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Evaluation of *In Vivo* Proteolytic Activity

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1. Introduction

Osteoarthritis (OA) is a degenerative articular disease primarily observed in older adults, and is the leading cause of physical disability and impaired quality of life. OA is characterized by softening, fibrillation, erosion, defect of the articular cartilage, bone hypertrophy at the margins with osteophyte formation, subchondral sclerosis, and chronic inflammation of the synovial membrane and joint capsule (Zhang et al., 2010).

These alterations are thought to be caused by biochemical and biomechanical factors leading to a failure in the balance of synthesis and degradation (Martel-Pelletier et al., 2010). Pathogenesis of OA is based on an imbalance of the functional requirements and morphologic alterations, and these statuses progressively and chronically evoke subsequent alterations (Lorenz et al., 2005).

Although the etiology of OA is not completely understood, the accompanying biochemical, structural, and metabolic alterations of the articular cartilage have been well described (de Seny et al., 2011; Kraus 2010; Reeves et al., 2011; Sobczak et al., 2010). Recently, it has been revealed that proteolytic enzymes, cytokines, biomechanical stress, and altered genetics are involved in its pathogenesis, and proteolytic activity is particularly important in regards to the morphologic alterations of the articular structures, and is considered as an internal-factor of the disease (Takei et al., 1999; Kevorkian et al., 2004). It has been suggested that proteolytic activity in the constituent of the joint, such as synovium, joint fluid, cartilage, is continuously involved in the articular alterations of the disease, as its progression is gradual.

Although it is extremely difficult to accurately predict future articular OA alterations, it is possible to evaluate the present phenomenon, which may lead to speculation of possible further alterations by evaluating proteolytic activity in the joint.

2. Evaluation of *in vivo* proteolytic activiy

2.1 *In situ* zymography

There have been many studies of proteolytic activity, using gelatin zymography. This method is a valuable and effective tool for examining and analyzing proteolytic activity, as gelatin degrades over the course of the disease (Hattori et al., 2003; Cha et al., 2004; Sun et al., 2003). However, most current zymography methods are used to qualitatively examine this activity, and are thus not adequate for histological evaluation or quantification.

In situ zymography was developed to determine the *in vivo* proteolytic activity and determine its histological location. However, this method has only been used to demonstrate a qualitative analysis as the gelatin does not sufficiently coat the film with a uniform thickness of substrate to allow precise quantification of the *in vivo* proteolytic activity (Senzaki et al., 2000; Yi et al., 2001; Galis et al., 1995; Viemard-Barone et al., 2000; Goodall et al., 2001).

A newly method, "film *in situ* zymography (FIZ)", has been developed specially to evaluate the histological distribution the *in vivo* proteolytic activity (Ikeda et al., 2000; Takano et al., 2001; Zheng et al., 2002; Kaji et al., 2003) .

This new method works by applying unfixed frozen tissues (or fluid) to the recently developed FIZ film (Fuji Film. Co., Tokyo, Japan) which is uniformly coated with cross-bridge gelatin at a thickness of 7 μm. In our study, the synovial tissue specimens were embedded in Tissue-Tek OCT Compound (Lab-Tek Products, Elkhart, IN, USA) and frozen in the cryostat's refrigerated chamber. Then, frozen sections were cut at 4 μm, and applied to the FIZ film, followed by flushing with water for a few seconds. After incubation for 6 hours at 37°C, the film was stained with 0.2% Ponceau solution (which is commonly used for protein staining), (Sigma-Aldrich, St. Louis, MO, USA) for 3 minutes and fixed with 1% acetate for 5 minutes. After flushing with water for 15 minutes, the film was stained with hematoxylin for nuclear staining. Gelatinolyzed areas caused by the proteolytic activity in the synovium were detected as pale in color, and non-gelatinolyzed areas were stained red (Figure 1). In several studies, this method was successful in achieving reproducible quantification of gelatinolyed areas (Iwata et al., 2001; Furuya et al., 2001; Yamanaka et al., 2000).

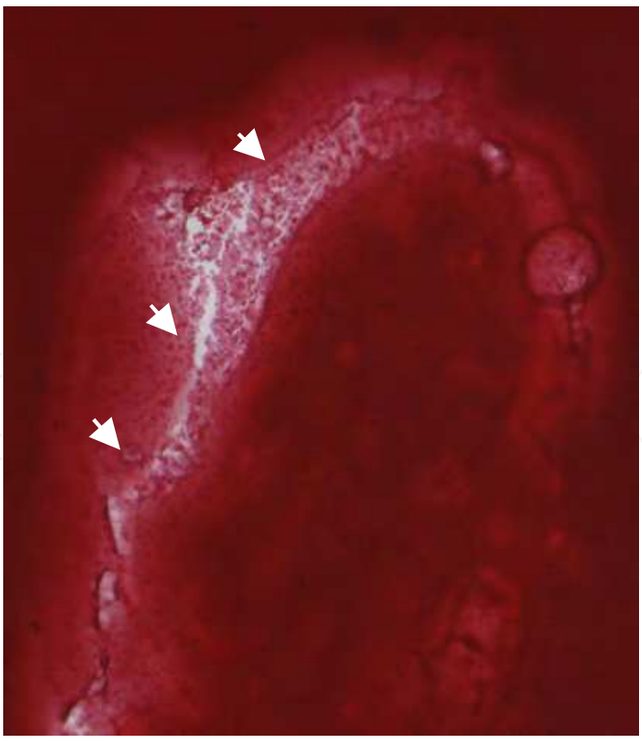

Fig. 1. Film *in situ* zymography of OA synovium. Arrows indicate the proteolytic lesions as pale in color in the Ponceau-stained FIZ film.

2.2 Quantification by image analyzer

Furthermore, it is possible to quantify the degree of this activity using a digital image analyzer (Image Processor for Analytical Pathology, IPAP, Sumitomo Tech, Osaka, Japan). The IPAP system is comprised of a conventional microscope, a CCD color video camera, an IBM-compatible microcomputer and a specialized image analysis board, Matrox Image-1280 (Dorval, Quevec, Canada) to convert microscopic photographic images into digital images, and allows us to analyze many samples, fields and parameters (Figure 2). For each Ponceau-stained FIZ film image, the analyzer can measure the approximate optical density of gelatinolyzed area as optical density of gelatinolyzed area (ODG) and ratio of gelatinolyzed area (RGA). The ODG is the mean optical density of the red-stained component at 50 random points in the gelatinolyzed area. The RGA is the ratio of the gelatinolyzed area to the entire synovium stained on the FIZ films as background reference (ODG and RGA were measured blindly at a magnification of ×4). As such, implementing both FIZ and IPAP enable the histological evaluation and quantification of the *in vivo* proteolytic activity to analyze the gelatinolyzed area (Uzuki et al., 1999; Yoshida et al., 2009).

Fig. 2. IPAP system. This system is comprised of a microscope, a CCD color video camera and an analyzing computer for converting microscopic photographic images into digital images.

3. *In vivo* proteolytic activity on OA synovium

It was revealed through FIZ the *in vivo* proteolytic activity on OA synovium was mainly distributed in the layer of the lining rather than in the stroma, although this histological feature is predominant and consisted of uniform fibrous proliferation with chronic inflammation. Furthermore, distribution of the proteolytic area in rheumatoid arthritis (RA) synovium, which showed obvious inflammatory changes, also detected in layer of the lining (Yoshida et al., 2009).

Comparing the *in vivo* proteolytic activity using FIZ and IPAP, there was a significant difference between OA and RA synovium in regards to the ODG (Figure 3) and RGA (Figure 4)(Yoshida et al., 2009). These findings suggest that there is also a difference on the proteolytic potential per one active-cell and the proteolytic-cell number between OA and RA synovium, and this might reflect that the articular alteration in OA is less progressive than in RA.

Furthermore, the proteolytic area is mostly localized in the layer of the lining and similar to both OA and RA articular disease, although they have different degrees of activity. The proteolytic area is constantly exposed to the articular space, and this finding suggests that the *in vivo* proteolytic activity in synovium might be affected in the interaction with the constituents of articular space as an internal-factor of the articular alterations.

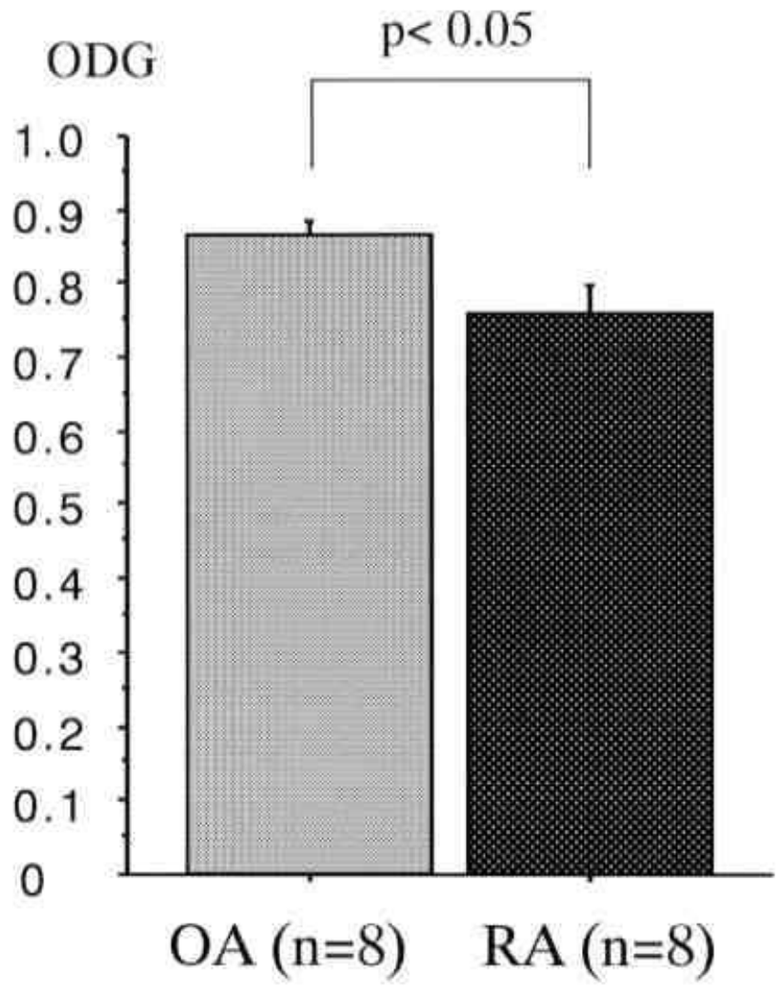

Fig. 3. Optical density of the gelatinolyzed area (ODG) as produced by FIZ and IPAP the synovium of OA and RA. OA synovium had a significantly higher ODG (0.864±0.037) than RA synovium (0.758±0.019). All OA cases were classified as grade 4 using Kellgren and Lawrence classification and all RA cases were classified as stage IV using the Steinbrocker classification.

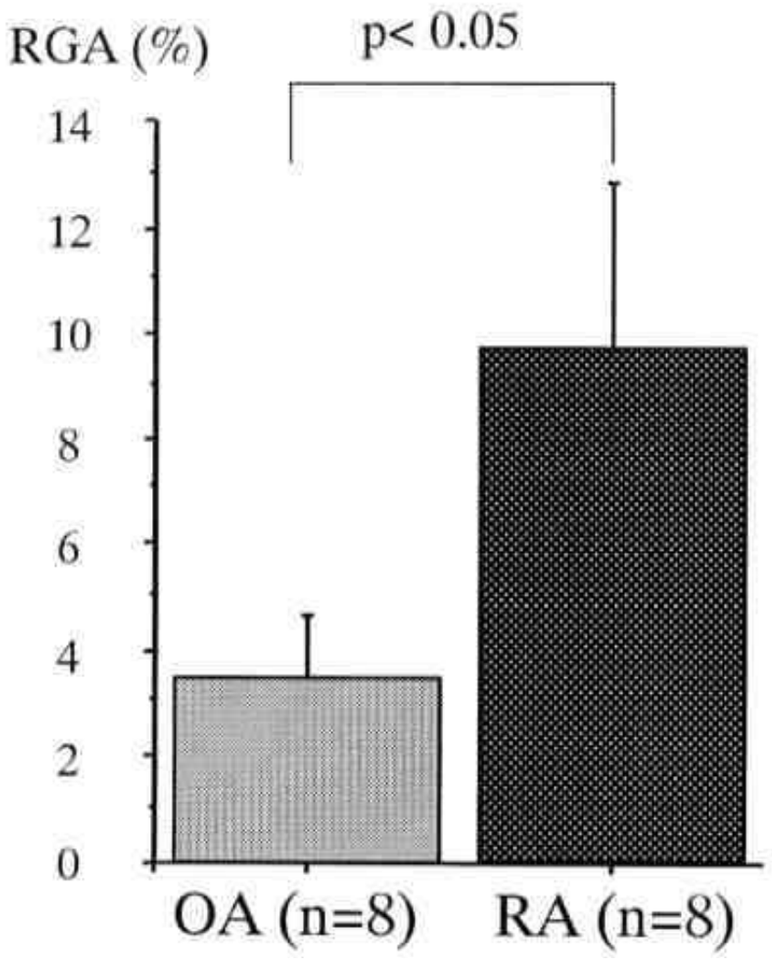

Fig. 4. Ratio of gelatinolyzed areas (RGA) as produced by FIZ and IPAP the synovium of OA and RA. The synovium of OA had a significantly lower RGA (3.5±1.1%) than RA synovium (9.7±3.1%). All OA cases were classified as grade 4 using Kellgren and Lawrence classification and all RA cases were classified as stage IV using the Steinbrocker classification.

In examination of enzyme expression by immunohistochemistry using serial sections, matrix metalloproteinase (MMP)-2, MMP-9, also known as gelatinase-A and -B, were mainly expressed by fibroblast- or macrophage-like cells of the synovial-lining layer (Figures 5a and 5b). Interestingly, these same cells also expressed tissue inhibitor of metalloproteinase (TIMP) -1 and TIMP-2 (Figures 5c and 5d). In addition, the distribution of cells expressing MMPs and TIMPs corresponded to the proteolytic areas detected by FIZ investigation (Figure 5a, 5b, 5c, and 5d as serial sections showed the expression of the enzymes by immunohistochemisty in lining layer of RA synovium). These findings indicate that synovial cells simultaneously produce proteolytic enzymes and their inhibitors, and suggest that the *in vivo* proteolytic activity might be dependent on "imbalances" in enzymes-inhibitors production of the individual cells.

Utilizing FIZ and IPAP may further help to understand biological enzymatic activity on articular manifestations of OA.

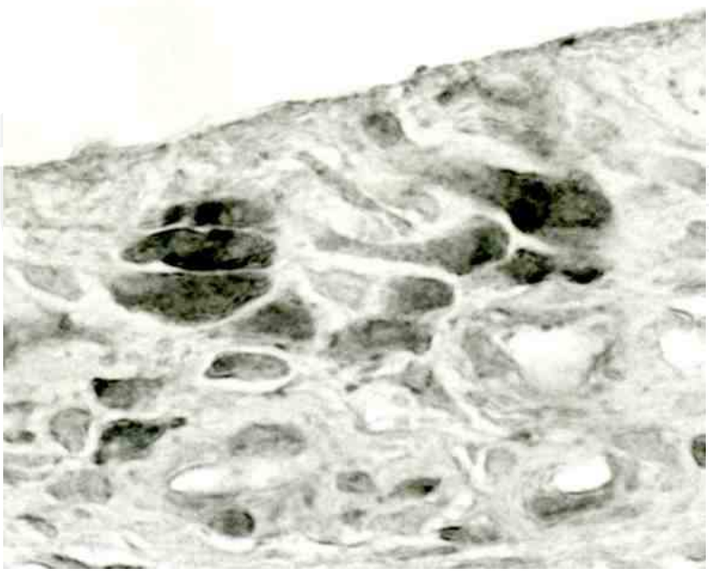

Fig. 5a. Expression of MMP-2 by immunohistochemistry.

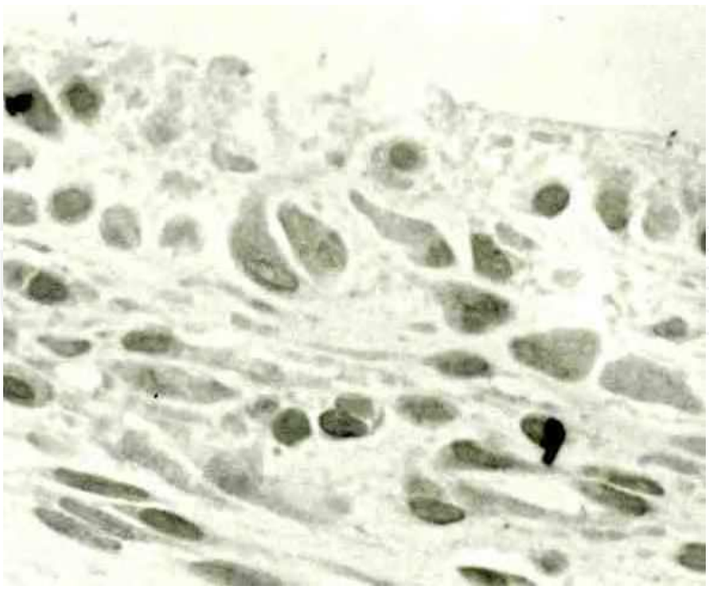

Fig. 5b. Expression of MMP-9 by immunohistochemistry.

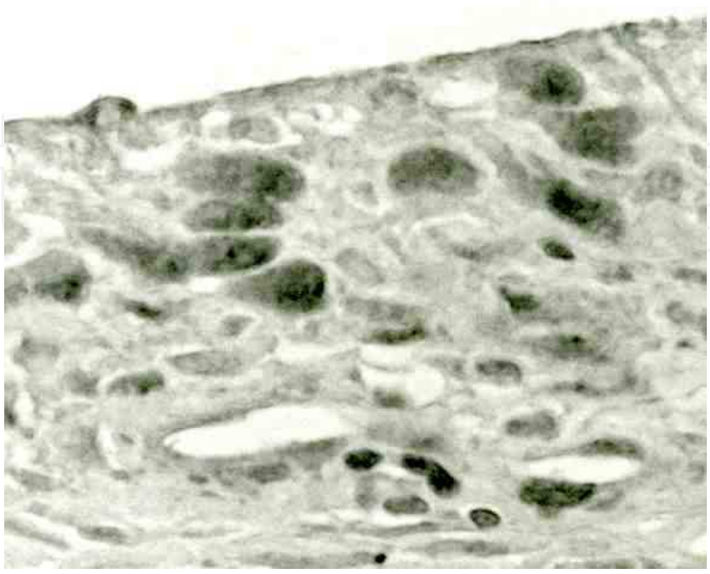

Fig. 5c. Expression of TIMP-1 by immunohistochemisry.

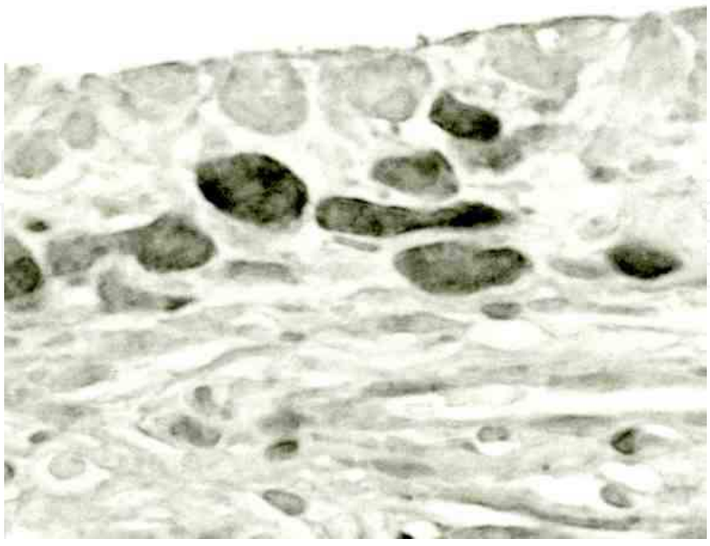

Fig. 5d. Expression of TIMP-2 by immunohistochemisry.

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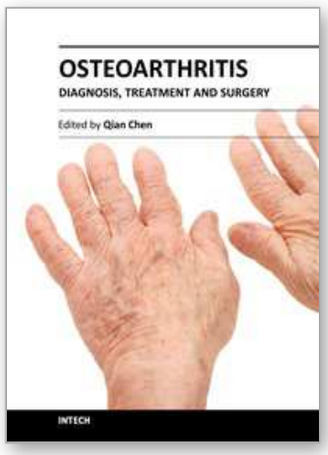

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Osteoarthritis is one of the most debilitating diseases affecting millions of people worldwide. However, there is no FDA approved disease modifying drug specifically for OA. Surgery remains an effective last resort to restore the function of the joints. As the aging populations increase worldwide, the number of OA patients increases dramatically in recent years and is expected to increase in many years to come. This is a book that summarizes recent advance in OA diagnosis, treatment, and surgery. It includes wide ranging topics from the cutting edge gene therapy to alternative medicine. Such multifaceted approaches are necessary to develop novel and effective therapy to cure OA in the future. In this book, different surgical methods are described to restore the function of the joints. In addition, various treatment options are presented, mainly to reduce the pain and enhance the life quality of the OA patients.

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