

Comparative Study of Matrix Metalloproteinases in Synovial Fluid from Rheumatoid Arthritis and Arthrosis Patients

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Matrix metalloproteinases (MMPs) are the most important enzymes involved in joint cartilage degradation, both in healthy and ill persons, mainly in inflammatory joint diseases (rheumatoid arthritis - RA). We assessed the synovial fluid MMPs from RA and arthrotic patients by means of a modified Manicourt-Lefevre zymogen method. Our findings suggest that synovial MMPs and proMMPs can differentiate between RA and arthrosis. In RA patients we found that certain MMPs types correlated with the activity and severity of disease, as well as with treatment regimens.

Keywords: Matrix metalloproteinases, rheumatoid arthritis, arthrosis

The joints cartilage owes its stability to a balance between the rate of collagen and proteoglycans synthesis and degradation. During the phases of tissue growing and development the synthesis rate surpasses by far the degradation rate, but in pathologic conditions this ratio is reversed. The main cause of joint cartilage and subchondral bone degradation is the high local level of active proteinase that affect extracellular matrix collagens and proteoglycans. Several proteinases can attack the joint cartilage: aspartic proteinases, cystein proteinases, serine proteinases, matrix metalloproteinases [1-3]

Extracellular matrix metalloproteinases (MMPs) are the most important group of enzymes involved in extracellular matrix degradation. All MMPs share several common proprieties:

- the enzymes have 3 main structural domains: the NH₂ terminal propeptidic domain, the catalytic domain, the COOH-terminal hemopexin-like domain;
- the catalytic domain has 1 zinc atom connected with 3 histidin radicals, which lead to the term of metalloproteinases;
- the C-terminal domain influences the MMPs substrate specificity;
- MMPs are synthesized as proenzymes (proMMPs) which are subsequently activated by proteinases;
- MMPs activation is made by eliminating a highly conserved sequence containing cystein from the propeptidic domain;
- MMPs are inhibited by tissue inhibitors of MMPs (TIMPs) and by alpha₂-macroglobulin [1-3].

Most MMPs are not expressed in physiological conditions. Some of them, like MMP 13, are secreted only in pathological conditions like rheumatoid arthritis (RA) neoplasms etc. [1,2] Interleukin 1 (IL1), Tumor Necrosis Factor alpha (TNF α), Epidermal Growth Factor (EGF), basic Fibroblast Growth Factor (basic FGF), interferons stimulate MMPs secretion, while Transformation Growth Factor beta (TGF β), retinoic acid and corticoids inhibit it [1,2,4]. In joints MMPs are secreted by chondrocytes,

synovial fibroblasts, macrophages, lymphocytes, polymorphonuclears, mast cells, endothelial cells etc.

MMPs activation involves the splitting of a sequence from the NH₂-terminal domain by proteinases (plasmin, chimase, cathepsin G). MMPs activity is blocked by tissue inhibitors of MMPs (TIMP), which binds to the zinc atom. TIMP synthesis is stimulated by TGF β, IL-6, IL-11, steroids and is inhibited by lipopolysaccharides, cytochalasine etc. [2, 5]

High level and excessive MMPs activity are encountered in many rheumatic diseases. MMPs are considered to be the key mediators of joint damage in RA [6-8]

Experimental part

Our study had the following objectives:

- to assess the main MMPs found in the synovial fluid in patients with RA, comparing to arthrotic patients;
- to correlate the types of MMPs in RA patients synovial fluid with the activity and severity of the disease.

Material and methods: there were studied 19 patients - 17 females and 2 males- of whom 14 (12 females and 2 males) with RA and 5 with primary arthrosis. The patients were hospitalized from 1997 to 2001 in the Internal Medicine and Rheumatology Department of Dr. I. Cantacuzino Hospital from Bucharest. The patients were between 22 and 79 years old; the mean of the 2 groups were similar (56.41 +/- 16.27 years in RA patients, 51.75 +/- 8.22 years in arthrosis patients). The classification of patients as RA and primary arthrosis was based on the American College of Rheumatology criteria.

For the assessment of MMPs we used synovial fluid extracted by articular puncture. The fluid was processed in the laboratory of the National Institute of Biological Sciences of Bucharest. The synovial fluid was treated with type VI hyaluronidase from ox testicles at a final concentration of 30U/mL for an hour at ambient temperature, to reduce viscosity, and centrifuged at 2000g for 10 minutes at 4°C.

The cellular sediment was processed by an original procedure of extraction by cellular lysis. The supernatant

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- cell-free synovial fluid - was studied for MMPs specific gelatinase activity using SDS-PAGE substrate zymography (sodium dodecyl sulfate polyacrylamide gel electrophoresis), using Manicourt and Lefevre method modified by Cîmpean *et al* [9,10]. This method is an electroforetic technique used for assessing specific proteolytic activity for enzymes separated in polyacrilamid gel (PAGE) in non-reduction conditions. This method has a good sensibility allowing to identify a certain enzyme out of a complex mix of proteinases, as well as its mollecular mass of latent and active forms, by means of denaturation and renaturation process which takes place in the gel.

The lysis stripes can be induced by proMMPs and MMPs inactivated by TIMP, because the possibility of disociation of the MMPs/TIMP complexes in the denaturated tampons used in zimography has been demonstrated. This method doesn't allow TIMP identification, nor determination of their activity in the fluid sample. The denaturated type I collagen (type A gelatin from pig skin) was added to the standard Laemmle mixture for acrylamide polymerization from the separation gel, at a final concentration of 1mg/mL. The synovial fluid samples were combined in a 3:1 ratio with the sample's buffer (10% SDS; 4% Sucrose; 0,25 M Tri-HCl, pH 6,8) and immediately applied on the support gel, at a proteic concentration of 10 µg. The concentration of poliacylamide (PAA) in the separation gel was 9% and in the concentration gel was 4%. The migration was done at 4°C and 90 V for a duration between 90-120 min, until the migration front represented by the bromphenol blue reached the lower part of the gel. The polymerization of PAA gels was done using ammonium persulphate as initiator and N,N,N',N'-tetramethyldiamide (TEMED) as a catalyst. Migration was performed in a Hoefer vertical electrophoresis system (fig. 1).

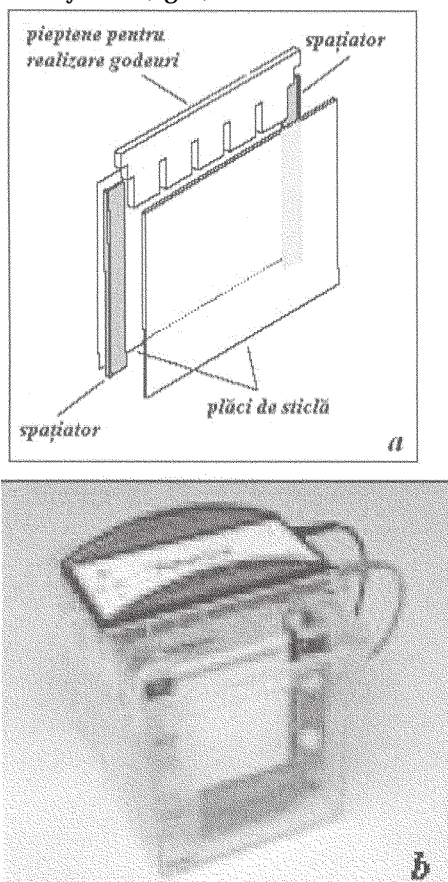


Fig. 1. a) Device for poliacylamide (PAA) gel preparation for vertical electrophoresis. b) Hoefer device for vertical gel electrophoresis

In order to eliminate the SDS and to renaturate the enzymes, after electrophoresis the gels were washed for one hour in a solution of Tris-HCl 50 mM, CaCl₂ 5 mM, ZnCl₂ 1 mM, pH 7.6, which contained 2.5% Triton X-100. The solution was changed at 30 min. Then the gels were incubated at 37°C in the same buffer but without the Triton X-100, for 16 h, then coloured for 1 h with 0.1% Coomassie Brilliant Blue R-250 in methanol 30%, acetic acid 7%, distilled water 63% and finally discoloured in a solution of methanol: acetic acid: distilled water (3:1:6). The MMPs activity was visualized as colorless stripes on the blue background of the gels. Samples were co-migrated in the presence of a marker of high molecular mass (36-205 kDa). For checking if the detected gelatinolytic activities were owed to MMPs, the samples were examined by electrophoresis in the same conditions and then incubated in the same buffer with or without 1,10-phenantrolyne, a specific inhibitor of MMPs. The same method was used to assess MMPs in the cell lysate.

The cell lysate was obtained by re-suspending the cell sediment obtained by centrifugation of the synovial fluid in PBS, centrifugation at 2000 g for 10 minutes, followed by cell lysis at 4°C for 24 h in Tris-HCl tampon 50 mM at pH 7.5 containing 0.5% Triton X-100, 0.001% Brij-35, 1 mM CaCl₂ and 2 µM ZnCl₂, in a cells:lysis media ratio of 1:4. The lysate was then centrifuged at 5000 g (4°C) for 10 min and the supernatant was examined by zymographic study for MMPs and serin-proteases. The protein quantity applied on the gel was 5 µg.

The main MMPs assessed by this method are: MMP 1 (collagenase 1), MMP 2 (gelatinase A), MMP 3 (stromelysin 1), MMP 8 (collagenase 2), MMP 9 (gelatinase B). Because the substrate is a gelatine, the method is more sensitive in detecting gelatinases than collaganases or stromelysines. The reference molecular masses of these MMPs and of the corresponding proenzymes are: MMP 1 – 42 kDa; proMMP 1 – 51 kDa; MMP 2 – 62 kDa; proMMP 2 – 70 kDa; MMP 3 – 48 kDa; proMMP 3 – 57 kDa; MMP 8 – 58 kDa; proMMP 8 – 75 kDa; MMP 9 – 92 kDa; proMMP 9 – 98 kDa.

Results and discussions

All the patients with RA presented with active disease: more than 6 swollen and painful joints, inflammatory syndrome (erythrocyte sedimentation rate – ESR above 30 mm/h and/or C reactive proteine – CRP above 2mg/dL and/or fibrinogen above 400mg/dL), morning joint stiffness for at least 60 min.

The patients with arthrosis had mainly knee modifications, but these were also present in other joints (distal interphalanganian, proxymal metatarso-phalanganian) and typical arthrosic radiologic modifications.

Synovial fluid zymogram showed several stripes of degradation (fig. 2):

- the stripe at 180-205 kDa corresponds to several MMP 9 dimers;
- the stripe at 116 kDa assesses a gelatinolytic activity complex presumed to be a complex between MMP 9 (gelatinase B) and neutrophil α-2 microglobuline or some MMP 2 (gelatinase A) dimers;
- the stripe at 96-98 kDa represents proMMP 9 (progelatinase B);
- in the 75-84 kDa stripe appears the active form of MMP 9, with a molecular mass between 84-92 kDa, and proMMP 8 (procollagenase 2) with a molecular mass of 75kDa;
- the stripe at 68-72 kDa represents proMMP 2 (progelatinase A);
- the stripe at 64 kDa represents the intermediate form, partially active, of MMP 2;

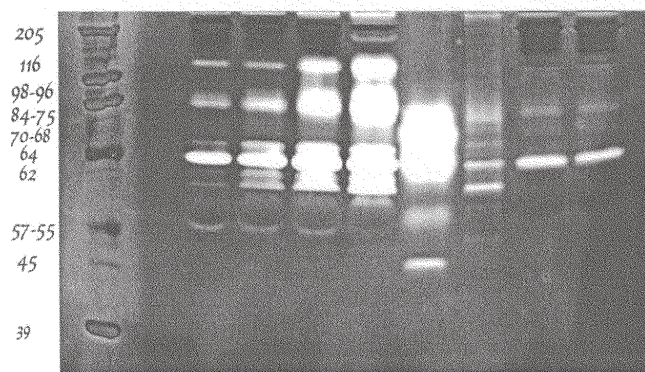


Fig. 2. MMPs in synovial fluid in the study group (overview), substrate zymography

- the active form of MMP 2 appears in the 62 kDa stripe;
- the stripe at 55-57 kDa represents proMMP 3 (prostromelysin 1) and proMMP 1 (procollagenase 1, usually at 51 kDa);
- at 45 kDa appears the active form of MMP 1 (collagenase 1, known with a 42 kDa molecular mass), MMP 3 (stromelysin 1, known with a 48 kDa molecular mass) and MMP 8 (cartilage collagenase 2);
- small autolysate MMPs fractions appear in the 39 kDa stripe.

The differences between the MMPs molecular masses found in our study and those quoted in the literature appear because of the migration of markers used for MMPs assessment. The activity of all the enzymes and enzymatic complexes could not be noticed in samples where we added 1,10 phenantrolyn 10 mM, which is a specific MMPs inhibitor. This test demonstrated that the migrated fractions represent indeed different MMPs and not other type of enzymes.

Even though the method we used is not a quantitative one, we can estimate MMPs level on the thickness and intensity of the corresponding stripes, at the same quantity of protein applied on gel and submitted to electrophoresis migration. The main MMPs assessed through this method were MMP 1, 2, 3, 8, 9 and their proenzymes (fig.3).

For statistics we used the SPSS 11 program and we performed mainly the Chi square test.

- MMP 1 (collagenase 1) has 42 kDa. This enzyme is produced by chondrocytes and fibroblasts. It is the main enzyme which biodegrades fibrillar collagens, including type II collagen – the main matrix component of the joint cartilage. Our laboratory method is less sensitive for collagenases, this is why we did not obtain lysis stripes in the 45 kDa region in synovial fluid, nor in the sera or cell lisate.

- proMMP1 has 51 kDa. In our study we considered it to migrate in the 55 kDa stripe, along with proMMP 3. Five out of 14 patients with RA have shown an activity in this stripe versus none in the arthrosis group. Although without statistical value, because of the small study groups ($p = 0.11$), we can point out that the presence of proMMP 1 and proMMP 3 (which migrates in the same area) in the synovial fluid differentiates the patients with PR from those with arthroses.

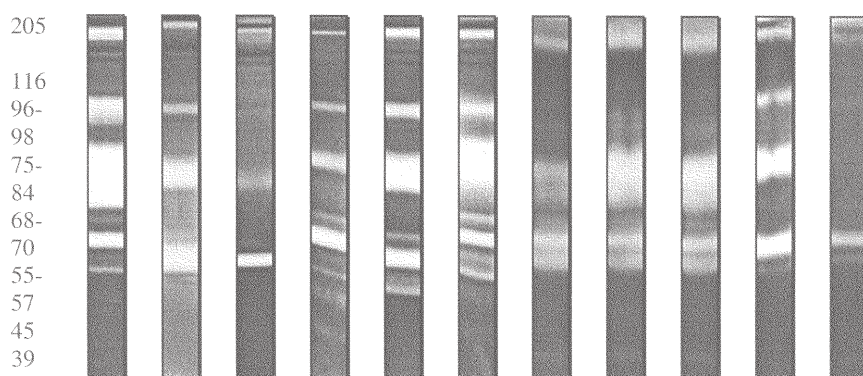
- MMP 2 (gelatinase A) is secreted by chondrocytes and fibroblasts. It has 62 kDa. In our study it was present in 13 out of 14 patients with RA versus 2 out of 5 arthrosis patients. MMP 2 was more frequently present in the synovial fluid in RA patients ($p < 0.05$).

- proMMP 2 has 70 kDa. In the synovial fluid the corresponding band is at 68-70 kDa. We noticed it in 10 out of 14 RA patients and in only one out of 5 arthrosis patients. Similar to the MMP 2, this proenzyme is more frequent in the synovial fluid of RA patients than in those with arthrosis ($p < 0.05$).

- proMMP 3 has 57 kDa. It has been noticed, together with proMMP 1, in the 55-57 kDa band in 5 out of 14 RA patients. The proenzyme has not been noticed in any synovial fluid sample from the arthrosis group. As we said, these data have no statistic significance, but we can notice that proMMP 1 and proMMP 3 stripe (55-57 kDa) can differentiate RA versus arthrosis patients.

- MMP 8 (collagenase 2) is secreted by neutrophils. It has 58 kDa. Our study method did not allow to obtain interpretable data because of the lack of sensibility. It is possible that MMP 8 may migrate together with proMMP 1

Rheumatoid arthritis patients



Rheumatoid arthritis patients

Arthrosis patients

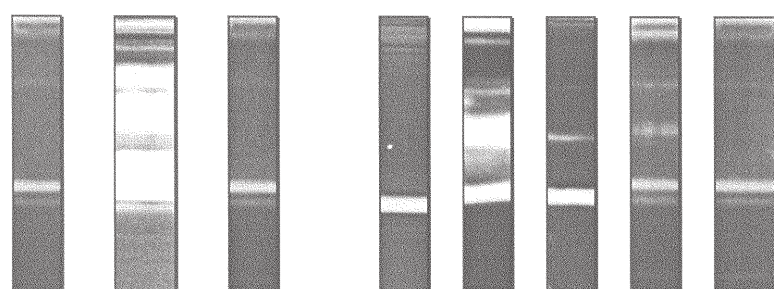


Fig. 3. MMPs in rheumatoid arthritis and arthrosis patients (each case presented separately), substrate zymography

Table 1
MMPs PROFILE ACCORDING TO THE RHEUMATOID ARTHRITIS (RA) STAGE

| Evolutionary stage of RA | Nr. of patients | MMPs profile – nr. of patients |
|--------------------------|-----------------|--|
| Incipient stage | 1 patient | proMMP 2 and 9 |
| Stage II | 6 patients | proMMP 1 and 3 (3), proMMP 2 (5), MMP 2 (6), proMMP 9 (5), MMP 9 (2) |
| Stage III | 6 patients | proMMP 1 and 3 (2), proMMP 2 (3), MMP 2 (6), proMMP 9 (4), MMP 9 (2) |
| Stage IV | 1 patient | proMMP 2, 9 and MMP 2 |

Table 2
MMPs PROFILE ACCORDING TO TREATMENT IN THE RHEUMATOID ARTHRITIS (RA) PATIENTS

| RA treatment | Nr. of patients | MMPs profile – nr. of patients |
|-----------------------------|-----------------|--|
| DMARDs + NSAIDs | 7 patients | proMMP 1 and 3 (4), proMMP 2 (4), MMP 2 (6), proMMP 9 (6), MMP 9 (3) |
| NSAIDs + corticosteroids | 6 patients | proMMP 1 and 3 absents, proMMP 2 (5), MMP 2 (6), proMMP 9 (4), MMP 9 (1) |
| No treatment | 1 patient | proMMP 1, 2, 3, 9 and MMP 2 and 9 |

DMARDs = disease modifying anti-rheumatic drugs, NSAIDs = non-steroidal anti-inflammatory agents

and proMMP 3. This could explain the large size of the corresponding lysis band. This statement is also corroborated with the presence of numerous neutrophils in the cell sediment of the synovial fluid in RA patients, demonstrated in a previous study [10].

- proMMP 8 has a molecular mass of 75 kDa. The proenzyme has been faintly noticed in 2 patients with RA.

- MMP 9 (gelatinase B) has 92 kDa. It is produced by neutrophils, macrophages, fibroblasts. It has been detected in the synovial fluid of 5 out of 14 RA patients, but in none of the arthrosis group. Although there is no statistical significance ($p > 0.05$, like proMMP 1 and 3), we consider that the importance of MMP 9 as a marker of cartilage degradation and of RA activity is obvious. In our study MMP 9 distinguished the patients with RA from those with arthrosis. In 3 out of 5 cases we have detected the simultaneous presence of the 55-57 kDa band, which suggests the implication of stromelysin 1 (MMP 3) in activating proMMP 9.

- proMMP 9 has 98 kDa. It has been identified in 11 out of 14 RA patients, and in 3 out of 5 arthrosis patients. There were no significant statistical differences ($p > 0.05$) between the two groups.

The patients had different MMPs profiles according to the evolutionary stage of RA, as well as according to their treatment (tables 1 and 2).

The small number of cases renders the interpretation of these data difficult. We can appreciate that absence of MMP 9 in the stage IV patient might suggest the diminution of the number of neutrophils involved in the rheumatoid process in the later stages of disease. The significantly greater activity of enzymes and proenzymes in the stages II-III of RA, characterized by intense inflammatory activity

and extensive joint destructions, is to be noticed.

The method we used is more sensitive for gelatinases than for the other MMPs. The study group was small and not all of the presented data have statistical significance. The presence of proMMP 1 and 3, MMP 2 and 9 in the synovial fluid differentiates RA patients from arthrosis ones and may be useful in the differential diagnosis. For the moment zymography is not a routine method of studying the synovial fluid.

The most significant aspect of the zymogram was that of the patient without treatment. We noticed the presence in the synovial fluid of all the aforementioned enzymes and proenzymes. The smaller number of patients with MMP 9 from the group treated with corticosteroids might be due to their direct action of diminishing some MMP activity.

In order to assess the relations between disease activity (estimated by ESR, CRP and fibrinogen serum levels) and MMPs profile, we grouped the patients with RA according to the presence or absence of proMMP 1 and 3, respectively MMP 9 in the synovial fluid (table 3).

We noticed a significant statistical association ($p = 0.017$) between MMP 9 and higher values of ESR (we already know the role of MMP 9 as a marker of RA activity) and the association of proMMP 1 and 3 with higher fibrinogen values ($p < 0.01$). The study group was small so we cannot draw definitive conclusions, but we can appreciate the confirmation of the marker role for RA activity in the studied group.

Three patients had simultaneously shown the presence of MMP 9 and pro MMP 1 and 3. We expected that these patients have marked inflammatory phenomena in comparison to the other patients, but we did not find any difference ($p < 0.05$) between these patients and the rest

Table 3
CORELLATIONS BETWEEN AVERAGE VALUES OF ESR, CRP AND FIBRIOGEN AND MMPs PROFILE

| Average values | proMMP 1,3 positive | proMMP 1, 3 negative | p | MMP 9 positive | MMP 9 negative | p |
|----------------|---------------------|----------------------|--------|----------------|-------------------|-------|
| ESR | 71 +/- 8,94 | 52,88 +/- 22,92 | 0,12 | 76 +/- 15,16 | 50,11 +/- 17,68 | 0,017 |
| CRP | 3,15 +/- 1,53 | 2,54 +/- 2,1 | 0,58 | 3,1 +/- 2,11 | 2,57 +/- 1,84 | 0,63 |
| Fibrinogen | 593 +/- 48,16 | 451 +/- 83,28 | < 0,01 | 556 +/- 83,68 | 485,11 +/- 105,31 | 0,21 |

of the group for any of the evaluated parameters (ESR, CRP, fibrinogen).

RA is characterized by a massive production of proinflammatory cytokines, intense local cell activation and proliferation, bone and articular destructive processes. According to Firestein MMPs are the main destructive component [7]. MMPs are also involved in the rheumatoid synovial neoangiogenesis. The method we used in our study, SDS-PAGE zymography, highlights especially gelatinases (MMP 2 and 9). A similar study by Makowski and Ramsbi highlighted the association between different types of MMP 9 and neutrophil joint infiltrate [11].

Another study by Matsuno *et al.* highlighted significant higher levels of MMP 1, 3 and 9 in RA synovial fluid than in arthrosis [12]. We did not find high MMP 1 and 3 levels in our RA group, maybe because of the method's lack of sensibility but also because of the enzymes substrate fixation.

One of the aims of our study consisted in establishing MMPs profile in different stages of RA. We found a maximum of MMPs secretion in the II and III stages, while we found a diminution of MMPs 1, 3 and 9 secretion in stage IV.

The presence of MMP 9 and proMMP 1 and 3 in the synovial fluid correlated with higher values of inflammatory tests, and with more active forms of RA. In the future we intend to study larger groups, and to make correlations between MMPs profile and RA activity.

Conclusions

The method we used (SDS-PAGE gelatin zymography) highlights especially gelatinases (MMP 2 and 9).

The presence of MMP 2 and 9 and also of proMMP 1 and 3 in the synovial fluid differentiated in our study group RA and arthrosic patients.

MMPs production reaches its maximum in the II and III stages of RA, whereas in the I and IV stages it tends to be more limited.

The lack of treatment was associated with a large production of MMPs and subsequently with a significant articular destruction.

The presence of MMP 9 and proMMP 1 and 3 in the synovial fluid correlated with more active forms of the disease and with inflammatory tests.

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