ 1%BSA for 1 hr at 37°C in a plate incubator (LabSystems, Helsinki, Finland). α_2M specific monoclonal antibodies #5850-1004 (ANAWA Trading, SA, USA) were bound to the F(ab)' fragments overnight at 4°C. A sample aliquot (100 μl) was added and incubated for 2 hrs at 37°C in the plate incubator. The plates were washed 5 times with PBS/0.5% Tween-20/1%BSA and 100 μl secondary antibody #5850-0304 (α_2M specific sheep-anti-mouse polyclonal, HRP-conjugated; ANAWA Trading, SA, USA) was added and incubated for 2 hrs at 37°C. Color reagent (100 μl) was added and the reaction was stopped after 15 min. by the addition of 10% H_2SO_4. The yellow colored product was measured using a Multiskan® MCC/340 (LabSystems, Helsinki, Finland) at 450 nm wavelength.

FPLC

Human plasma or purified human α_2M (Sigma-Aldrich Corp., St. Louis, MO, USA) in MMP buffer were spiked with 10 nM (final concentration) active MMP-13. Samples were incubated for 1 hr at room temperature and analyzed by the FPLC system (Superose® 6HR 10/30 column; Pharmacia Fine Chemicals, Uppsala, Sweden). The optical density was measured using a spectrophotometer at 280 nm. Fractions of 0.5 ml were collected and MMP activity was measured using the TNO211-F substrate as described above. α_2M levels were determined using the α_2M sandwich ELISA as described above. The same fractions were measured for MMP activity using High Molecular Weight substrate UKcol, as described above.

MMP activity in α_2M/MMP complexes versus ESR in serum samples of early arthritis clinic patients

For this study, a selection was made from the Early Arthritis Clinic (EAC) cohort, that was started at the Department of Rheumatology of the Leiden University Medical Center, The Netherlands in 1992 and described in detail by Lard *et al.* (21). From this large population-based inception cohort 50 patients with a diagnosis RA according to the 1987 ACR criteria (22) were selected. Serum samples used in the present study were prepared after blood collection and were stored at -20°C prior to analysis. MMP activity in α_2M/MMP complexes was measured as described above, the erythrocyte sedimentation rate (ESR) was determined upon blood collection.

Statistical analysis

Differences between the groups were analyzed with the (un)paired Student's t-test. Correlations were sought by calculating the correlations coefficients with SPSS software (Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

MMP-13 mediated degradation of HMW substrates in the presence or absence of α_2M

In biological fluids α_2M inhibits serine, cysteine, aspartic and metalloproteinases (15) by molecular trapping (13, 23, 24). MMPs entrapped within the

α_2M molecule lose their ability to degrade any natural substrates such as collagen type II (15). In the present work MMP-13 was used to study MMP activity in the presence or absence of α_2M because of its high activity towards both HMW (Collagen type I and UKcol) and LMW (fluorogenic peptides) substrates. To confirm the inhibitory ability of α_2M towards MMPs, the degradation of the natural HMW MMP substrate, collagen type I, was studied. Collagen type I breakdown by MMP-13 was analyzed by SDS-PAGE in the presence or absence of α_2M. MMP-13 solutions were prepared in buffer containing α_2M. Three possible test conditions were achieved: (a) an excess of activated MMP-13 over α_2M; (b) equal amounts of α_2M and MMP-13; and (c) an excess of α_2M over MMP-13. Active MMP-13 degraded collagen type I into the characteristic TC_A and TC_B fragments (27% of collagen was degraded during overnight incubation at 30°C). Similar results were seen when the MMP-13 concentration exceeded the α_2M concentration (29% degradation). However, no TC_A and TC_B fragments were seen when the α_2M concentration was equal to or higher than the MMP-13 concentration.

Similar experiments were performed using HMW substrate UKcol. UKcol is a modified urokinase in which the plasmin activation site has been replaced by a general MMP cleavage site (25). UKcol activation by MMP-13 was detected (11.2 Units/ml, 1.25 nM MMP-13) in the absence of α_2M, whereas no activation was seen after pre-incubation of MMP-13 with α_2M (0.2 Units/ml, 1.25 nM α_2M/MMP-13). These results show that the activity of MMP-13 towards the HMW substrates collagen type I and UKcol is inhibited by α_2M.

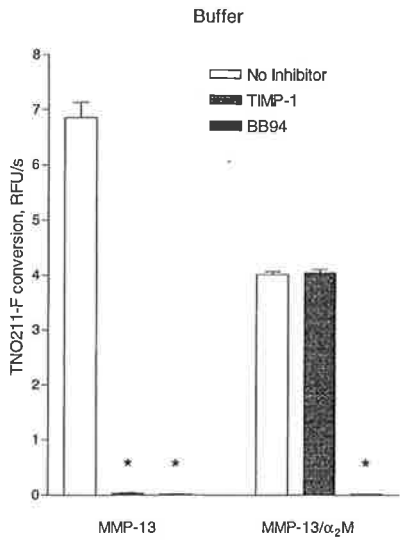

Fig. 1. MMP mediated Low Molecular Weight substrate conversion in the presence of α_2M in buffer.
Activated MMP-13 was pre-incubated in buffer in the absence or presence of α_2M for 2 hrs at 30°C, inhibitors were added and the incubation was continued for 1 hr at 30°C. Bars show mean (± SD) of measurements performed in triplicate. MMP activity in the absence of inhibitor is shown by open bars. 0.1 μM TIMP-1 (gray bars) and 0.1 μM BB94 (solid bars) completely inhibited MMP activity in buffer in the absence of α_2M. In the presence of α_2M no inhibition by TIMP-1 was seen, whereas 100% inhibition by BB94 was achieved.
* indicates $p < 0.05$ when compared to the MMP activity measured in the absence of the inhibitor.

MMP-13 mediated degradation of LMW substrate in the presence or absence of α_2M

To investigate whether LMW fluorogenic substrates could be used to detect MMPs in the presence of α_2M, conversion of TNO211-F (a LMW fluorogenic substrate) by MMP-13 was studied in buffer in the presence or absence of α_2M and in normal human serum (contains endogenous α_2M) spiked with MMP-13. As shown in Figure 1 (MMP-13 and MMP-13/α_2M: "no inhibitor"), MMP-13 mediated TNO211-F conversion was detectable in buffer in the absence and presence of α_2M. Possible explanations for the lower TNO211-F conversion rate by MMP-13 in the presence of α_2M are a low diffusion rate of the substrate into the α_2M/MMP complexes, lower substrate availability (protein binding) or lower MMP-13 activity inside α_2M. MMP-13 spiked to serum was also able to degrade LMW substrate TNO211-F (Fig. 2, MMP-13 and MMP-13/α_2M: "no inhibitor").

Furthermore, the inhibitory activity of HMW MMP inhibitor TIMP-1 and LMW MMP inhibitor BB94 towards MMPs in the presence of α_2M was analyzed. MMP-13 was incubated in buffer in the presence or absence of α_2M

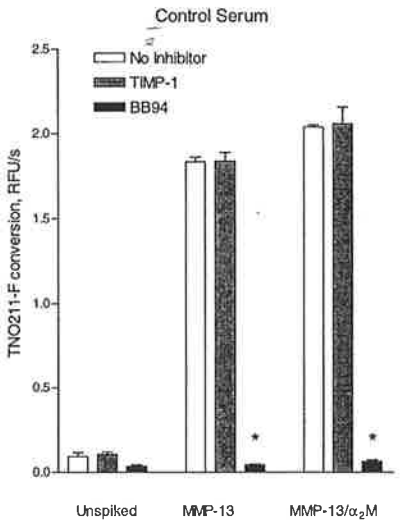

Fig. 2. MMP mediated Low Molecular Weight substrate conversion in serum spiked with MMP-13.
Activated MMP-13 was pre-incubated in human serum for 2 hrs at 30°C, inhibitors were added and the incubation was continued for 1 hr at 30°C. All of the MMP-13 spiked to human serum (regardless of the pre-incubation with α_2M) was inhibited by BB94 (solid bars), whereas no inhibition by TIMP-1 (gray bars) was seen.
* indicates $p < 0.05$ when compared to the MMP activity measured in the absence of the inhibitor.

or in normal human serum and its activity was measured using LMW fluorogenic substrate TNO211-F. Incubations with BB94 and TIMP-1 in buffer showed that MMPs are effectively inhibited by BB94, both in the presence and absence of α_2M (Fig. 1, MMP-13, MMP-13/α_2M: "BB94"). TIMP-1 fully inhibited MMPs in the absence of α_2M, but no inhibition was achieved in the presence of α_2M (Fig. 1, MMP-13, MMP-13/α_2M: "TIMP-1"). Subsequently, similar inhibition experiments were performed with active MMP-13 spiked to normal human serum. Again, all spiked MMPs were effectively inhibited by BB94 whereas no inhibition by TIMP-1 was found (Fig. 2, MMP-13, MMP-13/α_2M: "BB94" and "TIMP-1").

This pattern of substrate conversion shows that the presence of α_2M in the solution prior to the addition of the inhibitor prevents MMP/TIMP-1, but not MMP/BB94 complex formation. Altogether, these findings suggest that in fluids that contain α_2M active MMPs are mostly present in the form of α_2M/MMP complexes.

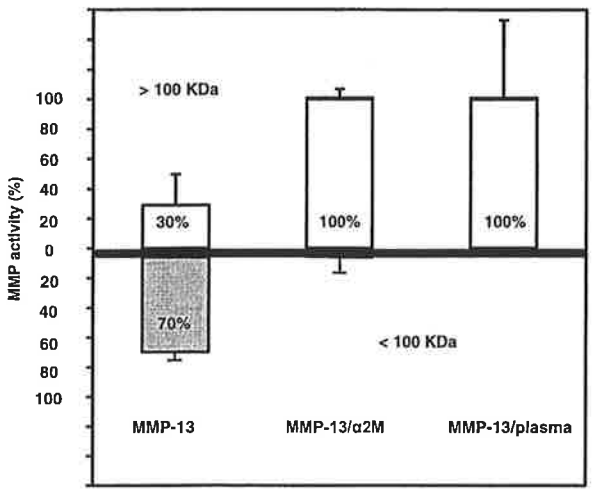

Fig. 3. Fractionation by 100 kDa cut-off filters: Activity measurements of MMP-13 spiked to buffer or normal human plasma.
Activated MMP-13 (final concentration of 0.2 nM) was pre-incubated in buffer in the presence or absence of α_2M or in normal human plasma for 1 hr at 30°C. Solutions were ultra-filtrated at 1000 g for 10 min and MMP activity was determined using TNO211-F in the flow-through and supernatant, the total MMP activity was set at 100%. 70% of the MMP-13 activity in buffer was found in the < 100 kDa fraction, indicating the passage of free MMP-13 through the filtration membrane. In the buffer containing α_2M, 100% of MMP-13 activity was recovered in the > 100 kDa fraction; the same pattern was seen for MMPs spiked to plasma, indicating α_2M/MMP-13 complex formation in plasma.

Analysis of α_2M/MMP complex formation: size exclusion analysis
Fractionation by 100 kDa cut-off filters. To confirm that active MMPs indeed form complexes with α_2M in the systemic circulation, another approach was used: size separation analysis. Based on the estimated MW of α_2M/MMP complexes of ~ 775 kDa and the MW of activated MMP-13 of 48 kDa, a 100 kDa cut-off filter was used. Free MMPs should pass the filtration membrane, whereas HMW α_2M/MMP-13 complexes should not. Solutions were ultra-filtrated and MMP activity was determined using LMW substrate TNO211-F in < 100 kDa and > 100 kDa fractions.

When dissolved in buffer in the absence of α_2M (Fig. 3, MMP-13), the majority of the MMP-13 activity was detected in the < 100 kDa fraction showing that free active MMP-13 was indeed ultra-filtrated. MMP activity in the > 100 kDa fraction may be explained by aggregate formation of activated MMP-13 molecules which prevents passage through the membrane. When active MMP-13 was incubated with α_2M prior to ultra-filtration (Fig. 3, MMP-13/α_2M, all MMP activity was found in the > 100 kDa fraction. As such this data indicates that ultrafiltration provides an adequate tool for discrimination between free and α_2M entrapped MMPs.

Filtration of human control plasma spiked with MMPs (Fig. 3, MMP-13/plasma) resulted in 100% activity measured in the > 100 kDa fraction, showing the same pattern as was obtained with MMPs in α_2M containing buffer. Altogether, these results support the

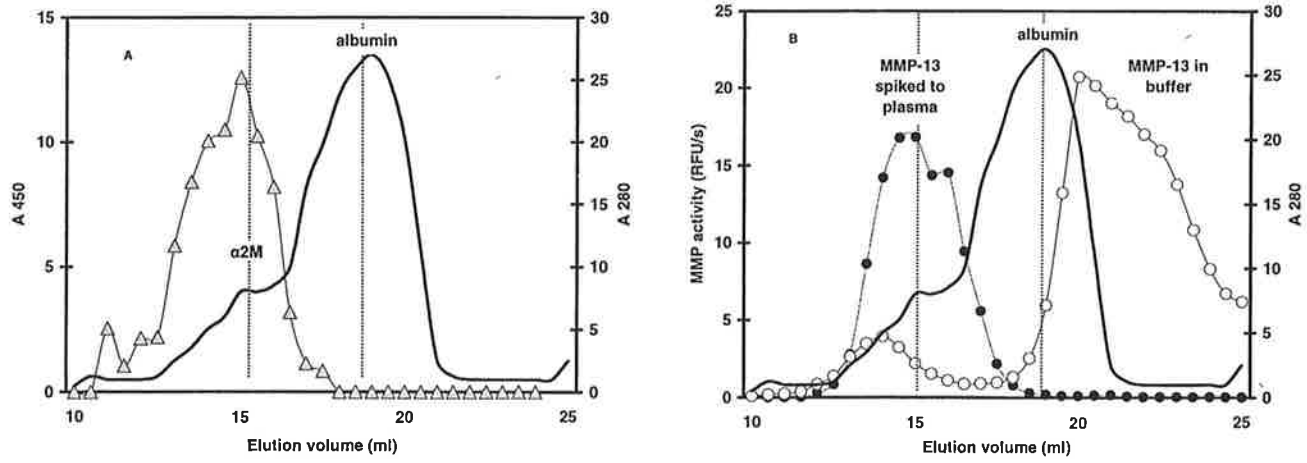

Fig. 4. Size exclusion FPLC.
(**a**) To establish the elution pattern of free active MMP-13 (MW 48 kDa) and α_2M/MMP-13 (MW ~ 775 kDa), a Superose 6 column was calibrated ($A_{280\ nm}$, solid black line, with α_2M (MW 725 kDa) and albumin (MW 69 kDa); to verify the elution position of α_2M, an in-house developed α_2M ELISA ($A_{450\ nm}$, gray triangles, was used to detect α_2M in the fractions. α_2M eluted earlier than the albumin peak.
(**b**) After calibration, free activated MMP-13 dissolved in buffer in the presence or absence of α_2M was analyzed, all fractions were collected and MMP activity was measured using TNO211-F. Analysis of MMP-13 (○) in buffer showed a major peak of enzyme activity at the tail of the albumin peak (retention 20 ml). Activity of MMP-13 in α_2M containing buffer was measured in fractions at the identical position as α_2M. Similarly, enzyme activity of plasma spiked with MMP-13 (●) showed an MMP enzyme activity peak at the α_2M and α_2M/MMP-13 positions (retention 14.5 ml).
These findings show a shift in MMP-13 activity from LMW into the HMW fractions, after pre-incubation in α_2M containing buffer or in normal human plasma.

hypothesis that the surplus of active MMPs in the systemic circulation is entrapped in α_2M.

FPLC size exclusion analysis: α_2M/MMP-13 complex formation.

As another size-exclusion approach to determine whether active MMPs are indeed entrapped in α_2M, FPLC size exclusion analysis of MMP-13 spiked human plasma was performed. The Superose 6 column was first calibrated with α_2M (MW 725 kDa) and albumin (MW 69 kDa). The elution position of α_2M was determined by α_2M ELISA (Fig. 4A). As expected, α_2M (725 kDa) eluted earlier than albumin (determined by protein absorption at 280 nm, confirmed by ELISA).

After calibration, active MMP-13 dissolved in buffer in the presence or absence of α_2M was analyzed. All fractions were collected and MMP activity was measured using LMW fluorogenic substrate TNO211-F (Fig. 4B). Albumin was spiked to all solutions to serve as a reference point. FPLC analysis of the MMP-13 in buffer showed a major peak of enzyme activity at the tail of the albumin peak. An additional peak of enzyme activity eluted earlier than albumin (MW > 100 kDa), which may be explained by aggregate formation of active MMP-13 molecules as was also seen in the experiment using the 100 kDa cut-off filters (Fig. 3). Activity of MMP-13 in α_2M-containing buffer was found in the fractions at the α_2M position. Similarly, enzyme activity in plasma spiked with MMP-13 showed an MMP activity peak at the α_2M and α_2M/MMP-13 elution position. To investigate whether the MMPs are indeed in complex with α_2M and therefore are not able to breakdown HMW substrates, all fractions were measured for MMP activity using HMW substrate UKcol (20). No MMP mediated UKcol conversion was detected at the elution position of α_2M/MMP complexes where it was detectable with LMW substrate TNO211-F, suggesting α_2M/MMP complex formation.

Altogether these findings show a shift in MMP activity into the HMW fraction, i.e. to the α_2M elution position, after incubation of MMPs with α_2M.

MMP activity levels in serum of RA patients and healthy controls.

To establish the feasibility of MMP activity measurements to discriminate between normal and pathological situations, MMP mediated TNO211-F substrate conversion was determined in serum of RA patients and healthy controls (n = 8 and n = 15, respectively). MMP activity could be detected in both populations, but was significantly increased in serum of RA patients as compared with healthy controls ($P < 0.001$ RA vs. controls), indicating a measurable surplus of active MMPs in the systemic circulation in this pathological situation (Fig. 5).

Furthermore, to investigate the feasibility of MMP activity measurements as a marker of disease activity in RA, MMP activity in serum of 50 RA patients was compared to ESR. The analysis showed a significant correlation between the two parameters ($r=0.72$, $P<0.001$, Fig. 6).

To study the potential clinical use of MMP activity measurements for the evaluation of the treatment efficacy, MMP activity was determined in serum of leflunomide-treated RA patients (n = 4) at baseline and after 16 weeks of treatment. Leflunomide has previously been shown to influence the MMP/TIMP balance in favor of TIMP *in vitro* (26). If this is the case *in vivo* as well, leflunomide would be expected to decrease the surplus of active MMPs in the circulation of these patients. In this pilot experiment, all 4 patients showed a 40% decrease in MMP activity levels after 16 weeks of treatment (mean ± SD): from 0.038 ± 0.007) to 0.022 ± 0.014), P = 0.015).

Discussion

The present study shows that MMP activity can be measured in the systemic circulation using Low Molecular Weight fluorogenic substrates. Further analysis showed that measured MMP activity originates from α_2Macroglobulin/MMP (α_2M/MMP) complexes. The results of this study also show that MMP activity measurements in serum of RA patients may provide an interesting new tool for evaluation of the disease process in RA.

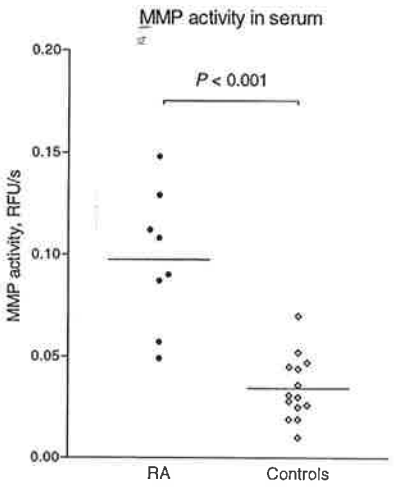

Fig. 5. MMP activity in serum of rheumatoid arthritis patients versus healthy controls. MMP activity was determined in serum of the RA patients and healthy controls (n = 8 and n = 15, respectively, symbols represent individual values (mean of duplicate measurements). MMP activity could be detected in both subsets, but was significantly higher in serum of RA patients ($p < 0.001$ RA versus controls, unpaired Student's t-test).

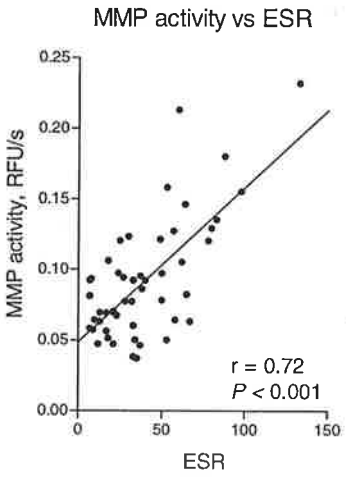

Fig. 6. MMP activity in the serum of rheumatoid arthritis patients versus erythrocytes sedimentation rate (ESR). MMP activity and ESR was measured in the serum of 50 RA patients. A significant correlation was found between the two parameters ($r = 0.72$, $P < 0.001$, Spearman's rho), indicating a relationship between the inflammatory status and the activity of the proteolytic system.

It has previously been shown that activated MMPs can form complexes with α_2M in biological fluids (13, 15, 24). In general, α_2M acts as a proteinase scavenger and can inhibit serine, cysteine, aspartic and metalloproteinases by molecular trapping. After proteinases are enclosed in α_2M, they are rapidly

eliminated from the circulation (23). MMPs entrapped within the α_2M molecule loose their ability to break down natural HMW substrates such as collagen type II (15), but are still capable of degrading LMW peptide substrates, provided that the substrate reaches the active site of the MMPs in α_2M/MMP complexes (23). Our results on the degradation of a natural substrate of MMPs (collagen type I) or a modified HMW protein substrate (UKcol) confirm these findings. Neither collagen type I nor UKcol was degraded by MMPs in the presence of α_2M, whereas full degradation was achieved in the absence thereof. On the contrary, LMW fluorogenic substrates were easily degraded by MMPs in the presence or absence of α_2M, as such demonstrating the feasibility of using LMW fluorogenic substrates for MMP activity detection in the presence of α_2M.

Previously we have shown that MMP activity measured in the systemic circulation using LMW fluorogenic substrates is likely to originate from α_2M/MMP complexes (Beekman *et al.*, 1999). In the present study, we provided further evidence of α_2M/MMP complex formation in the systemic circulation using size exclusion analysis. According to our working hypothesis, activated MMP form stable complexes with α_2M (approximate MW of 725 kDa). Using 100 kDa cut-off filters we showed that after spiking of activated MMP to buffer in the presence of α_2M, MMP activity is indeed found in the HMW (> 100 kDa) fraction, indicating α_2M/MMP complex formation. Similar results were seen after the filtration of normal human plasma, which was spiked with activated MMP-13. Furthermore, MMP activity was measured after size separation by FPLC. This analysis showed a switch of MMP activity from the LMW fraction to HMW fractions after incubation of activated MMPs in buffer in the presence of α_2M. The same pattern of MMP activity distribution was found in normal human plasma spiked with activated MMPs. Moreover, the results showed that MMPs in the HMW fraction were still active towards a LMW substrate and not towards the HMW synthetic substrate UKcol. Taken together, these results confirm that in the systemic circulation activated MMPs are present in the form of α_2M/MMP complexes.

In addition, the results of the present study show that MMP activity levels in the systemic circulation of RA patients are increased when compared to those in healthy controls. As such, these findings are in line with the current view on the pathological process in RA. Martel-Pelletier *et al.* (8) suggested, that at the tissue level, the differential regulation of MMP and TIMP synthesis by IL-1 may promote cartilage degradation in RA by creating an imbalance between the level of MMPs and their tissue inhibitors. If this situation is reflected in the circulation, the excess of activated MMPs will result in α_2M/MMP complex formation, which could explain the increased levels of α_2M/MMP complexes found in the serum of RA patients.

It can be questioned where the increased MMP levels present in the systemic circulation found in this study originate from. Firstly, it is possible that MMPs are produced as a systemic response to the joint inflammation (27). Secondly, a leakage of MMPs may occur from the inflamed joints into the systemic circulation (28). Based on measurements of MMP activity in plasma after spiking with active MMPs we conclude that the surplus of active MMPs will be entrapped in α_2M regardless their origin. Additional studies are needed to investigate the origin of the MMPs in complexes with α_2M, i.e. to investigate whether the MMPs present in the systemic circulation of RA patients represent the local situation in the inflamed joint.

Nowadays, serum MMP antigen levels are used to study the status of the proteolytic system, which is directly involved in joint tissue degradation. Correlations have been found between antigen levels of proMMP-3 and the development of radiological damage in early arthritis (8), proMMP-2 levels and joint erosion during early synovitis (29), and proMMP-1 levels and the number of new joint erosions (30). However, the proMMP antigen levels represent mainly the potential of the proteolytic system to degrade joint tissues. The present study shows that MMP activity measurements in α_2M/MMP complexes in the systemic circulation may in fact reflect the end status of the proteolytic system, e.g. the end status of the system after production, activation and inhibition of MMPs. Moreover, the results of the present study show that MMP activity levels in the circulation are correlated with an inflammatory marker, ESR, which is widely used to asses the disease activity. These results imply that activity of the proteolytic system is related to the inflammatory process in RA. Further studies will give insight into the association between cartilage degradation and systemic MMP activity levels.

Assessment of MMP activity in serum of leflunomide-treated RA patients indicates, to our knowledge for the first time, the effect of therapy on net MMP activity. MMP activity in serum was significantly reduced after 16 weeks of leflunomide treatment, implicating lower amounts of α_2M/MMP complexes present in the systemic circulation, i.e. lower surplus of active MMPs. These findings are consistent with in vitro experiments showing a decrease in MMP and an increase in TIMP production by Leflunomide, leading to lower surplus of active MMP (26).

In conclusion, the present study shows that MMPs form complexes with α_2M in the systemic circulation of RA patients and that levels of α_2M/MMP complexes are also increased in patients with higher inflammatory activity, as shown by correlation between α_2M/MMP levels and ESR. Our data provide a sufficient basis for further exploration of α_2M/MMP activity measurements as a biomarker for disease activity in RA.

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References

1. NAGASE H, WOESSNER JFJ: Matrix metalloproteinases. *J Biol Chem* 1999; 274: 21491-4.
2. NAGASE H, OKADA Y: Proteinases and matrix degradation. *In* KELLEY WN, HARRIS ED, RUDDY S and SLEDGE CB (Eds.): *Text-*

book of Rheumatology. Philadelphia, W.B. Saunders, 1998: 323-41.
3. BRESNIHAN B: Pathogenesis of joint damage in rheumatoid arthritis. *J Rheumatol* 1999; 26: 717-9.
4. MURPHY G, KNAUPER V, ATKINSON S *et al.*: Matrix metalloproteinases in arthritic disease. *Arthritis Res* 2002; 4 (Suppl. 3): S39-49.
5. KONTTINEN YT, AINOLA M, VALLEALA H *et al.*: Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. *Ann Rheum Dis* 1999; 58: 691-7.
6. YAMANAKA H, MATSUDA Y, TANAKA M *et al.*: Serum matrix metalloproteinase 3 as a predictor of the degree of joint destruction during the six months after measurement, in patients with early rheumatoid arthritis. *Arthritis Rheum* 2000; 43: 852-8.
7. POSTHUMUS MD, LIMBURG PC, WESTRA J *et al.*: Serum matrix metalloproteinase 3 in early rheumatoid arthritis is correlated with disease activity and radiological progression. *J Rheumatol* 2000; 27: 2761-8.
8. MARTEL-PELLETIER J, MCCOLLUM R, FUJIMOTO N *et al.*: Excess of metalloproteases over tissue inhibitor of metalloprotease may contribute to cartilage degradation in osteoarthritis and rheumatoid arthritis. *Lab Invest* 1994; 70: 807-15.
9. BURGER D, REZZONICO R, LI JM *et al.*: Imbalance between interstitial collagenase and tissue inhibitor of metalloproteinases 1 in synoviocytes and fibroblasts upon direct contact with stimulated T lymphocytes: Involvement of membrane-associated cytokines. *Arthritis Rheum* 1998; 41: 1748-59.
10. VAN DER LAAN WH, GOOSSENS PH, PIETERMAN EJ *et al.*: More profound inhibitory effect of TIMP-3 gene transfer than TIMP-1 gene transfer on synovial fibroblast invasion. *Arthritis Rheum* 2000; 43, 9 (Suppl.): S170.
11. YOSHIHARA Y, NAKAMURA H, OBATA K *et al.*: Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. *Ann Rheum Dis* 2000; 59: 455-61.
12. MOESTRUP SK, HOLTET TL, ETZERODT M *et al.*: Alpha 2-macroglobulin-proteinase complexes, plasminogen activator inhibitor type-1-plasminogen activator complexes, and receptor-associated protein bind to a region of the alpha 2-macroglobulin receptor containing a cluster of eight complement-type repeats. *J Biol Chem* 1993; 268: 13691-6.
13. GRINNELL F, ZHU M, PARKS WC: Collagenase-1 complexes with alpha2-macroglobulin in the acute and chronic wound environments. *J Invest Dermatol* 1998; 110: 771-6.
14. EKEROT L, OHLSSON K: Protease inhibitors in rheumatoid synovial fluid. Analyses of electrophoretic homogeneity and protease inhibitory capacity. *Rheumatol Int* 1982; 2: 21-6.
15. BEEKMAN B, DRIJFHOUT JW, RONDAY HK *et al.*: Fluorogenic MMP activity assay for plasma including MMPs complexed to alpha 2-macroglobulin. *Ann NY Acad Sci* 1999; 878: 150-8.
16. BEEKMAN B, DRIJFHOUT JW, BLOEMHOFF W *et al.*: Convenient fluorometric assay for matrix metalloproteinase activity and its application in biological media. *FEBS Lett* 1996; 390: 221-5.
17. DRIJFHOUT JW, NAGEL JBB, TEKOPPELE JM, BLOEMHOFF W: Solid phase synthesis of peptides containing the fluorescence energy transfer Dabcyl-EDANS coupe. *In* KAUMAYA PTP and HODGES RS (Eds.): *Peptides, Chemistry, Structure and Biology*. Mayflower Scientic Ltd., 1996: 129-31.
18. MURPHY G, WILLENBROCK F: Tissue inhibitors of matrix metalloendopeptidases. *Methods Enzymol* 1995; 248: 496-510.
19. DEGROOT J, VERZIJL N, WENTING-VAN WIJK MJ *et al.*: Age-related decrease in susceptibility of human articular cartilage to matrix metalloproteinase-mediated degradation: the role of advanced glycation end products. *Arthritis Rheum* 2001; 44: 2562-71.
20. HANEMAAIJER R, VISSER H, KONTTINEN YT *et al.*: A novel and simple immunocapture assay for determination of gelatinase-B (MMP-9) activities in biological fluids: Saliva from patients with Sjögren's syndrome contain increased latent and active gelatinase-B levels. *Matrix Biol* 1998; 17: 657-65.
21. LARD LR, BOERS M, VERHOEVEN A *et al.*: Early and aggressive treatment of rheumatoid arthritis patients affects the association of HLA class II antigens with progression of joint damage. *Arthritis Rheum* 2002; 46: 899-905.
22. ARNETT FC, EDWORTHY SM, BLOCH DA *et al.*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
23. BARRETT AJ: Alpha 2-macroglobulin. *Methods Enzymol* 1981; 80 Pt C: 737-54.
24. WU SM, PIZZO SV: Mechanism of hypochlorite-mediated inactivation of proteinase inhibition by alpha 2-macroglobulin. *Biochemistry* 1999; 38: 13983-90.
25. VERHEIJEN JH, NIEUWENBROEK NM, BEEKMAN B *et al.*: Modified proenzymes as artificial substrates for proteolytic enzymes: colorimetric assay of bacterial collagenase and matrix metalloproteinase activity using modified pro-urokinase. *Biochem J* 1997; 323: 603-9.
26. DEAGE V, BURGER D, DAYER JM: Exposure of T lymphocytes to leflunomide but not to dexamethasone favors the production by monocytic cells of interleukin-1 receptor antagonist and the tissue-inhibitor of metalloproteinases-1 over that of interleukin-1beta and metalloproteinases. *Eur Cytokine Netw* 1998; 9: 663-8.
27. MANICOURT DH, FUJIMOTO N, OBATA K *et al.*: Levels of circulating collagenase, stromelysin-1, and tissue inhibitor of matrix metalloproteinases 1 in patients with rheumatoid arthritis. Relationship to serum levels of antigenic keratan sulfate and systemic parameters of inflammation. *Arthritis Rheum* 1995; 38: 1031-9.
28. CHEUNG NT, MATTEY DL, DAWES PT *et al.*: Serum pro-matrix metalloproteinase 3 in rheumatoid arthritis: A reflection of local or systemic inflammation ? *Arthritis Rheum* 1996; 39: 884-6.
29. GOLDBACH-MANSKY R, LEE JM, HOXWORTH JM *et al.*: Active synovial matrix metalloproteinase-2 is associated with radiographic erosions in patients with early synovitis. *Arthritis Res* 2000; 2: 145-53.
30. CUNNANE G, FITZGERALD O, BEETON C *et al.*: Early joint erosions and serum levels of matrix metalloproteinase 1, matrix metalloproteinase 3, and tissue inhibitor of metalloproteinases 1 in rheumatoid arthritis. *Arthritis Rheum* 2001; 44: 2263-74.