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Simple Method Using Gelatin-Coated Film for Comprehensively Assaying Gelatinase Activity in Synovial Fluid

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1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that leads to the irreversible destruction of cartilage and bone. An important step in the destruction of tissue is the degradation of extracellular matrix (ECM), whose major components are type II collagen and aggrecan in cartilage, and type I collagen in bone. The synovial fluid of RA patients often contains high concentrations of proteinases, such as those of the matrix metalloproteinase (MMP) and a disintegrin-like and metalloproteinase with thrombospondin type 1 motif (ADAMTS) families, which induce ECM degradation either directly or indirectly and participate in joint destruction. MMP-1, MMP-8, and MMP-13, which function as collagenases, have proteolytic activity towards type I and type II collagen (Chakraborti et al., 2003; Murphy & Nagase, 2008). MMP-2 and MMP-9, termed gelatinases, degrade denatured collagen, while MMP-3 degrades several ECM proteins (Chakraborti et al., 2003). All of them have the gelatinase activity. ADAMTS-4 and ADAMTS-5 are major enzymes involved in the degradation of aggrecan (Arner, 2002) and have low levels of gelatinase activity (Gendron et al., 2007; Lauer-Fields et al., 2007).

The concentration and activity of MMP and ADAMTS enzymes in synovial fluid and serum are measurable using ELISA or enzyme-specific substrates. In particular, MMP-3 is often the target of laboratory tests as a biomarker for disease progression. However, for the elucidation of actual RA progression, it is important to measure the comprehensive activity of all enzymes.

In situ zymography is a method for assessing endogenous protease activity in tissue section (Yan & Blomme, 2003). Film *in situ* zymography (FIZ) using gelatin-coated film is a useful method for assessing the enzymes involved in arthritis (Yoshida et al., 2009). In this method, a frozen tissue section is adhered onto a gelatin-coated film and gelatin degradation loci on the film are then analyzed after a suitable incubation period. A locus of gelatin degradation represents an area where comprehensive enzymatic activity is high. This chapter describes a method for assaying the comprehensive gelatinase activity in synovial fluid using gelatin-coated films for FIZ.

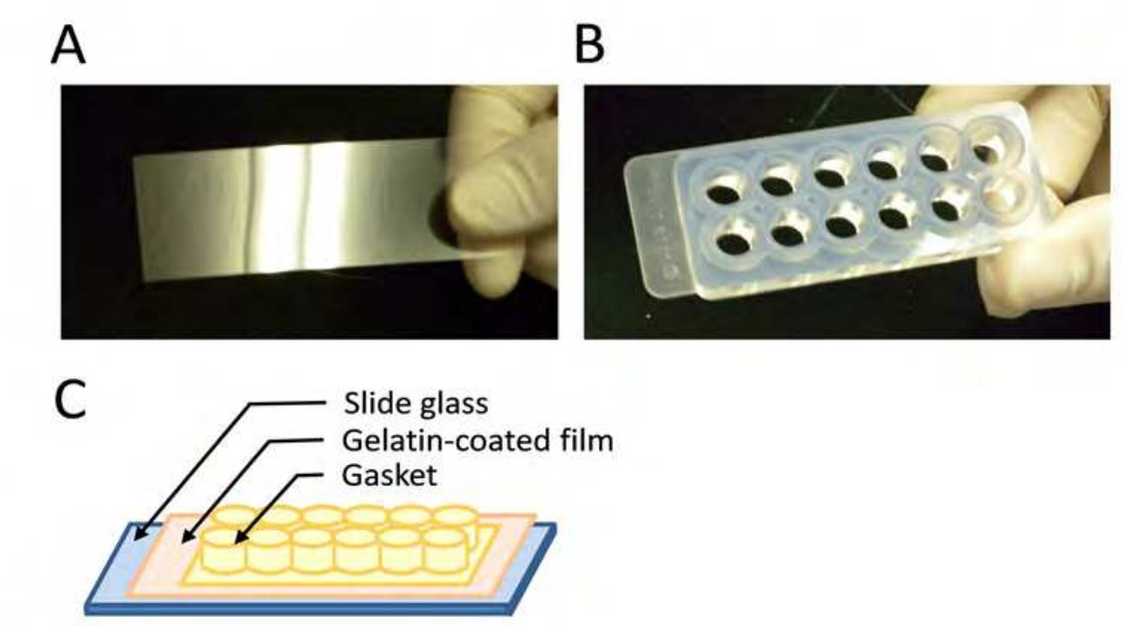
2. Method for synovial fluid analysis using gelatin-coated film

2.1 Synovial fluid samples

Synovial fluid samples were obtained from RA patients, osteoarthritis (OA) patients. Patients provided written consent for collection of synovial fluid samples before the procedure. All RA patients fulfilled the American College of Rheumatology criteria for the classification of RA. OA was diagnosed according to clinical findings. All the OA samples we examined were grade III or IV according to the Kellgren/Lawrence radiographic grading system. This study were approved by the ethics committees at Iwate Medical University

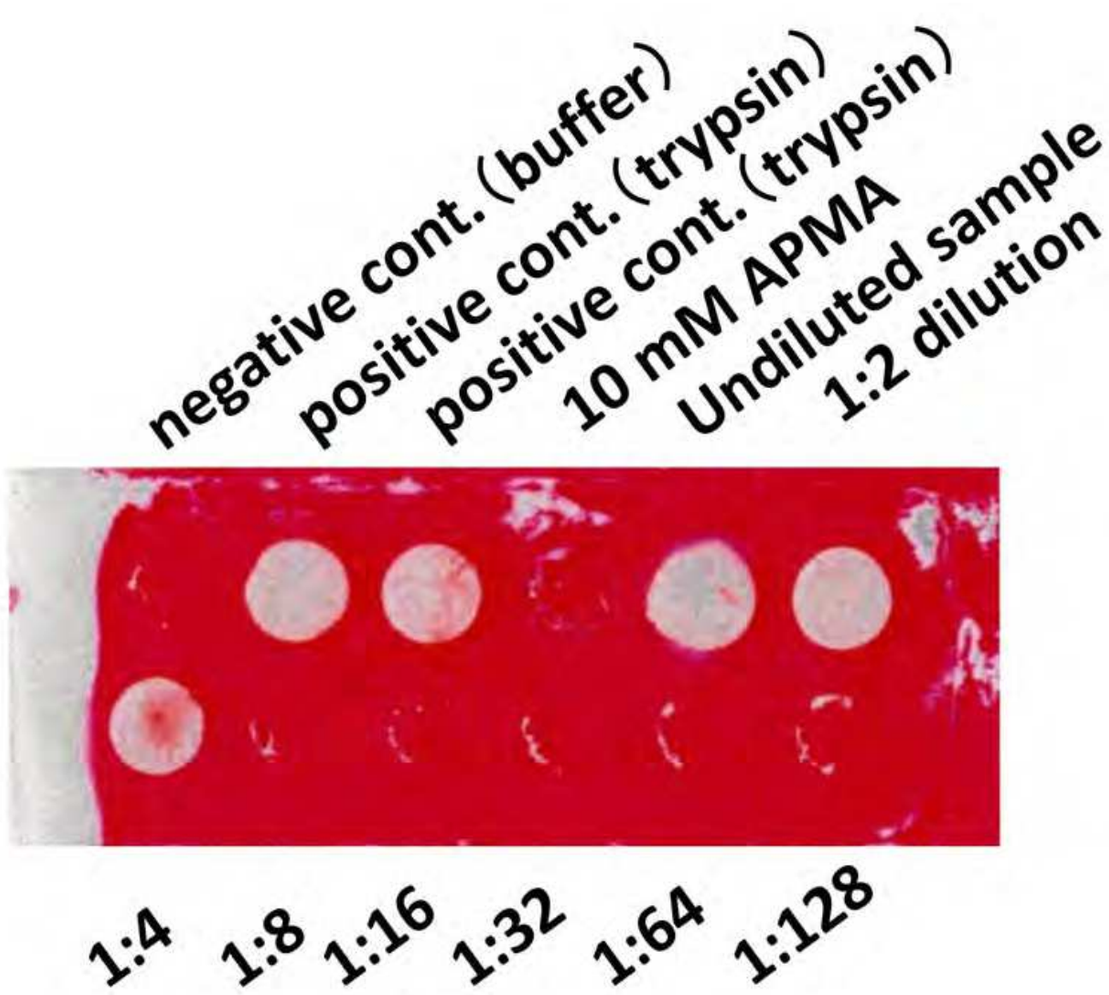
2.2 Method

Gelatin-coated film (Zymo-film; Fuji Film Co., Tokyo, Japan) is adhered onto a slide glass and a silicon gasket with 12 wells (flexi PERM; Greiner Bio-One GmbH, Frickenhausen, Germany) is then attached onto the film using Vaseline (Fig. 1). Two-hundred microliters of a two-fold serial dilution of synovial fluid sample is added into each well. To prevent the sample from evaporating, a lid is placed on the gasket and a moist chamber is used for the incubation period. After overnight incubation at 37 °C, the gelatin-coated film is washed twice with PBS, stained with a 0.2% Ponceau S solution, and then dried at room temperature (Fig. 2).



A: gelatin-coated film, B: gasket, C: illustration of the assembled detection system

Fig. 1. Gelatin-coated film and gasket

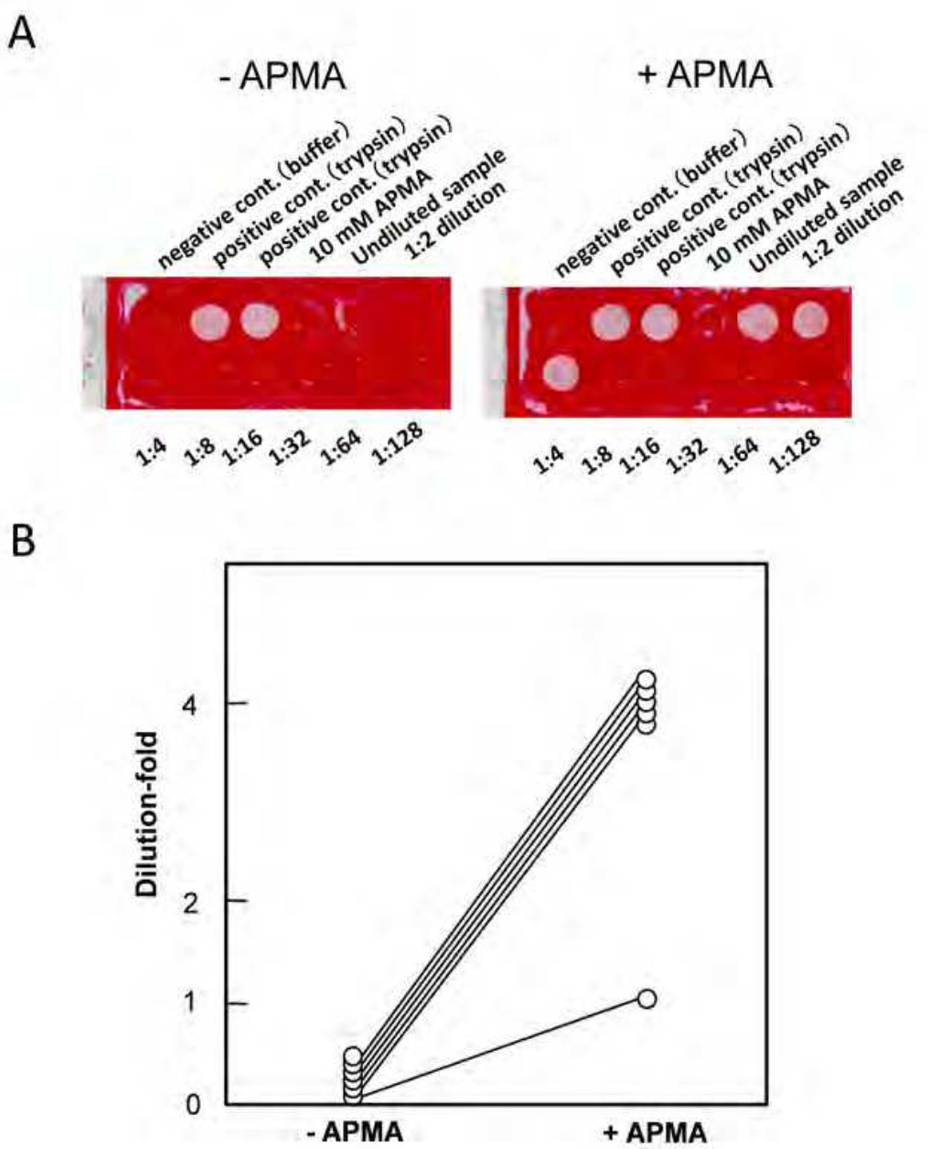


Gelatin-degraded spots are optically transparent. In this case, gelatinase activity was detected in the synovial fluid samples diluted up to 1:4.

Fig. 2. Ponceau S stained gelatin-coated film

PBS is used as a negative control, and a trypsin solution (1 and 10 μg/ml**)** is used as a positive control. To lower the viscosity of the synovial fluid, 15 units/μl hyaluronidase from *Streptomyces hyalyticus* (Sigma-Aldrich, St. Louis, MO, USA) is added to the synovial fluid samples. As p-aminophenylmercuric acetate (APMA) activates MMPs (Nagase et al., 1990, 1991), APMA is also added to the samples.

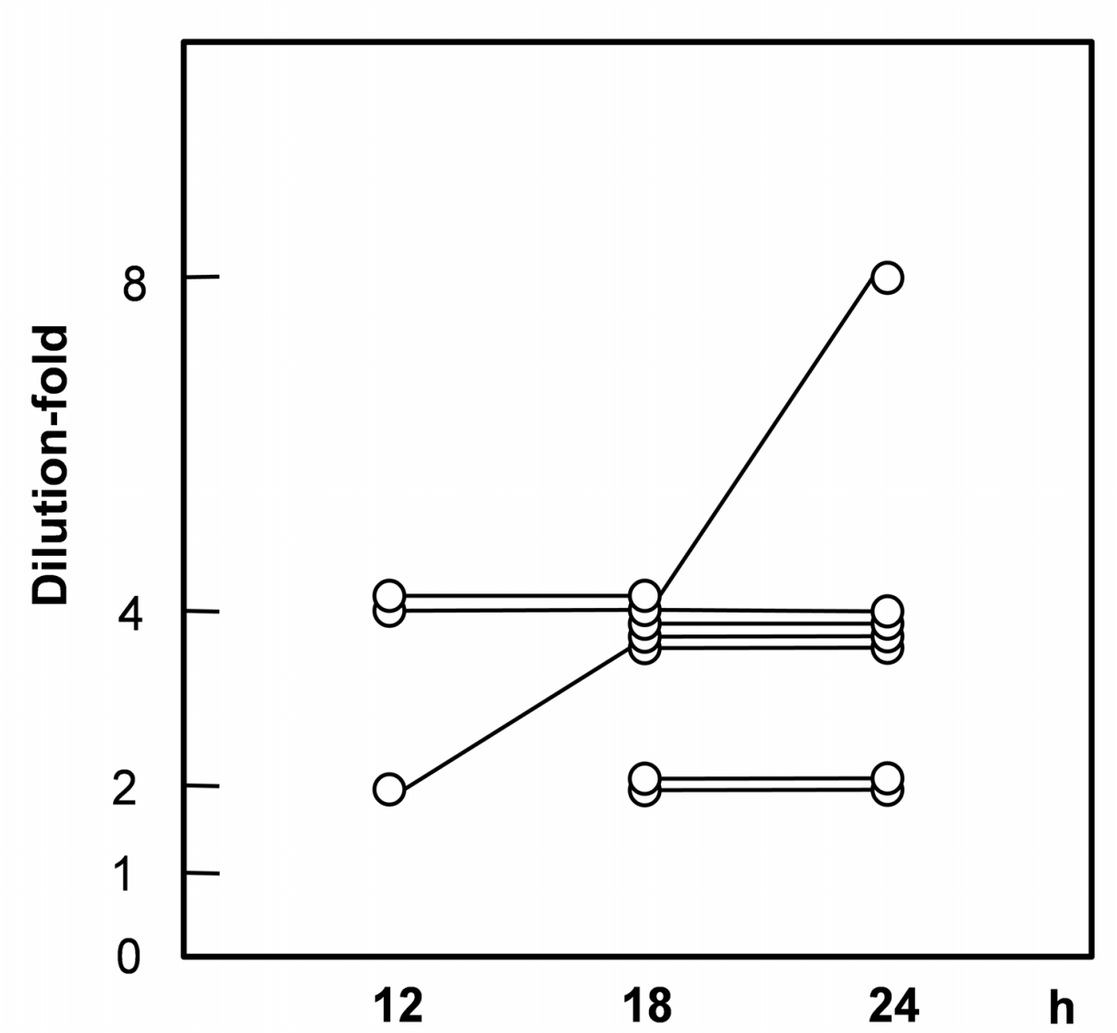
We demonstrated that six synovial fluid samples obtained from RA patients did not show gelatin degradation without APMA treatment (Fig. 3A, left, and Fig. 3B). However, treatment of the same samples with a final concentration of 1 mM APMA resulted in gelatin degradation (Fig. 3A, right, and Fig. 3B).



A. Gelatin-coated film incubated with synovial fluid treated with (right) and without (left) APMA. Without APMA treatment, even undiluted sample did not degrade the coated gelatin. With APMA treatment, samples diluted up to 1:4 showed gelatinase activity.
B. Change of gelatinase activity for six synovial fluid samples from RA patients following APMA treatment. The vertical axis shows the highest dilution-fold of samples that was positive for gelatinase activity. Zero indicates negative gelatinase activity.

Fig. 3. Gelatinase activity in synovial fluid samples with or without APMA treatment.

The optimal incubation time for the detection of gelatinase activity was determined by incubating synovial fluid samples for 3, 6, 12, 18, and 24 h on the gelatin-coated film. Only low levels of degradation levels were detected at 3 and 6 h (data not shown). The degradation levels at 18 and 24 h were constant for many samples (Fig. 4). In only one of seven samples, the degradation levels were elevated from 18 to 24 h. In the following experiments, the incubation time was fixed at 24 h.

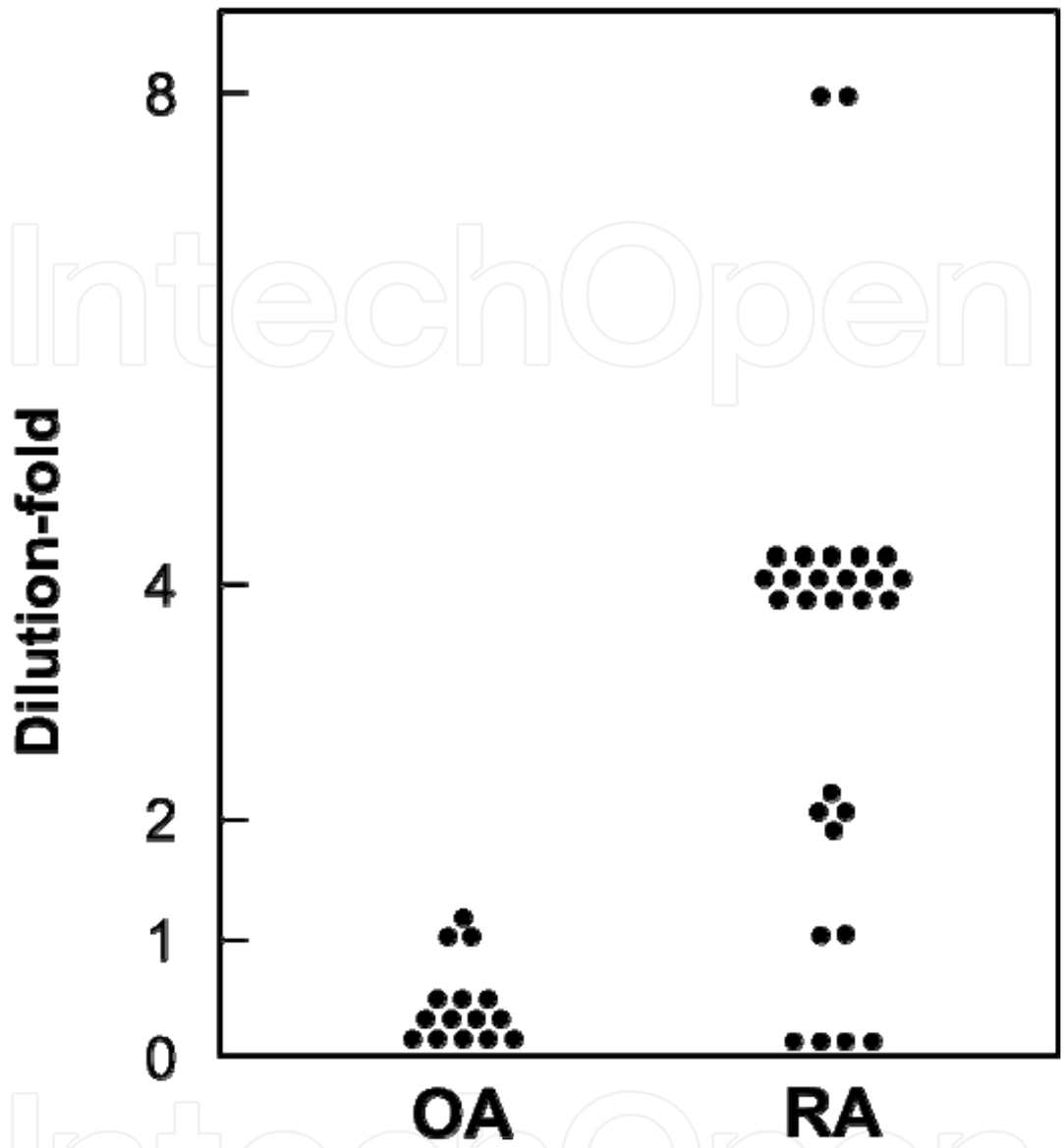


The vertical axis shows the highest dilution-fold of samples that was positive for gelatinase activity. Zero indicates negative gelatinase activity. Gelatin-coated films with synovial fluid samples were incubated for 12, 18, and 24 h.

Fig. 4. Gelatin degradation levels at various incubation times.

3. Comparison of gelatinase activity of RA and OA synovial fluid

Osteoarthritis (OA) is a disease of the joints and is caused by mechanical stress and an imbalance between catabolic and anabolic activities for cartilage (Sun, 2010). Although OA has a different etiology from RA, their mechanisms of pathogenesis are partly shared, with MMPs and ADAMTSs also playing a role in the development of OA (Huang & Wu, 2008; Murphy & Nagase, 2008; Sun, 2010). Therefore, the gelatinase activities of synovial fluid from RA and OA patients were analyzed and compared using the gelatin-coated film assay method. The synovial fluid from RA patients displayed higher proteinase activity than that from OA patients (Fig. 5), a result that is consistent with a previous study (Mahmoud et al., 2005).

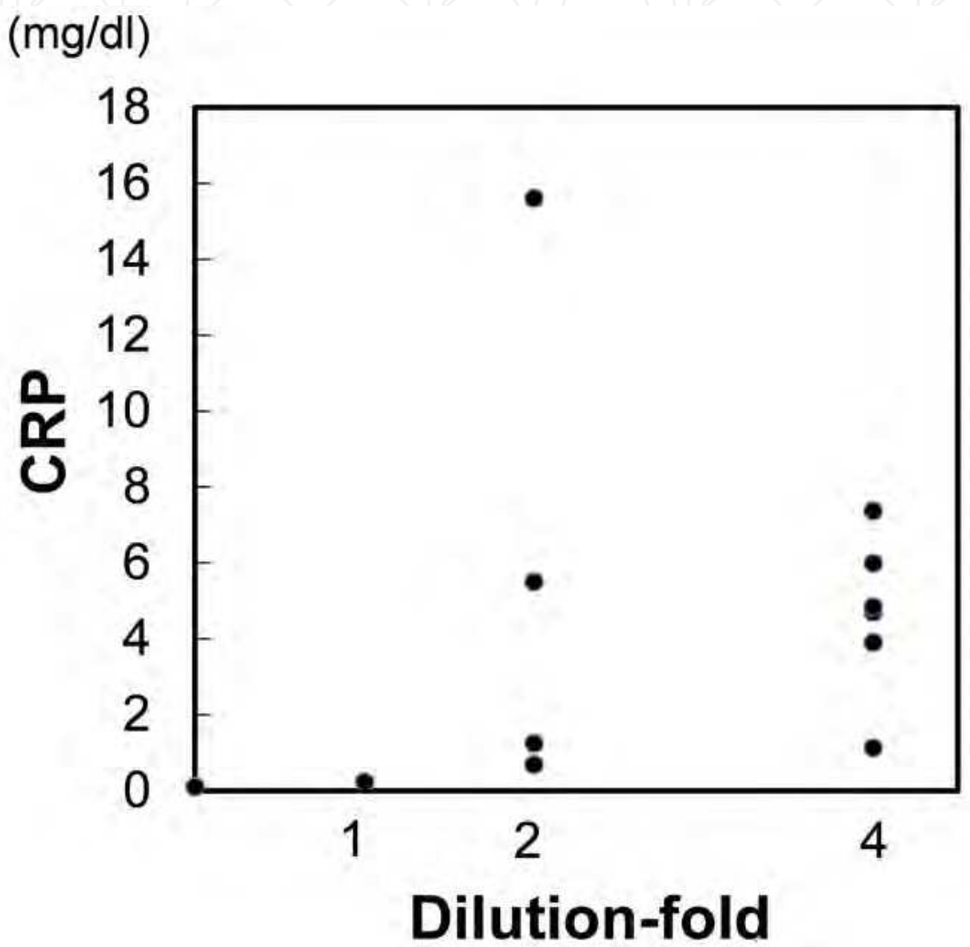


The vertical axis indicates the highest dilution-fold of sample that was positive for gelatinase activity. A value of zero indicates negative gelatinase activity. While only 3 of 15 (20%) samples from OA patients exhibited gelatinase activity, activity was detected in 24 of 28 (86%) samples from RA patients. Gelatinase activity was detected in two samples from RA patients diluted 1:8.

Fig. 5. Gelatinase activity of synovial fluid from OA and RA patients.

4. Correlation between gelatinase activity and biological marker

The concentration of proteases in serum and synovial fluid often correlates with that of biological markers. For example, MMP-3 levels significantly correlate with those of C-reactive protein (CRP) (Posthumus et al., 2003; Wassilew et al., 2010). On comparison of gelatinase activity with biological markers, it was demonstrated that samples with high gelatinase activity tended to have high CRP levels (Fig. 6).



The horizontal axis represents the highest dilution-fold of synovial fluid sample that was positive for gelatinase activity from RA patients. The vertical axis indicates the CRP level.

Fig. 6. Correlation between gelatinase activity and CRP levels.

5. Conclusions

The protease MMP-3, which degrades several ECM proteins and activates a number of MMPs, is often a useful predictor of joint destruction (Mamehara et al., 2010; Yamanaka et al., 2000). However, other proteases present in synovial fluid are also involved in cartilage destruction and warrant measurement. Our method using gelatin coated-film allows the comprehensive assay of gelatinase activity, including MMP-3 and other proteases, and is thought to accurately reflect the pathological condition of RA and OA and serve as a useful tool for the prediction of joint destruction. Notably, the observed gelatinase activities of the synovial fluid from RA and OA patients assayed by this method were consistent with a previous report. Gelatinase activity measured by this method also correlated with CRP levels.

The benefits of this assay method include the capability to measure comprehensive enzyme activity and simplicity, as special instruments are not required. Despite these advantages, the detection sensitivity of the proposed method is not sufficient to detect the enzyme activities of many OA samples. Improvement of the detection sensitivity is the most important issue to address, and will provide detailed information about the gelatinase activity in synovial fluid and more closely reflect clinical conditions.

The developed assay method is expected to be a useful tool for predicting the effect of therapy for not only RA, but also OA.

6. Abbreviations

The abbreviations used are: RA, rheumatoid arthritis; OA, osteoarthritis; ECM, extracellular matrix; MMP, matrix metalloproteinase; ADAMTS, a disintegrin-like and metalloproteinase with thrombospondin type 1 motif; FIZ, film in situ zymography; APMA, p-Aminophenylmercuric acetate; CRP, C-reactive protein.

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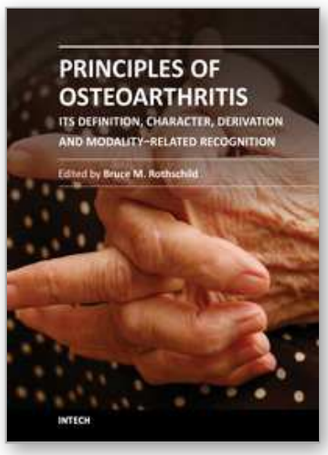

Principles of Osteoarthritis- Its Definition, Character, Derivation and Modality-Related Recognition

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This volume addresses the nature of the most common form of arthritis in humans. If osteoarthritis is inevitable (only premature death prevents all of us from being afflicted), it seems essential to facilitate its recognition, prevention, options, and indications for treatment. Progress in understanding this disease has occurred with recognition that it is not simply a degenerative joint disease. Causative factors, such as joint malalignment, ligamentous abnormalities, overuse, and biomechanical and metabolic factors have been recognized as amenable to intervention; genetic factors, less so; with metabolic diseases, intermediate. Its diagnosis is based on recognition of overgrowth of bone at joint margins. This contrasts with overgrowth of bone at vertebral margins, which is not a symptomatic phenomenon and has been renamed spondylosis deformans. Osteoarthritis describes an abnormality of joints, but the severity does not necessarily produce pain. The patient and his/her symptoms need to be treated, not the x-ray.

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