Serum Levels of Matrix Metalloproteinase 3 (Stromelysin 1) for Monitoring Synovitis in Rheumatoid Arthritis

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• ***Context.*—Matrix metalloproteinase 3 (MMP-3) is expressed in synovial tissues and involved in cartilage destruction in rheumatoid arthritis and osteoarthritis.**

***Objective.*—To study whether measurement of MMP-3 serum concentrations is useful to monitor the activity of rheumatoid synovitis.**

***Design.*—Levels of MMP-3 in serum and synovial tissue samples obtained from 29 rheumatoid arthritis patients and 20 osteoarthritis patients were measured by the 1-step sandwich enzyme immunoassay system.**

***Results.*—Levels of MMP-3 in the serum and synovial samples were significantly higher in rheumatoid arthritis than in osteoarthritis ($P < .001$), and the levels correlated directly with each other ($r = 0.712$, $P < .001$; N = 49). Immunohistochemistry demonstrated almost exclusive localization of MMP-3 to the lining cells in rheumatoid synovium. The immunoreactivity correlated directly with the scores of synovial inflammatory cell infiltration ($r = 0.606$, $P < .001$; n = 29) and the MMP-3 levels in the synovial tissues ($r = 0.564$, $P = .001$; n = 29) and those in the serum samples ($r = 0.529$, $P = .003$; n = 29) in rheumatoid arthritis. Levels of MMP-3 in rheumatoid serum samples dropped to low values at 1 and 2 weeks after total knee arthroplasty, while the levels of C-reactive protein increased at 1 week and the erythrocyte sedimentation rate and counts of white blood cells and platelets were unchanged at 1 and 2 weeks postoperative.**

***Conclusions.*—Our results demonstrate that MMP-3 levels in the serum of rheumatoid arthritis patients correlate with the levels produced by the synovial lining cells and suggest that the activity of rheumatoid synovitis can be monitored by measuring serum levels of MMP-3.**

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Proteinases belonging to all of the proteinase classes are produced in joint tissues of individuals with rheumatoid arthritis (RA) or osteoarthritis (OA).[1,2] Among them, matrix metalloproteinases (MMPs) are believed to play a central role in the destruction of articular cartilage.[1,2] The MMPs are a gene family of neutral metalloproteinases composed of 23 members in humans and include secreted-type MMPs and membrane-anchored MMPs.[2–4] Secreted-type MMPs can be classified into 6 subgroups according to their substrate specificity and structural differences[2]: (1) collagenases, including tissue collagenase (MMP-1), neutrophil collagenase (MMP-8), and collagenase 3 (MMP-13); (2) gelatinases, such as gelatinase A (MMP-2) and gelatinase B (MMP-9); (3) stromelysins, including stromelysin 1 (MMP-3) and stromelysin 2 (MMP-10); (4) matrilysins, such as matrilysin 1 (MMP-7) and matrilysin 2 (MMP-26); (5) furin-activated MMPs, including stromelysin 3 (MMP-13) and epilysin (MMP-28); and (6) other MMPs such as metalloelastase (MMP-12), MMP-19, enamelysin (MMP-20), MMP-21, and MMP-27. Membrane-anchored MMPs include MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17), MT5-MMP (MMP-24), MT6-MMP (MMP-25), and MMP-23. These MMPs are capable of digesting almost all the components of extracellular matrix in various tissues, including articular cartilage, when they act in concert.

One of the major MMPs expressed in rheumatoid synovial tissue is MMP-3.[5] It can digest a broad spectrum of substrates such as aggrecan, type IX collagen, link protein, and gelatins[5–8] and also can activate various proMMPs including proMMP-1, proMMP-7, proMMP-8, proMMP-9, and proMMP-13,[2,9] most of which are expressed in rheumatoid joint tissues.[10–14] Serine proteinases such as plasmin, leukocyte elastase, and cathepsin G readily activate proMMP-3 itself.[15–17] Matrix metalloproteinase 3 is considered to play essential roles in the degradation of both aggrecan and collagen fibrils in the cartilage, the latter of which may be digested through the degradation of perifibrillar extracellular matrix components such as type IX collagen[6,7] and the activation of proMMP-1,[18] proMMP-9,[19] and proMMP-13.[20] Previous studies including our own have demonstrated that MMP-3 is expressed by the synovial lining cells in RA.[21–23] The lining cells of osteoarthritic synovitis also produce MMP-3 in the advanced stages of OA.[24] We have demonstrated that the steady-state levels of MMP-3 in sy-

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novial fluids are remarkably higher in RA than in OA[25] and have suggested that MMP-3 accumulates in the synovial fluids as a consequence of overproduction by synovial tissues in RA.[25] In addition, MMP-3 is detectable in serum samples, and the levels are higher in RA patients than in OA patients or normal subjects.[26–29] However, little or no information is available on the levels of MMP-3 in the synovial tissues of RA and OA, or whether there is any correlation between the levels in the serum and synovial tissue samples. In addition, only a few studies have monitored the serum levels of MMP-3 in RA patients who underwent arthroplasty or were treated with drugs targeting the synovitis.[30,31]

In the present study, we determined levels of MMP-3 in samples of serum and synovium from patients with RA or OA. The serum levels were also monitored in RA patients who underwent total knee arthroplasty. Our results in the present study suggest that MMP-3 levels in serum samples are useful measure of the activity of synovitis in RA.

MATERIALS AND METHODS

Patients

Twenty-nine patients with RA (3 men and 26 women; 57.6 ± 18.1 years old [mean ± SD]) and 20 patients with unilateral knee OA (3 men and 17 women; 74.9 ± 6.6 years old) were studied. Diagnosis of the RA patients was based on the 1987 revised American College of Rheumatology Criteria.[32] The patients were in the advanced stages, corresponding to Larsen grade III or IV and Steinbrocker stage III, which were based on the radiographic findings of tibiofemoral joints. All patients were treated with nonsteroidal anti-inflammatory drugs or disease-modifying antirheumatic drugs, including gold thiomalate, auranofin, D-penicillamine, or bucillamine. Twenty-three of these RA patients also received low-dose steroid treatment (predonisolone, maximum 7.5 mg/d) and 7 of them had methotrexate (maximum 8 mg/wk). Knee OA was diagnosed by clinical and radiologic evaluations based on the American College of Rheumatology Criteria for OA.[33] Patients who presented with obvious joint injury were excluded from the study. All the OA patients received various nonsteroidal anti-inflammatory drugs for knee pain. Patients with RA or OA were not treated with intraarticular injection of steroids, chondroitin polysulfate, or hyaluronic acid for at least 1 month prior to this study.

Serum samples were collected by venous puncture from the RA (n = 29) or OA (n = 20) patients about 1 week before the operation of total knee arthroplasty, and stored at −20°C before being used for the assay. Synovial samples were also obtained when these patients underwent total knee replacement surgery accompanied with total synovectomy and were subjected to histological evaluation and homogenization. For time-course evaluation of laboratory data, serum samples were collected from patients with RA at the times of 1, 2 (n = 9), and 4 weeks (n = 4) after the surgery. Informed consent was obtained from the patients for the experimental use of the serum and surgical samples according to hospital ethical guidelines.

Histology and Immunohistochemistry

Histologic diagnosis was made by standard light microscopic evaluation of the sections stained with hematoxylin-eosin. Rheumatoid synovial specimens (n = 29) were analyzed according to our grading system, as follows[24]: (1) Synovial lining cell hyperplasia was graded from 0 to 3+. One to 2 layers of cells was graded as 0; 3 to 4 layers, 1+; 5 to 6 layers, 2+; and 7 or more layers, 3+. (2) Cellular infiltration (degree of infiltration by lymphocytes, plasma cells, and mononuclear and polymorphonuclear leukocytes) was graded from 0 to 4+. No infiltration present was graded as 0; mild infiltration, 1+; moderate and focal infiltration, 2+; moderate and diffuse infiltration, 3+; and marked and diffuse cellular infiltration, 4+. (3) Fibrosis of the sublining cell layer was graded from 0 to 3+, where 0 was normal; 1+, mild; 2+, moderate; and 3+, marked fibrosis.

Rheumatoid synovial tissues (n = 29) were treated with monensin for 3 hours and fixed with periodate-lysine-paraformaldehyde fixative for 18 to 24 hours at 4°C according to our method.[21,24] Paraffin sections were reacted with the monoclonal antibody to MMP-3 (8 μg/mL; clone 55-2A4, Daiichi Fine Chemical Co Ltd, Takaoka, Japan) or nonimmune mouse immunoglobulin G (IgG; 8 μg/mL, Dako Denmark A/S, Glostrup, Denmark). After reactions with biotinylated horse IgG to mouse IgG (Vector Laboratories Inc, Burlingame, Calif) and an avidin-biotin-peroxidase complex (Dako Denmark A/S), color was developed with 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Co, St Louis, Mo), as described previously.[24] The specificity of the monoclonal antibody (55-2A4) has been previously demonstrated.[26] The ratio (%) of immunostained synovial lining cells to total lining cells was measured by observing 5 different fields at magnification of ×200 without knowledge of MMP-3 assay or clinicopathologic data.

Preparation of Tissue Homogenates and Measurement of MMP-3

Synovial samples from RA (n = 29) and OA (n = 20) were cut into small pieces and homogenized on ice in 50mM Tris-HCl buffer, pH 7.5 containing 0.15M NaCl, 10mM $CaCl_2$, 0.02% NaN_3, and 0.05% Brij 35. The homogenates were centrifuged at 4°C for 20 min at 10000*g*, and protein concentrations in the supernatants were determined by the dye-binding method according to the manufacturer's instructions (Bio-Rad Laboratories Inc, Hercules, Calif). Levels of MMP-3 in the homogenate supernatants and sera were assayed by a 1-step sandwich enzyme immunoassay system according to our method.[26] The assay system for MMP-3 uses 2 simultaneous immunoreactions of a solid-phase monoclonal antibody and a horseradish peroxidase–labeled Fab′ fragment of another monoclonal antibody, and measures both precursor and active forms of MMP-3 and active MMP-3 complexed with tissue inhibitors of metalloproteinases.[26] The detection limit of the enzyme immunoassay is 0.63 ng/mL. For synovial tissues, values were calculated as mg/g protein and nmol/g protein using the molecular weight of 52220 for proMMP-3, as described previously.[25]

Time-Course Evaluation of Laboratory Data From RA Patients After Arthroplasty

Laboratory parameters for patients with RA were monitored for up to 4 weeks after total knee arthroplasty. These included MMP-3, C-reactive protein (CRP), erythrocyte sedimentation rate (Westergren method), and counts of white blood cells and platelets.

Statistical Analyses

Differences between the 2 diagnostic groups were analyzed by the Mann-Whitney *U* test. Correlations were sought using the Spearman rank correlation coefficient. Differences between laboratory data obtained before and after surgery were tested by the Friedman repeated measure analysis of variance on ranks. Significances of individual differences were evaluated using the Scheffé test if the Friedman test was significant. Statistical analyses were conducted using STATVIEW 5.0 (SAS Institute Inc, Cary, NC).

RESULTS

Levels of MMP-3 in Serum and Synovial Tissue Samples From Patients With RA or OA

Using the enzyme immunoassay system, MMP-3 was measurable in all the serum samples from RA and OA, and the level was significantly higher in RA (287.3 ± 199.9 ng/mL [mean ± SD]) than in OA (44.8 ± 32.4 ng/mL) ($P < .001$) (Figure 1, A). However, it was impossible to analyze the correlation between the MMP-3 levels and clinical stages

of RA and OA because all the patients were in the advanced stages. No definite correlation was obtained between the groups of patients treated with different drugs.

Matrix metalloproteinase 3 in the supernatants of synovial tissue homogenates from RA and OA was also detectable in all the samples examined. As shown in Figure 1, B, the level was significantly higher in RA (1.28 ± 1.26 mg/g protein; 24.5 ± 23.7 nmol/g protein) than in OA (0.12 ± 0.08 mg/g protein; 2.3 ± 1.6 nmol/g protein) ($P < .001$). When correlations between the MMP-3 levels in the serum and synovial tissue samples were examined, a direct correlation was observed ($r = 0.712$, $P < .001$; N = 49) (Figure 1, C).

Immunostaining of MMP-3 in RA Synovial Tissues and Its Correlation With MMP-3 Levels

By immunohistochemistry, MMP-3 was localized with anti–MMP-3 antibody almost exclusively to the lining cells of rheumatoid synovium (Figure 2, A through C), whereas no or negligible staining was obtained with nonimmune mouse IgG (Figure 2, D). When the immunoreactivity (percentage of immunoreactive cells to total lining cells) was determined by counting immunoreactive cells, about 22% of the total lining cells (21.7 ± 15.5%) were positively immunostained in each case. The immunoreactivity appeared to correlate with the activity of rheumatoid synovitis and the MMP-3 levels in rheumatoid synovium (Figure 2, A through C). Thus, we further analyzed synovial pathology according to our grading system (lining cell hyperplasia, inflammatory cell infiltration, and fibrosis of the rheumatoid synovial tissues), and compared the degree of these factors with the immunoreactivity in the synovial lining cells. As shown in Figure 3, A, the immunoreactivity was well correlated with scores of inflammatory cell infiltration in the sublining cell layer ($r = 0.606$, $P < .001$; n = 29). However, no correlations were obtained between the immunoreactivity and synovial cell hyperplasia, fibrosis, or total scores (data not shown). On the other hand, the MMP-3 levels in the synovial tissue homogenates tended to correlate with the scores of inflammatory cell infiltration ($r = 0.265$, $P = .004$; n = 29) (Figure 3, B). There was a direct correlation between the MMP-3 immunoreactivity and the MMP-3 levels in the synovial tissue homogenates ($r = 0.564$, $P = .001$; n = 29) (Figure 3, C). In addition, the immunoreactivity directly correlated with serum levels of MMP-3 in RA patients ($r = 0.529$, $P = .003$; n = 29) (Figure 3, D).

Changes in Serum MMP-3 Levels in RA Patients Following Knee Joint Replacement

Serum levels of MMP-3 in RA patients (n = 9) were monitored for up to 4 weeks after total knee arthroplasty. As shown in Figure 4, A, the levels observed in each case before surgery (408.1 ± 236.1 ng/mL) dropped at 1 week and 2 weeks after the surgery (124.0 ± 63.2 ng/mL and

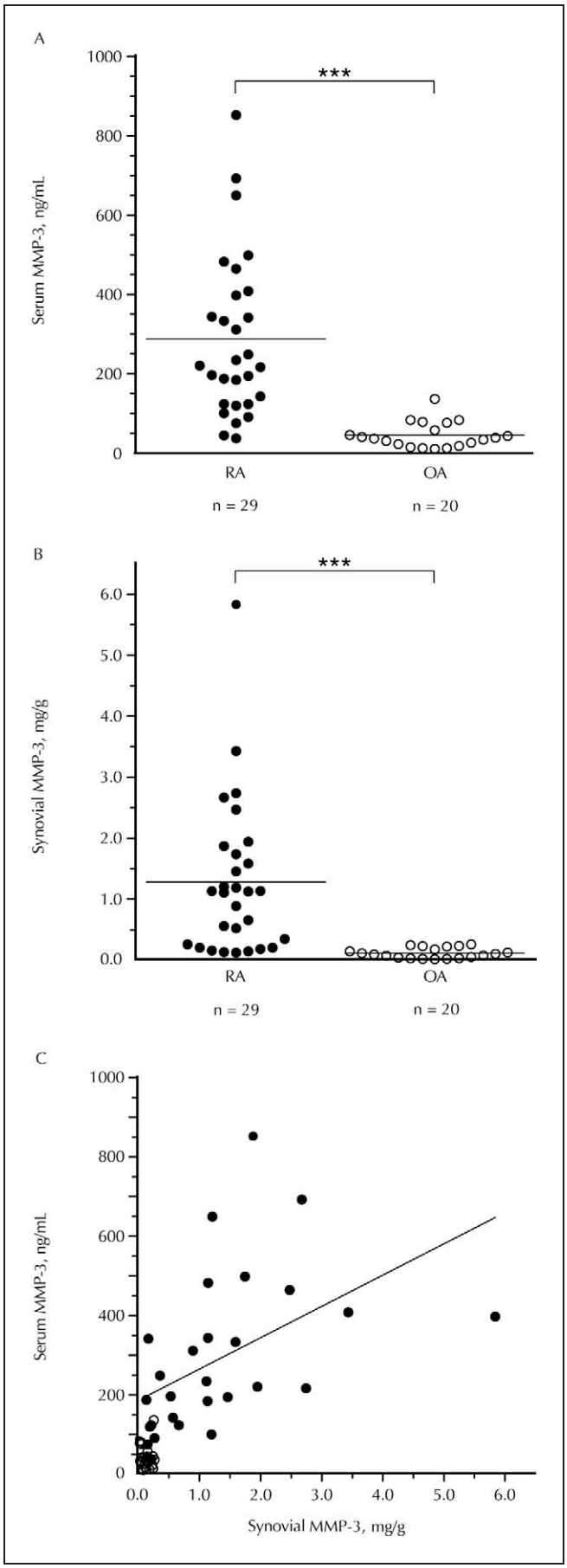

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Figure 1. *Matrix metalloproteinase 3 (MMP-3) levels in serum and synovial samples from rheumatoid arthritis or osteoarthritis patients and their correlation. The levels in the serum (A) and synovial tissue (B) samples from rheumatoid arthritis (RA) (n = 29) or osteoarthritis (OA) (n = 20) patients were measured as described in "Materials and Methods." Note a direct correlation between the levels in serum samples and synovial tissues (r = 0.712, P < .001; N = 49) (C). ***, P < .001.*

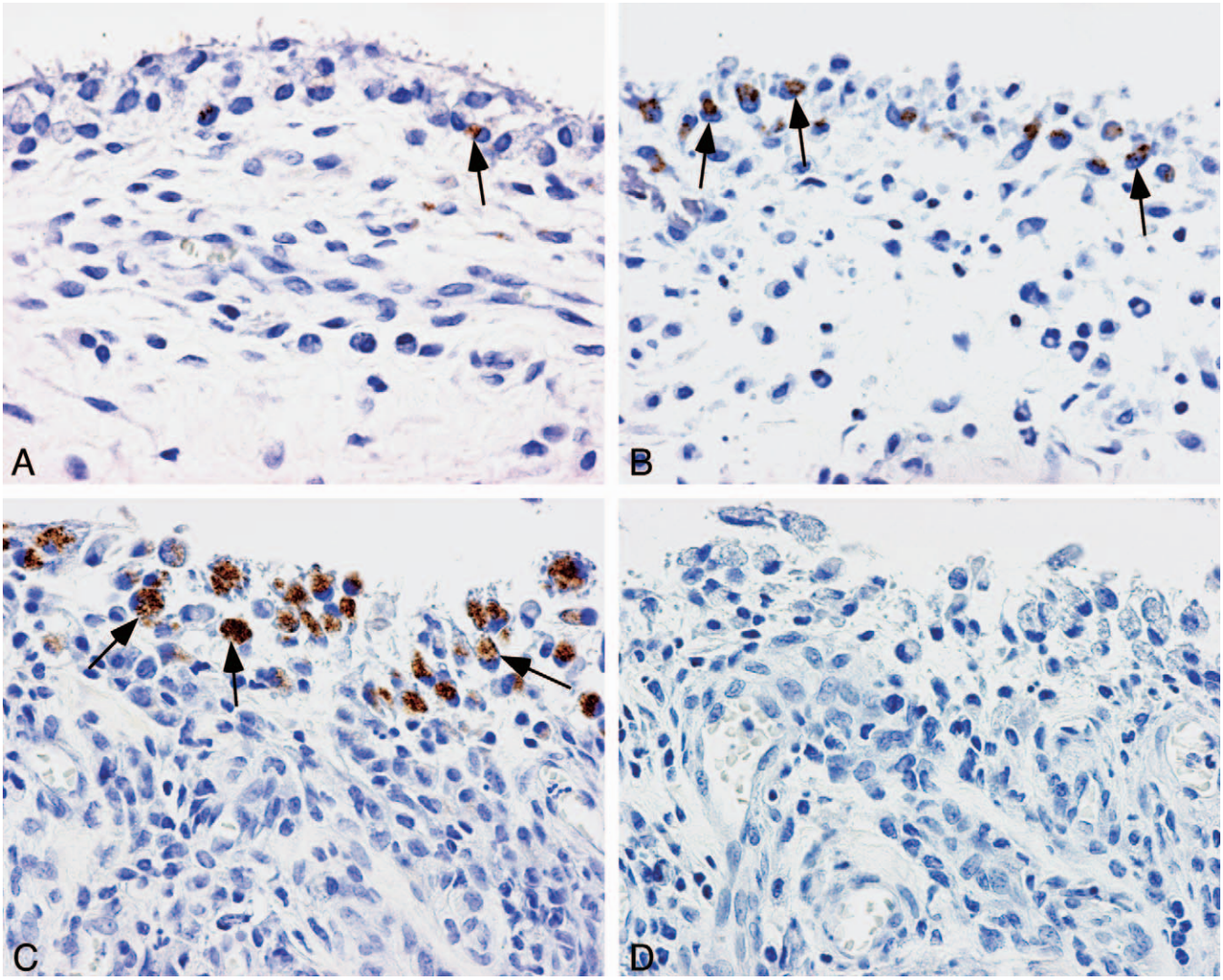

Figure 2. *Immunolocalization of matrix metalloproteinase 3 (MMP-3) in rheumatoid synovial tissues with different MMP-3 levels. Immunostaining and measurement of MMP-3 were performed as described in "Materials and Methods." A through C, Representative synovial tissues with low (0.21 mg/g protein) (A), moderate (1.74 mg/g protein) (B), or high (2.67 mg/g protein) (C) levels of MMP-3, immunostained with anti–MMP-3 antibody. D, Synovial tissue with high MMP-3 level (2.67 mg/g protein) stained with nonimmune immunoglobulin G, showing no staining. Note that the number of MMP-3–immunostained lining cells (arrows in A, B, and C) appears to be related to the MMP-3 levels measured by enzyme immunoassay (MMP-3 immunohistochemical staining [A through C] and nonimmune immunoglobulin G immunohistochemical staining [D], original magnification ×400 [A through D]).*

138.7 ± 65.3 ng/mL, respectively); the levels after surgery were significantly lower than those before surgery (P = .001 and P = .002, respectively). The levels appeared to be maintained for at least 4 weeks after the operation (153.5 ± 36.0 ng/mL; n = 4), although statistical analysis was not possible because of small numbers of samples (data not shown). In contrast to the alteration of MMP-3 levels, CRP levels before surgery (3.5 ± 3.4 mg/dL) were not significantly different from those after 1 week (5.5 ± 5.0 mg/dL), although they tended to increase in most cases (7/9 cases; Figure 4, B), a finding probably related to the operation trauma. The CRP levels decreased after 2 weeks (1.9 ± 1.4 mg/dL; Figure 4, B) and after 4 weeks (1.7 ± 1.8 mg/dL; data not shown). Erythrocyte sedimentation rate levels observed before surgery (66.4 ± 19.8 mm/h) were unchanged at 1 week (74.0 ± 21.7 mm/h) and 2 weeks (65.7 ± 29.7 mm/h) after surgery (Figure 4, C). Counts of white blood cells (9586 ± 2860 cells/μL) and platelets (39 000 ± 8212 cells/μL) before surgery were not significantly different from those at 1 week (7757 ± 1494 cells/μL and 33 814 ± 9265 cells/μL, respectively), at 2 weeks (6829 ± 1151 cells/μL and 37 686 ± 11 762 cells/μL, respectively), and at 4 weeks (6100 ± 1267 cells/μL and 37 080 ± 6278 cells/μL, respectively) after surgery (data not shown).

COMMENT

The present study has demonstrated that the levels of MMP-3 in both serum and synovial tissue samples from RA patients are significantly higher than those in OA patients. Previous studies by our and other groups have shown that MMP-3 is expressed by the synovial tissues in both RA and OA.[21–24] However, no information has been available about the levels produced in synovial tissues. In the present study, we have provided the first evidence that the MMP-3 level is 10.7-fold higher in RA synovium than

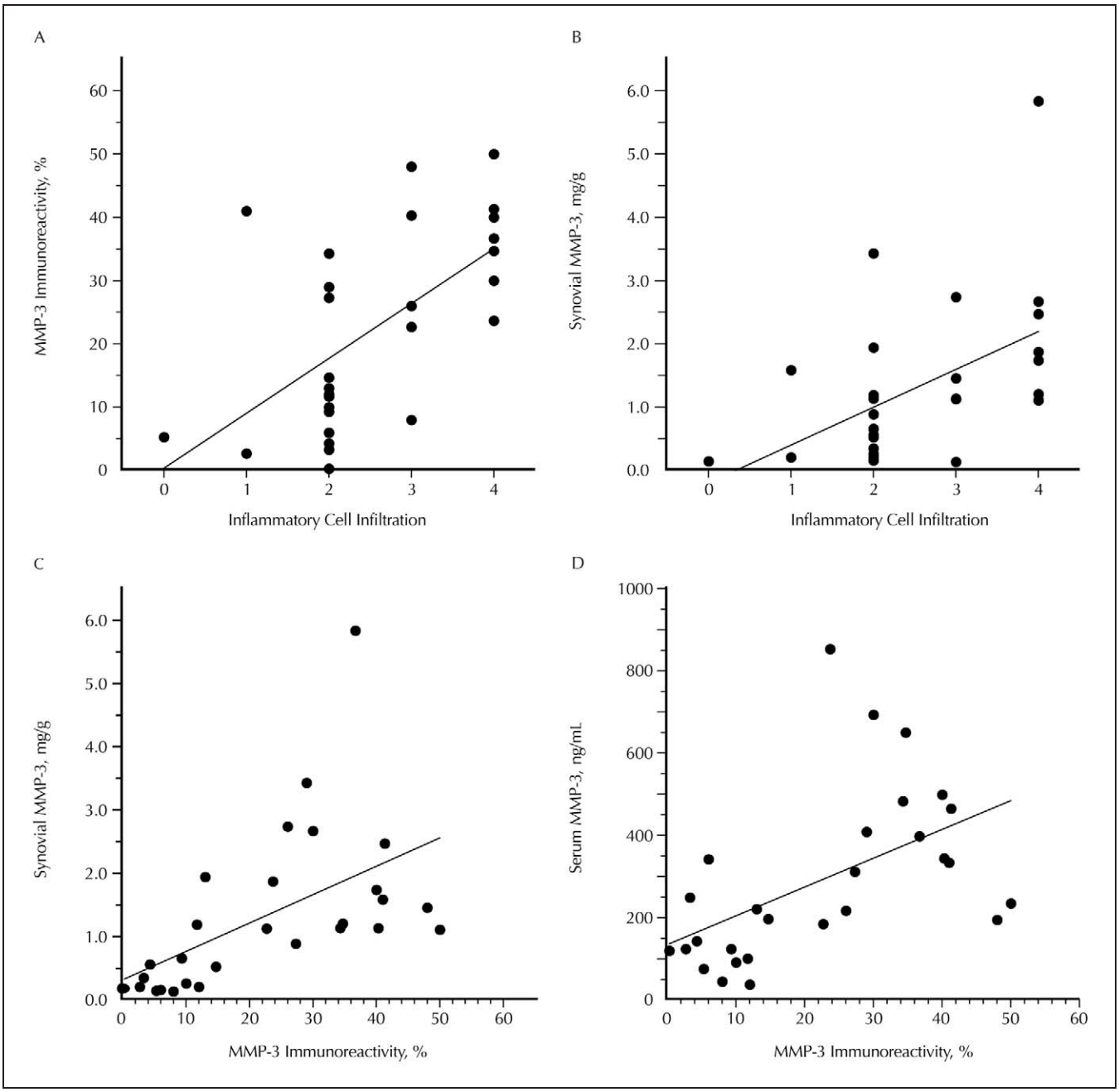

Figure 3. *Correlations between matrix metalloproteinase 3 (MMP-3) immunoreactivity and MMP-3 levels in synovial tissue and serum samples. A and B, Correlations of inflammatory cell infiltration in rheumatoid synovial tissues with MMP-3 immunoreactivity or MMP-3 levels in the synovial tissues, respectively. C and D, Correlations of MMP-3 immunoreactivity in rheumatoid synovial tissues with MMP-3 levels in the synovial tissues or MMP-3 levels in serum samples, respectively.*

in OA synovium. Using the same assay system for MMP-3, we have determined the levels of MMP-3 in human malignant tumor tissues including carcinomas of the breast,[34] thyroid,[35] oral mucosa,[36] stomach,[37] endometrium,[38] salivary gland,[39] and gliomas,[40] and the corresponding nonneoplastic control tissues.[34–40] The results have shown that both these malignant tumors and nonneoplastic tissues produce only trace amounts of MMP-3.[34–41] When the levels were compared, RA synovial tissues produced more than approximately 30-fold and approximately 170-fold higher levels of MMP-3 than did the malignant tumor and nonneoplastic tissues, respectively. Although limited information is so far available for the MMP-3 levels in various inflammatory tissues other than RA and OA synovium, it seems likely that MMP-3 is selectively and highly produced in inflammatory synovial tissues, especially rheumatoid synovium.

Previous immunohistochemical and in situ hybridization studies[21–23] have shown that MMP-3 is localized to the lining cells of rheumatoid synovium. The present immunohistochemical and enzyme immunoassay analyses have further demonstrated that more than 20% of the lining cells are producing MMP-3 in RA synovium and that the immunoreactivity (percentage of immunoreactive cells to

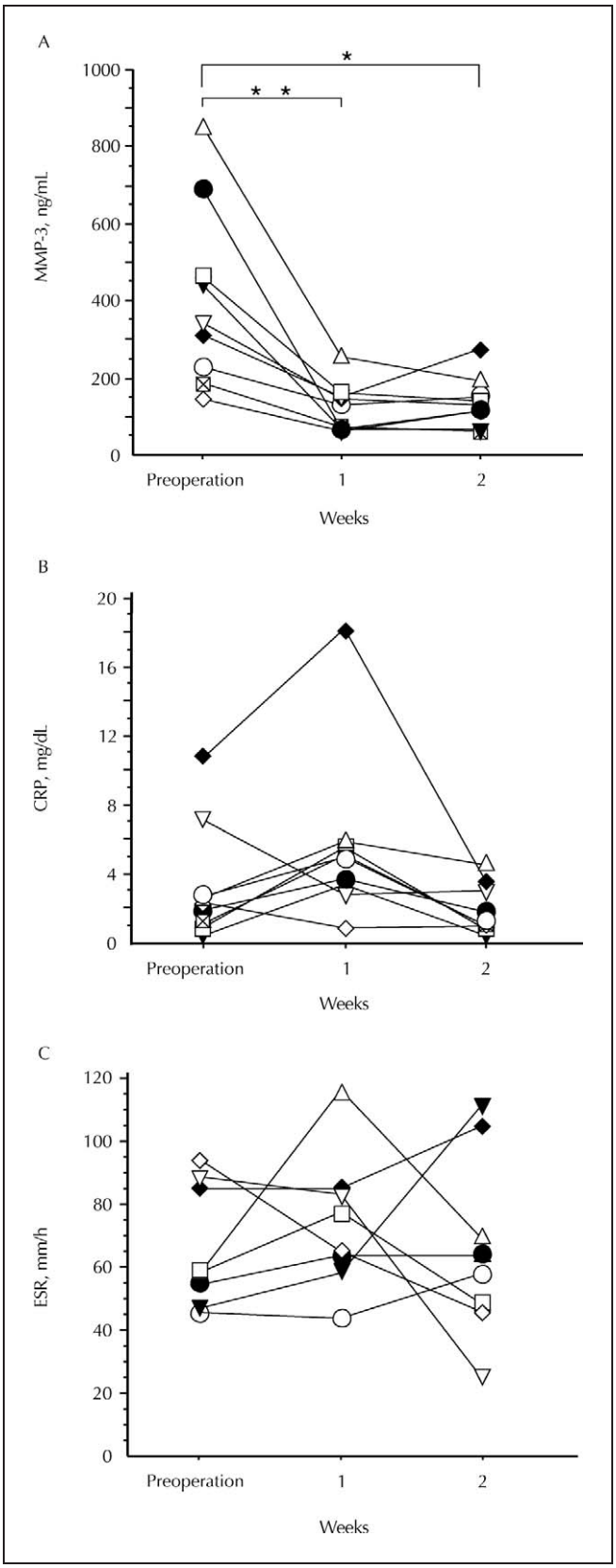

Figure 4. *Time-course changes in the levels of matrix metalloproteinase 3 (MMP-3) and C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) after total knee arthroplasty. Matrix metalloproteinase 3 (A) and C-reactive protein levels (B) in the serum samples and erythrocyte sedimentation rate (C) were measured in 9 rheumatoid arthritis patients as described in "Materials and Methods." *, P = .002; **, P = .001.*

total lining cells) directly correlates with the levels of MMP-3 in the RA synovial tissues, suggesting that the lining cells are responsible for the production. Matrix metalloproteinase 3 is induced by proinflammatory cytokines such as interleukin 1 and tumor necrosis factor α in rheumatoid synovial fibroblasts in culture.[5,42,43] A previous study by Klimiuk et al[44] has reported that RA patients with severe synovitis (follicular synovitis in their study) exhibit high serum concentration of MMP-3. Although their study did not analyze the MMP-3 production in the synovium, our study has shown direct correlation between the MMP-3 immunoreactivity and the MMP-3 levels in the synovium. Thus, these findings suggest that cytokines including interleukin 1 and tumor necrosis factor α derived from synovial inflammatory cells act to induce MMP-3 biosynthesis in rheumatoid synovitis. Although the lining cell hyperplasia and MMP-3 production appear to be coordinately regulated by cytokines such as interleukin 1 and tumor necrosis factor α,[45] the present study showed no correlation between the immunoreactivity and the lining cell hyperplasia. Therefore, the hyperplasia appears to be controlled by more complicated mechanisms involving various factors other than the stimulators of MMP-3 expression.[45]

Our previous study[25] demonstrated that among MMP-1, -2, -3, -7, -8, -9, and -13, MMP-3 is most abundantly present in the synovial fluids of RA patients and the levels are about 7-fold higher in RA than in OA. Matrix metalloproteinase 3 is produced by the synovial lining cells, and the level in the synovial tissues is 10.7-fold higher in RA than in OA, as shown in the present study. Moreover, in RA patients, serum MMP-3 levels correlate with corresponding levels in synovial fluid,[46] showing levels 250-fold lower than those in synovial fluid.[47,48] Thus, it is reasonable to think that MMP-3 produced by the synovial lining cells is secreted into synovial fluids within the knee joint cavities and then gains access into blood circulation in the patients. Our data showing a direct correlation between synovial and serum levels of MMP-3 further support the hypothesis.

To study the applicability of MMP-3 serum levels as a marker for rheumatoid synovitis, we longitudinally monitored serum levels in RA patients who underwent total knee arthroplasty. Importantly, MMP-3 decreased to lower levels at 1 week after surgery, and the reduced levels seemed to be maintained for up to 4 weeks. Thus, the data indicate that joint replacement surgery of even a single knee joint affects serum MMP-3 levels in RA patients. This is probably because the knee joint is the major joint possessing a large amount of synovial tissue. Similar decreases in plasma MMP-3 levels are reported with RA patients who received total knee or total hip joint replacements.[30] Levels of CRP appeared to be normalized at 2 weeks after the surgery, but the levels tended to transiently increase at 1 week after the operation. Because CRP levels are known to increase when RA patients are exposed to nonspecific local and/or systemic inflammation caused by factors such as surgery and infection,[49] it is likely that the transient increase in CRP level is due to the local tissue injury by arthroplasty. Erythrocyte sedimentation rate and counts of white blood cells and platelets were not significantly different before and after the operation. Therefore, among the parameters examined in the present study, only serum MMP-3 levels reflect a dynamic process of the rheumatoid joint, and measurement of the serum MMP-3

levels is considered to be useful to monitor the activity of rheumatoid synovitis.

It is a further limitation of this study that no information is available about how quickly serum MMP-3 in the patients with early RA increases to the levels sufficient for diagnosis of RA. In addition, enhanced serum MMP-3 levels are reported in some patients with systemic lupus erythematosus,[50] connective tissue diseases,[28] or glomerulonephritis,[51] and corticosteroid therapy of patients with RA, vasculitis, systemic sclerosis, or lupus erythematosus increases the levels of MMP-3 in serum.[52] On the other hand, previous prospective studies on serum concentrations of MMP-3 in early RA patients have demonstrated that the serum MMP-3 level is a useful marker for predicting joint damage progression.[29,53] In addition, attempts have been made to use serum MMP-3 level as a marker for the effectiveness of antirheumatic therapy such as anti–tumor necrosis factor α antibody treatment.[54,55] Thus, all these data suggest that determination of serum MMP-3 levels is a simple and noninvasive method for monitoring synovial inflammation and pathologic processes underlying joint destruction in RA, although the data should be carefully evaluated concerning disease specificity and therapeutic effects.

In conclusion, our results demonstrate that MMP-3 levels in serum samples of RA patients correlate directly with the tissue levels produced by the synovial lining cells and degree of inflammatory cell infiltration in rheumatoid synovial tissues and decrease after total knee arthroplasty. These data suggest that the activity of rheumatoid synovitis can be monitored by measuring serum levels of MMP-3.

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