Concise report

Leflunomide and methotrexate reduce levels of activated matrix metalloproteinases in complexes with α_2 macroglobulin in serum of rheumatoid arthritis patients

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ABSTRACT

Objective: To analyse the effects of leflunomide and methotrexate treatment on matrix metalloproteinase (MMP) activity levels in α_2 macroglobulin/MMP (α_2M/MMP) complexes in the systemic circulation of rheumatoid arthritis (RA) patients.

Methods: A total of 102 RA patients from a prospective, double-blind, randomised clinical trial comparing leflunomide and methotrexate were selected; clinical data and blood samples were collected at baseline, at 4 months and at 1 year. Serum MMP activity levels in α_2M were quantified using low molecular weight fluorogenic substrates, indicating the proportion of activated MMPs that were not inhibited by specific tissue inhibitors of MMP (TIMP).

Results: Patients had active disease as shown by high disease activity score (DAS, mean of 6.9 and 7.0 for methotrexate and leflunomide patients respectively), which was reduced over the study period (4.2 and 5.2 respectively, $p<0.001$). In leflunomide-treated patients a significant reduction of MMP activity levels was observed as early as at the 4 months timepoint persisting thereafter, whereas in methotrexate-treated patients the reduction was seen at 1 year.

Conclusion: The results show that systemic levels of activated MMPs are reduced in RA patients upon exposure to leflunomide or methotrexate.

Rheumatoid arthritis (RA) is a chronic disease characterised by systemic inflammation and joint damage mediated by various mechanisms among which increased activity of proteolytic systems. Of these proteolytic systems, matrix metalloproteinases (MMPs) play a key role. MMPs are a group of Zn^{2+} dependent extracellular enzymes that are involved in a normal and pathological tissue remodelling.[1] MMPs are produced as pro-enzymes and most of them are activated extra-cellularly after secretion.[1] The proteolytic action of activated MMPs is controlled by their specific inhibitors (tissue inhibitors of MMPs, TIMPs) and a general protease scavenger, α_2 macroglobulin (α_2M). In pathological joint conditions such as RA, there is an imbalance between the levels of MMPs and TIMPs in favour of MMPs.

RA is in general controlled by disease modifying anti-rheumatic drugs (DMARDs), which have effects on both inflammation and structural joint damage. One of the widely used DMARDs, leflunomide, has been shown to be effective on both signs and symptoms and radiological damage in patients with RA.[2 3] In vivo, leflunomide acts as a pro-drug and is quickly metabolised into the active metabolite A77-1726 in the gut wall and liver. In vitro, A77-1726 was shown to inhibit dihydroorotate dehydrogenase (DHODH), by which leflunomide influences the de-novo pyrimidine biosynthesis, and to interact with primary and secondary signalling events.[4 5] The gold standard DMARD for the treatment of RA in the past decade has been methotrexate (MTX), and as such it was used as the comparator drug for leflunomide in phase III clinical trials. Currently, the mechanism of action of methotrexate in RA is not completely understood.[6]

As leflunomide has been proven to effectively slow down radiological joint damage progression and MMPs are involved in joint degradation, the present study was designed to investigate the in vivo effects of leflunomide on MMP levels. The level of activated (but not TIMP inhibited) MMP, which forms complexes with α_2M were measured in systemic circulation of RA patients participating in a randomised, controlled clinical trial comparing leflunomide to methotrexate.

MATERIALS AND METHODS

In vivo study

A total of 102 (53 leflunomide and 49 methotrexate treated) patients were selected from 999 RA patients who participated in a prospective, double-blind, randomised clinical trial comparing leflunomide and methotrexate.[7] Only sites with large numbers of patients enrolled were selected. For all 102 patients serum samples from baseline, after 4 months, and after 1 year were available and tested. Clinical variables included the disease activity score (DAS) and C-reactive protein (CRP), measured at identical timepoints. Patients were treated with either leflunomide 20 mg/day (after a loading dose of 100 mg/day for the first 3 days) or methotrexate 15 mg/week (initial dose 7.5 mg/week, increase to 10 mg/week 4 weeks after baseline, and increase to 15 mg/week 8 weeks after baseline).

MMP activity measurements in α2M/MMP complexes in serum

MMP activity was measured using 6.25 μM (all concentrations are final) TNO211-F substrate in the presence or absence of 5 μM BB94 (a general MMP inhibitor) as described previously.[8] In short, serum samples were diluted in MMP buffer and

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EDTA-free Complete™ (serine and cysteine proteases inhibitor, Roche, Mannheim, Germany) was added to all conditions. 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, California, USA). All MMP activity measurements were performed in a total volume of 80 µl in black clear-bottom 384 well plates (Dynatech, Denkendorf, Germany).

Statistics

MMP levels per treatment group in time were analysed using the Friedman test and Wilcoxon signed ranks test. Leflunomide and methotrexate groups were compared using a Mann–Whitney U Test. Correlations for non-parametric data were evaluated by calculating the Spearman rank correlation coefficients. All tests were two-tailed and $p \leqslant 0.05$ was considered significant. The statistical analysis was performed using SPSS V.12.0.1 (Chicago, Illinois) statistical software.

RESULTS

Study patients

Demographic and clinical data are depicted in table 1. All patients had very active disease at baseline, as measured by the disease activity score (DAS) of 6.9 (1) (mean (SD)) for the leflunomide patients and 7.0 (0.9) for the methotrexate patients, with a significant reduction after 4 months, and after 1 year in leflunomide and methotrexate-treated patients (table 1). The C-reactive protein (CRP) levels were significantly reduced in leflunomide and methotrexate patients (table 1). In line with previous results, the CRP levels were significantly lower after 1 year of treatment in methotrexate group as compared to leflunomide treatment group ($p = 0.014$).[9]

MMP activity levels in α_2M/MMP complexes

Table 1 shows MMP activity levels in α_2M/MMP complexes (median, 25th–75th percentiles) in leflunomide and methotrexate-treated patients. At baseline, MMP levels were slightly higher in leflunomide patients, although the difference just did not reach the level of statistical significance ($p = 0.05$, Mann–Whitney U test). After 4 months treatment, MMP levels were significantly reduced in leflunomide-treated patients, whereas methotrexate patients group showed no changes. At 1 year, MMP levels remained decreased in leflunomide-treated patients. In methotrexate-treated patients there was a significant reduction in MMP levels at 1 year as compared to baseline; MMP levels at the 1-year timepoint were comparable between MTX and leflunomide groups.

MMP levels at baseline were significantly correlated with DAS in MTX and leflunomide treatment groups (table 1, $p = 0.021$ and $p<0.001$, respectively). No correlations were seen at later time-points. No relationship was found between high CRP levels and MMP activity levels in both groups.

DISCUSSION

The present study shows that levels of α_2M/MMP complexes are significantly reduced in RA patients upon treatment with leflunomide or methotrexate after 1 year of treatment. A significant decrease of 30% in α_2M/MMP levels in leflunomide-treated RA patients was seen as early as 4 months after the initiation of the treatment and remained at this level for the duration of the study. In the methotrexate group, MMP activity levels did not change in the first 4 months but were significantly reduced at 1 year; at this timepoint MMP levels measured in the leflunomide and methotrexate groups were similar.

The observed differences in time of the reduction reflect the early onset of leflunomide compared to methotrexate as observed in phase III clinical trials and in two small studies focusing on MMP-1/TIMP-1 activity in synovial tissue samples and MRI.[3 9 10] Although these differences could be compound specific, the precise nature of the detected differences needs further investigation.

The 1-year results imply that methotrexate and leflunomide activity is comparable with regard to the reduction of MMP levels in the systemic circulation of RA patients. The observation of lower baseline levels of MMP activity in α_2M/MMP complexes in MTX-exposed patients as compared to the leflunomide group is unlikely to influence the overall results as patients were randomly assigned to the study treatment groups.

The mode of action of the active metabolite of leflunomide (A77–1726) in relation to effects on MMPs has been extensively studied in vitro.[4 5] Addition of A77–1726 to synovial fibroblast culture leads to reduction in the amount of proMMP-3 produced by these cells and A77–1726 induces reduction in proMMP-1 production by synoviocytes.[10 11] In RA patients, immunohystochemical techniques used on synovial tissue biopsy samples identified that leflunomide not only reduces

Table 1 Demographics, clinical data and markers measurements of the studied rheumatoid arthritis (RA) patients

	Leflunomide, n = 53					Methotrexate, n = 49				
Age (years, mean (SD))*	62 (8)					58 (12)				
Disease duration (months, mean (SD))	49 (39)					42 (36)				
Gender (m/f)	43/10					36/13				
	Baseline	4 Months	p_1	1 Year	p_2	Baseline	4 Months	p_1	1 Year	p_2
DAS (mean (SD)	6.9 (1)	5.6 (1.6)	<0.001	5.2 (1.5)	<0.001	7.0 (0.9)	5.2 (1.6)	<0.001	4.4 (1.4)	<0.001
CRP (mg/L)	2.7 (0.7–5.8)	0.5 (0.2–1.6)	<0.001	0.6 (0.1–1.9)	<0.001	2.6 (0.8–6.6)	1.1 (0.6–2.1)	<0.001	0.6 (0.6–1.1)	<0.001
α_2M/MMP (RFU/s)	17.8 (13–25)	11.6 (9–17)	<0.001	12.0 (7–19)	<0.001	14.2 (10–22)	14.4 (9–20)	0.353	11.7 (9–15)	0.007
Correlations of α_2M/MMP levels with:										
DAS, r (P)	0.498 (<0.001)	0.230 (0.108)		0.284 (0.053)		0.329 (0.021)	0.219 (0.135)		−0.36 (0.810)	
CRP, r (P)	0.222 (0.113)	0.23 (0.869)		0.44 (0.760)		−0.38 (0.797)	0.271 (0.69)		0.109 (0.460)	

C-reactive protein (CRP) and MMP activity in α_2M/MMP complexes: median (25th–75th percentiles). p_1 And p_2 show significance of the found differences at 4 month and 1 year, respectively, vs baseline. Inter-group differences were studied using Friedman test and Wilcoxon signed ranks test for non-parametric data. One-way ANOVA with post-hoc analysis was used for normally distributed data. Correlations were calculated using Spearman rho. DAS, disease activity score.

proMMP-1 levels in synovial tissue but also seems to restore MMP/TIMP imbalance.[3] In this latter study the beneficial effects of MTX on restoration of this balance as previously reported were confirmed.[12] The results of the present study collaborate and further extend these findings by showing that treatment of RA patient indeed results in a decreased amount of activated but not TIMP inhibited MMPs in complex with α_2M.

With other proteolytic enzymes, MMPs are thought to play a crucial role in joint tissue degradation. The importance of tissue inhibitors of MMPs and MMP/TIMP balance in arthritic diseases has been reported previously.[12 13] Based on molar ratios it was calculated that TIMP levels would be insufficient to counteract the increased production of the proMMPs, which in turn may lead to excessive cartilage degradation by proteolytic enzymes.[14 15] Thus, restoration of MMP/TIMP imbalance could be a valid goal in the treatment of RA patients.

Although MMP levels were decreased in the present study, as well as levels of systemic inflammatory marker C-reactive protein, no correlation was found between these parameters. These results indicate the need to further investigate the effects of the study compounds on the CRP and MMP activity levels as a marker of not only disease activity but also treatment efficacy.

In conclusion, the present study provides evidence of the decrease of α_2M/MMP complexes in the systemic circulation of the RA patients upon treatment with either leflunomide or methotrexate.

Competing interests: None declared

REFERENCES

1. **Murphy G,** Knauper V, Atkinson S, Butler G, English W, Hutton M, *et al*. Matrix metalloproteinases in arthritic disease. *Arthritis Res* 2002;**4**(Suppl 3):S39–49.
2. **Weinblatt ME,** Kremer JM, Coblyn JS, Maier AL, Helfgott SM, Morrell M, *et al*. Pharmacokinetics, safety, and efficacy of combination treatment with methotrexate and leflunomide in patients with active rheumatoid arthritis. *Arthritis Rheum* 1999;**42**:1322–8.
3. **Kraan MC,** Reece RJ, Barg EC, Smeets TJ, Farnell J, Rosenburg R, *et al*. Modulation of inflammation and metalloproteinase expression in synovial tissue by leflunomide and methotrexate in patients with active rheumatoid arthritis. Findings in a prospective, randomized, double-blind, parallel-design clinical trial in thirty-nine patients at two centers. *Arthritis Rheum* 2000;**43**:1820–30.
4. **Chong AS,** Finnegan A, Jiang X, Gebel H, Sankary HN, Foster P, *et al*. Leflunomide, a novel immunosuppressive agent. The mechanism of inhibition of T cell proliferation *Transplantation* 1993;**55**:1361–6.
5. **Manna SK,** Aggarwal BB. Immunosuppressive leflunomide metabolite (A77 1726) blocks TNF-dependent nuclear factor-kappa B activation and gene expression. *J Immunol* 1999;**162**:2095–102.
6. **Cronstein BN.** Molecular therapeutics. Methotrexate and its mechanism of action. *Arthritis Rheum* 1996;**39**:1951–60.
7. **Emery P,** Breedveld FC, Lemmel EM, Kaltwasser JP, Dawes PT, Gömör B, *et al*. A comparison of the efficacy and safety of leflunomide and methotrexate for the treatment of rheumatoid arthritis. *Rheumatology* 2000;**39**:655–65.
8. **Tchetverikov I,** Lard LR, DeGroot J, Verzijl N, TeKoppele JM, Breedveld FC, *et al*. Matrix metalloproteinases-3,-8,-9 as markers of disease activity and joint damage progression in early rheumatoid arthritis. *Ann Rheum Dis* 2003;**62**:1094–9.
9. **Smolen JS,** Emery P. Efficacy and safety of leflunomide in active rheumatoid arthritis. *Rheumatology* 2000;**39**(Suppl 1):48–56.
10. **Burger D,** Begue-Pastor N, Benavent S, Gruaz L, Kaufmann MT, Chicheportiche R, *et al*. The active metabolite of leflunomide, A77 1726, inhibits the production of prostaglandin E(2), matrix metalloproteinase 1 and interleukin 6 in human fibroblast-like synoviocytes. *Rheumatology* 2003;**42**:89–96.
11. **Elkayam O,** Yaron I, Shirazi I, Judovitch R, Caspi D, Yaron M. Active leflunomide metabolite inhibits interleukin 1beta, tumour necrosis factor alpha, nitric oxide, and metalloproteinase-3 production in activated human synovial tissue cultures. *Ann Rheum Dis* 2003;**62**:440–3.
12. **Firestein GS,** Paine MM. Stromelysin and tissue inhibitor of metalloproteinases gene expression in rheumatoid arthritis synovium. *Am J Pathol* 1992;**140**:1309–14.
13. **Martel-Pelletier J,** McCollum R, Fujimoto N, Obata K, Cloutier JM, Pelletier JP. Excess of metalloproteases over tissue inhibitor of metalloprotease may contribute to cartilage degradation in osteoarthritis and rheumatoid arthritis. *Lab Invest* 1994;**70**:807–15.
14. **Ishiguro N,** Ito T, Obata K, Fujimoto N, Iwata H. Determination of stromelysin-1, 72 and 92 kDa type IV collagenase, tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-2 in synovial fluid and serum from patients with rheumatoid arthritis. *J Rheumatol* 1996;**23**:1599–604.
15. **Yoshihara Y,** Nakamura H, Obata K, Yamada H, Hayakawa T, Fujikawa 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.