

# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

185,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index  
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.  
For more information visit [www.intechopen.com](http://www.intechopen.com)



## Evaluation of *In Vivo* Proteolytic Activity

Wataru Yoshida, Akihisa Kamataki, Miwa Uzuki and Takashi Sawai  
*Iwate Medical University,  
Japan*

### 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 activity

#### 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  $\mu\text{m}$ . 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  $\mu\text{m}$ , 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 gelatinolyzed areas (Iwata et al., 2001; Furuya et al., 2001; Yamanaka et al., 2000).

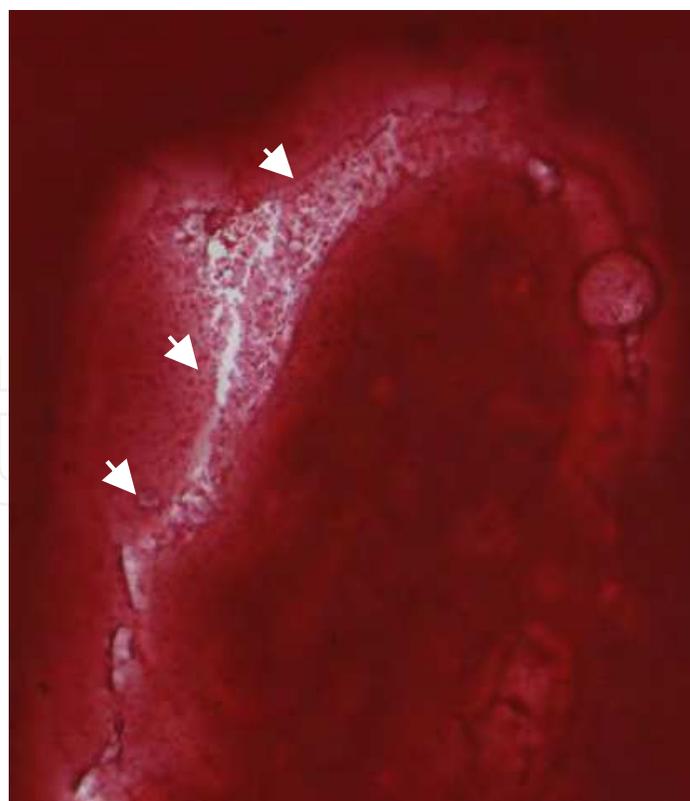


Fig. 1. Film *in situ* zymography of OA synovium. Arrows indicate the proteolytic lesions as pale in color in the Ponceau-stained FIZ film.

## 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, Quebec, 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  $\times 4$ ). 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.

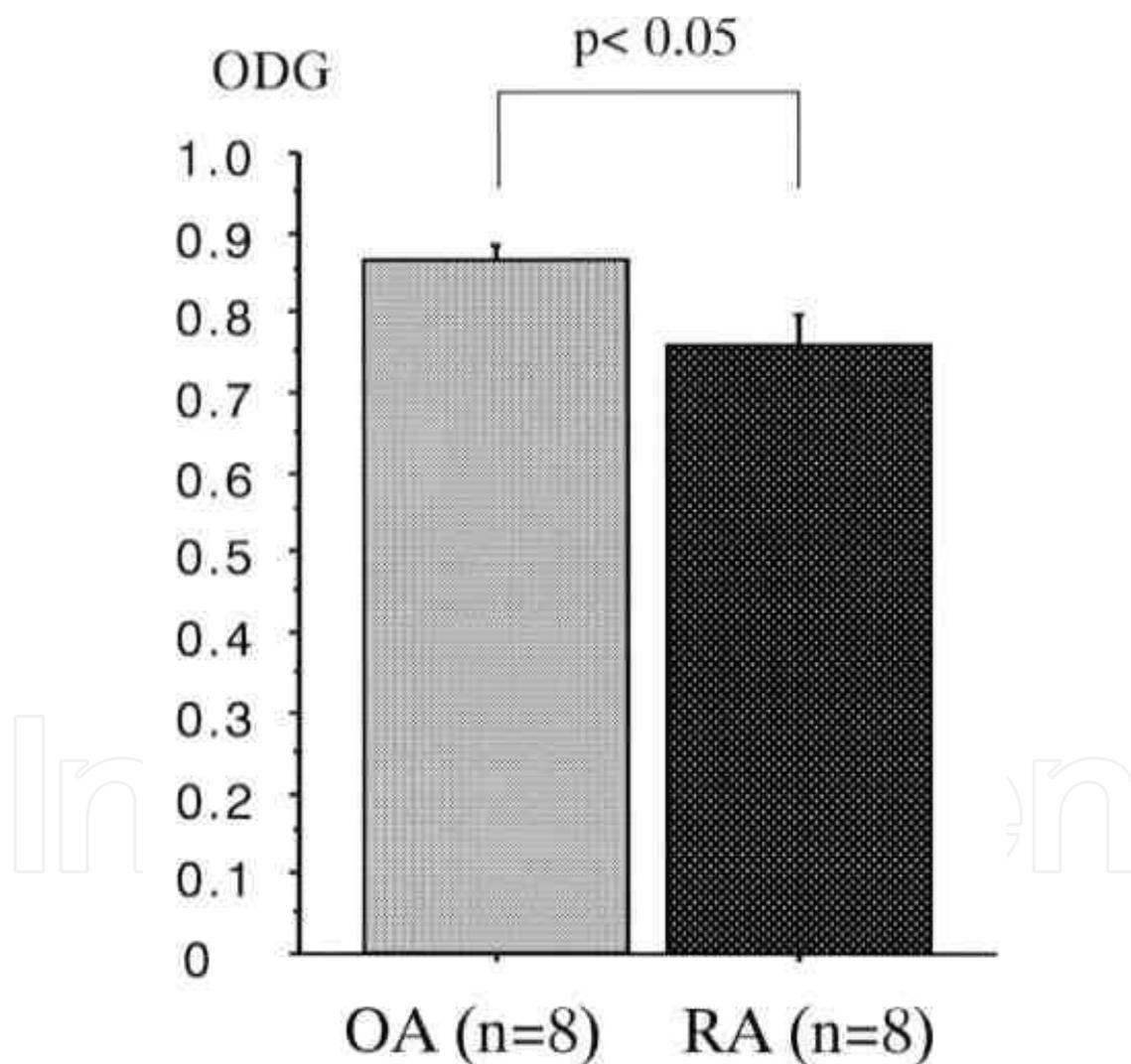


Fig. 3. Optical density of the gelatinolyzed area (ODG) as produced by FIZ and IPAP the synovium of OA and RA. OA synovium had a significantly higher ODG ( $0.864 \pm 0.037$ ) than RA synovium ( $0.758 \pm 0.019$ ). All OA cases were classified as grade 4 using Kellgren and Lawrence classification and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

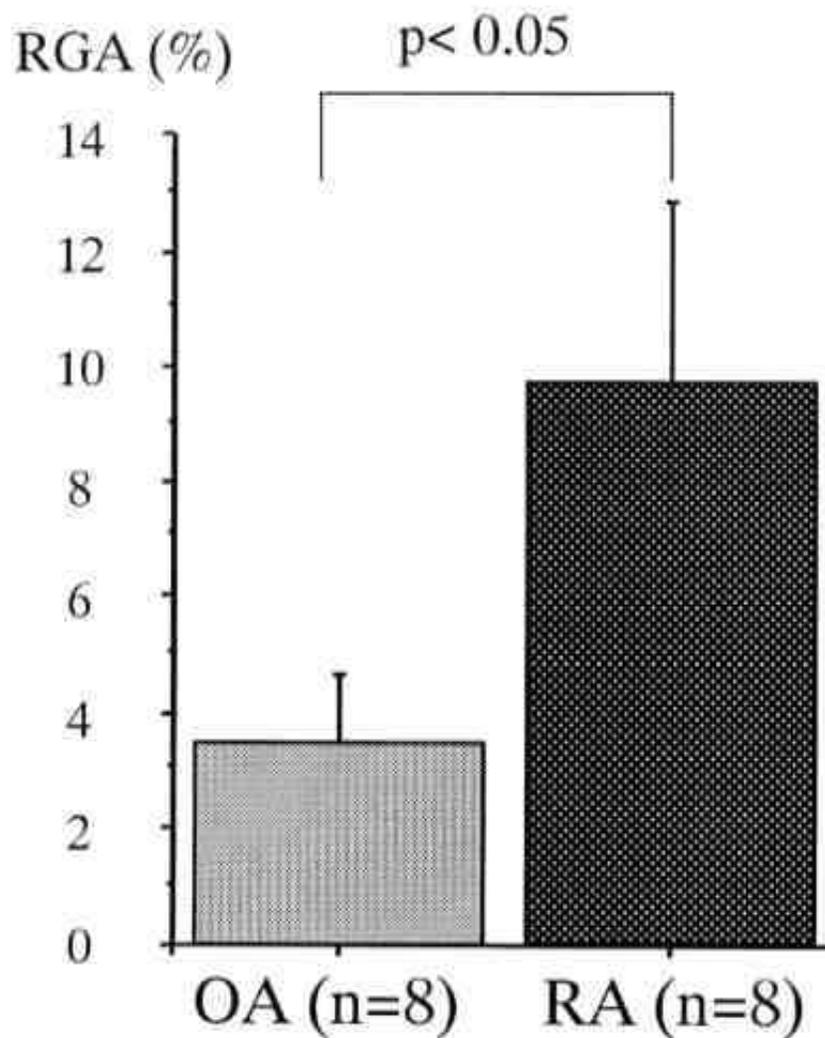


Fig. 4. Ratio of gelatinolyzed areas (RGA) as produced by FIZ and IPAP the synovium of OA and RA. The synovium of OA had a significantly lower RGA ( $3.5 \pm 1.1\%$ ) than RA synovium ( $9.7 \pm 3.1\%$ ). All OA cases were classified as grade 4 using Kellgren and Lawrence classification and all RA cases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

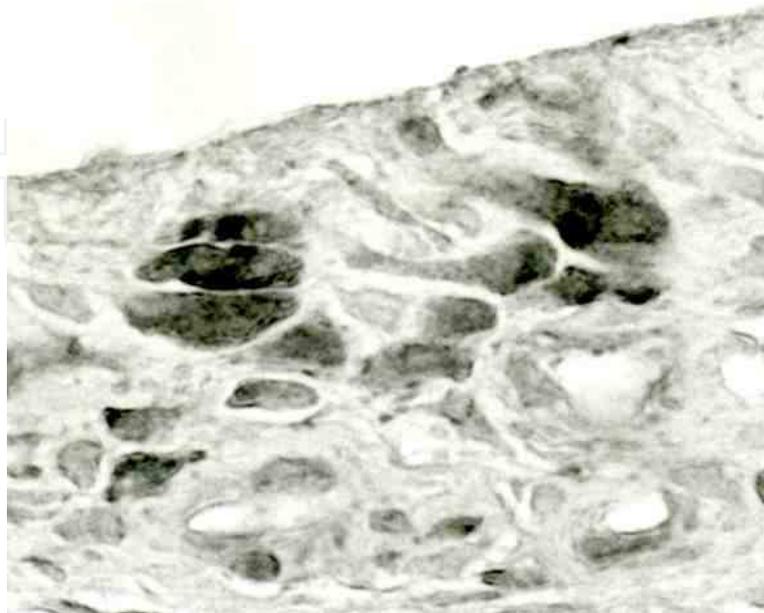


Fig. 5a. Expression of MMP-2 by immunohistochemistry.

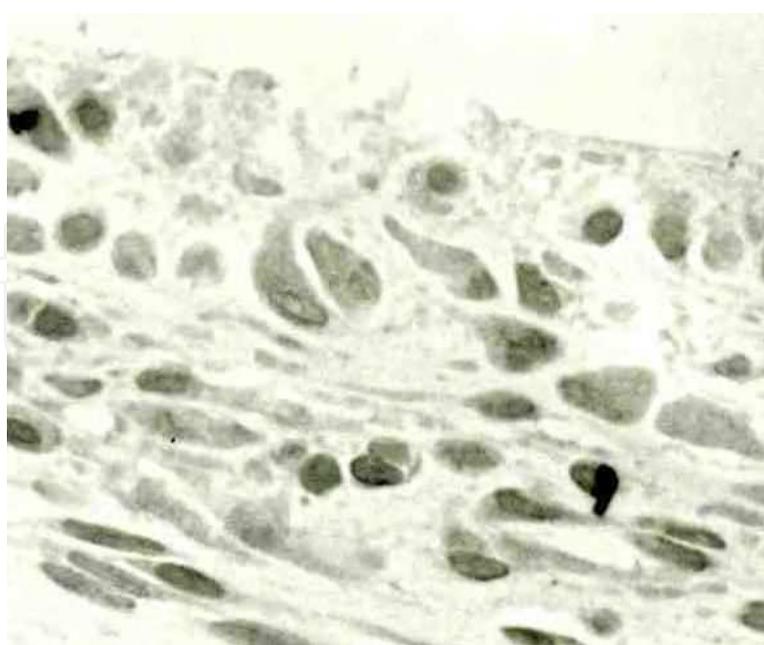


Fig. 5b. Expression of MMP-9 by immunohistochemistry.

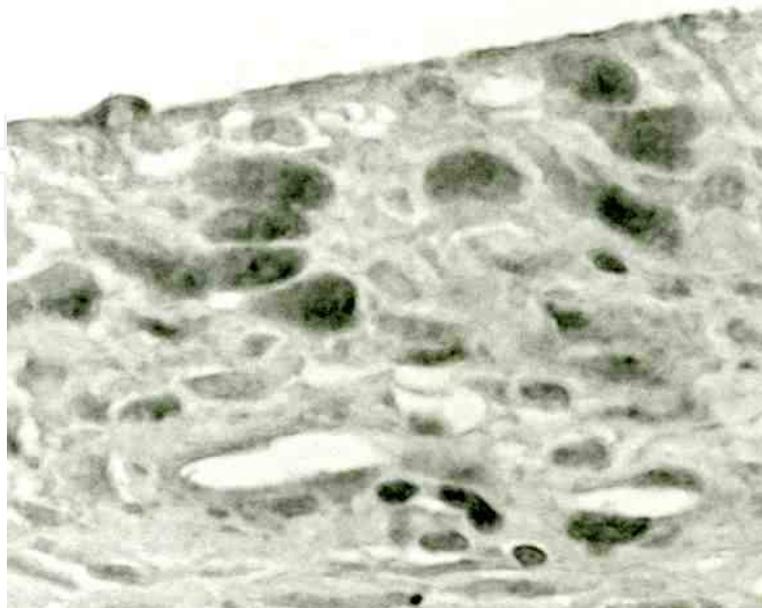


Fig. 5c. Expression of TIMP-1 by immunohistochemisry.

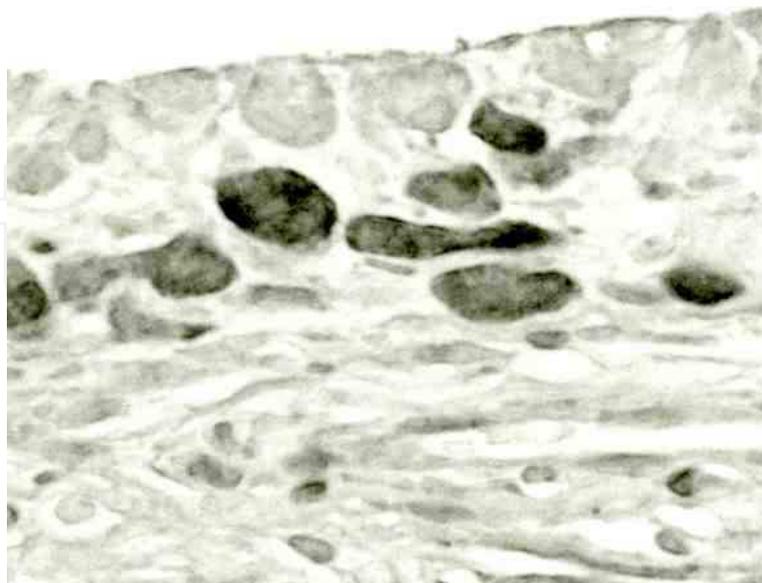


Fig. 5d. Expression of TIMP-2 by immunohistochemisry.

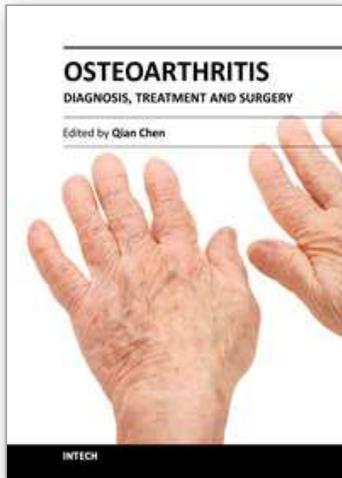
#### 4. References

- Cha H.S., Ahn K.S., Jeon C.H., Kim J., Koh E.M. (2003). Inhibitory effect of cyclo-oxygenase-2 inhibitor on the production of matrix metalloproteinases in rheumatoid fibroblast-like synoviocytes. *Rheumatol Int*, Vol. 24, No. 4, (Jul 2004) pp. 207-211, ISSN 0172-8172
- de Seny D., Sharif M., Fillet M., Cobraiville G., Meuwis M.A., Marée R., Hauzeur J.P., Wehenkel L., Louis E., Merville M.P., Kirwan J., Ribbens C., Malaise M. (2011). Discovery and biochemical characterisation of four novel biomarkers for osteoarthritis. *Ann Rheum Dis*, Vol., 70, No. 6, (Jun 2011) pp. 1144-1152, ISSN 0003-4967
- Furuya M., Ishikura H., Nemori R., Shibata M., Fujimoto S., Yoshiki T. (2001). Clarification of the active gelatinolytic sites in human ovarian neoplasms using in situ zymography. *Hum Pathol*, Vol. 32, No. 2, (Feb 2001) pp. 163-168, ISSN 0046-8177
- Galis Z.S., Sukhova G.K., Libby P. (1995). Microscopic localization of active proteases by in situ zymography: detection of matrix metalloproteinase activity in vascular tissue. *FASEB J*, Vol. 9, No. 10 (Jul 1995) pp. 974-980, ISSN 0892-6638
- Goodall S., Crowther M., Hemingway D.M., Bell P.R., Thompson M.M. (2001). Ubiquitous elevation of matrix metalloproteinase-2 expression in the vasculature of patients with abdominal aneurysms. *Circulation*, Vol. 104, No. 3, (Jul 2001) pp.304-309, ISSN 0009-7322
- Hattori T., Kawaki H., Kubota S., Yutani Y., de Crombrughe B., von der Mark K., Takigawa M. (2003). Downregulation of a rheumatoid arthritis-related antigen (RA-A47) by ra-a47 antisense oligonucleotides induces inflammatory factors in chondrocytes. *J Cell Physiol*, Vol. 197, No. 1, (Oct 2003) pp. 94-102, ISSN 0021-9541
- Ikeda M., Maekawa R., Tanaka H., Matsumoto M., Takeda Y., Tamura Y., Nemori R., Yoshioka T. (2000). Inhibition of gelatinolytic activity in tumor tissues by synthetic matrix metalloproteinase inhibitor: application of film in situ zymography. *Clin Cancer Res*, Vol. 6, No. 8, (Aug 2000) pp. 3290-3296, ISSN 1078-0432
- Iwata H., Yamamoto M., Nemori R., Mizutani M., Iwase T., Miura S., Obata Y., Hara Y., Omoto Y., Toyama T., Yamashita H., Iwase H., Kobayashi S. (2001). Localization of gelatinolytic activity can be detected in breast cancer tissues by film in situ zymography. *Breast Cancer*, Vol. 8, No. 2, (2001) pp. 111-115, ISSN 1340-6868
- Kaji M., Moriyama S, Sasaki H., Saitoh Y., Kiriya M., Fukai I., Yamakawa Y., Mitsui A., Toyama T., Nemori R., Fujii Y. (2003). Gelatinolytic activity of matrix metalloproteinase in lung cancer studied using film in situ zymography stamp method. *Lung Cancer*, Vol. 39, No. 2, (Feb 2003) pp. 125-130, ISSN 0169-5002
- Kevorkian L., Young D.A., Darrah C., Donell S.T., Shepstone L., Porter S., Brockbank S.M., Edwards D.R., Parker A.E., Clark I.M. (2004) Expression profiling of metalloproteinases and their inhibitors in cartilage. *Arthritis Rheum*, Vol. 50, No. 1, (Jan 2004) pp. 131-141, ISSN 0004-3591
- Kraus V.B. (2010). Osteoarthritis year 2010 in review: biochemical markers. *Osteoarthritis Cartilage*, Vol 19, No. 4, (Apr 2011) pp. 346-353, ISSN 1063-4584
- Lorenz H., Wenz W., Ivancic M., Steck E., Richter W. (2005). Early and stable upregulation of collagen type II, collagen type I and YKL40 expression levels in cartilage during early experimental osteoarthritis occurs independent of joint location and

- histological grading. *Arthritis Res Ther*, Vol. 7, No. 1, (2005) pp. 156-165, ISSN 1478-6354
- Martel-Pelletier J., Pelletier J.P. (2010). Is osteoarthritis a disease involving only cartilage or other articular tissues? *Eklem Hastalik Cerrahisi*, Vol. 21, No. 1, (Apr 2010) pp. 2-14, ISSN 1305-8282
- Reeves N.D., Bowling F. L. (2011). Conservative biomechanical strategies for knee osteoarthritis. *Nat Rev Rheumatol*, Vol. 7, No. 2, (Feb 2011) pp. 113-122, ISSN 1759-4790
- Senzaki H., Paolucci N., Gluzband Y.A., Lindsey M.L., Janicki J.S., Crow M.T., Kass D.A. (2000). Beta-blockade prevents sustained metalloproteinase activation and diastolic stiffening induced by angiotensin II combined with evolving cardiac dysfunction. *Circ Res*, Vol. 86, No. 7, (Apr 2000) pp. 807-815, ISSN 0009-7330
- Sobczak S., Baillon B., Feipel V., Van Sint Jan S., Salvia P., Rooze M. (2010). In vitro biomechanical study of femoral torsion disorders: effect on tibial proximal epiphyseal cancellous bone deformation. *Surg Radiol Anat*, Vol. 33, No. 5, (Jul 2010) pp. 439-449, ISSN 0930-1038
- Sun H.B., Nalim R., Yokota H. (2003). Expression and activities of matrix metalloproteinases under oscillatory shear in IL-1-stimulated synovial cells. *Connect Tissue Res*, Vol. 44, No. 1, (2003) pp. 42-49, ISSN 0300-8207
- Takano S., Tsuboi K., Matsumura A., Sato H., Nose T. (2001). Localization of gelatinase activities in glioma tissues by film in situ zymography. *Brain Tumor Pathol*, Vol. 18, No. 2, (2001) pp. 145-150, ISSN 1433-7398
- Takei I., Takagi M., Santavirta S., Ida H., Hamasaki M., Ishii M., Fukushima S., Ogino T., Konttinen Y.T. (1999). Matrix metalloproteinases and tissue inhibitors of metalloproteinases in joint fluid of the patients with loose artificial hip joints. *J Biomed Mater Res*, Vol. 45, No. 3, (Jun 1999) pp. 175-183, ISSN 0021-9304
- Uzuki M., Watanabe T., Katsura Y., Sawai T. (1999). Quantitative histochemical study of hyaluronic acid binding protein and the activity of uridine diphosphoglucose dehydrogenase in the synovium of patients with rheumatoid arthritis. *Anal Quant Cytol Histol*, Vol. 21, No. 1, (Feb 1999) pp. 75-80, ISSN 0884-6812
- Vieillard-Baron A., Frisdal E., Eddahibi S., Deprez I., Baker A.H., Newby A.C., Berger P., Levame M., Raffestin B., Adnot S., d'Ortho M.P. (2000). Inhibition of matrix metalloproteinases by lung TIMP-1 gene transfer or doxycycline aggravates pulmonary hypertension in rats. *Circ Res* 2000, Vol. 87, No. 5, (Sep 2000) pp. 418-425. ISSN 0009-7330
- Yamanaka H., Makino K., Takizawa M., Nakamura H., Fujimoto N., Moriya H., Nemori R., Sato H., Seiki M., Okada Y. (2000). Expression and tissue localization of membrane-type 1, 2, and 3 matrix metalloproteinases in rheumatoid synovium. *Lab Invest*, Vol. 80, No. 5, (2000) pp. 677-687, ISSN 0023-6837
- Yi C.F., Gosiewska A., Burtis D., Geesin J. (2001). Incorporation of fluorescent enzyme substrates in agarose gel for in situ zymography. *Anal Biochem*, Vol. 291, No.1, (Apr 2001) pp. 27-33, ISSN 0003-2697
- Yoshida W., Uzuki M., Nishida J., Shimamura T., Sawai T. (2009). Examination of in vivo gelatinolytic activity in rheumatoid arthritis synovial tissue using newly developed in situ zymography and image analyzer. *Clin Exp Rheumatol*, Vol. 27, No. 4, (Jul-Aug 2009) pp. 587-593, ISSN 0392-856X

- Zhang W., Doherty M., Peat G., Bierma-Zeinstra M.A., Arden N.K., Bresnihan B., Herrero-Beaumont G., Kirschner S., Leeb B.F., Lohmander L.S., Mazières B., Pavelka K., Punzi L., So A.K., Tuncer T., Watt I., Bijlsma J.W. (2010). EULAR evidence-based recommendations for the diagnosis of knee osteoarthritis. *Ann Rheum Dis*, Vol. 69, No. 3, (Mar 2010) pp. 483-489, ISSN 0003-4967
- Zheng K., Nagai Y., Kishimoto T., Yamazawa K., Tate S., Nemori R., Hirai Y., Sekiya S., Ishikura H. A quantitative evaluation of active gelatinolytic sites in uterine endometrioid adenocarcinoma using film in situ zymography: association of stronger gelatinolysis with myometrial invasion. *Jpn J Cancer Res*, Vol. 93, No. 5, (May 2002) pp. 516-522, ISSN 0910-5050

IntechOpen



## **Osteoarthritis - Diagnosis, Treatment and Surgery**

Edited by Prof. Qian Chen

ISBN 978-953-51-0168-0

Hard cover, 404 pages

**Publisher** InTech

**Published online** 02, March, 2012

**Published in print edition** March, 2012

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.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Wataru Yoshida, Akihisa Kamataki, Miwa Uzuki and Takashi Sawai (2012). Evaluation of In Vivo Proteolytic Activity, Osteoarthritis - Diagnosis, Treatment and Surgery, Prof. Qian Chen (Ed.), ISBN: 978-953-51-0168-0, InTech, Available from: <http://www.intechopen.com/books/osteoarthritis-diagnosis-treatment-and-surgery/evaluation-of-in-vivo-proteolytic-activity>

**INTECH**  
open science | open minds

### **InTech Europe**

University Campus STeP Ri  
Slavka Krautzeka 83/A  
51000 Rijeka, Croatia  
Phone: +385 (51) 770 447  
Fax: +385 (51) 686 166  
[www.intechopen.com](http://www.intechopen.com)

### **InTech China**

Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

IntechOpen

IntechOpen