Examination of *in vivo* gelatinolytic activity in rheumatoid arthritis synovial tissue using newly developed *in situ* zymography and image analyzer

W. Yoshida[1], M. Uzuki[2], J. Nishida[3], T. Shimamura[3], T. Sawai[2]

[1]Department of Orthopedic Surgery, Iwate Prefectural Kamaishi Hospital, Kamaishi, Japan;
[2]Department of Pathology, Iwate Medical University, School of Medicine, Morioka, Japan;
[3]Department of Orthopedic Surgery and Rheumatology, Iwate Medical University, School of Medicine, Morioka, Japan.

Abstract

Objective

The aim of this study was to examine in vivo gelatinolytic activity of rheumatoid arthritis (RA) synovium using a newly developed in situ zymography (ISZ) method and pathological image analyzer, and to evaluate the relationship between this activity and several features on RA.

Methods

A total of 8 samples of synovium were obtained from RA patients during surgery, and 8 samples from osteoarthritis (OA) patients were examined as controls. Furthermore, total 14 samples of syovium were obtained for comparison among radiographical classifications as Larsen grade (4 cases of grade III, 5 cases of grade IV and 5 cases of grade V). These specimens were frozen with OCT compound immediately after surgery. Frozen sections were applied to a newly developed gelatin-coated FIZ film (Fuji Film Co.Tokyo.Japan) designed for use ISZ, and incubated at 37° C for 6 hours. Using an image analyzer (image processor for analytical pathology; IPAP), two variables were measured as indicators of in vivo gelatynolytic activity: optical density of gelatinolyzed area (ODG), and ratio of gelatinolyzed area (RGA). Also, we investigated the relationship between these indicators and the following variables: radiographic changes (Larsen grades), clinical data (C-reactive protein concentration), histological score of synovial tissue (modified Rooney's score), and expression of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 (assessed by immunohistochemistry).

Results

RA synovium had significantly higher RGA and lower ODG than OA, indicating higher gelatinolytic activity in RA. Synovium from cases with Larsen grade IV or V had significantly lower ODG than cases with grade III, but there was no significant difference in RGA between grades. There was no significant correlation between gelatinolytic activity (ODG or RGA) and either CRP or modified Rooney's Histological Score. The results of ISZ indicate that the gelatinolyzed areas were mainly localized in the lining area, with a small amount scattered throughout the stroma. The results of immunohistochemistry indicate that MMP-2, MMP-9, TIMP-1 and TIMP-2 were expressed in areas of gelatinolysis.

Conclusions

The present results indicate that in vivo gelatinolytic activity of synovium is stronger in RA than in OA. They also indicate that gelatinolytic activity of RA synovial cells is stronger in cases with Larsen grade IV or V than in cases with grade III, although the gelatinolyzed area is similar. Gelatinolytic activity, as indicated by optical density and the gelatinolyzed area, differed between regions, even within the same specimen, suggesting an imbalance between production of proteinases and their inhibitors. We believe that the present zymography method can contribute to the elucidation of biological enzymatic activity of RA synovium.

Key words
Rheumatoid arthritis, in situ zymography, gelatinolytic activity.

Wataru Yoshida, MD, PhD
Miwa Uzuki, MD, PhD
Jun Nishida, MD, PhD
Tadashi Shimamura, MD, PhD
Takashi Sawai, MD, PhD, Professor

Please address correspondence and reprint requests to:
Prof. Takashi Sawai, MD, PhD,
Department of Pathology,
Iwate Medical University,
School of Medicine,
19-1 Uchimaru, Morioka,
Iwate 020-8505, Japan.
E-mail: tsawai@iwate-med.ac.jp

Received on December 1, 2008; accepted in revised form on March 20, 2009.

Abbreviations:
ISZ: *in situ* zymography
IPAPA: image processor for analytical pathology
ODG: optical density of gelatinolyzed area
RGA: ratio of gelatinolyzed area

Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is a chronic, progressive disease characterized by inflammation and structural damage to the joint. However, in patients with arthritis, the development of joint destruction is unpredictable (1-3). The production of proteolytic enzymes by the inflamed synovium is thought to be critical in the pathogenesis of RA articular damage. In the absence of disease, these proteolytic enzymes are involved in normal tissue remodeling (4-6). There have been numerous studies of proteolytic enzymes, particularly matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), the latter being specific MMP inhibitors that form non-covalent, tight-binding complexes with active MMPs. MMPs and TIMPs are thought to play key roles in joint destruction (7-15). There is a great need to elucidate the proteolytic activity involved in the joint destruction of RA. Zymography is a valuable method for examining this activity, and its effectiveness has been assessed in several studies (16-18). However, most current zymography methods are used to qualitatively examine proteolytic activity, and are not adequate for histological evaluation or quantification. Consequently, little is currently known about the degree of *in vivo* histological proteolytic activity of RA synovium. The aim of the present study was to examine the distribution of *in vivo* gelatinolytic activity using a newly developed method of *in situ* zymography in which unfixed frozen tissues are applied to a gelatin-coated film, and to measure the degree of this activity as *in vivo* proteolytic activity of RA using a pathological digital-image analyzer.

In the present study, we compared *in vivo* gelatinolytic activity between synovium from RA and osteoarthritis (OA) patients, and compared activity among radiographic classifications of RA (Larsen grades). We evaluated the correlation of gelatinolytic activity with C-reactive protein concentration and histological inflammation of synovium (modified Rooney's score). Also, we examined the localization of cells expressing MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which degrade components of the extracellular matrix with high specificity for denatured collagen (gelatin), and cells expressing their specific inhibitorsTIMP-1 andTIMP-2, in order to examine the relationship between the localization of these cells and the distribution of gelatinolyzed areas, as determined by immunohistochemistry using serial sections.

Materials and methods

Patients and specimens

To compare RA and OA synovium, we examined 8 cases of RA and 8 cases of OA (Table I). These RA cases were classified as stage IV using the Steinbrocker system.

For comparison among RA cases with different radiographic appearances classified as Larsen grade, we examined 4 cases of grade III, 5 cases of grade IV, and 5 cases of grade V (Table II). We compared gelatinolytic activity among Larsen grades and evaluated the relation of gelatinolytic activity to Rooney's score, C-reactive protein concentration and expression of proteolytic enzymes (Table III). All RA cases were diagnosed according to the 1987 revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association). All synovial specimens were obtained during total knee replacement surgery, and the serum samples assayed for C-reactive protein concentration were obtained 1 day before the joint surgery. Examination of all specimens with informed consent from all patients was authorized by the Ethical Committee of Iwate Medical University.

In situ zymography

To detect histologically the gelatinolytic activity of synovial tissue, we used a newly developed *in situ* zymography film (FIZ film; Fuji Film.Co. Tokyo,

Table I. Subjects for comparison between RA and OA.

	n	Age (years ± SD
RA	8	55.8 ± 10.9
OA	8	71.9 ± 3.1

RA: rheumatoid arthritis; OA: osteoarthritis, and all RA cases were Stage IV.

Table II. Characterisitics of cases examined among RA cases.

Larsen grade	Number of cases	Age (years±SD)	CRP (mg/dl±SD)	Duration of disease (years ± SD)
III	4	50.0 ± 6.6	2.0 ± 2.5	19.0 ± 8.2
IV	5	52.8 ± 14.1	2.9 ± 1.7	14.8 ± 6.4
V	5	54.8 ± 19.7	2.9 ± 1.6	12.2 ± 5.2

Japan). This film is uniformly coated with cross-bridge gelatin at a thickness of 7 μm. All synovial specimens were embedded in Tissue-Tek OCT Compound (Lab-Tek products, Elkhart, IN, USA) immediately after surgery. Then, 4-μm cryostat sections were cut and applied to the film, followed by washing with water for a few seconds. After incubation for 6 hours at 37°, the film was stained with 0.2% pansaou solution (which is commonly used for protein staining; Sigma, USA) for 3 minutes and fixed with 1% acetate for 5 minutes. After washing with water for 15 minutes, the film was stained with hematoxylin for nuclear staining. Gelatinolyzed areas caused by gelatinolytic activity of synovium were detected as pale areas, and non-gelatinolyzed areas were uniformly stained red.

Quantization of gelatinolytic activity by image analyzer

To quantify the degree of gelatinolytic activity, we used a digital image analyzer (image processor for analytical pathology, IPAP, Sumitomo Tech, Osaka, Japan), which combines a microscope, a CCD camera and an analyzing computer. For each ponsaou-stained FIZ film, we measured two variables: optical density of gelatinolyzed area (ODG) and ratio of gelatinolyzed area (RGA). ODG is the mean optical density of the red component at 50 random points in the gelatynolyzed area. RGA is the ratio of the gelatinolyzed area to the entire synovium. ODG and RGA were measured blindly at a magnification of ×4.

Histological score

For each case, we scored the degree of inflammation of RA synovium according to Rooney's histological score as local assessment (19). The scoring technique used for all 6 features is shown in Table III.

Synoviocyte hyperplasia. A normal synoviocyte monolayer was given a score of 0. As the depth of the synoviocyte lining layer increased, the score increased accordingly. If the cell depth of the section varied, the grade corresponding to the predominant cell depth was recorded.

Fibrosis. The degree of fibrosis was estimated as the amount of fibrous tissue that had replaced the normal loose connective tissue present beneath the synovial lining layer. All fields of the section were assessed. Sections containing <10% fibrous tissue in the sublining layers were considered normal and graded 0. As the percentage of fibrosis in the section increased, the score increased accordingly, to a maximum of 10, which was equivalent to >80% fibrosis in the section.

Proliferating blood vessels. Endothelial cells forming a solid tube or enclosing a lumen were considered to constitute a vessel. If <4 vessels were observed per high-power field (HPF), the section was considered normal and was scored 0. As the number of vessels per HPF increased, the score increased accordingly, and >22 vessels per HPF was scored the maximum 10. If the number of vessels per HPF varied, the score corresponding to the predominant number of vessels per HPF was recorded.

Perivascular infiltrates of lymphocytes. Perivascular infiltrates were characterized as aggregates of lymphocytes that were contiguous with the vessel wall and were <10 cells in diameter. The final score for perivascular infiltrates was based on two factors: the number of vessels involved, and the diameter of the perivascular infiltrate. If no vessels were involved, the grade was 0. The greater the percentage of vessels surrounded by lymphocytes, the higher the score. The maximum grade of 10 corresponded to involvement of 100% of vessels. The diameter of the perivascular infiltrate was assessed and was graded as mild (2-4 cells in diameter), moderate (5-7 cells in diameter) or severe (8-10 cells in diameter). The number of vessels involved was the dominant variable and was the basis of the initial score. If the cellular infiltrate around the vessels was considered

Table III. Method of modified Rooney's scoring features in RA synovium.

	Score										
	0	1	2	3	4	5	6	7	8	9	10
Synoviocyte hyperplasia*	1	2	3	4	5	6	7	8	9	10	>10
Fibrosis†	<10	<15	<20	<25	<30	<40	<50	<60	<70	<80	>80
Proliferating blood vessels‡	0-3	4-5	6-7	8-9	10-11	12-13	14-15	16-17	18-19	20-22	>22
Perivascular infiltrates of lymphocytes§	<5	10	20	30	40	50	60	70	80	90	100
Focal aggregates of lymphocytes¶	11	15	20	25	30	35	40	45	50	55	>55
Diffuse infiltrates of lymphocytes#	0	10	20	30	40	50	60	70	80	90	100

*Predominant cell depth of synovial lining layer; † Percentage of fibrosis in subsynovial layer; ‡ Number of vessels per high power field (HPF); § Percentage of vessels per HPF; ¶ Number of cells in diameter; # Percentage of diffuse lymphocytes per HPF.

moderate, the initial score remained unchanged. If the infiltrate was considered mild or severe, the initial score was lowered or raised by 1 point, respectively.

Focal aggregates of lymphocytes. Focal aggregates of lymphocytes were defined as aggregates that were not intimately related to a synovial vessel or in which the perivascular cuff of lymphocytes was >10 cells in diameter. Scoring of focal aggregates was based on size, rather than the number of aggregates. Absence of focal aggregates was scored 0. As the diameter (measured as number of cells) of the focal aggregates increased, the score for the section increased accordingly. An aggregate with a diameter of >55 cells received the maximum score of 10.

Diffuse infiltrates of lymphocytes. Lymphocytes that were not part of perivascular or focal aggregates were considered to be diffuse infiltrates. Quantification of diffuse infiltrates was relatively subjective. We estimated the percentage of cells per HPF that were lymphocytes, with higher percentages corresponding to higher scores. If the field was entirely occupied by lymphocytes, it was given a score of 10. If the percentage of infiltrating lymphocytes varied between HPFs, the score that corresponded to the predominant percentage was recorded.

Immunohistochemistry

We assayed expression of gelatinase A (MMP-2), gelatinase B (MMP-9), TIMP-1 and TIMP-2 by immunohistochemistry using monoclonal antibodies (anti-MMP-2, -MMP-9, -TIMP-1, -TIMP-2; Fuji Chemistry, Tokyo, Japan). Frozen sections of synovial tissue (thickness, 4 μm) were embedded in OCT compound and fixed in 100% alcohol for 10 minutes. All sections were then washed with phosphate-buffered saline (PBS), followed by blocking of endogenous peroxidase activity with 3% hydrogen peroxide in methanol for 30 minutes. Next, 10% normal goat serum was applied to the sections for 30 minutes, which were then reacted with primary antibodies at 4° for 24 hours.

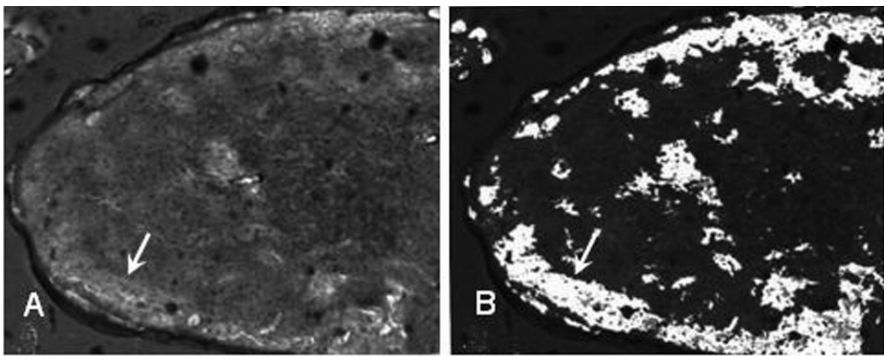

Fig. 1. Arrow indicates the gelatinolized area of RA synovim (**A**) detected by *in situ* zymography. The arrow indicated pale area was recognized as the measurable region by IPAP analysis (**B**).

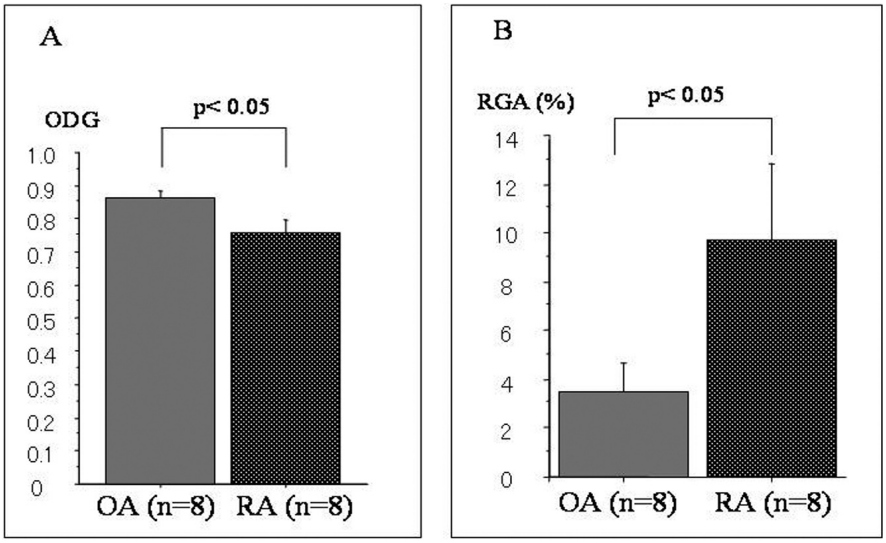

Fig. 2. Comparison with RA and OA synovium. RA synovium demonstrated significantly lower ODG (0.758±0.019) than OA (0.864±0.037) (p<0.05, **A**). RA synovium demonstrated significantly higher RGA (9.7±3.1 %) than OA (3.5±1.1 %) (p<0.05, **B**).

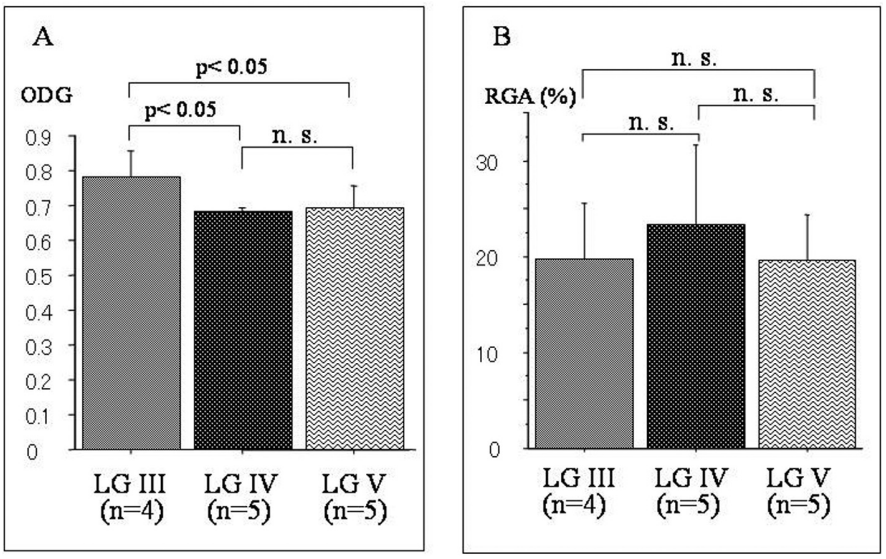

Fig. 3. Difference of gelatinolytic activity among Lrsen grades. There was significant difference between grade III (0.781±0.075) and IV (0.679±0.016), III and V (0.691±0.064) in ODG (p<0.05, **A**). However no significant difference was shown among Larsen grades in RGA (grade III; 19.8±5.8 %, grade IV; 23.4±8.2 %, grade V; 19.5±4.8%, **B**).

After washing with PBS, all sections were reacted at room temperature for 30 minutes with rabbit immunoglobulins conjugated to a peroxidase-labeled amino acid polymer (Histofine simplestain Multi Po, Nichirei, Tokyo, Japan), and finally were treated with 3,3'-diaminobenzidine (DAB, Sigma Chemical Co., USA). Negative control sections were reacted with normal mouse, rabbit and sheep serum instead of the primary antibody.

Statistical methods

Significance of the differences between the two groups was evaluated using Fisher's PLSD test, and correlation of gelatinolytic activity (ODG and RGA) with clinical assessment (C-reactive protein concentration) or the histological score (Rooney's score) was evaluated using Spearman's test.

Results

Gelatinolyzed areas detected by *in situ* zymography were chiefly localized at whole of the lining layer of RA synovium (Fig. 1A, gelatinolyzed pale area indicated by arrow) rather than in the stroma. All RA cases exhibited the same pattern of localization. The gelatinolyzed areas detected barely in OA synovium localized at the lining layer. The arrow indicated area (Fig. 1B) was considered suitable for measurement of ODG and RGA using the IPAP system as the gelatinolyzed area.

Comparison of gelatinolytic activity between RA and OA synovium

RA synovium had a significantly lower ODG (0.758±0.019) than OA synovium (0.864±0.037) (Fig. 2A: Comparison with RA and OA synovium in ODG; $p<0.05$). RA synovium had a significantly higher RGA (9.7±3.1%) than OA synovium (3.5±1.1%) (Fig. 2B: Comparison with RA and OA in RGA; $p<0.05$).

Comparison of gelatinolytic activity among Larsen grades

Grade III had a significantly higher ODG (0.781±0.075) than grades IV (0.679±0.016) and V (0.691±0.064) (Fig. 3A: Comparison of ODG among Larsen grades; $p<0.05$). However there was no significant difference in RGA among Larsen grades (grade III, 19.8±5.8%; grade IV, 23.4±8.2%; grade V, 19.5±4.8%) (Fig. 3B: Comparison of RGA among Larsen grades).

Correlation of gelatinolytic activity with C-reactive protein (CRP) concentration and histological score.

There was no correlation of CRP concentration with ODG or RGA, and there was no correlation of modified Rooney's score with ODG or RGA. In addition, there was no significant difference between Larsen grades in C-reactive protein concentration or Rooney's score.

Relation between enzyme expression and gelatinolyzed area

In immunohistochemistry using serial sections, MMP-2and MMP-9 were mainly expressed by fibroblast-like or macrophage-like cells of the synovial lining layer. These same cells also expressed TIMP-1 and TIMP-2. The distribution of cells expressing MMPs and TIMPs corresponded to the gelatinolyzed areas detected by zymography. Some cells expressing MMPs and TIMPs were also detected scattered throughout the non-gelatinolyzed area, but at a markedly lower concentration than in gelatinolyzed areas. There were no marked differences among Larsen grades in the distribution of cells expressing MMPs and TIMPs.

Discussion

There have been many studies of proteolytic activity, including gelatynolytic activity, using techniques including gelatin zymography (16-18, 20-23). *In situ* zymography was developed to determine the localization of proteolytic activity *in vivo* or histologically. However, *in situ* zymography has been restricted to qualitative analysis because of the inability to coat film with substrate at a sufficiently uniform thickness to allow precise quantification of *in vivo* proteolytic activity (24-28). The present *in situ* zymography method utilizes a film developed at Fuji Photo Film Co., Ltd, Tokyo, which is uniformly coated with a 7-μm layer of gelatin and cross-linking agent. In several studies, reproducible quantification of areas of gelatynolysis has been achieved using this film (29-31). The aim of the present study was to quantify histologically the degree of gelatinolytic activity of RA synovium using the IPAP image analyzer. The IPAP system, which consists of a microscope and computer, converts microscopic photographic images into digital images, measures optical density and counts cell numbers under various conditions (32). In the present study, we measured ODG and RGA of gelatinolyzed areas, using Ponsaou-stained FIZ films as background reference. ODG reflects the degree of gelatinolytic activity per cell. RGA reflects the number of gelatinolytic cells on the synovium.

In the present study, the gelatinolyzed areas detected by *in situ* zymography were primarily localized at the lining layer of the synovium and rather than in the stroma. This is consistent with the previous finding that many enzyme activators are present in the joint fluid, and that they stimulate enzyme production by synovial cells of the lining layer or activate these enzymes, playing a crucial role in the pathogenesis of articular damage (33, 34).

In the present study, RA synovium had significantly greater ODG and RGA than OA synovium, indicating that RA synovial cells have stronger *in vivo* gelatinolytic activity and RA synovial tissue contains more gelatinolytic cells.

In the present study, specimens with Larsen grade III had significantly higher ODG than grades IV and V, but there was no significant difference in RGA among Larsen grades. This suggests that the *in vivo* gelatinolytic activity of individual RA synovial cells is stronger at later stages of joint destruction, but that the number of gelatinolytic cells does not markedly increase.

In the present study, CRP concentration and histological score did not correlate with ODG, RGA or Larsen grade. This indicates that the levels of inflammatory variables such as CRP concentration and Rooney's score do not reflect the *in vivo* gelatinolytic activity of RA synovium or the degree of joint destruction. This suggests that the patho-physiologic mechanisms of joint

inflammation are partially independent from the mechanisms of joint destruction (3, 35-40). RA is a systemic or local inflammatory disease caused by disorders of the immune system, but the degree of joint destruction in RA appears to be more accurately reflected by the *in vivo* gelatinolytic activity of synovial cells than by markers of inflammation such us CRP concentration or histological score of synovium.

High MMP levels in arthritis are thought to result from increased production by inflamed joints (41). Gene expression of several MMPs has been observed in the synovial lining layer, in scattered cells in the sublining area, and in activated synovial endothelial cells. In the present study, gelatinolyzed areas were mainly localized at the lining layer of the synovium, and the gelatinolytic enzymes MMP-2 and MMP-9 and their inhibitors TIMP-1 and TIMP-2 were mainly expressed in these areas, as indicated by immunohistochemistry. These MMPs and TIMPs were also expressed, to a lesser degree, by cells scattered throughout non-gelatinolyzed areas. These results indicate that RA synovial cells simultaneously produce proteolytic enzymes and their inhibitors, and suggest that differences *in vivo* gelatinolytic activity among cells are due to imbalances in enzyme production of individual cells. That is, proteolytic enzyme production is greater than inhibitor production (positive balance) in the gelatinolyzed areas, and inhibitor production is greater than proteolytic enzyme production (negative balance) in the non-gelatinolyzed areas. This is consistent with the present finding that cases of joint destruction with Larsen grade IV or V have a more positive balance between MMPs and TIMPs than cases with grade III (6, 21, 40, 42-44).

Conclusion

In the present study, we used *in situ* zymography and IPAP analysis to examine *in vivo* gelatinolytic activity of RA synovial tissue. This activity differed considerably among RA types, reflecting differences in the degree of joint destruction. The present results indicate that these differences in gelatinolytic activity are caused by differences in the balance of enzyme production by RA synovial cells. The newly developed methods used in the present study can contribute to a better understanding of biological enzymatic activity of RA synovium.

Authors' contributions

W. Yoshida, J. Nishida, T. Shimamura, and T. Sawai participated in the design of this study. W. Yoshida and M. Uzuki participated in the immnunohistological study. W. Yoshida and T. Sawai participated in the *in situ* zymography, pathological analysis and statistical analysis. T. Sawai coordinated and helped to draft the manuscript. All the authors read and approved the final manuscript.

Acknowledgments

We thank Akira Kurose MD, PhD and Masaaki Yoshida MD, PhD from Iwate Medical University.

References

1. CUNNANE G, FITZGERALD O, BEETON C *et al.*: Synovial tissue proteinase gene expression and joint erosions in early rheumatoid arthritis. *Arthritis Rheum* 2001, 44: 1744-53.
2. BALLARA S, TAYLOR PC, REUSCH P *et al.*: Raised serum vascular endothelial growth factor levels are associated with destructive change in inflammatory arthritis. *Arthritis Rheum* 2001, 44: 2055-64.
3. CUNNANE G, FITZGERALD O, BEETON C *et al.*: Early joint erosions and serum levels of matrix metalloproteinase 1, matrix metalloproteinase 3, and tissue inhibitor of metalloproteinase 1 in rheumatoid arthritis. *Arthritis Rheum* 2001, 44: 2263-74.
4. YATSUGI N, TSUKAZAKI T, OSAKI M *et al.*: Apoptosis of articular chondrocytes in rheumatoid arthritis and osteoarthritis: correlation of apoptosis with degree of cartilage destruction and expression of apoptosis-related proteins of p53 and c-myc. *J Orthop Sci* 2000, 5: 150-56.
5. KIM HA, SONG YW: Apoptotic chondrocyte death in rheumatoid arthritis. *Arthritis Rheum* 1999, 42: 1528-37.
6. MACNAUL KL, CHARTRAIN N, LARK M *et al.*: Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinases-1 in rheumatoid human synovial fibroblast. *J Biol Chem* 1990, 265: 17238-45.
7. KRANE SM, BYRNE MH, LAMEITRE V *et al.*: Different collagenase gene products have different roles in degradation of type I collagen. *J Biol Chem* 1996, 271: 28509-15.
8. KONTTINEN YT, SALO T, HANEMAAIJER R *et al.*: Collagenase-3 (MMP-13) and its activators in rheumatoid arthritis: localization. *Matrix Biol* 1999, 18: 401-12.
9. AHRENS D, KOCH AE, POPE RM *et al.*: Expression of matrix metalloproteinase 9 (96-kd gelatinase B) in human rheumatoid arthritis. *Arthritis Rheum* 1996, 39: 1576-87.
10. MAEDA S, SAWAI T, UZUKI M *et al.*: Determination of interstitial collagenase (MMP-1) in patients with rheumatoid arthritis. *Ann Rheum Dis* 1995, 54: 970-75.
11. WILL H, HINZMANN B: cDNA sequence and mRNA tissue distribution of a novel human matrix metalloproteinase with a potential transmembrane segment. *Eur J Biochem* 1995, 231: 602-8.
12. VINCENTI MP, COON CI, MENGSHOL JA *et al.*: Cloning of the gene for interstitial collagenase-3 (matrix metalloproteinase-13) from rabbit synovial fibroblast: differential expression with collagenase-1 (matrix metalloproteinase-1). *Biochem J* 1998, 331: 341-46.
13. KEISZER G, LAMBIRI I, NAGEL R *et al.*: Circulating levels of matrix metalloproteinase MMP-3 and MMP-1, tissue inhibitor of metalloproteinase 1 (TIMP-1), and MMP-1/TIMP-1 complex in rheumatic disease. Correlation with clinical activity of rheumatoid arthritis versus other surrogate markers. *J Rheumatol* 1999, 26: 251-58.
14. STRACKE JO, FOSANG AJ, LAST K *et al.*: Matrix metalloproteinases 19 and 20 cleave aggrecan and cartilage oligometric matrix protein (COMP). *FEBS Letters* 2000, 478: 52-6.
15. BIRKEDAL-HANSEN H, MOORE WGI, BODDEN MK *et al.*: Matrix metalloproteinases: A review. *Crit Rev Oral biol Med* 1993, 4: 197-250.
16. SUN HB, NALIM R, YOKOTA H: Expression and activities of matrix metalloproteinases under oscillatory shear in IL-1-stimulated synovial cells. *Connect Tissue Res* 2003, 44: 42-9.
17. HATTORI T, KAWAKI H, KUBOTA S *et al.*: Down regulation of a rheumatoid arthritis-related antigen (RA-A47) by ra-a47 antisense oligonucleotides induces inflammatory factors in chondrocytes. *J Cell Physiol* 2003 Oct, 197: 94-102.
18. CHA HS, AHN KS, JEON CH *et al.*: Inhibitory effect of cyclo-oxygenase-2 inhibitor on the production of matrix metalloproteinases in rheumatoid fibroblast-like synoviocytes. *Rheumatol Int* 2003 Jul 26.
19 ROONEY M, CONDELL D, QUINLAN W *et al.*: Analysis of the histologic variation of synovitis in rheumatoid arthritis. *Arthritis Rheum* 1988, 31: 959-63.
20. NAGAYA H, YAMAGATA T, YAMAGATA S *et al.*: Expression of synovial fluid and serum hyaluronidase activity as a joint marker in rheumatoid arthritis and osteoarthritis patients (by zymography). *Ann Rheum Dis* 1999, 58: 186-88.
21. MORODOMI T, OGATA Y, SASAGURI Y *et al.*: Purification and characterization of matrix metalloproteinase 9 from U937 monocytic leukaemia and HT1080 fibrosarcoma. *Biochem J* 1992, 285: 603-11.
22. LE J, DAUCHOT P, PERROT J *et al.*: Quantitative zymography of matrix metalloproteinases by measuring hydroxyproline: Application to gelatinase A and B. *Electrophoresis* 1999, 20: 2824-29.

23. TAKEI I, TAKAGI M, SANTAVIRTA S *et al.*: Matrix metalloproteinases and tissue inhibitors of metalloproteinases in joint fluid of the patients with loose artificial hip joints. *J Biomed Mater Res* 1999, 45: 175-83.
24. SENZAKI H, PAOLOCCI N, GLUZBAND YA *et al.*: β-Blockade prevents sustained metalloproteinase activation and diastolic stiffening induced by angiotensin II combined with evolving cardiac dysfunction. *Circ Res* 2000, 86: 807-15.
25. YI CF, GOSIEWSKA A, BURTIS D *et al.*: Incorporation of fluorescent enzyme substrates in agarose gel for in situ zymography. *Anal Biochem* 2001, 291: 27-33.
26. GALIS ZS, SUKHOVA GK, LIBBY P: Microscopic localization of active protease by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue. *FASEB J* 1995, 9: 974-80.
27. VIEILLARD-BARON A, FRISDAL E, EDDAHIBI S *et al.*: Inhibition of matrix metalloproteinases by lung TIMP-1 gene transfer or doxycycline aggravates pulmonary hypertension in rat. *Circ Res* 2000, 87: 418-25.
28. GOODALL S, CROWTHER M, HEMINGWAY DM *et al.*: Ubiquitous elevation of matrix metalloproteinase-2 expression in the vasculature of patients with abdominal aneurysms. *Circulation* 2001, 104: 304-09.
29. IWATA H, YAMAMOTO M, NEMORI R *et al.*: Localization of gelatinolytic activity can be detected in breast cancer tissue by film in situ zymography. *Breast Cancer* 2001, 8: 111-15.
30. FURUYA M, ISHIKURA H, NEMORI R *et al.*: Clarification of the active gelatinolytic sites in human ovarian neoplasms using in situ zymography. *Hum Pathol* 2001, 32:163-8.
31. YAMANAKA H, MAKINO K, TAKIZAWA M *et al.*: Expression and tissue localization of membrane-type 1, 2, and 3 matrix metalloroproteinases in rheumatoid arthritis. *Lab Invest* 2000, 80: 677-87.
32. UZUKI M, WATANABE T, KATSURA Y *et al.*: Quantitative histochemical study of hyaluronic acid binding protein and the activity of uridine diphosphoglucose dehydrogenase in the synovium of patients with rheumatoid arthritis. *Analyt Quant Cytol Histol* 1999, 21: 75-80.
33. YOSHIHARA Y, NAKAMURA H, OBATA K *et al.*: Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. *Ann Rheum Dis* 2000, 59: 455-61.
34. MATSUNO H, YUDOH K, WATANABE Y *et al.*: Stromelysin-1 (MMP-3) in synovial fluid of patients with rheumatoid arthritis has potential to cleave membrane bound fas ligand. *J Rheumatol* 2001, 28: 22-8.
35. LEHTINE JT, KAARELA K, KAUPPI MJ *et al.*: Valgus deformity and proximal subluxation of the rheumatoid elbow: a radiographic 15 year follow up study of 148 elbows. *Ann Rheum Dis* 2001, 60: 765-9.
36. ISHIGURO N, ITO T, MIYAZAKI K *et al.*: Matrix metalloproteinases, tissue inhibitors of metalloproteinases, and glycosaminoglycans in synovial fluid from patients with rheumatoid arthritis. *J Rheumtol* 1999, 26: 34-40.
37. ISHIGURO N, ITO T, OGUCHI T *et al.*: Relationship of matrix metalloproteinases and their inhibitors to cartilage proteoglycan and collagen turnover and inflammation as revealed by analyses of synovial fluids from patients with rheumatoid arthritis. *Arthritis Rheum* 2001, 44: 2503-11.
38. ISHIGURO N, ITO T, OBATA K *et al.*: Determination of stromelysin-1, 72 and 92 kDa type IV collagenase, tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-2 in synovial fluid and serum from patients with rheumatoid arthritis. *J Rheumatol* 1996, 23: 1599-1604.
39. EMERY P AND SALMON M: Early rheumatoid arthritis: time to aim for remission? *Ann Rheum Dis* 1995, 54: 944-47.
40. MILLIS AJ, HOYLE M, MCCUE HM *et al.*: Differential expression of metalloproteinase and tissue inhibitor of metalloproteinase gene in aged human fibroblast. *Exp Cell Res* 1992, 201: 373-9.
41. KONTTINEN YT, AINOLA M, VALLEALA H *et al.*: Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. *Ann Rheum Dis* 1999, 58: 691-97
42. BURGER D, REZZONICO R, LI J *et al.*: Imbalance between interstitial collagenase and tissue inhibitor of metalloproteinases-1 in synoviocytes and fibroblasts upon direct contact with stimulated T lymphocytes. *Arthritis Rheum* 1998, 41:1748-59.
43. JOVANOVIC DV, MARTEL-PELLETIER J, DI BATTISTA JA *et al.*: Stimulation of 92-kd gelatinase (matrix metalloproteinase 9) production by interleukin-17 in human monocyte/macrophages. *Arthritis Rheum* 2000, 43: 1134-44.
44. MILTENBURG AM, LACRAZ S, WELGUS HG *et al.*: Immobilized anti-CD3 antibody activates T cell clones to induce the production of interstitial collagenase, but not tissue inhibitor of metalloproteinases, in monocytic THP-1 cells and dermal fibroblasts. *J Immunol* 1995 Mar 15, 154: 2655-6.