

A correlation between knee cartilage degradation observed by arthroscopy and synovial proteinases activities☆

Stefano Marini^a, Giovanni Francesco Fasciglione^a, Giovanni Monteleone^b, Marco Maiotti^b, Umberto Tarantino^b, Massimiliano Coletta^{a,*}

^aDepartment of Experimental Medicine and Biochemistry Science, University of Rome TorVergata, Rome, Italy

^bDepartment of Surgery, University of Rome TorVergata, Rome, Italy

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Abstract

Objective: A novel study has been carried out to characterize the amount and activity levels of metalloproteinases (i.e., MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13) and of their inhibitors (i.e., TIMP-1 and TIMP-2) in synovial fluid from patients ($n = 56$) with different degrees of either chondral lesions or knee arthritis identified and classified by arthroscopy.

Design and methods: Zymographies, Western blotting and ELISA tests have been used to correlate the disease stage, as determined by arthroscopy, and both the amount and the activation state of different MMPs and of their inhibitors.

Results: Analysis of data obtained demonstrates that the degree of cartilage degradation, as seen by arthroscopy, is strictly related to the activity of some synovial MMPs, in particular MMP-2 and MMP-13 and on reduced inhibitory effect of MMP-2 by TIMP-2; in addition, a serine protease weighing about 125 kDa appears only in patients with severe cartilage degradation, i.e., with knee arthritis.

Conclusions: On the whole, this is the first study in which an analysis of synovial MMPs/other proteinases activity and TIMPs has been strictly related to arthroscopy results in patients with different degrees of osteoarthritis. Results indicate that an imbalance between specific MMP activities and the amount of TIMPs and of its inhibitory efficiency is crucial for the disease evolution and it is related to the disease stage. © 2003 The Canadian Society of Clinical Chemists. All rights reserved.

Keywords: Matrix metalloproteinases; Knee arthritis; Arthroscopy

1. Introduction

In recent years, computer tomography and magnetic resonance have allowed a more precise evaluation of cartilage degeneration [1,2], even although these techniques often identify arthritic lesions only when the cartilage is deeply damaged. One possibility of evaluating joint damage and monitoring chondral lesions in chronic pain may be related to the analysis of chondral degeneration markers (enzymes and cytokines) in the joint microenvironment, i.e., in the synovial fluid. As a matter of fact, there is increasing evidence that abnormalities in connective tissue remodelling, which characterize several diseases, such as rheumatoid

arthritis or osteoarthritis, may be referred to alterations in the synovial production of cytokines as well as of matrix metalloproteinases [3,4].

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that can degrade all components of the extra-cellular matrix. Enzymes of this family, such as tissue collagenase (MMP-1), stromelysin-1 (MMP-3), type IV collagenase (or gelatinase A, MMP-2) and gelatinase B (MMP-9) are secreted as zymogens, and once activated, they are able to remove connective tissue during normal turnover and pathologic breakdown [5]. The latent form (proenzyme) can be activated, at least “*in vitro*”, by other proteases (e.g., plasmin, plasma kallikrein, tissue plasminogen activator, which are present in the inflammatory micro-environment), as well as by mercurials (e.g., 4-aminophenylmercuric acetate) [6]. MMPs appear to be crucial for connective tissue remodelling in physiologic processes, including wound healing [7], angiogenesis [8], cytrophoblast implantation [9], embryonic development [10], in the

* Corresponding author. Tel.: +39-0672596365; fax: +39-0672596353.

E-mail address: coletta@seneca.uniroma2.it (M. Coletta).

☆ Abbreviations: IL; interleukin; MMP; matrix metalloproteinase; PBS; phosphate buffered solution; TIMP; tissue inhibitor of metalloproteinase; TNF; tumor necrosis factor.

cycling of endometrium [11] and in pathologic conditions, such as tumor invasion and metastasis [12], progressive joint destruction [13], inflammation [14], Alzheimer's disease [15] and atherosclerosis [16]. The source of degradative proteinases may depend on the type of disease: in rheumatoid arthritis enzymes may arise from the pannus that proliferates over the cartilage and from inflammatory cells, such as neutrophils and macrophages, that attack the cartilage surface. In osteoarthritis, migration of inflammatory cells is minimal at the early stages of the disease, so that enzymes secreted from chondrocytes are most likely responsible for cartilage degradation.

Among factors influencing MMPs activities in cartilage remodelling and/or destruction, tissue inhibitors and cytokines are the most important ones.

Specific tissue inhibitor of metalloproteinases (TIMPs) [17–20], several of which have been recently described, can inhibit activated MMPs. Among them, the best known is TIMP-1, a glycoprotein of Mr 28,000 containing 12 cysteine residues which form 6 disulphide bonds [19,20].

Cytokines also play a critical cross-role in the regulation and repair during the normal and pathologic turnover of the cartilage matrix [21–26]: preliminary studies demonstrated a relationship between IL-6 amount and arthritis disease stage [27]. The complex interrelationship described above is however further complicated by the observation that growth regulatory cytokines, such as insulin-like growth factor-I, tumor growth factor- β , β -fibroblast growth factor and epidermal growth factor, primarily contribute to the preservation of cartilage matrix integrity by increasing the synthesis of cartilage matrix, by stimulating chondrocyte proliferation and, in some cases, by inhibiting the synthesis of MMPs and increasing the synthesis of protease inhibitors [3–4].

The aim of this study is both to characterize gelatinases, MMPs and their inhibitors and to analyze their amount and activity in synovial fluids from a statistically significant number of patients with different degrees of either chondral lesions or knee arthritis identified and classified by arthroscopy.

The analysis of the amount and activity of native MMPs and of their inhibitors at different stages of arthritis is important to identify the role and the function of such molecules in joint diseases and to give some contribution to the elucidation of the complex network of interconnections between different pathologic processes. Moreover, the results obtained could be used to find early markers related to different disease degrees useful to increase diagnosis accuracy and therapy development.

2. Materials and methods

2.1. Patients

The study has been performed on a group of 56 patients (28 males and 28 females) never tested before with a me-

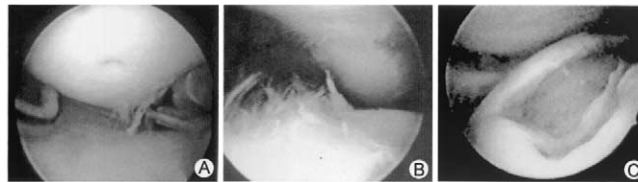


Fig. 1. Arthroscopic appearance of knee cartilage from a patient with mild damage. 1a: the surface of the cartilage appears intact, but palpation produces depressions indicative of edema; 1b: moderate cartilage damage: open lesions without exposure of subchondral bone; 1c: severe cartilage damage: full-thickness lesions that expose the subchondral bone.

dian age of 52 yr (range 20–79); all patients consumed NSAIDs during the last year. Arthroscopies have been performed during either routine outpatient therapeutic procedures or surgery. Based on the various classification systems [27], that have been applied to cartilage lesions of the knee observed during arthroscopy, patients have been divided into the following four groups:

- Group I (mild damage, i.e., closed lesions: the surface of the cartilage appeared intact, but palpation produced depressions indicative of edema, see Figure 1a): 13 patients (7 males and 6 females) with a median age of 39 yr (range 22–64);
- Group II (moderate damage, i.e., open lesions without bone exposure: ranging from superficial fibrillation to disruption of deeper layers without exposure of the subchondral bone, see Figure 1b): 14 patients (8 males and 6 females) with a median age of 58 yr (range 50–67);
- Group III (severe damage, i.e., open lesions with bone exposure: full-thickness lesions that exposed the subchondral bone, see Figure 1c): 14 patients (7 males and 7 females) with a median age of 48 yr (range 20–72);
- Group IV (knee arthritis group): 15 patients (6 males and 9 females) with a median age of 61 yr (range 50–79).

The groups were pooled taking into account only the results obtained by arthroscopy without any reference to symptoms, pain intensity, mobility reduction or histopathological grade of cartilage degradation.

All patients were negative for rheumatoid disease (i.e., rheumatoid factor, antistreptolysin titers, mucoprotein levels, Waaler-Rose test, c-reactive protein) and showed hematocchemical and renal parameters within the normal range and similar among groups. No crystals were observed in the joints during arthroscopy. No patient experienced previously septic or psoriatic arthritis. Associated lesions included 19 ruptured menisci, 5 lesions of the anterior cruciate ligament, 2 meniscal cyst and 2 loose body; the time-interval between trauma and arthroscopy ranged between 4 weeks and 6 months.

2.2. Sample preparation

Synovial fluid (from 5–15 mL) was withdrawn from knee joints just before or during every arthroscopy. The synovial fluid samples were collected into sterile plastic tubes and centrifuged at 3,000 g for 20 min at 4°C. Cell-free supernatants were stored at –70°C before use.

2.3. Substrate zymography

Gelatin substrate zymography was used for the evaluation and characterization of synovial proteinases [28]. Synovial fluid (1 µg of proteins/lane; protein determination following Bradford 1976) was mixed with a fivefold excess of sample buffer (0.25 mol/L Tris, 0.8% SDS, 10% glycerol and 0.05% bromophenol blue) and electrophoresis was run on 12% SDS-polyacrylamide gels (SDS-PAGE) containing 1 mg/mL of gelatin, as described [29]. Some gels were performed using α-casein (1 mg/mL) (Sigma) or bovine collagen type I (0.5 mg/mL) (a generous gift of prof. Menegatti, Univ. of Ferrara, Italy) as substrate. These two substrates have been used to identify MMP-1, MMP-3, MMP-8 and MMP-13 activities; it should be underlined that, even through also MMP-2 and MMP-9 can express a slight hydrolytic activity vs. SDS-treated type I collagen, the analysis of the whole data allow us to discriminate between gelatinolytic and collagenolytic activities.

After electrophoresis, SDS from gels was removed by four 15-min washings in 2% Triton X-100. The gelatin gels were then incubated at 37°C for 18 h in incubation buffer (50 mM Tris-HCl buffer pH 7.6, 0.15 mol/L NaCl, 10 mM CaCl₂, 2% Triton X-100). The gels were then stained with 0.5% Coomassie blue and destained in 10% acetic acid and 40% methanol until proteinase bands were clearly visible. Proteinase bands were further characterized by adding 20 mM EDTA or 0.3 mM 1,10-phenanthroline (MMPs inhibitors), or 1 mM PMSF (serine proteinase inhibitor) to the incubation buffer. Protein markers (Sigma) were used as molecular weight standards. Images of the gels were video-digitised by a CCD camera (NEC T1-24A, Japan) and image software (Image Version 1.42, NIH, Bethesda, MD). Gel imaging was standardized by capturing them at the same focal length and exposure. The intensity and area of gelatinolytic activity were then quantified. An arbitrary unit scale (A.U.) was used and results were statistically analyzed. To confirm the data obtained, some experiments have been performed by using serially diluted samples.

2.4. Western immunoblots

To identify proteinases, synovial fluid (1 µg proteins/lane; protein determination following Bradford [49] 1976) of each sample was mixed with a fivefold excess of sample buffer (0.25 mol/L Tris, 0.8% SDS, 10% glycerol and 0.05% bromophenol blue) and run on 12% polyacrylamide gel either after 2 min of boiling in presence of 2-mercapto-

ethanol or, to identify TIMP-2, under native conditions. The gels were then transferred to nitro-cellulose membrane (Schleicher and Schuell, Dassel, Germany) in TRANS-BLOT (Biorad, Italy) using Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol). Aspecific binding sites were blocked with 5% dry low-fat milk in PBS for 1 h at 37°C; membranes were then washed once with PBS and further incubated overnight at 4°C with the right concentration of rabbit polyclonal (Triple Point Biologics, U.S.A.) or mouse monoclonal (Oncogene, U.S.A.) antibodies directed against human tissue collagenase (MMP-1), both forms of human gelatinase A (MMP-2), human stromelysin-1 (MMP-3), human neutrophil collagenase (MMP-8), human gelatinase B (MMP-9), human collagenase-3 (MMP-13), and TIMP-1/TIMP-2. Some preliminary experiments using anti MT-MMP (MMP-14) have also been performed. Thereafter, 3 washes (5 min each) with PBS/Tween-20 (0.2%) were performed and membranes were then incubated with a 1/1000 dilution of horseradish peroxidase-labeled goat antirabbit or antimouse antibodies (Biorad) in PBS for 2 h, followed by further washings (3 washes, 5 min. each) with PBS/Tween-20 (0.2%). The bands were visualized by incubation of the blots with substrate (4-chloronaphtol 0.6 mg/mL and 30% H₂O₂, 0.6 µL/ml) or by using ECL detection systems (Amersham, U.K.). Results were analyzed by a CCD camera (NEC T1-24A, Japan) and image software (Image Version 1.42, NIH, Bethesda, MD). The intensity and area of the bands were then quantified. An arbitrary unit scale was used and results were statistically analyzed.

2.5. ELISA

Enzyme Linked ImmunoSorbent Assay (ELISA) was used to determine TIMP-1/TIMP-2 levels in synovial fluids. Synovial fluid (each sample containing the same amount of proteins) was diluted 1/10 in 0.05 mol/L carbonate buffer pH 9.5 and used to coat 96-well PVC plates o.n. at 4°C (100 µL/well). After 3 washes with 100 µL/well of PBS-Tween 0.05%, plates were blocked with 5% dry low-fat milk/0.1 mg/mL nonspecific goat Ig in PBS for 45 min and then, after washing, incubated with a 1/500 dilution of anti-TIMP-1 antibody (Triple Point Biologics) in PBS (100 µL/well) or anti-TIMP-2 (Oncogene) in PBS 1:2500 for 2 h at 4°C; plates were then washed three times with PBS-Tween. A 1/1000 dilution of horseradish peroxidase-labeled goat antirabbit or anti mouse antibodies (Biorad) in PBS was added to each well; plates were then incubated for 2 h at 4°C. After 4 washes with PBS-Tween 0.05%, plates were incubated with the chromogen substrate (0.4 mg/mL 1,2 phenyldiamine and 0.4 µL/ml H₂O₂ in citrate buffer, pH 4.7). Preliminary experiments, performed using recombinant TIMP-1/2 as standard, confirmed that, under the conditions described above, both molecules, even in the presence of large amounts of active or pre-MMPs, were specifically recognized by antibodies with a statistically significant linearity ($r = 0.97$) (data not shown). The data obtained were

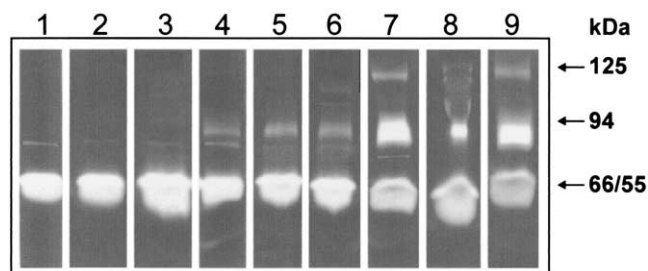


Fig. 2. Zymography of synovial fluids by using gelatin as substrate. Lane 1 to 3: groups I/II (closed lesions and open lesions without bone exposure); lane 4 to 6 group III (open lesions with bone exposure); lane 7 to 9 group IV (knee arthritis group). For details see Materials and Methods section. In each lane typical zymographies obtained from 3 patients/group are reported. Similar results have been obtained in all patients belonging to the same group.

further confirmed by using a commercially available TIMP-1/2 Immunoassay kit (Chemicon International, U.S.A.). A standard curve obtained by using recombinant TIMP-2 has shown that the concentration of TIMP-2 ranged from 140 (0.65 O.D.) to 180 (1.3 O.D.) ng/mL.

2.6. Statistical analysis

A computer database containing all data measured from patients was created. These data were analyzed using the Stat View 512+ program from Brain Power Inc. (Calabasa, CA). Regression analysis, correlation test and “t” Student’s test have been used.

3. Results

3.1. Identification of proteinases in the synovial fluids

Synovial fluids were withdrawn during routine outpatient therapeutic procedures (for groups I and II) ($n = 27$) or during surgery (group III, IV) ($n = 29$) (Figure 1). Liquids were centrifuged, aliquoted in 2-mL samples and frozen at -70°C until use. The amount of hemoglobin present in the synovial fluid (indicating the possibility of blood, and thus

of blood proteinases contamination) was checked, on a regular basis, by measuring synovial liquid optical absorbance at 415 to 430 nm; all fluids containing more than $0.01 \mu\text{g/mL}$ hemoglobin were discarded. Preliminary analysis showed the presence of specific gelatinolytic activity with different lytic patterns in all synovial fluids tested. The amount of proteolysis for each band was analyzed and results were plotted and statistically analyzed (Figure 2).

The results reported in all the figures are referred to the same, representative patients. Similar results have been obtained in each group.

Groups I and II (mild damage with closed lesions and moderate damage with open lesions without bone exposure respectively) gave similar results both in terms of MMPs involved and in values. For the sake of simplicity these two groups have been joined in statistical analysis.

Although proteins with molecular weights of 66/55-kDa with gelatinolytic activity were found in 100% of cases, this activity greatly differed among the groups: zymograms from patients in groups I-II ($n = 27$) showed a mean gelatinolytic activity of enzymes with molecular weights 66/55-kDa of 10095 ± 1743 A.U., while in patients with open lesions and subchondral bone exposure (i.e., group III) ($n = 14$), the mean proteolytic activity with the same molecular weight increased up to 14492 ± 1152 A.U. ($p < 0.05$ vs. groups I-II); group IV patients ($n = 15$) showed a gelatinolytic activity of 19024 ± 1035 A.U. ($p < 0.001$ vs. groups I-II and $p < 0.02$ vs. group III) (see Figure 2 and Table 1). A pale band weighting 84 kDa (activated MMP-9) was also observed in the same small amount in all patients (see Figure 2).

A proteolytic activity related to a 94-kDa enzyme was present in 84% of patients with open lesions and bone exposure (belonging to group III) (Figure 2), displaying a mean activity of 2045 ± 1022 A.U. (see Table 1), and in 75% of patients with knee arthritis (i.e., group IV), showing a mean activity value of 12991 ± 1932 A.U. ($p < 0.02$ vs. group III of chondritis patients, see Table 1). On the other hand, only one patient with closed chondral lesions (i.e., from group I) and no patients in group II showed activity related to the 94-kDa enzyme. Therefore, such an outcome

Table 1
Correlation between lesion severity and marker presence

Markers	Chondral lesions		Gonarthrosis
	Groups I–II	Group III	Group IV
66/55 kDa	100% (10095 ± 1743)	100% (14492 ± 1152)* ^o	100% (19024 ± 1035) [^]
94 kDa	3.7% (1027)	84% (2045 ± 1022)	75% (12991 ± 1932) ^{^^}
125 kDa	N.D.	N.D.	30.8 (8035 ± 1819)
TIMP-1	0.617 ± 0.15 O.D. ^{oo}		0.849 ± 0.218 O.D.
TIMP-2	0.912 ± 0.135 O.D. ^{oo}		0.899 ± 0.145 O.D.

The intensities of band zymography have been measured by a CCD camera (NEC T1-24A, Japan) and image software (Image Version 1.42, NIH, Bethesda, MD). The intensity and area of the bands were then quantified. An arbitrary unit scale was used. These data were analysed using the Stat View 512+ program from Brain Power Inc. (Calabasa, CA). N.D.: not detected; ** $p < 0.05$ groups I–II vs. group III; ^o $p < 0.02$ group III vs. group IV; [^] $p < 0.001$ groups I–II vs. group IV; ^{^^} $p < 0.02$ group III vs. group IV; ^{oo} $p < 0.02$ groups I–II vs. groups III–IV.

clearly indicates that the occurrence of this activity is strongly related to the disease degree.

Proteinase activities corresponding to enzymes weighing about 125 kDa was found only in 30.8% of arthritic patients (belonging to group IV, see Table 1) with a mean value of 8035 ± 1819 A.U.

In conclusion, the band weighing 66/55 kDa is present in all patients, but the activity appears to be related to the disease degree. A small activity at about 84 kDa was also seen in all patients with clinically relevant osteoarthritis and was found to be unrelated to the disease stage. The activity associated to the band weighing 94 kDa is present only in the majority of patients from the group III and IV, but with a mean activity which is again related to the severity of disease. Finally, the proteolytic activity coupled to the band weighing about 125 kDa is only present in (30% of patients with knee arthritis (i.e., from group IV, see Table 1). All data obtained have also been confirmed in experiments performed with serially diluted samples (data not shown).

Similar results with an additional pale band weighting about 50 kDa present in similar amount in all samples, later identified as activated MMP-3, have been obtained by using casein as zymography substrate. In collagen zymography, similar patterns (although less evident) were also observed; in some experiments an additional band, weighing about 45 kDa, whose intensity was strictly related to the severity of the chondromalacia (top of Figure 5) has also been detected. This band was characterized by Western blot experiments as MMP-1 and/or MMP-13 (see below).

3.2. Characterization of proteinase activity

The large, diffuse band at 66/55-kDa with strong gelatinolytic activity is always completely inhibited when gels have been incubated in a buffer containing 0.3 mM 1,10-phenanthroline or 10 mM EDTA; therefore, this activity has been definitely attributed to metalloproteinases. Since several MMPs show a M.W. of about 55 to 65 kDa, Western blot experiments have been performed, employing antibodies against collagenase type I (MMP-1, M.W. 52–43 kDa), gelatinase A (MMP-2, M.W. 72–62 kDa), stromelysin-1 (MMP-3, M.W. 59–56 kDa), neutrophil collagenase (MMP-8, M.W. 64 kDa) and collagenase-3 (MMP-13, M.W. 51–43 kDa). It should be pointed out that data obtained in Western blot allow us to identify not only the specific active MMPs present in synovial fluids but also to measure their relative amounts (determined by analyzing band intensities, Table 2). Results obtained show the presence, in all fluids tested, of MMP-2 (total amount of both forms) (Figure 3), MMP-3 and MMP-8 in similar amounts (Figure 4) even although activated MMP-2 amount was higher in groups III and IV than in groups I-II (Figure 3 and Table 2). Further, an increase of immunoreactive MMP-1 related to the disease progression was also observed (bottom Fig. 5 and Table 2). In addition, the amount of MMP-13 immunoreactive protein (corresponding as MMP-1 to the

Table 2
Intensity of bands detected by Western blot

Proteinase	Chondral lesions		Gonarthrosis
	Groups I–II	Group III	Group IV
ProMMP-2	87708 \pm 3985	67960 \pm 2366	56639 \pm 5484
MMP-2	66517 \pm 5803	57569 \pm 5241	85614 \pm 6446
MMP-1	66088 \pm 7152	83436 \pm 10267	148611 \pm 3966
MMP-13	111733 \pm 8565	160327 \pm 7624	241607 \pm 22893

The intensities have been measured by a CCD camera (NEC T1-24A, Japan) and image software (Image Version 1.42, NIH, Bethesda, MD). The intensity and area of the bands were then quantified. An arbitrary unit scale was used. The data have been analysed using the Stat View 512+ program from Brain Power Inc. (Calabasa, CA).

band weighting about 42 kDa with collagenolytic activity, see zymography section) has been found to be directly related to the severity of the chondromalacia increasing from group I to group IV (Figure 6 and Table 2). Finally, a pale zymographic band (see Figure 2), further identified in Western blot experiments as activated MMP-9, was also detected in similar amount in all samples (data not shown). It should be underlined that, under our conditions, we always find, in Western blot, only the mature forms of MMP-1, MMP-3, MMP-8 and MMP-13.

The 94-kDa and the 125-kDa activities are completely inhibited by 0.1 mM PMSF or 4 mM aprotinin in the incubation buffer, whereas no effect is observed upon addition of 1,10-phenanthroline or EDTA, indicating that this activity should be related instead to a serine proteinase activity. This is further supported by a Western blot observation: 125-kDa and 94-kDa bands do not react with any antibody against MMPs used. To identify the activity at 125-kDa, we have performed different zymographies using some serine proteases, namely plasmin 0.1 U, kallikrein 1 U, urokinase 0.1 U and plasminogen 0.1 U (data not shown). None of these enzymes has shown a zymographic band of 125-kDa band, rendering still undetermined the identification of this enzyme. We can only claim that these bands were not produced by blood contamination, as suggested by the following observations:

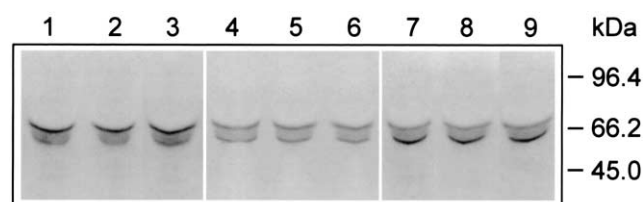


Fig. 3. Western blots of synovial fluids obtained by using anti MMP-2 antibodies. Lane 1 to 3: groups I/II (closed lesions and open lesions without bone exposure); lane 4 to 6 group III (open lesions with bone exposure); lane 7 to 9 group IV (knee arthritis group). In each lane typical results obtained from 3 patients/group are reported. Similar results have been obtained in all patients belonging to the same group. For details see Materials and Methods section. Biorad Molecular Weights Low Range Standard were used.

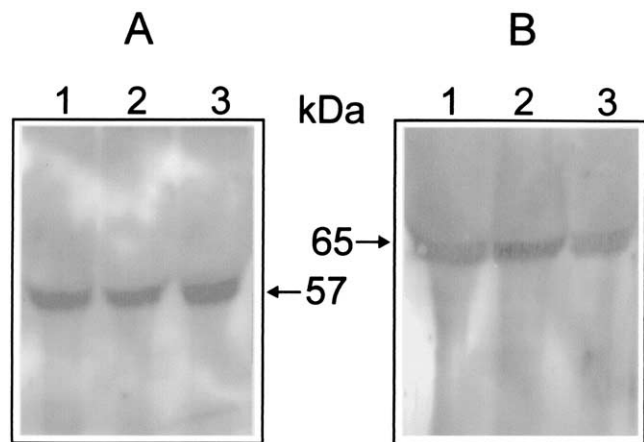


Fig. 4. Western blot of synovial fluid: lane 1: groups I/II (closed lesions and open lesions without bone exposure); lane 2 group III (open lesions with bone exposure); lane 3 group IV (knee arthritis group). Panel A: anti MMP-3 antibodies; panel B: anti MMP-8 antibodies. In each lane typical results obtained from 3 patients are reported. Similar results have been obtained in all patients belonging to the same group. For details see Materials and Methods section. Biorad Molecular Weights Low Range Standard were used.

1. blood gelatinases presented a completely different proteolytic pattern;
2. zymographies of sera harvested from all patients revealed no difference among patients both for active MMPs amount and proteolytic patterns; and
3. by using EDTA, all proteolytic bands present in sera were inhibited and only a band weighting about 94 kDa (possibly plasmin) was found.

3.3. Identification of proteinase inhibitors (TIMP-1/2)

The variation of the enzymatic activity of MMPs under the pathologic conditions outlined above has been also compared to the amounts of tissue inhibitors of MMPs (i.e., TIMPs), and in particular of TIMP-1 and TIMP-2, which can be observed in synovial fluids of these patients. ELISA demonstrated that the mean total TIMP-1 value, expressed as O.D. was 0.617 ± 0.15 in synovial fluid harvested from groups I and II and 0.849 ± 0.218 in fluid from groups III and IV ($p < 0.02$), thus indicating, on the whole, a slight increase of the total amount of TIMP-1 could be related to disease progression, as previously demonstrated [30]. However, synovial fluid Western blot analysis, performed under native conditions, has shown that very little TIMP-1 was bound to MMPs while more than 95% of the molecule was observed as a single band weighting about 20 kDa (data not shown).

No internal correlation was observed between mean synovial fluid activities of MMPs and mean synovial fluid levels of TIMP-1 in all patients ($r = 0.638$, $p > 0.2$ for groups I and II and $r = 0.021$, $p > 1$ for groups III-IV). This result suggests that the role of TIMP-1 in regulating the MMPs activity is very modest in relation to these pathologic

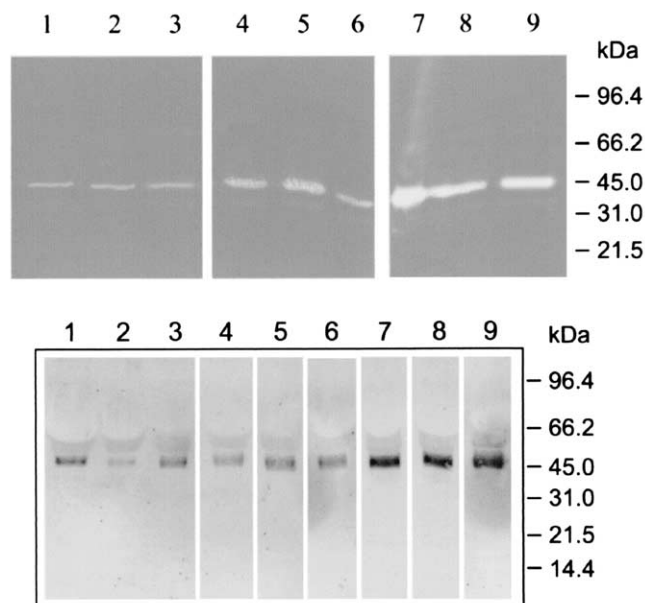


Fig. 5. Top: Zymography of synovial fluids by using collagen as substrate. Lane 1 to 3: groups I/II (closed lesions and open lesions without bone exposure); lane 4 to 6 group III (open lesions with bone exposure); lane 7 to 9 group IV (knee arthritis group). For details see Materials and Methods section. In each lane typical zymographies obtained from 3 patients/group are reported. Similar results have been obtained in all patients belonging to the same group. Bottom: Western blots of synovial fluids obtained by using anti MMP-1 antibodies. Lane 1 to 3: groups I/II (closed lesions and open lesions without bone exposure); lane 4 to 6 group III (open lesions with bone exposure); lane 7 to 9 group IV (knee arthritis group). In each lane typical results obtained from 3 patients/group are reported. Similar results have been obtained in all patients belonging to the same group. For details see Materials and Methods section. Biorad Molecular Weights Low Range Standard were used.

situations, a statement which seems also confirmed by the observation that mean levels of TIMP-1 are only slightly varying for different patients groups, independently of the variation of MMP activity observed going from patients of group I to patients from group IV.

The total amount of TIMP-2 was found similar in all patients, independently on the severity of the disease; the values, found in ELISA, ranged from 0.912 ± 0.135 O.D. to 0.899 ± 0.145 in all groups (140–180 ng/mL).

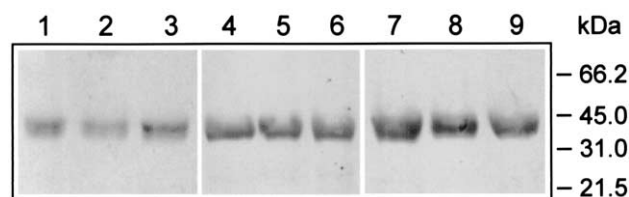


Fig. 6. Western blots of synovial fluids obtained by using anti MMP-13 antibodies. Lane 1 to 3: groups I/II (closed lesions and open lesions without bone exposure); lane 4 to 6 group III (open lesions with bone exposure); lane 7 to 9 group IV (knee arthritis group). In each lane typical results obtained from 3 patients/group are reported. Similar results have been obtained in all patients belonging to the same group. For details see Materials and Methods section. Biorad Molecular Weights Low Range Standard were used.

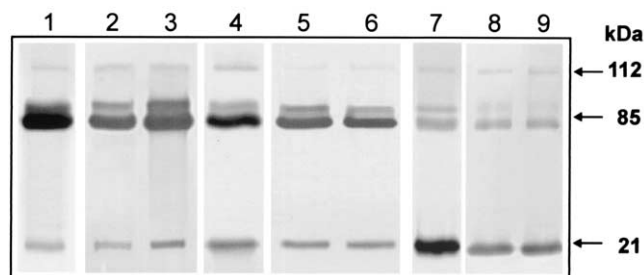


Fig. 7. Western blots of synovial fluids obtained by using anti TIMP-2 antibodies. Lane 1 to 3: groups I/II (closed lesions and open lesions without bone exposure); lane 4 to 6 group III (open lesions with bone exposure); lane 7 to 9 group IV (knee arthritis group). In each lane typical results obtained from 3 patients/group are reported. Similar results have been obtained in all patients belonging to the same group. Electrophoresis was performed in native conditions. For details see Materials and Methods section. Biorad Molecular Weights Low Range Standard were used.

However, it is important to outline that Western blot experiments, performed in native conditions, using anti-TIMP-2 antibodies, showed that in synovial fluids from patients of different groups the ratio between MMP-2-bound and free TIMP-2 differs significantly. As a matter of fact, despite the fact that the total amount of TIMP-2 is essentially the same for all patients, in synovial fluids from patients of group I most of TIMP-2 is bound to MMP-2, while in patients of group IV most of TIMP-2 is free (Figure 7); the situation is intermediate for patients belonging to groups II and III.

A denaturing gel performed by using same synovial fluids confirmed, as demonstrated in ELISA, that TIMP-2 amount is similar among different groups (Figure 8).

4. Discussion

The degradation of the articular cartilage seriously impairs joint functions. Such a damage occurs frequently in various joint diseases, mainly arthritis, such that in severe cases a widespread destruction of the cartilage can be observed, mainly concerning the portion which faces articular surfaces. The primary cause of the pathologic destruction of cartilage, especially under chronic conditions, is the ele-

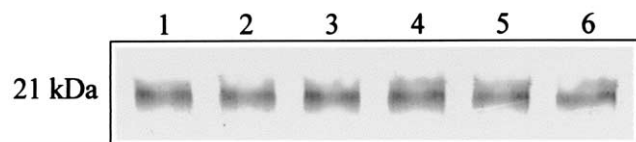


Fig. 8. Western blots of synovial fluids obtained by using anti TIMP-2 antibodies. Lane 1 to 2: groups I/II (closed lesions and open lesions without bone exposure); lane 3 to 4 group III (open lesions with bone exposure); lane 5 to 6 group IV (knee arthritis group). In each lane typical results obtained from 2 patients/group are reported. Similar results have been obtained in all patients belonging to the same group. For details see Materials and Methods section. Biorad Molecular Weights Low Range Standard were used.

vated proteolytic activity. In osteoarthritis, the main pathologic event is thought to be the mechanical stress, which increases in ageing joints, and it is followed by both a reduction of chondrocyte-matrix association and an altered response of chondrocytes. On the other hand, in disorders, such as rheumatoid arthritis, it is generally accepted that erosion of cartilage matrix occurs in areas, which are close or contiguous to the proliferating synovial pannus and, to some extent, to the cartilage surface exposed to synovial fluid.

Under conditions which accompany inflammatory joint disorders and changes in the composition of its extra-cellular matrix, chondrocytes undergo significant alterations which are not yet fully understood [3,31–34]. Therefore, we focused our interest on the behavior of some molecules, such as MMPs and TIMPs, which are proposed to be active agents during joint degeneration processes.

Different studies have described the amount of MMPs, TIMPs and some interleukins in both synovial fluid and serum of patients affected by rheumatoid arthritis and osteoarthritis [35–38]. However, no attempt has ever been made before to correlate the activity of the molecules with the severity of the joint lesions. In addition, in the last two years several studies have been performed to identify the amount and the role of MMPs on different joint pathologies but no paper has correlated the disease's stage, as scored by arthroscopy, and both the protease activities and the amount of bound/free inhibitors [39].

The present study was performed by analyzing synovial fluid harvested from 56 patients (28 males and 28 females), all of them having undergone arthroscopy, with an age ranging between 20 to 79 yr, in order:

1. to analyze the amount and the activity of different MMPs and of their inhibitors in synovial fluid harvested from patients with different acute/inflammatory/degenerative joint pathologies (severe gonarthrosis and chondral lesions of the I, II and III degrees of a classification obtained upon arthroscopy);
2. to correlate the stage of the disease with the activity and the amount of MMPs, and their inhibitors.

On the whole, the results obtained show that the activity of MMPs present in synovial fluids is strictly related to the disease severity and progression (Table 1) while preliminary results demonstrate that synovial fluid harvested from healthy joints express a low MMP activity; this activity and, on the whole, the proteolytic patterns were unrelated to the patient's age (data not shown).

Our first interest was focused on the increased gelatinolytic activity observed at 66 to 55 kDa. Immunoblots have been then performed to identify the molecule responsible for this increased activity. Antibodies against MMP-2 (M.W. 72–62 kDa) and MMP-3 (M.W. 56–59 kDa), have allowed the identification of these MMPs in samples even although no

relationship has been observed between the total amount of immunoreactive enzymes and the progression (arthroscopically determined) of chondromalacia, as previously reported for MMP-3 [40]. However, it should be pointed out that although the total amount of MMP-2 is similar among different samples, the relative amount of pre-MMP-2 decreases during disease progression (see Figures 3, 7).

It must be remarked that the finding of different MMP activity patterns (as shown by zymography) despite a close similarity for the overall amount of active MMPs present in different synovial fluids (as shown by Western blot experiments performed by using anti-MMPs antibodies, see Figures 2–7) [41] suggests that the severity of the disease must be associated to the production of a MMP with either enhanced activity or reduced inhibition or with both. In this respect, it is very important to outline that, even although the total amount of TIMP-2 (as confirmed in ref. [41]) and MMP-2 were similar among different groups, the extent of binding of TIMP-2 to MMP-2 (as from Western blot data obtained under native conditions by using anti TIMP-2 antibodies) turns out to be much less in patients of group IV with respect to patients from other groups (see Figure 7). This finding, which is very clearcut and might be used as a marker of the disease severity, could account for the increased MMP activity observed in the synovial fluids of these patients, allowing to propose MMP-2 as the most likely responsible for this phenomenon. As a whole, the increased MMP-2 activity, observed during disease progression, without a parallel increase of its total amount and the less efficient inhibition by TIMP-2 indeed suggest that the increase of disease severity must be related to the increased MMP-14-mediated activation [42] of MMP-2 leading to a form which could also have a reduced affinity for TIMP-2 (Figure 7). We can thus suppose that during osteoarthritis progression, the amount of active MMP-2 progressively increases, reaching the highest concentration when the pathology becomes clinically relevant. At this stage, disease progression is related to increased activation of MMP-2 associated with the production of a MMP-2 form with a reduced TIMP-2 affinity constant. Preliminary data obtained in our laboratory by using MMPs harvested from tumor cells seems to confirm this hypothesis: pathologic progression is often associated with the production of different forms of MMPs, usually expressing higher activity due to a super-activation.

Preliminary experiments performed by using antibodies anti-MT1-MMP (MMP-14, M.W. 66–54 kDa) to detect the amount of such an enzyme in the synovial fluid did not show any difference among different samples, thus indicating that the amount of MMP-2 released in synovial fluid is not directly related to

disease progression. Therefore, we can conclude that the most likely hypothesis is that the disease progression is related not to the total amount of MMP-2, as also supported by previous data (see above), but to the activation of a MMP-2 which is less efficiently inhibited by TIMP-2 or it is superactivated.

To determine also the role of collagenases in this pathologic process we have analyzed both immunoreactive and activity levels of synovial MMP-1, MMP-8 and MMP-13, which are the only proteinases currently known to be able of initiating the denaturation of fibrillar collagens, such as type I, by first cleaving the triple helix of this molecule.

Other authors demonstrated that IL-1 β can up-regulate “in vitro” MMP-8 production in human cartilage [43–44]; our data show that the same amount of MMP-8 has been found in different groups, thus indicating that this molecule could be unrelated to disease progression in these patients assuming from a long time NSAIDs.

On the other hand, the amount of MMP-13, as detected both as an immunoreactive protein and by the protein activity, has been found directly related to the degree of cartilage degradation determined arthroscopically (see Figure 6); in addition, a slight increase of MMP-1, related to the severity of chondromalacia, was observed, confirming previous observations [31].

It should be also emphasized the role of MMP-13 in joint degradation [45–47]. As a matter of fact, MMP-13 is the strongest MMP candidate in cartilage tissue for the degradation of matrix components, including the core protein of aggrecan. Our data strongly support this hypothesis, since a larger amount of immunoreactive protein was observed in patients of groups III–IV while just a little amount of MMP-13 was found in synovial fluids harvested from normal or group I patients.

Two other proteolytic bands (94 kDa and 125 kDa) were found to be related to the disease degree: the former one was found in groups II–III, while the latter was found only in group IV patients (i.e., in subjects with the highest degree of cartilage degeneration). Different experiments were performed to identify these proteins, but they only allowed to rule out possibilities. Thus, as an example, the possibility that the 94 kDa band is gelatinase B (M.W. 92–84 kDa) has been excluded since antibodies against MMP-9 slightly reacted in Western blot with a band weighing 84-kDa; this band, similar in all tested patients, was found to be unrelated to disease progression. A zymography performed using purified enzymes ruled out also the possibility that the activity at 94 kDa could be due to plasmin, kallikrein, urokinase and plasminogen. A recent observation has shown the existence of a serine protease, which has been called matriptase, and it displays the same MW (i.e., 95

kDa) and the capability of degrading matrix proteins [48]; however, its presence in the synovial fluid has never been reported before, and we have no evidence that this is the 94-kDa protein observed in our zymographies.

The 125-kDa band did not react with anti-MMP antibodies, while its proteolytic activity is strongly inhibited by PMSF, again suggesting a serine protease activity. Also in this case the identification of the enzyme is not possible at this stage, even although we cannot rule out the possibility of a multimeric form of a smaller macromolecule.

On the whole, our data indicate that cartilage degradation is strictly related to both an increase in synovial activity of MMP-2 and immunoreactive MMP-13 amounts and the appearance of at least two serine proteinases, one of them, weighing about 125 kDa, is present only in those patients with knee arthritis (group IV). It is useful to underline that serine protease may be important in the activation of many pro-MMPs, such as pro-MMP-1 (tissue collagenase), pro MMP-3 (stromelysin-1) and pro-MMP-9 (gelatinase B), even although none of them is involved, in our work, as a disease progression marker. Pro-MMP-2 is unique in that its activation is not caused by serine proteinases but it is achieved by membrane-associated activators, such as MT1-MMP (similar among different synovial fluids but differing in terms of cell-surface bound protein, as stated in ref. [42]) and TIMP-2.

Preliminary studies demonstrated that the alteration of MMP-2 activity is mirrored by an increase of cytokines, namely IL-6, in the synovial fluids of patients with enhanced severity degree of the disease [27]. Like in many other pathologic processes [22], there is an unbalancing between TIMPs and cytokines, even although it has not been confirmed whether the unbalance between IL-6 and TIMPs and the increase of IL-6 is the origin or the result of the cartilage destruction, and therefore of the mechanical stress. Krane [3,4] suggested that the production of “inappropriate” cytokines or alterations in the temporal sequence or amounts of release of these soluble cell products, as well as of matrix metalloproteinases or of their inhibitors, significantly contributes to connective tissue alterations and cartilage destruction. Our data further confirm their observations, indicating that an unbalance of MMP activities and the amount of MMPs inhibitors are crucial for the disease evolution. However, in our case we have shown for the first time that this unbalance may be related to the presence of a likely modified MMP-2 or a modified TIMP-2 and this can be a key element in triggering the pathologic evolution of this disease [43].

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