

Inhibitory effects of leflunomide therapy on the activity of matrix metalloproteinase-9 and the release of cartilage oligomeric matrix protein in patients with rheumatoid arthritis

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Abstract

Objective

To determine the effects of the disease modifying antirheumatic drug (DMARD) leflunomide on the expression of the matrix metalloproteinase MMP-1 (collagenase) and the activity of MMP-9 that are believed to play a major role in cartilage destruction associated with inflammation in patients with rheumatoid arthritis (RA). Serum concentrations of cartilage oligomeric matrix protein (COMP) should offer promise for monitoring tissue degradation in the RA joints during a 6-month therapy with leflunomide.

Methods

Thirty-six patients with RA meeting the ACR-criteria were recruited for the study in a multicentre trial. A dose of 20 mg leflunomide/day (after a 3-day 100 mg/day loading dose), an isoxazole derivate and inhibitor of the "de novo" pyrimidine synthesis, was administered for a study period of 6 months. MMP-1, the activity of MMP-9 and COMP values were measured in serum by enzyme immuno assay. The very sensitive acute phase protein serum amyloid A (SAA) was also determined by EIA. The measurements were performed before and after 3 and 6 months of leflunomide therapy.

Results

High levels of active MMP-9, COMP and SAA were detected in the sera of the patients with RA prior to the start of the leflunomide therapy compared to normal control sera. A significant reduction of the MMP-9 activity levels was seen after 3 months immunomodulation with leflunomide and was maintained after 6 months ($p < 0.01$). The degradation marker COMP and the inflammation marker SAA decreased significantly after 6 months ($p < 0.04$, respectively $p < 0.01$). There was also an insignificant tendency of MMP-1 reduction in serum after 6 months.

Conclusion

This study demonstrated that a DMARD therapy with leflunomide can cause positive effects on cartilage degradation and inflammation achieving reductions in the acute phase protein SAA, the enzymatic attack of MMPs and the loss of the cartilage matrix component COMP.

Key words

Leflunomide, cartilage oligomeric matrix protein, metalloproteinases, rheumatoid arthritis, serum amyloid A.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease with systemic disturbances of the immunosystem and inflammatory mechanisms resulting in an erosive synovitis, cartilage degeneration and joint destruction. Both inflammation and destruction lead to functional impairment and disability. RA is associated with premature morbidity leading to an immense socioeconomic burden (1). There is, therefore, a need for new effective agents that can provide high efficacy combined with good safety. Therapeutic intervention early in the disease course of RA with disease modifying antirheumatic drugs (DMARDs) can lead to disease control and less joint damage. In the last years there has been a development of new therapeutic agents for RA like the inhibitor of the de novo pyrimidine synthesis leflunomide (2, 3, 4). Leflunomide is now an approved safe and effective agent in the treatment of RA - its clinical benefit being sustained over 24 months (5, 6).

In vitro findings on cultured human articular cartilage suggest that leflunomide is able to protect cartilage matrix from degradative factors induced by interleukin-1 β (7).

The destruction of cartilage is thought to involve the actions of the matrix metalloproteinases (MMPs), which are proteolytic enzymes released by a wide range of cells including fibroblasts, mononuclear phagocytes like monocytes/macrophages and chondrocytes in response to proinflammatory cytokines (8).

Expression of MMPs like MMP-1 and MMP-9 is considerably enhanced in inflammatory joint diseases including RA (9). Especially human macrophages synthesize and secrete several MMPs that are structurally related and participate in the degeneration of extracellular components (10, 11). The MMPs secreted by macrophages include interstitial collagenase (MMP-1) and 92-kd gelatinase (MMP-9). MMP-9 degrades components of extracellular matrix with high specificity for denatured collagen (gelatin) and can cleave native collagens of types IV, V and XI and elastin but not proteoglycans and

type I collagen (10). MMP-9 also plays an active role in cellular diapedesis, augmentation of cellular invasion and tissue degeneration via inappropriate turnover of the connective tissue matrix (12). The MMP induced destruction of cartilage represents the consequence of an inflammatory process in which the normally fine tuned balance between matrix synthesis and degeneration is disturbed. Investigations on stimulated rheumatoid synovial fibroblasts (13) suggest that the suppression of MMP synthesis is a possible mechanism for the inhibitory activity of leflunomide against rheumatoid arthritis.

One approach to study cartilage turnover in RA is to identify molecules which are primarily present in cartilage matrix and to investigate whether serum levels of these molecules reflect tissue degeneration processes (14).

Cartilage oligomeric matrix protein (COMP), a component of the extracellular matrix of articular cartilage, is a pentameric non-collagenous glycoprotein belonging to the thrombospondin protein family (15); sometimes referred as TSP-5 (16, 17).

Increased serum concentrations of COMP are of prognostic value for the later course of RA (18). A high clinical disease activity (DAS) correlates with high COMP levels in serum and with increased proteolytic activity and joint destruction (19). Increased COMP levels may be indicative of synovitis which could directly or indirectly contribute to joint erosion (20).

The aim of the present study was to evaluate the effects of leflunomide therapy on the expression of MMPs and COMP to have more insight in possible therapeutic induced changes of the erosive process in patients with RA.

Methods

Patients

Thirty-six patients (8 male, 28 female) were included in the study. All fulfilled the criteria of the American College of Rheumatology (ACR) for the diagnosis of a definite or classic RA and all gave informed consent before entering the study. The study was conducted according to the principles of the World

Medical Association Declaration of Helsinki revised version of Venice, Italy in 1983 and Edinburgh, Scotland in 2000 and applicable regulatory requirements in each participating centre. Consenting patients were required to use adequate methods of contraception and pregnant or lactating women were excluded. We excluded patients who had been treated with biologicals.

Study design

This 6-month study was a multicentre open evaluation of the expression of metalloproteinases, the presence of COMP in serum and the clinical response upon the treatment of RA with leflunomide.

Leflunomide (Arava tablets; Aventis Pharma, Germany GmbH., Frankfurt/Main) was started with a dose of 100 mg daily for the first 3 days, then 20 mg/day for half a year. Concomitant non-steroidal anti-inflammatory drug (NSAID) treatment was allowed during the trial period.

Clinical outcome with respect to the response to leflunomide was controlled by the physician at each visit using a 4-point rating scale. None of the patients had overtaken leflunomide. Safety was monitored by clinical physical examination, radiography, sonography, and standard hematological and serum biochemical tests. The occurrence of adverse reactions was documented.

Blood samples

Blood for the laboratory determinations was taken before the first leflunomide dose (baseline), at 3 months and after 6 months therapy.

Analysis/immunoassays

Besides routine laboratory measurements, samples of patient sera were subjected to specific enzyme-linked immunosorbent assays (ELISAs) for the determination of serum amyloid A (human SAA; BioSource Europe SA., Belgium), MMP-1 (matrixmetalloproteinase-1; human Biotrak ELISA system, Amersham Biosciences Europe GmbH., Germany), active MMP-9 (matrixmetalloproteinase-9 activity assay system; Biotrak cellular communication assay; Amersham Biotech Europe GmbH., Germany). These three special tests were performed in the Boltzmann Institute Saalfelden to whom all patient recruiting centres sent the collected frozen samples.

Statistics

With the statistical program packages MedCalc Statistics for Biomedical Research Vs. 5.0 (MedCalc Software, Belgium) and Systat 9.0 Statistics (SPSS Inc., USA) parametric and non-parametric methods were used as appropriate (Pearson's Correlation (parametric), Spearman's Correlation (non-parametric), Wilcoxon, Mann-Whitney U-Test, student's t-test, descriptive statistics).

Results

The assessment of the clinical success of leflunomide therapy is listed in Table III. It shows clearly, that the efficiency of the therapy improves distinctly from 3 to 6 months. In nearly 40% of the RA patients the clinical success was rated with "very good" after half a year, poor success was only

found in 9.5% of the cases.

High concentrations of MMP-1, active MMP-9, COMP, and SAA were determined prior to therapy (Table I). The serum levels of MMP-9 (mean 176 ng/ml) were more than 4 times higher than in healthy probands who had values of about 40 ng/ml. Also the levels of MMP-1, which are usually below or about the sensitivity of the assay (1.7 mg/ml) in healthy controls, were distinctly elevated in the observed patients with RA having levels about 11 ng/ml. We measured high serum concentrations of the cartilage degradation marker COMP with 10.4 U/l in mean (Table I). After 3 months we found the high activity of MMP-9 to be significantly reduced ($p < 0.01$). This improvement persisted and after 6-month therapy MMP-9 was even reduced to a mean value of 122.6 ± 12.1 ng/ml. After half a year also the MMP-1 mean levels, staying unchanged during 3 months, decreased slightly (from 11.9 to 9.6 ng/ml) but not significantly. However, the COMP levels decreased continuously during leflunomide therapy with a significant result after 6 months ($p < 0.04$). As expected, the inflammation determined by the acute phase protein SAA was downregulated significantly after 26 weeks therapy (Table I).

The results of a correlation analysis at month 6 are shown in Table II. We found a closely correlation of MMP-1 and COMP with the acute phase protein SAA; active MMP-9, however, showed no correlation with SAA, MMP-1, or clinical success. A poor but significant relationship of the cartilage degradation marker COMP was found

Table I. Serum values (mean \pm standard error of the mean) of matrixmetalloproteinase-1 (MMP-1), active matrixmetalloproteinase-9 (MMP-9 activity), cartilage oligomeric matrix protein (COMP) and serum amyloid A (SAA) in patients with rheumatoid arthritis during 6 months therapy with leflunomide; significance to baseline: * $p < 0.05$ ** $p < 0.01$.

		LEFLUNOMIDE THERAPY						normal mean values (healthy controls)
		day 0 prior to therapy		after 3 months		after 6 months		
	units	mean	SEM	mean	SEM	mean	SEM	
MMP-1	ng/ml	11.9	2.1	11.6	1.9	9.6	1.8	2
MMP-9 activity	ng/ml	176	15.7	125.4**	13.6	122.6**	12.1	44.2
COMP	U/l	10.4	0.62	9.8	0.89	8.5*	0.92	8
SAA	ng/ml	72.6	15.4	59.2	23.4	46.6**	14.6	< 10

with clinical success and SAA. Also a weak correlation of the clinical outcome to the MMP-1 levels ($p < 0.05$) occurred.

Twenty-five percent of the patients suffered from undesired side effects such as diarrhea, nausea, stomatitis, alopecia, vertigo, or exanthema; only in 2 cases the DMARD therapy had to be stopped because of lack of efficacy.

Discussion

Metalloproteinases like MMP-9 play a key role in the destruction of joint structures, a phenomenon frequently observed in patients with inflammatory arthritides such as RA (21). In contrast to MMP-1 and MMP-3, which are produced in the synovial tissues, MMP-9 is produced by infiltrating cells of the monocyte/macrophage lineage (10, 22). A predominant 92-kd gelatinase B activity is evident in RA plasma samples and MMP-9 is increased in RA plasma versus normal plasma (12). Giannelli *et al.* (23) detected MMP-9 not only in latent but in its active form in patients with rheumatoid arthritis in serum and synovial fluids.

We found similar results with high concentrations of active MMP-9 in our RA patients prior to treatment with leflunomide, after 3 months therapy already the high activity of MMP-9 decreased significantly, improving again after 6 months leflunomide.

Elevated levels of MMP-9 have been demonstrated in patients with inflammatory diseases like RA; a correlation between the increased level of MMP-9 activity in RA and the severity of the disease has been found (9). In contrast to these results we were not able to verify a correlation of MMP-9 activity with the amount of the acute phase reactions or the clinical success despite of the MMP-9 reduction during the leflunomide therapy.

The increased presence of cartilage oligomeric matrix protein COMP in serum has been associated with accelerated joint damage in RA (20). Serum concentrations of fragments of the cartilage specific component COMP were found to be increased early in patients with RA who rapidly developed severe destructions in both large and small

Table II. Correlations between the values at month 6 for the main study parameters in RA patients receiving leflunomide DMARD-therapy.

CORRELATIONS		correlation coefficient (r)	significance (p)
from	to		
MMP-1	SAA	0.68	< 0.001
MMP-9 activity	SAA	0.15	n.s.
SAA	CRP	0.54	< 0.01
MMP-9 activity	MMP-1	0.04	n.s.
COMP	MMP-1	0.47	< 0.01
COMP	SAA	0.33	< 0.05
COMP	MMP-9 activity	0.52	< 0.01
Clinical Outcome	SAA	0.56	< 0.01
Clinical Outcome	COMP	0.36	< 0.05
Clinical Outcome	MMP-9 activity	0.15	n.s.
Clinical Outcome	MMP-1	0.40	< 0.05

joints (18). In an animal model with an experimental collagen arthritis high serum levels of COMP in rats that had developed a histopathologically proven strong arthritis with high arthritis scores demonstrate COMP to be a marker of tissue destruction (14). A chronic erosive disease course with acute stage in rats with pristane induced arthritis could be distinguished by higher serum concentrations of COMP (24). Since synovial cells like fibroblasts can synthesize COMP and contribute to the pool of COMP, increased COMP levels may be indicative of synovitis beside its function as a cartilage tissue degradation marker (20).

Due to the selection of patients in our study who failed in other DMARD therapies before the treatment with leflunomide, we found higher mean levels of COMP compared to own results of an other study with more than 100 RA patients (25). But the weak correlation between COMP and SAA (Table II) indicates that COMP does not reflect the inflammatory component of the RA. Such lack of a correlation between inflammation measured by C-reactive protein and COMP levels described Roux-Lombard *et al.* (26). However in our study circulating COMP as a marker for structural damage of cartilage decreases. The results of the present study with significant

reduced COMP values in serum after 6 months of treatment with leflunomide are demonstrating leflunomide to be an efficacious drug that interferes with the mechanisms involved in destruction of joint integrity.

We measured in our study serum amyloid A (SAA), known as the most sensitive acute phase protein in plasma (27). Prolonged high plasma levels of SAA, a serum precursor of amyloid A protein, in chronic inflammation may lead to deposition of AA proteins in tissues (28).

Evidence that leflunomide inhibits the production of SAA is derived from recent studies of Migita *et al.* (29) on human hepatocytes stimulated with IL-1 β . Our in vivo results with significant reduction of SAA serum levels from 72.6 ng/ml (baseline) to 46.6 ng/ml after 6 months leflunomide help to confirm these findings.

In an immunohistochemical analysis on synovial tissue biopsy samples Kraan *et al.* (30) could demonstrate that leflunomide can reduce cellular infiltration that was in conjunction with reduced expression of the inflammatory cytokine TNF α and adhesion molecules (ICAM-1 and VCAM-1). Compared to methotrexate-treated patients, the authors observed in the biopsies of the leflunomide-treated patients a more pronounced reduction in the MMP-1: tissue inhibitor (TIMP)-1 ratio. Our

Table III. Clinical success of DMARD-therapy with leflunomide in RA.

	after 3 months	after 6 months
very good	23.3 %	38.1 %
good	26.7 %	19.1 %
moderate	33.3 %	33.3 %
poor	16.7 %	9.5 %

measurements of MMP-1 done in serum and not in tissue could demonstrate only a tendency of MMP-1 reduction that could not reach significance due to the variance depending on the little number of cases.

However the significant correlation of MMP-1 with SAA (Table II) in the present study indicates beneficial changes in joint inflammation and the matrix-degrading process under therapy with leflunomide.

MMP-3 production in synovial tissue explants was inhibited *in vitro* after incubation with different doses of leflunomide to demonstrate the modulatory effects on the mechanism of action (31). It should be noted that Migita *et al.* (13) found that leflunomide markedly inhibited MMP-1, -3 and -13 secretions in IL-1 stimulated rheumatoid synovial fibroblasts together with a suppression of the mitogen-activated protein kinase (MAPK) signalling pathway. These *in vitro* studies can explain the reduced MMP serum levels in our RA patients.

In conclusion, it was observed that the therapy with leflunomide over a 6-month therapy inhibits the activity of MMP-9 and the serum concentration of the cartilage degradation marker COMP, suggesting a possible mechanism by which leflunomide slows down joint erosions in RA. The positive effects on SAA are demonstrating leflunomide can cause reductions in the inflammatory process, too.

Further studies are needed to confirm these results in a greater collective.

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