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

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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.

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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.

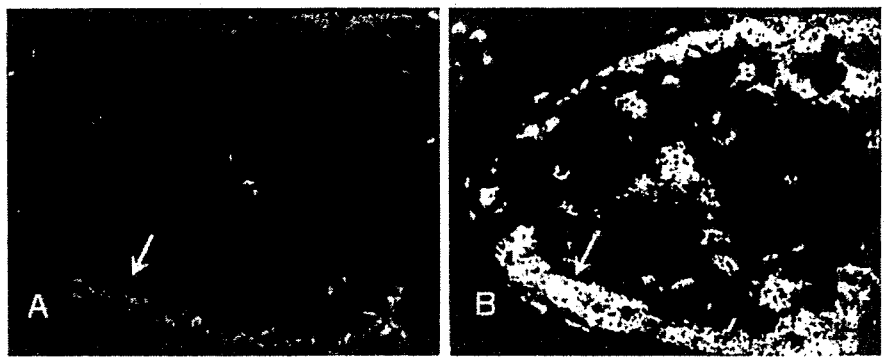

Fig. 1. Arrow indicates the gelatinolized area of RA synovim (A) detected by *in situ* zymography. The arrow indicated pale area was recognized as the measurable region by IPAP analysis (B).

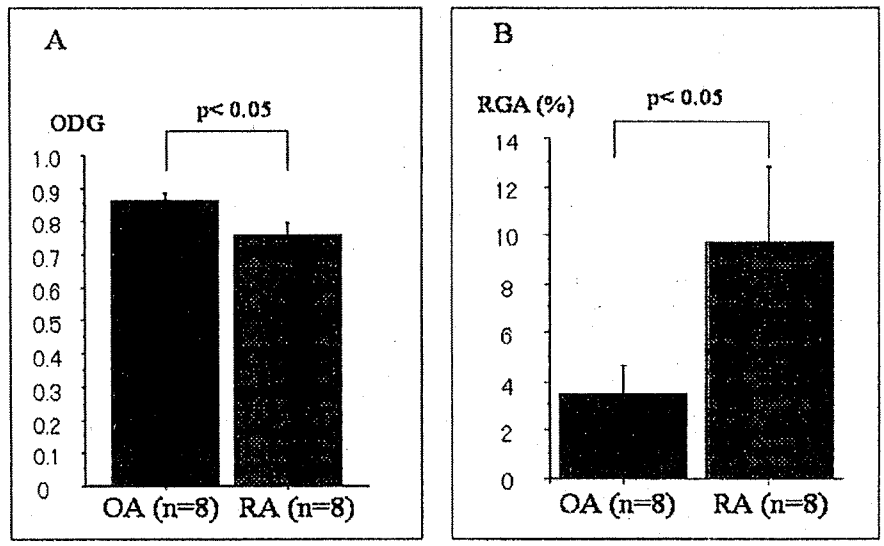

Fig. 2. Comparison with RA and OA synovium. RA synovium demonstrated significantly lower ODG (0.758±0.019) than OA (0.864±0.037) ($p<0.05$, A). RA synovium demonstrated significantly higher RGA (9.7±3.1 %) than OA (3.5±1.1 %) ($p<0.05$, B).

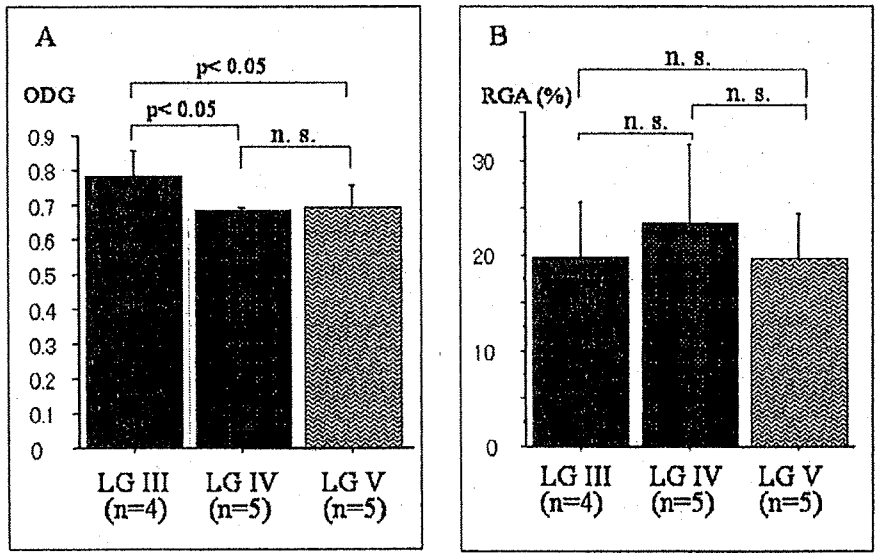

Fig. 3. Difference of gelatinolytic activity among Lrsen grades. There was significant difference between grade III (0.781±0.075) and IV (0.679±0.016), III and V (0.691±0.064) in ODG ($p<0.05$, A). However no significant difference was shown among Larsen grades in RGA (grade III; 19.8±5.8 %, grade IV;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

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関節リウマチにおける関節炎の破壊に関する最近の病理学的話題

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関節リウマチにおける関節炎の破壊に関する最近の病理学的話題

澤井 高志*1) 宇月 美和*2)

関節リウマチ（rheumatoid arthritis：RA）による関節の破壊については，病理組織像が従来に比べて大きく変化しているわけではなく，滑膜炎で始まり，軟骨の破壊を経て骨吸収に至り，最終的には関節の変形をきたすという組織像は変わっていない。これら炎症と骨吸収に関する新しい因子や機能が発見されると，病態についての解釈が変化すると同時に，それらの因子が最近の治療によって大きな影響を受け，関節の破壊像が変ってきているのではないかということで注目されることになる。一方，それらのタンパクに関連した遺伝子の機能も次第に明らかにされつつあり，ノックアウトやトランスジェニックなどの手法によって遺伝子学的，細胞内でのタンパクの役割に関する解明が行われている。現在，関節破壊の過程がすべて明らかにされたわけではないが，今回は，その過程を追いながら数多くの症例の経験からその形態学的特徴を述べてみたい。

Rheumatoid arthritis in the context of bone and cartilage.

Recent topics of histopathology associated with joint destruction in rheumatoid arthritis.

Division of Leading Pathophysiology, Department of Pathology, School of Medicine, Iwate Medical University.

Takashi Sawai, Miwa Uzuki

Histopahological features of rheumatoid arthritis, beginning from synovitis through deteriorating cartilage and bone to joint destruction has basically unchanged since the old days. On the other hand many inflammatory factors initiating, sustaining and/or activating inflammation such as cytokines and proteolytic enzymes, were successively detected, and followed by genetic analysis using animal models such as transgenic and knockout methods. Newly developed therapies by biological products remarkably have influenced the inflammatory these factors and genes, and seemed to modify the histopathological features.

This article refers the histopathlogical features of RA in topics such as places involved in early stage, and the cellular origin, especially about the fibroblast like cells (FLS) which have been paid attention recently

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as key cells presenting immunological, histiocytic and fibroblastic properties, furthermore, participating the bone destruction in part as well as osteoclast in RA.

We also introduce the several animal models of RA applied by many researchers for therapeutic and genetic analyses in RA.

はじめに

関節リウマチ（rheumatoid arthritis：RA）は，全身の関節をおかす慢性炎症性疾患であり，原因は免疫異常によるとされている（図1）。

最近のRAの動向をみた場合，大きく二つの特徴があげられる。一つは生物学的製剤の開発・投与によって，病像が大きく変わりつつあることであり，当然，その変化は滑膜組織の炎症や軟骨・骨の吸収像の変化にも微妙な影響を与えている。もう一つは画像診断の進歩である。小さな病変の観察が可能となり，きわめて早期の変化が捉えられるようになった。こういうなかで，病理学的所見の果たす役割は，正確な細胞・組織学としての裏付けと動態に関する考察ではないかと思われる。それだけに，ヒトの検体を用いた解析の限界に対しては，動物モデルを用いらざるを得ないこともある。ここでは，筆者らが用いてきたいくつかの動物モデルをとりあげながら，最近のRAの話題をとりあげてみた。

RAの初期変化は骨髄に始まる

RAが，最初にどこから始まるかという点については長年の課題であった。最近は骨髄で最初に変化がみられるという説が強いが，それがどのような変化であるかという説が定着したわけではない。越智らは大きな胞体を有する顆粒球系細胞がRA患者の骨髄内，それも骨破壊の高度な関節の近くに数多くみられ，小さな隙間を通って滑膜に至るという所見を述べている[2]。RAの滑膜組織においてはHLA/DR$^+$，CD14$^+$の線維芽細胞様細

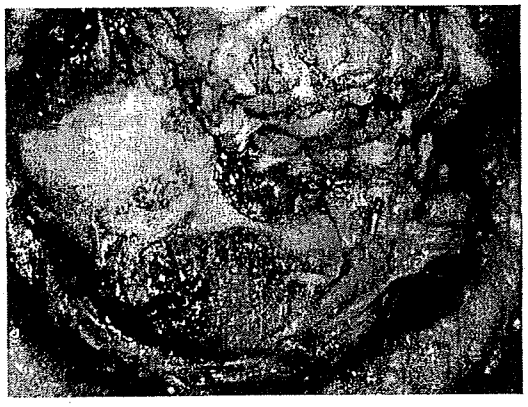


図1　RAの膝関節

RAではパンヌスの増生が認められ，軟骨，骨を侵食している。

（文献1より引用）

図2　関節滑膜の初期の電顕像

初期には血管周囲に紡錘形の線維芽細胞様細胞（FLS）が出現してくる。この細胞はRAの病変を形成する主要な細胞であると思われる。

（筆者ら提供）

FLC：線維芽細胞様細胞，RA：rheumatoid arthritis（関節リウマチ）

胞（FLC）の増加がみられるが（図2），骨髄細胞からこのFLCへの変換については解明すべき点が多い。問題は，骨髄で初めに増える細胞は如何なる性質を有する細胞で，滑膜にどのような形で移動し，細胞の機能，マーカーをどのように変化，獲得していくかということである。LiとMakarovは，FLSは間葉系細胞の幹細胞であり，NF-κBがこのFLSをosteogenic cellやadipogenic cellへの分化を調節しているという証明をGFPマウスの培養細胞を用いておこなっている[3]。最近，MRI（magnetic resonance imaging）などで骨髄浮腫と診断される例があり[4]，抗CCP抗体の高値とともに，予後に関係しそうだと報告されているが[5]，これも浮腫だけでなく，ある種の細胞が増加していることも十分考えられる。

滑膜の初期病変はbare areaから始まる？

ヒトでの滑膜での初期病変は滑膜と軟骨の移行部であるbare areaから始まるといわれてきた。しかし，ヒトで早期RAの滑膜を入手することは倫理上からもますます難しくなってきており，まして，bare areaの組織所見をみることは，ほとんど不可能である。RA発症1カ月例の滑膜組織では，滑膜表層細胞の増加傾向がみられ（図3），その下の毛細血管の周囲には，HLA/DR陽性のFLCが出現し，次にTリンパ球の浸潤，集簇がみられ，Bリンパ球はかなり時間を経てから出現して集簇するのが特徴であった。従って，FLSは初期の状態からRAの病変形成・進行の大きな鍵を握っている可能性があるといえる[6]。しかし，滑膜，軟骨移行部のbare areaがどのようになっているかをヒトで観察するのは不可能であり，今後，ますます状況は厳しくなるものと思われる。そこで，このような解析には，動物モデルが必要となってくる。

関節炎の初期病変を観察するために，自己免疫現象を緩徐に自然発症するMRL/Mp-lpr/lpr（MRL/1）マウスを選択し，免疫組織学的検討を行った。その結果，生後4週の早期にbare areaに近い軟骨下骨髄に炎症性細胞が集積し始め，週

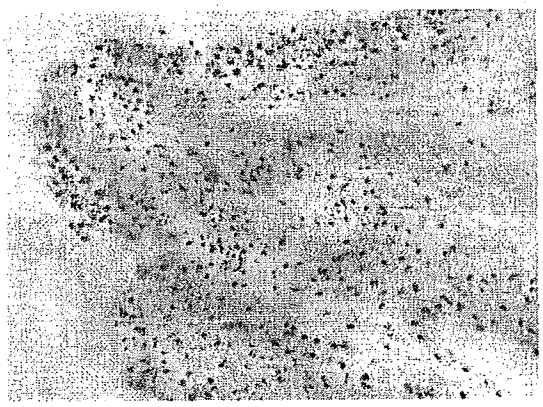
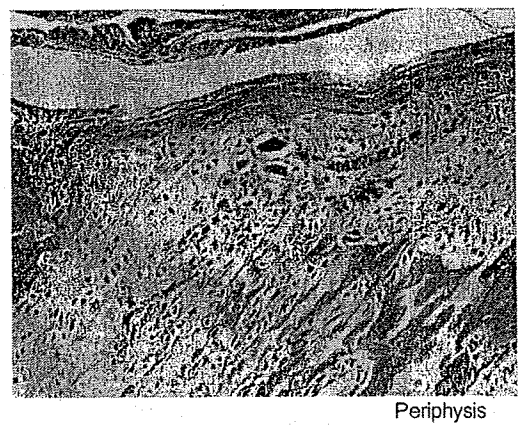


図3　RA初期の組織像

滑膜の表層や深部には軽度のCD14，HLA/DR陽性の細胞を認める。Bリンパ球の浸潤は時間的に遅くなる。

（文献6より引用改変）



Periphysis

図4　MRLマウスの初期病変

マウスにおいては軟骨，滑膜，骨の移行部のperiphysisには，IgGの沈着やマクロファージの浸潤を認める。ヒトのbare areaに相当するものと思われる。

（文献7より引用改変）

MRI：magnetic resonance imaging

齢とともに，成長軟骨，骨髄に向かって次第に拡大する傾向が認められた[7]（図4）。さらに，この部位の血管の周囲のIgGや補体の沈着，Mac-1陽性で未消化の貪食物（dense body）を有するマクロファージ，酒石酸抵抗性酸ホスファターゼ（TRAP）陽性の多核大型細胞が認められ，これらの細胞も週齢とともに増加した。なお，コントロールに用いたDBA/1Jマウスでは，同部の炎症性変化は全く観察されなかった。この領域は，Oestreichらにより periphysis と名づけられて

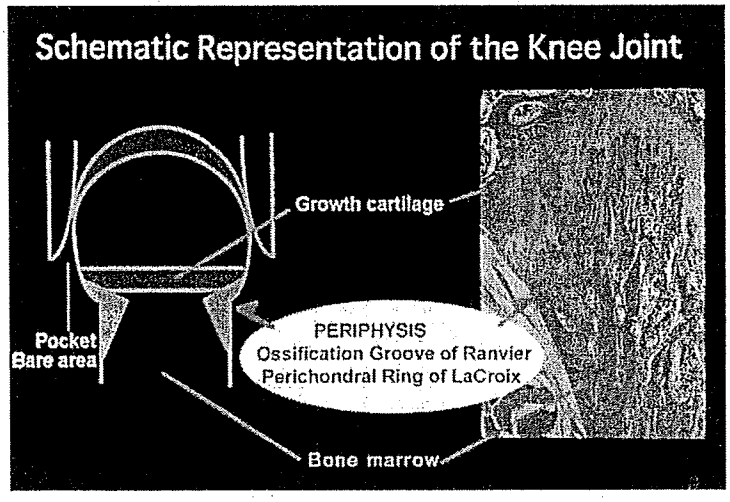


図5　Periphysisの部分のシェーマとマウスの病変

Priphysisの部分は移行部のため柔らかい結合組織と血管が認められる。

（文献7，8より引用改変）

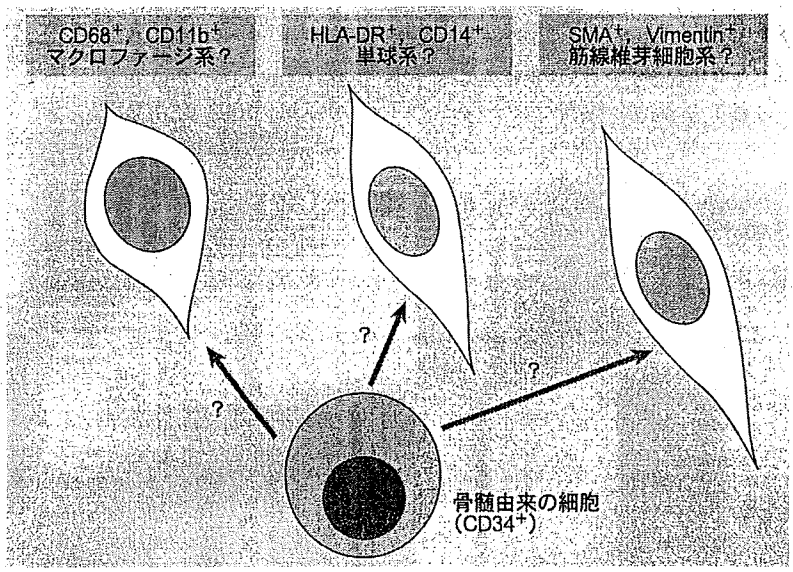


図6　線維芽細胞様細胞（FLS）の性質

現在，FLSには①本来の線維芽細胞，②免疫担当細胞，③マクロファージ的細胞の3種類が考えられる。これらは主に形と免疫染色をもとに考えられているが，同一の起源を有する細胞かどうかは不明である。

（文献1より引用改変）

TRAP：酒石酸抵抗性酸ホスファターゼ

おり[8]（図5），数種の未分化な細胞の存在するossification groove of Ranvier とⅡ型コラーゲンがネットワークを形成するperichondral ring of Lacroix とから構成される。MRL/1 マウスのperichondral ring における変化は，Ⅱ型コラーゲンに対する免疫反応に引き続く軟骨破壊に関連したものと考えられるが，その根拠は，①periphysis の細胞はマウス血清中の抗Ⅱ型コラーゲン抗体と同時期に出現し，炎症性変化は抗体価と相関して変動したこと[7]，②発生の早期から無血管性組織として血液とは隔離されていた軟骨が，組織移行部での血管が侵入するperiphysis で新たに抗原として認識されることなどである。従って，モデル動物であるMRL/1 マウスのperiphysis の変化は，ヒトRA における初期病変と共通した所見を呈することが示唆される。

滑膜組織の細胞はどこから

旺盛な炎症を示すRA の滑膜組織をみるとリンパ球，血管に混じって多くのFLS が認められ，このFLS はRA の滑膜炎を特徴づける重要な細胞であろうと思われるが，今のところ，このFLS にはいくつかの種類があると考えられる。一つは通常の線維芽細胞（間葉系細胞としての役割）であり，二つには情報伝達に関与する細胞であり（免疫系担当細胞），そして，三つには骨破壊に関与する細胞（マクロファージ系細胞）である（図6）。従来，RA はTリンパ球が主体の疾患でT Cell Disease ともいわれてきた。しかし，最近は細胞動態や治療との関係からB Cell がRA の病態の形成に大きな比重をしめているのではないかといわれている[9][10]。以前，NK 細胞の研究が盛んだったころは，滑膜組織にNK 細胞がほとんどいないにもかかわらず，RA はNK 細胞によって支配されているという説が流れたことがあった。形態学的にRA の組織像が時代とともに大きく変化することはないが，さまざまな因子の関与が証明されるにつれて考え方が左右されることは珍しくない。従って，我々形態学に従事しているものの責任は，きちんと物をみて正確に記録しておくことであると思っている。

軟骨・骨破壊への過程とその因子

軟骨・骨の破壊は病理学的にみていくつかの原

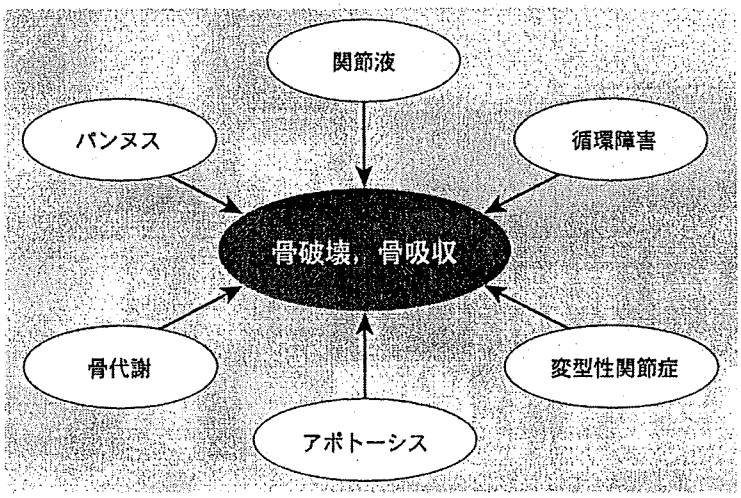


図7　軟骨・骨破壊に影響を与える因子

軟骨，骨の吸収，破壊にはいくつかの原因が考えられる。

（筆者ら作成）

因からなる。関節の破壊となる因子を図7にあげた。もちろん，このなかで破壊的な作用として持続的なもの，あるいは破壊に対して比重の大きなものなど因子によって異なるが，やはり，大きな影響を与えるのは炎症性肉芽組織(パンヌス)ではないかと思われる(図8)。このパンヌスには多くの炎症性細胞や毛細血管と，またそれぞれの細胞が産生，放出するサイトカインやタンパク分解酵素，増殖因子などが含まれている。そして，最近の生物学的製剤による治療との関係で示唆されるのはNF-αの影響が大きいという可能性であろう[11]。TNF-αを抑えることで炎症が抑制され，手術件数が減少しているという最近の傾向を考えると，この効果をだれしもが目で確認したくなるのではないかと思う。そして，最近は抗CD20抗体[12]，抗IL-6 receptor抗体[13]などに関連した生物学的製剤なども効果が認められることから，我々はここでもう一度原点に返って，これらのサイトカインによって支配されているRAの炎症というものを見直す必要があるのではないかと思われる。

図8 RAのパンヌス

炎症性の肉芽組織であるパンヌスは腫瘍と同じように浸潤しながら軟骨，骨を破壊していく。

(文献1より引用)

最近の治療による組織像の変化

前述のごとく軟骨・骨の吸収，破壊の像が大き

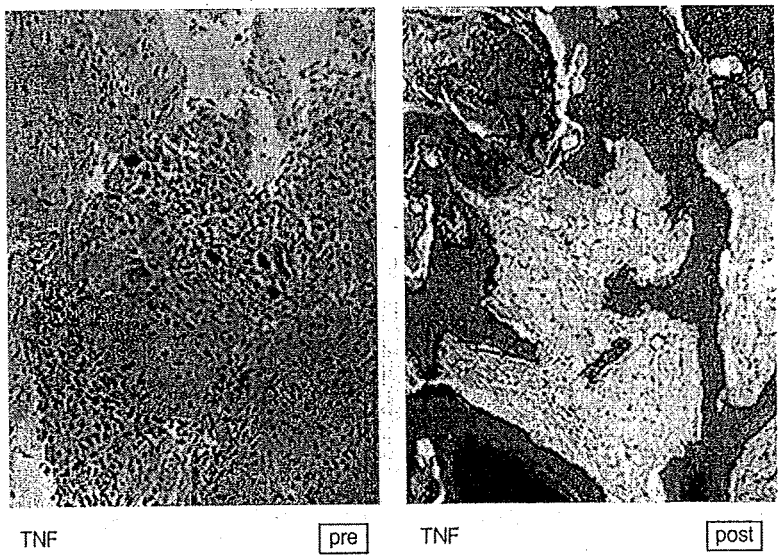


図9 SCIDマウスを利用した生物学的製剤，抗TNF-α抗体の治療効果の実験

ヒトの滑膜組織（左）をSCIDマウスに移植して抗TNF抗体を投与したところ，炎症性細胞浸潤が消失し（右），治療効果が動物で実証された。

(筆者ら提供)

く変わったわけではない。ただ，最近生物学的製剤の投与とともにその炎症の特徴に違いが現れてきているような印象を受ける。**図9**はRA患者の滑膜組織を移植したSCIDマウスに抗TNF-α抗体を投与した組織像である。写真左のように滑膜組織にみられた炎症性細胞は，写真右のように細胞の種類を問わずほとんど消失してしまっている。決して組織が壊死になっているわけではない[14]。**図10**は最近，我々が経験した抗TNF-α抗体を投与したRA患者の滑膜組織である。マクロファージなどの炎症性細胞はほとんど消失している。**図11**は炎症反応は治まったものの変形と痛みのために手術をおこなった症例であり，滑膜組織は絨毛性を保ったまま炎症性細胞が減少し，

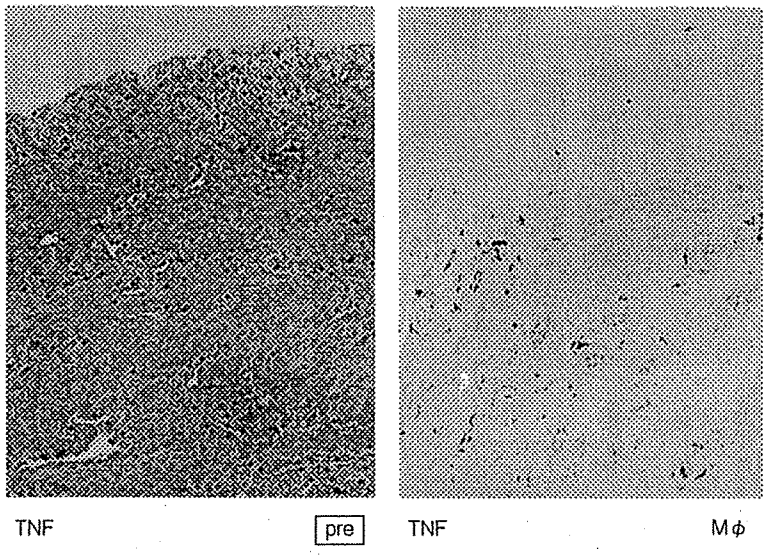


図10　RA患者に抗TNF-α抗体を投与したあとの滑膜組織

HE染色では，細胞数の減弱が認められ（左），抗CD68抗体で免疫染色をおこなってもマクロファージはほとんど認められない。

（文献15より引用）

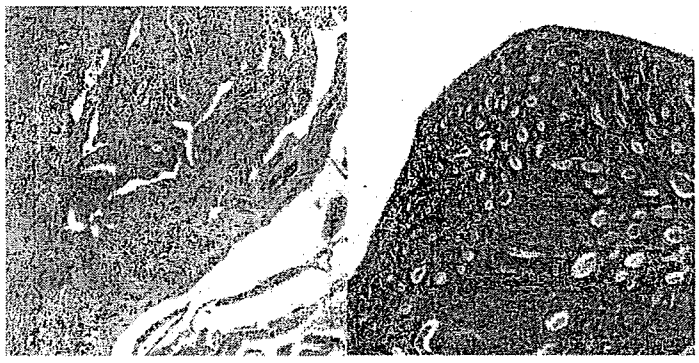


図11　生物学的製剤（抗TNF-α抗体）を投与したヒトの滑膜組織

図10と同様，炎症性細胞はほとんどみられなくなる（左），あるいは血管だけが残って，炎症性細胞がなくなる（右）。いずれにせよ炎症性細胞は減少している。

（筆者ら提供）

硝子化あるいは線維化し，毛細血管だけが目立っている。つまり，治療によって滑膜組織の炎症性細胞がなくなってしまうということである。滑膜組織に何が起こったのであろうか。単純に考えれば，炎症性細胞-血管内皮細胞との接着関係が薬剤によって阻害されたとみるべきかもしれない。その機序が接着因子の発現，Rolling，遊走の抑制などによるものかどうか，もっと他の影響があるか等などは今後の課題である。いずれにせよ，これまでみてきた炎症の慢性化による滑膜組織の萎縮とは異なり，絨毛状の形を保ったまま"化石"のように炎症性細胞が消褪してしまったと考えざるをえない。病理学的にみての問題は，滑膜の炎症と軟骨・骨破壊の進行について解離しているものがあるが，その原因については，今後，まだ解析の余地がある。

関節炎と動物モデル

ここで若干，関節炎の動物モデルについて触れる。従来，関節炎モデルは大きく，自然発症と誘発による関節炎に分類され，その後プリスタン，SKJマウスなどの新しいモデル関節炎も追加され，病態の解明や治療の開発に利用されている。**表1**はこれまで利用されてきた関節炎モデルである。これまでは関節炎のモデルとしては，特にRAを対象にして誘発関節炎と自然発症のモデル動物が用いられてきた。前者は主に治療，後者は病態解明などで利用されることが多い。しかし，最近は遺伝子との関係が注目されるとノックアウト，トランスジェニックを用いた解析が利用されている。また，軟骨，骨破壊についても破壊される側である軟骨，骨などの基質の異常だけでなく，サイトカインやタンパク分解酵素の作用，これに影響を与える因子，さらにアポトーシスなど細胞内のシグナル伝達の面からも解析されている。

表1　関節炎モデル

A．自然発症関節炎モデル
1．MRLマウス関節炎
2．NZB/KNマウス関節炎
3．SKJマウス関節炎
B．誘発関節炎モデル
1．アジュバント関節炎
2．コラーゲン関節炎ラット
3．Ⅱ型コラーゲン関節炎モデルマウス
4．大腸菌関節炎ウサギ
5．プリスタン関節炎
6．レンサ球菌関節炎ラット
7．塩化水銀関節炎ラット
8．結晶誘発性関節炎
最近の関節炎モデル
1．SCID細胞移入関節炎
2．SCIDマウス組織移植関節炎
3．ノックアウトマウスを用いた関節炎モデルの解析
4．トランスジェニックマウス
5．カクテル関節炎

（筆者ら作成）

また，抗リウマチ薬が開発されると，その機序，効果については，開発されてきたモデル動物の利用がおこなわれている。動物モデルを用いての利点はその解析に時間の因子や薬剤の投与量，投与方法などを自由に組み込むことができることであり，ヒトでは解析の難しくなってきた初期病変からの組織変化の推移の観察にも有用である。その点では，動物モデルの利用はMRIなどの画像の利用と並んで，今後も関節炎解析の大きな方法である。

しかし，これらのモデル関節炎とヒトの関節炎を比較してみると組織像からみて全く同じように論じていいということではない。むしろ炎症という概括的な表現を除けば異なる面もいろいろみられる。例えば，アジュバント関節炎には顆粒系細胞はみられるもの，リンパ球がみられないし，MRL/1マウスでも滑膜に出ている多くは，リンパ球というよりはマクロファージ系の細胞である。従って，利用する場合はそれらを念頭において使っていく必要がある。ある薬剤をモデル動物に投与して関節炎を抑制したからヒトにも効果があると断定するのは早計であり，慎重な扱いが必要である。以下，我々が扱った関節炎モデルについて簡単に紹介したい。

1．MRL/1マウスの関節炎の解析[16]（図12）

MRL/lマウスの関節炎の組織像を検討した結果，滑膜組織にはマクロファージ様の細胞が多数出現し，免疫複合体を貪食していた。滑膜の炎症はみられるもののヒトのようなリンパ濾胞の形成をみることはなく，滑膜細胞の多層化もヒトの多層化のような高度なものではなかった。

2．MRL/lマウスの骨髄の変化[17]（図13a，b）

MRL/1マウスの骨髄の時間による変化をMRL/nマウスとの比較で検討した。その結果，MRL/Lマウスでは時間の変化とともにI-A^k，Thy1，2陽性細胞が増殖し，これが自己免疫の病態あるいは関節炎に大きく関与している可能性が示唆された。

3．SCIDマウスによる抗IL-6抗体の影響[18]（図14）

抗TNF-α抗体投与と類似して，SCIDマウスに移植したRA患者の滑膜の炎症性細胞はほとんど消失する。

MRL/lマウス滑膜のマクロファージ（免疫複合体を貪食した像）

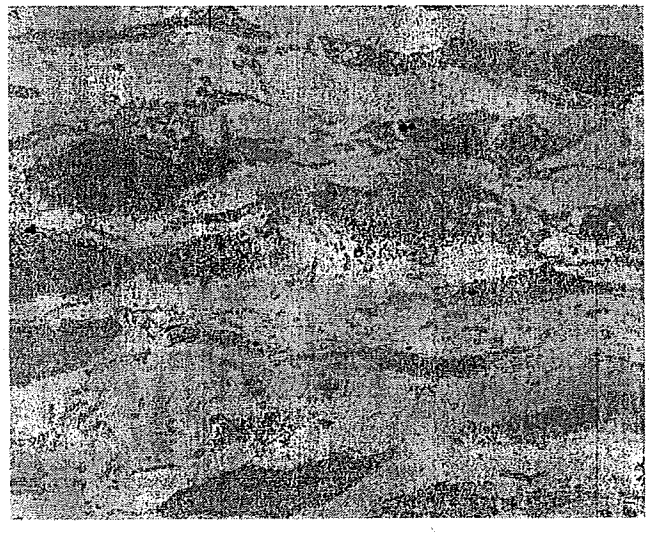


図12　MRL/1マウスの滑膜組織

MRL/1マウスの滑膜組織には紡錘形の細胞が多数認められ，免疫複合体を貪食している像が認められる。ヒトのRA滑膜のようなリンパ球の浸潤は目立たない。

（文献16より引用改変）

4．アジュバント関節炎[19]（図 15）

Freund Conjugate Adjuvant（FCA）の投与後，24 時間ぐらいで関節には炎症がおこり，初期は顆粒系，後期は単球系が出現してパンヌスを形成する。動物モデルとしてはもっとも激しい関節炎を呈するが，リンパ球よりは顆粒細胞が目立つ炎症である。

5．プリスタン関節炎[20]（図 16）

鉱物油の一成分であるプリスタンを腹腔内投与して関節炎を発症し，軟骨・骨破壊が起こり，炎症性細胞としてリンパ球，形質細胞，多核巨細胞，

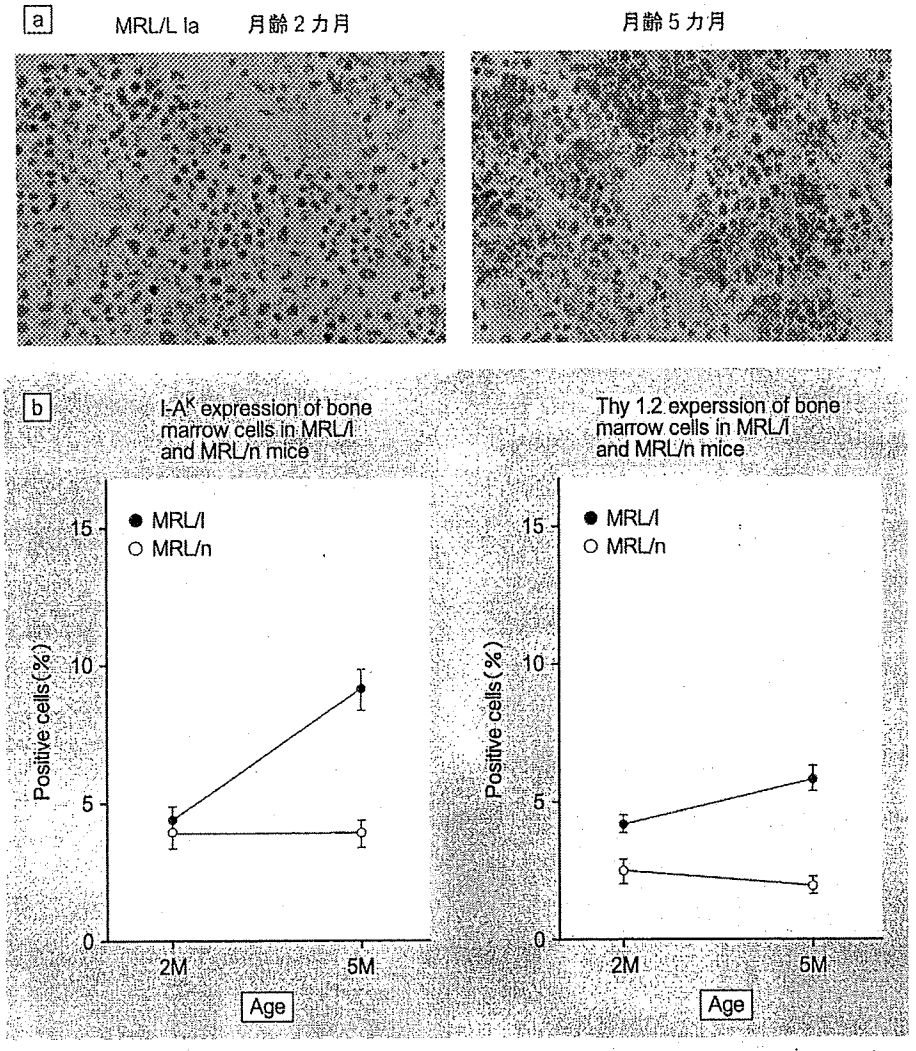


図 13a, b　MRL/1 マウスの骨髄像の月齢 2 カ月と 5 カ月の変化（a は写真，b はグラフ）

I-A^k を免疫染色で検討した結果，5 カ月例になると I-A^k 陽性の細胞が増加する。I-A^k はヒトの HLA/DR に相当する。同じ系統で免疫異常を発症しない MRL/n マウスにはこのような I-A^k 陽性細胞の増殖は全くみられない。Thy 1，2 についても I-A^k ほど顕著ではないが同じ傾向がみられる。（文献 17 より引用改変）

FCA：Freund Conjugate Adjuvant，GFP：Green fluorescent Protein

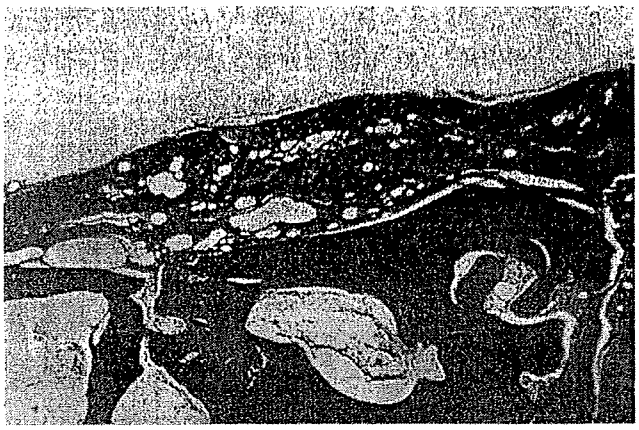


図 14　RA 患者の滑膜組織を SCID マウスに移植して抗 IL-6receptor 抗体を投与したあとの滑膜組織像

滑膜組織では，細胞数の減少が目立つ。

（文献 18 より引用改変）

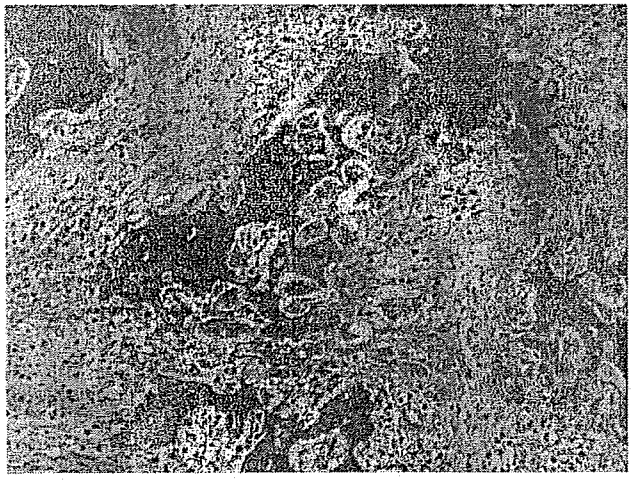


図 15　ウサギで発症させたアジュバント関節炎

顆粒球を主体とする激しい炎症性変化と破骨細胞の活性化により，高度の骨吸収が認められる。抗リウマチ薬の開発のために作成したアジュバント関節炎である。

（文献 19 より引用改変）

図 16　プリスタン関節炎

プリスタン投与によって誘発した関節炎を用いて，接着分子発現の実験を抑制することで抗リウマチ薬の開発中に利用した。プリスタン関節炎は，炎症がアジュバント関節炎に比較し穏やかで，発症までの時間がかかるために経過をみながらの薬剤の投与実験が可能となる。

（文献 20 より引用改変）

組織球の浸潤がみられる。自己抗体も出現し，SLE モデルの解析としても扱われている。

6．Green fluorescent Protein (GFP) による細胞の解析（図 17）

関節炎における骨髄と滑膜に浸潤する細胞の関