

Stromelysin-1 (MMP-3) in Synovial Fluid of Patients with Rheumatoid Arthritis Has Potential to Cleave Membrane Bound Fas Ligand

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ABSTRACT. *Objective.* To investigate the relationship between matrix metalloproteinases (MMP) and the soluble form of Fas ligand (sFasL) in the synovial fluid (SF) of patients with rheumatoid arthritis (RA), and to determine which MMP have a major role in cleaving FasL.

Methods. The concentrations of sFas and sFasL in SF from 48 patients with RA and 43 patients with osteoarthritis (OA) were measured using specific ELISA. The levels of different MMP (MMP-1, 2, 3, 7, 9) in SF were also measured by ELISA. The active forms of gelatinases were detected by gelatin zymogram. Human FasL-expressing transfected cells (hFasL/L5178Y) were used to investigate whether FasL is cleaved from membrane bound FasL.

Results. Significantly higher levels of MMP-1, 3, and 9 were found in SF from RA patients compared to OA patients, but MMP-7 was not detectable in either group. The concentrations of sFas and sFasL in SF were also higher in RA than in OA patients. However, there was no relationship between the concentration of sFas and sFasL. Among MMP, MMP-3 concentrations in SF were closely correlated with the level of sFasL and with disease activity of RA. Enzymatic cleavage assay indicated that MMP-3 has potential to cleave the FasL expressed on hFasL/L5178Y cells and to produce sFasL.

Conclusion. There was significant correlation between the concentration of sFasL and MMP-3 in SF of patients with RA. In addition, our data indicate that the shedding of FasL may be regulated by MMP-3 in the joint of patients with RA. (J Rheumatol 2001;28:22–8)

Key Indexing Terms:

SOLUBLE Fas

RHEUMATOID ARTHRITIS

APOPTOSIS

MATRIX METALLOPROTEINASE

STROMELYSIN-1

Fas ligand (FasL) is a type II integral membrane protein homologous to tumor necrosis factor (TNF). Fas is a member of the TNF receptor family and acts as one of the main signaling systems with the specialized function of inducing apoptosis. Thus the relationship between FasL and Fas is similar to the relationship between TNF and TNF receptor¹. FasL is expressed on activated T and natural killer (NK) cells, and it induces apoptosis to effector cells via the Fas/FasL system.

The soluble form of FasL (sFasL) is anchored on the cell surface and inhibits cytotoxicity of the membrane bound FasL². Matrix metalloproteinase (MMP) inhibitors block the release of TNF- α and sFasL^{1,2}.

In rheumatoid arthritis (RA), attention is focused on the relationship between insufficient apoptosis and the exacerbation of RA³. We have reported that synovial proliferation in RA could be inhibited by the induction of apoptosis mediated by Fas/FasL interaction^{4,5}. In the RA animal model, synovial proliferation occurs when the balance between the degree of synovial inflammation and apoptosis is broken⁶. Indeed, the level of sFas and sFasL, which antagonize apoptosis via the Fas/FasL system, are significantly increased in the synovial fluid (SF) of patients with RA^{7,8}. These results suggest that accumulation of sFas and sFasL in the joints of patients with RA may inhibit apoptosis and exacerbate the inflammatory process. However, the mechanism of accumulation of these molecules in the soluble form is still unknown. Moreover, it is well known that large amounts of various MMP are present in the SF of patients with RA and these MMP are closely related to joint destruction in RA. We investigated the relationship between MMP and sFasL in the SF of patients with RA, and determined which MMP had a major role in cleaving FasL.

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MATERIALS AND METHODS

Patients. Forty-eight patients with RA that fulfilled the revised criteria of the American Rheumatism Association were studied⁹. Forty-three patients with osteoarthritis (OA) were studied as a control. Both groups of patients were matched for age, sex, and disease duration (RA: age 62.2 ± 12.2 yrs, 41 women, 7 men, disease duration 8.7 ± 6.2 yrs; OA: age 64.5 ± 9.8 yrs, 39 women, 4 men, disease duration 6.5 ± 5.7 yrs). We obtained informed consent from each patient in writing before entry into the study. Patients with RA or OA were not treated with intraarticular steroid injections prior to the study. In patients with RA, the following laboratory and clinical assessments were determined: C-reactive protein (CRP) level, erythrocyte sedimentation rate (ESR), rheumatoid factor (RF) titer, duration of morning stiffness, and joint score^{10,11}. SF was collected from all the patients in both groups.

Analysis of MMP in SF. The analysis of MMP was conducted as described¹². Briefly, 3.2 μ l SF was used for the gelatin zymogram; a 5-fold dilution of SF was electrophoresed at 4°C, without reduction, on a 7.5% polyacrylamide gel impregnated with 0.3 mg/ml gelatin. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 for 15 min and was then incubated in a buffer containing 50 mM Tris-HCL, 5 mM CaCl₂, 1 μ M ZnCl₂, 1% Triton X-100, and 0.02% NaN₃. After incubation for 16 h at 37°C, the gel was stained for proteins with 0.1% Coomassie brilliant blue in 40% (v/v) isopropanol and destained in 7% (v/v) acetic acid. Three microliters SF was used for the immunoblotting assay of MMP-3. The membranes were treated with 5 μ g/ml human anti-stromelysin-1 monoclonal IgG (Cosmo Bio, Tokyo, Japan); goat anti-mouse Ig (G+L) horseradish peroxidase (HRP; Tago, Burlingame, CA, USA) was used as the secondary antibody. Bound HRP was detected by a chemiluminescence technique, using ECL Western blotting reagents. The membranes were exposed to Hyperfilm-³H autoradiography film. Control samples were collected from healthy volunteer controls (n = 5) with informed consent.

Concentrations of MMP were measured by the ELISA system according to the instructions of the manufacturer. MMP-1, 2, and 7 were measured by a human MMP ELISA system (Amersham Pharmacia Biotech, Buckinghamshire, UK) as described¹³⁻¹⁵. The concentrations of MMP-3 and 9 were measured by a human stromelysin-1 and gelatinase-B enzyme immunoassay (EIA) system (Fuji Chemical Industries Co. Ltd., Toyama, Japan) as described^{16,17}. In measuring MMP-1, 2, and 7, a 100 μ l aliquot of diluted SF (1:50 in MMP-1, 1:200 in MMP-2, 1:25 in MMP-7) was transferred to each microplate well previously coated with monoclonal antibody (Mab), and the plate was left to stand for 1 h with MMP-7 and for 2 h with MMP-1 and 2 at room temperature without shaking. In measuring MMP-3 and 9, each SF (20 μ l of 1:2000 dilution for MMP-3; 50 μ l of 1:100 dilution for MMP-9) was mixed with polyethylene beads coated with Mab, and the mixture was allowed to stand at room temperature for 90 min. Peroxidase activity bound on the plate or beads was detected using *o*-phenylenediamine in MMP-9 and 3,3',5,5'-tetramethylbenzidine (TMB) in MMP-1, 2, 3, and 7. The reaction was stopped by addition of sulfuric acid, and the absorbance at 450 nm for MMP-1, 2, 3, and 7 and at 495 nm for MMP-9 was measured.

Concentrations of polymorphonuclear elastase. The concentration of polymorphonuclear (PMN) elastase in SF was determined by the Merck immunoassay as described¹².

Concentrations of sFas and sFasL. The concentrations of sFas and sFasL were determined by a sandwich ELISA (MBL Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. Briefly, for the Fas Mab 2 types of anti-Mab, DX-2 and DX-3, were used⁷. With FasL Mab, 2 types of anti-Mab, 4H9 and 4A5, were also used⁸. The DX-2 and 4H9 Mab were previously coated onto 96 well microplates and incubated at room temperature for 1 h. The sample was diluted 5 times with phosphate buffered saline (PBS) and 100 μ l aliquots were added to the wells after the blocking reaction. After washing 5 times with 0.05% Tween 20/PBS, biotin conjugate DX-3 or 4A5 in 0.05% Tween 20/PBS was added and incubated at room temperature for 1 h. After 5 additional washes, peroxidase activity was detected using TMB as a substrate. Light absorbance was determined in a micro ELISA reader at 450 nm. The levels of sFas and sFasL were determined by comparison to standard

curves obtained using recombinant sFas or sFasL. To exclude a possible reaction with other proteins, we examined the samples in a mouse IgG coated plate (PharMingen, San Diego, CA, USA). The samples did not react in this assay system.

Active forms of MMP. The active forms of MMP-1, 2, 7, and 9 were purchased from Yagai Co. Ltd. (Tokyo, Japan). These activated MMP were purified from human skin. The active form of MMP-8 was made from pro-MMP-8 (Chondrex, San Diego, CA, USA) as described^{18,19}. Briefly, 10 μ l of pro-MMP-8 was added to buffered bovine serum albumin and 10 μ l of trypsin solution (100 μ g/ml; Sigma, St. Louis, MO, USA), and incubated at 37°C for 20 min. After that, 10 μ l of trypsin inhibitor solution (500 μ g/ml; Sigma) was added and incubated at room temperature for 1 h. The pro-MMP-8 was changed to the active form by the above method.

The active form of recombinant MMP-3 was kindly provided by Ono Pharmaceutical Co. Ltd. (Osaka, Japan) and it was produced in *Escherichia coli* as described^{20,21}. Briefly, cDNA of pro-MMP-3 was amplified by polymerase chain reaction (PCR) from a cDNA library of T98G (human glioblastoma cell line; American Type Culture Collection, Burlingame, CA, USA). The PCR product was incorporated into the bacterial expression vector pET11a (Stratagene Cloning System, San Diego, CA, USA). The protein of pro-MMP-3 was expressed in the *E. coli* strain, BL21(DE3)(pLysS)²⁰. This produced protein was confirmed as the pro-MMP-3 since its molecular weight was 50 kDa as determined by Western blot analysis. This pro-MMP-3 was activated as described²¹.

FasL cleavage effect of MMP. The FasL cleavage effect of MMP was analyzed with L5178Y human FasL transfected cells (hFasL/L5178Y)², kindly provided by Dr. K. Okumura (Juntendo University, Tokyo, Japan). These cells express FasL and have an apoptosis induction effect on synovial cells of RA⁵. The hFasL/L5178Y cells (10⁶/ml) were incubated in RPMI-1640 containing 10% fetal calf serum with or without various active forms of MMP (20 mU/well) in 96 well plates for 30 min². After centrifugation of the medium at 1500 rpm for 20 min, the sFasL in the supernatant was quantitated as described above by ELISA. To reveal the role of the active form of MMP in the release of the membrane bound FasL, an MMP inhibitor (EDTA; Sigma)²² was added to the hFasL/L5178Y cells.

FasL cleavage effect of RA SF. To examine whether RA SF cleaves FasL directly, RA SF with or without MMP-3 was added to the hFasL/L5178Y cells, and the increased concentration of sFasL in culture supernatant was measured as described above. The SF contained with MMP-3 was confirmed by immunoblotting analysis.

Statistical analysis. Data are expressed as the mean \pm SEM. The Mann-Whitney U test was used to examine differences between 2 groups. Pearson's correlation coefficient was used to examine correlation between 2 variables and the deviations were examined by Fisher's Z exchange.

RESULTS

High concentrations of MMP in RA SF. As shown in Figure 1, concentrations of MMP-1, 3, and 9 in the SF of patients with RA were significantly higher than in patients with OA. However, the concentration of MMP-2 did not differ between RA and OA patients. Measurable levels of MMP-7 in RA and OA patients were not detected. When the relationship between the concentration of MMP-3 and the clinical variables of RA was examined, positive correlations with CRP and local knee joint scores were recognized. However, the systemic joint pain score showed no correlation with the concentration of MMP-3 (Table 1). In MMP-1, positive correlation with CRP was observed. In patients with RA, neither MMP-2 nor MMP-9 was shown to have any correlation with clinical variables, i.e., ESR, CRP, rheumatoid factor, joint score, duration of morning stiffness, or knee joint score. We also examined the

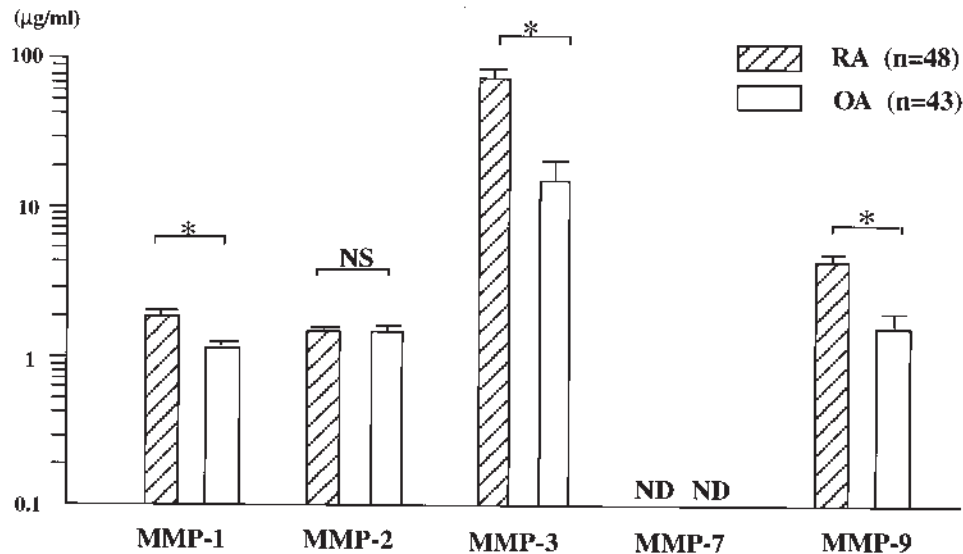


Figure 1. Concentration of MMP-1, 3, and 9 in SF of patients with RA were significantly higher than in patients with OA. However, the concentration of MMP-2 did not differ between RA and OA. MMP-7 was not detected in patients with RA or OA. Data are expressed as the mean \pm SEM. NS: not significant, ND: not detected; $p < 0.05$.

Table 1. Correlation of concentration of MMP in synovial fluid with clinical and laboratory markers of RA.

	MMP-1		MMP-2		MMP-3		MMP-9	
	r	p	r	p	r	p	r	p
ESR	0.132	0.533	0.188	0.371	0.480	0.141	0.361	0.076
CRP	0.482	0.032*	0.081	0.702	0.507	0.050*	0.289	0.163
RF	0.047	0.824	0.628	0.140	0.317	0.151	0.523	0.245
Systemic joint score	0.131	0.537	0.219	0.297	0.144	0.495	0.069	0.745
Morning stiffness	-0.273	0.186	0.051	0.811	-0.307	0.136	-0.080	0.0706
Knee joint score	0.182	0.388	0.250	0.231	0.397	0.048*	0.432	0.065

* $p < 0.05$.

Systemic joint score was determined previously¹⁰. Knee joint score was assessed previously¹¹. r: correlation coefficient, ESR: erythrocyte sedimentation rate, CRP: c-reactive protein, RF: rheumatoid factor.

relationship between the concentration of each MMP and the medication used (steroid or other antirheumatic drugs) as well as disease duration. No significant relationship between these factors and each MMP concentration was noted (data not shown). Moreover, there was no correlation between MMP-3 and other MMP.

MMP activity in SF of patients with RA. To determine whether SF MMP are activated in RA, a gelatin zymogram was performed. Based on the type of gelatinase determined by gelatin zymography analysis, SF could be classified into 3 distinct groups¹². That is, group I contained MMP-2 only, group II contained MMP-2 and inactive MMP-9, and group III contained MMP-2 and active MMP-9 (Figure 2A). All control SF were classified as group I. MMP-3 was detected by immunoblot analysis in group III SF, but it was not detectable in the other groups (Figure 2B). PMN elastase concentration was significantly higher in group III than in the other groups

($p < 0.01$; group III 123.2 ± 38.5 , group II 68.5 ± 21.7 , group I 0.8 ± 0.2 , controls 0.4 ± 0.7 $\mu\text{g/ml}$). A significantly higher number of RA patients than OA patients were classified into group III (RA:OA ratio, group I 4:30, group II 14:3, group III 30:10). Moreover, it was proved by immunoblotting analysis that MMP-3 was present in all group III SF, but was absent in the other groups (groups I and II). This result is in agreement with our previous reports^{12,14}.

Elevated levels of sFas and sFasL in SF of patients with RA. Concentrations of both sFas and sFasL in RA SF were significantly higher than those in patients with OA (Figure 3). However, there was no correlation between sFas and sFasL concentration ($r = 0.253$, $p = 0.60$). The relationship between the concentration of sFas and the RA clinical variables was also investigated (Table 2). The concentration of sFas correlated weakly with the serum CRP level, but with no other clinical variables. With regard to the correlation between sFasL

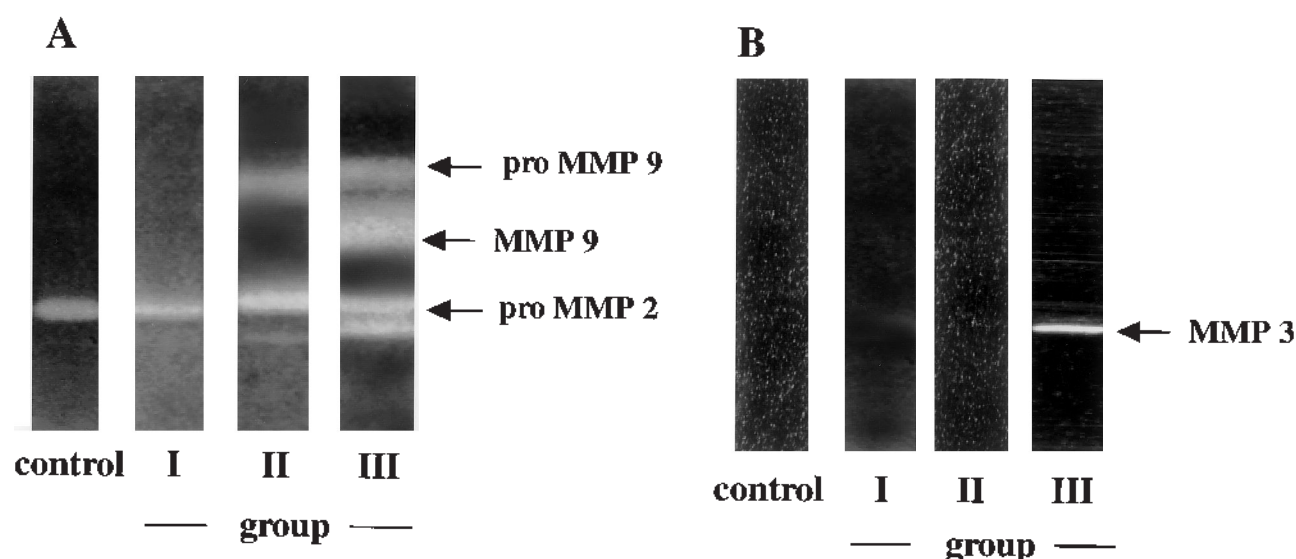


Figure 2. Synovial fluid condition. (A) Gelatin zymogram of SF: SF were classified into 3 distinct groups. Control samples were collected from healthy volunteers. Group I had MMP-2 (72 kDa) only, group II had MMP-2 and inactive MMP-9 (92 kDa), and group III had MMP-2 and active MMP-9 (82 kDa). (B) Immunoblotting of MMP-3: MMP-3 was only visualized in the SF of group III.

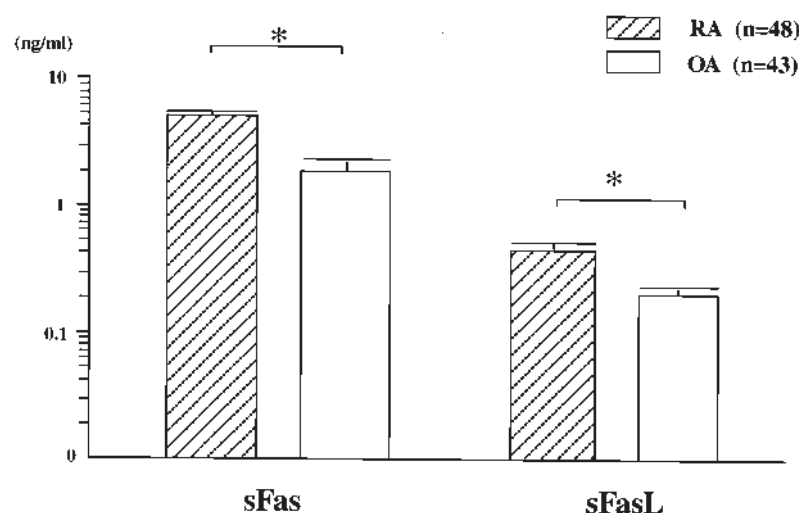


Figure 3. The concentrations of both sFas and sFasL in SF from patients with RA were significantly higher than in patients with OA. Data are expressed as mean \pm SEM. $p < 0.05$.

concentrations and RA clinical variables, there was a significant correlation between sFasL concentration and the knee joint score, but with no other clinical variable (Table 2). The relationship of sFas and sFasL levels and medications used, as well as disease duration, was investigated and no relationship was found (data not shown).

Relationship between MMP and sFas and sFasL concentrations in RA. A significant correlation between SF concentrations of sFasL and MMP-3 in patients with RA was revealed, although no such correlation was noted between sFasL concentrations and other MMP (Table 3). The sFas concentration had no relationship with any of the MMP. In OA patients, there was no correlation between all MMP and concentration of sFas and sFasL (data not shown).

Table 2. Correlation of synovial fluid sFas and sFasL concentration with clinical and laboratory markers of RA.

	sFAS		sFasL	
	r	p	r	p
ESR	0.181	0.390	0.208	0.322
CRP	0.393	0.050*	0.212	0.312
RF	0.111	0.823	0.170	0.420
Systemic joint score	0.150	0.477	0.416	0.077
Morning stiffness	-0.363	0.631	-0.174	0.409
Knee joint score	0.116	0.585	0.489	0.011*

* $p < 0.05$.

Systemic joint score was determined previously¹⁰. Knee joint score was assessed previously¹¹.

Table 3. Correlation of concentration of MMP with sFas and sFasL in RA synovial fluid.

	MMP-1		MMP-2		MMP-3		MMP-9	
	r	p	r	p	r	p	r	p
sFas	0.141	0.505	0.021	0.921	0.156	0.461	0.105	0.621
sFasL	0.151	0.474	0.027	0.897	0.398	0.048*	0.222	0.290

*p < 0.05.

Cleavage of membrane bound FasL and production of sFasL by MMP treatment. Concentrations of sFasL in the supernatant of cultured hFasL/L5178Y cells treated with active MMP-3 and 7 were significantly higher than those treated with other active MMP (Figure 4A). These data indicate that sFasL was released from the cell surface of hFasL/L5178Y cells by active MMP-3 and 7. To confirm that the sFasL release from hFasL/L5178Y cells was associated with active MMP-3, the active form of MMP-3 was cocultured with MMP inhibitor (EDTA) in hFasL/L5178Y cells. As illustrated in Figure 4B, EDTA inhibited by about 50% the release of sFasL from hFasL/L5178Y cells. This was also observed with the use of an active MMP-7 (data not shown).

Cleavage of membrane bound FasL and production of sFasL by RA SF. When we examined whether the SF of RA cleave FasL, we observed that the RA SF have a potential to cleave FasL directly, because the concentration of FasL increased by the addition of SF containing MMP-3. The cleavage of FasL from hFasL/L5178Y by RA SF was significantly higher with MMP-3 than without MMP-3 (Figure 5).

DISCUSSION

Large amounts of various MMP were detected in joints of patients with RA, and these MMP might play an important role in the pathological processes of RA associated joint destruction^{12,17,23-27}. We confirmed the elevation of various MMP in the SF of RA patients, with especially high concentrations of MMP-3 being observed. Among all MMP, MMP-3 has been considered to be the critical enzyme for cartilage matrix breakdown in RA, since it can directly act on native type II collagen, a principal collagen type in joint cartilage^{12,17,27,28}. Moreover, we previously reported that a large amount of MMP-3 in the SF of RA patients was activated²⁸. It has been suggested that activation of MMP-3 is influenced by the presence of PMN elastase, and subsequently MMP-9 is activated by the activators present in RA SF^{12,28}. In this study, we reconfirmed that a significantly higher number of RA patients were classified as having group III SF (activated MMP condition group) compared with OA patients. In addition, the concentrations of MMP-1 and 3 correlated significantly with the disease activity of RA, as reported^{12,17,27,29}. Furthermore, MMP-3 had a positive correlation with the local

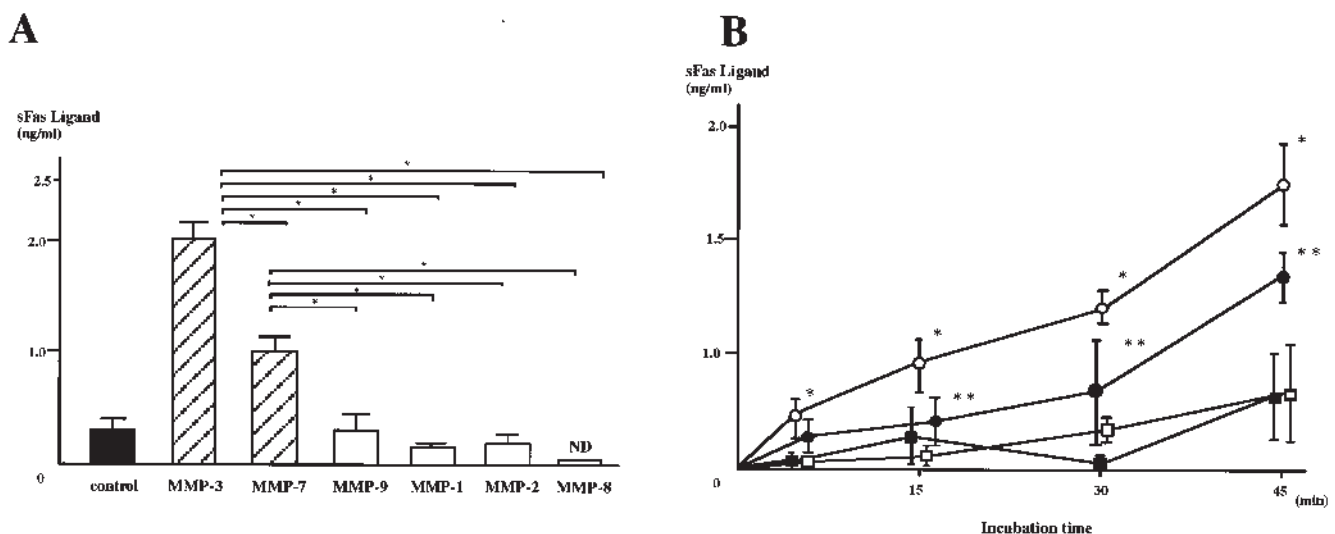


Figure 4. Shedding of FasL in hFasL/L5178Y cells. (A) Concentrations of sFasL in the supernatant of cultured hFasL/L5178Y cells treated with active MMP-3 and 7 were significantly higher than in the treated group with other active MMP. *Statistically significant, p < 0.05. (B) The active form of MMP-3 induced sFasL in the medium of hFasL/L5178Y cells. EDTA inhibited by about 50% the release of sFasL from hFasL/L5178Y cells. ○: MMP-3 (20 mUnit/well), ●: MMP-3 (20 mUnit/well) + EDTA (5 mM), □: none, ■: EDTA (10 mM). Data are expressed as mean ± SEM. *p < 0.05, MMP-3 treatment group versus other treatment group. **p < 0.05, MMP-3 + EDTA treatment group versus none or EDTA treatment group.

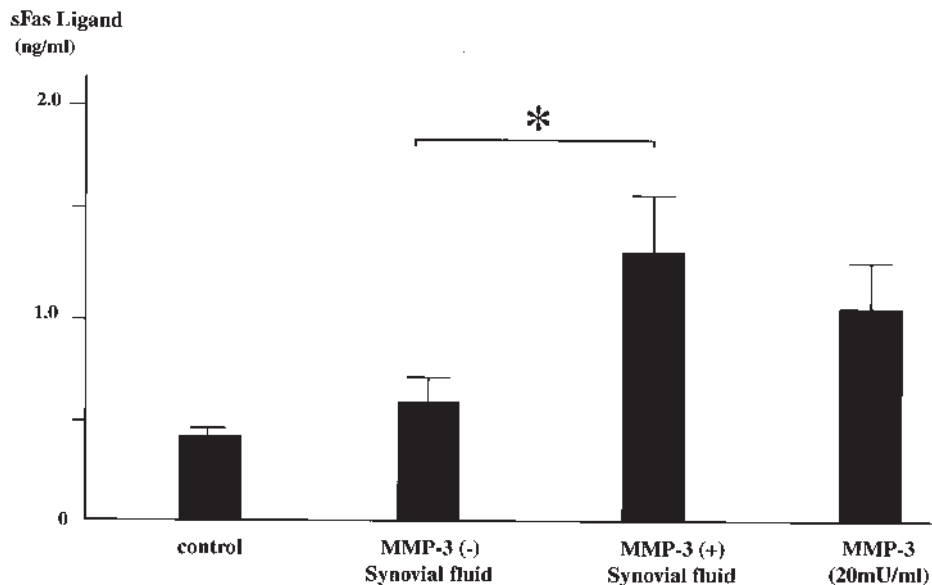


Figure 5. FasL cleavage effect of RA synovial fluid. The hFasL/L5178Y cells were incubated in RPMI containing FCS with or without MMP-3 for 30 min. Control: no treatment. MMP-3(-) SF: the group I or II SF that were classified by immunoblotting analysis. MMP-3(+) SF: the group III SF that were classified by immunoblotting analysis. MMP-3 (20 mU/ml): recombinant MMP-3. Each group, n = 15. Data are expressed as mean \pm SEM. *p < 0.01; MMP-3(+) SF group versus MMP-3(-) SF.

knee joint score but not with the systemic joint score. These results indicated that the presence of MMP-3 in SF might reflect an inflammatory condition in the joints in RA, and that activated MMP-3 is considered to play a major role in the degradation of articular cartilage. However, the presence of MMP-3 is not sufficient for the destruction of joint cartilage in RA, since MMP-3 gene deficient mice still maintain the ability to develop an RA-like condition with joint destruction³⁰. Therefore, MMP-3 appears to play a role in the destruction of joint cartilage, possibly by cooperating with other factors.

From the point of view of apoptosis, cartilage degradation is also regulated by Fas/FasL mediated chondrocyte apoptosis³¹. Fas/FasL mediated apoptosis is closely related to the development of the pathological manifestation of RA⁶⁻⁸. Previously, we showed that insufficient intraarticular apoptosis induced proliferation of synovium⁶, and that inducing apoptosis remitted the synovial proliferation^{4,5}. These data indicate that Fas/FasL mediated apoptosis is usually inhibited in the joints of patients with RA. The soluble forms of Fas and FasL are considered to be Fas/FasL mediated apoptosis inhibitors⁶⁻⁸. These soluble substances are significantly increased in the SF of RA patients. Therefore, high concentrations of sFas and sFasL, as well as MMP-3, might play an important role in manifestation of RA.

Recently, it was elucidated that MMP inhibitors have the potential to block the release of sFasL from the cell surface of FasL^{1,2}. This is of relevance to RA since both concentrations of activated MMP and sFasL were increased in the SF of patients with RA. The sFas was also increased in the RA SF;

however, sFas, unlike sFasL, is generated by alternative splicing of sFas mRNA³². Indeed, there was no correlation between sFas and sFasL concentration in the SF in patients with RA. On the other hand, the concentration of sFasL significantly correlated with the concentration of MMP-3. Therefore, we focused on the role of MMP-3 in the shedding of sFasL in RA.

When a variety of active forms of MMP were added to FasL transfected cells, sFasL was generated in the cultured supernatant by MMP-3 and 7, indicating that MMP-3 and 7 have the potential to cleave FasL from the cell surface. This was reconfirmed by the experiment using RA SF, in which the concentration of sFasL increased remarkably in the presence of MMP-3. Therefore, MMP-3 may be an essential MMP for shedding of FasL in RA, since MMP-7 was not detectable in the patients with RA. However, spontaneous shedding of FasL was not blocked by MMP inhibitor alone. In addition, a low concentration of sFasL was observed even when the SF without MMP-3 was added. These results support that MMP-3 may not be entirely responsible for shedding of FasL. Further investigation is needed for detection of the other factors involved in the cleavage of FasL^{33,34}.

We demonstrated that the concentration of MMP-3 and sFasL was increased in the synovial fluid of patients with RA, and a significant correlation between the concentration of sFasL and MMP-3 was found. Moreover, increased levels of MMP-3 may be responsible for the shedding of FasL from the cell surface in the joints of RA patients, but other factors play a role in this process.

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