Retrospective Article

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A personal journey with matrix metalloproteinases

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Abstract: I was given the honor of delivering the 2015 Lifetime Membership Award lecture at the International Proteolysis Society's annual meeting held in Penang, Malaysia in October 2015. It gave me an opportunity to look back on how I started my research on matrix metalloproteinases (MMPs) and how I continued to work on these proteinases for the next 42 years. This is a series of sketches from the personal journey that I took with MMPs, starting from the purification of metalloproteinases, cloning, structural studies, then to a more recent encounter, endocytic regulation of matrix-degrading metalloproteinases.

Keywords: aggrecanase; collagenase; endocytosis; low-density lipoprotein receptor-related protein 1 (LRP1); TIMPs.

Introduction

'Mammalian Proteases: A Glossary and Bibliography', published in 1980 by Alan Barrett and Ken McDonald, lists only 11 entries for metalloproteinases. Now we have learned that the human genome encodes 194 metalloproteases, the largest group in the proteolytic enzyme kingdom, with an estimated total of 566 (Overall and Blobel, 2007). The difficulty of finding metalloproteinases was largely due to the fact that many of them are regulated transcriptionally, post-transcriptionally, and post-translationally. For example, vertebrate 'collagenase' was discovered by Jerome Gross and Charles Lapière in the tissues of tadpoles undergoing metamorphosis (Gross and Lapiere, 1962). I was introduced to extracellular matrix (ECM)-degrading metalloproteinases, now known as 'matrix metalloproteinases' (MMPs)' when I joined Fred Woessner's lab in 1973 as a PhD student. Since then I have worked in this field throughout my scientific career. What I describe here is a personal journey that I took with MMPs. I owe this journey to two my great mentors, J Fred Woessner and Alan J Barrett who originally inspired me to enter into the world of proteases (Figure 1).

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From Japan to the USA (1973)

In 1973 I received a fellowship from the Japan Society for the Promotion of Science (JSPS) to pursue my PhD in Biochemistry in the USA. Professor Yo Mori at Tokyo College of Pharmacy arranged my study at Fred Woessner's laboratory at the University of Miami. At that time, I was nervous as I knew nothing about his projects, although I knew the well-known Woessner's hydroxyproline assay method.

Fred Woessner was then investigating the hormonal regulation of collagenase in post partum involuting uterus of rats and proteases that degrade cartilage proteoglycan (aggrecan). My PhD project was to purify the protease from bovine nasal cartilage that digests aggrecan at neutral pH. I first developed an assay to measure aggrecan-degrading activity. This was achieved by co-polymerizing large aggrecan into polyacrylamide bead particles, to which enzymes can freely diffuse and digested fragments released in to the buffer are measured. The enzyme was characterized as a metalloproteinase, but it was difficult to purify, as normal bovine cartilage contains only a minute amount of aggrecan-degrading enzyme. More than 10 years later, many of ECM-degrading enzymes were found to be regulated transcriptionally. We know now that ADAMTS-5 (adamalysin-like metalloproteinase with thrombospondin domains 5/aggrecanase 2) and MMP-13 (collagenase 3) constitutively produced in normal cartilage, but they are rapidly endocytosed by chondrocytes and degraded (Yamamoto et al., 2013, 2016), explaining the difficulty of detecting them in normal cartilage. Consequently, I published one paper on the aggrecan-bead assay, but not on the main body of my PhD thesis. Nonetheless, l learned many lessons from Fred. The two most

Figure 1: With my mentors.
J. Fred Woessner (left), the author (center) and Alan J. Barrett (right). The photograph was taken at the 1986 Gordon Research Conference on Proteolytic Enzymes and Their Inhibitors. In the summer of 1986, the author moved from New Jersey to Kansas city.

important ones are how to design control experiments and how to interpret data.

Strangeways research laboratory in Cambridge (1978–1980)

In spite of difficulties, I developed a strong interest in continuing the study of matrix-degrading proteinases, as I was convinced that the breakdown of ECM is important in biology and in diseases such as cancer, arthritis and atherosclerosis, and I wished to do postdoctoral studies at Strangeways Research Laboratory in Cambridge, UK, where a number of international leaders of the protease field were working together. Luckily, I was awarded a postdoctoral fellowship from the American Arthritis Foundations to spend 2 years in Alan Barrett's laboratory in Cambridge, then a third year at Edward (Ted) Harris' laboratory at Dartmouth College in the USA.

I went to Cambridge in the summer of 1978. It is a beautiful, old academic town. I met many friends. Guy Salvesen had just started his PhD work and Judy Bond was spending her sabbatical in Alan's lab. Strangeways had an aristocratic atmosphere. Daily ritual morning coffee break, lunch, and afternoon tea break gave us a good rhythm in our research life, as well as opportunities to meet with other members of the Laboratory. Alan Barrett talked science most of the time, even at lunchtime. We young students and postdocs enjoyed it, as we could learn so much about proteinases through osmosis.

I was attracted by the 'trap hypothesis' of α_2-macroglobulin that Alan Barrett and Phyllis Starkey put forward for its mechanism of protease inhibition (Barrett and Starkey, 1973), and wished to identify the 'bait' region where many different proteases attack. However, I had to give up, because the isolation of the peptide was too difficult. I however managed to purify plasma kallikrein and show it as an activator of latent collagenase in rheumatoid synovial fluids.

Those 2 years in Strangeways were very special in my career both in scientific experience and in making many long-term friendships.

Dartmouth medical school (1980–1983)

The E-I complex vs. proenzyme: a controversy about 'latent' collagenase

In August 1980 I left Cambridge and joined Ted Harris' lab at Dartmouth. There was then a serious debate about the latency of collagenase: an enzyme-inhibitor (E-I) complex vs. a proenzyme (zymogen)? The school supporting the E-I complex was based on the knowledge that the latent collagenase was activated by mercurial compounds such as 4-aminophenylmercuric acetate (APMA) or chaotropic agents, and it was proposed that those reagents dissociated the E-I complex.

I tackled this problem using the cell-free translation system with mRNAs from rabbit synovial fibroblasts. The time was right as Connie Brinckerhoff had just established the collagenase overexpressing system by treating the cells with phorbol ester and Carol Vater had just made a specific anti-rabbit collagenase antibody. We showed preprocollageanse, which was processed by microsomes to procollagenase. It was identical in molecular mass with 'latent collagenase' secreted from the cells in culture. It was converted to an active form by trypsin and plasma kallikrein in a test tube (Nagase et al., 1981).

Discovery of procollagenase activator and its unique activation process

Soon after, Carol Vater purified an activator of procollagenase from the same culture media from which procollagenase was purified (Vater et al., 1983). It was another prometalloproteinase and it required AMPA or trypsin to be activated, but it did not have collagenolytic activity. The activation process of procollagenase by the activator turned out to be unconventional: (i) activated activator could not readily activate procollagenase; and (ii) for full collagenase activation both procollagenase and proactivator needed to be present in the solution before adding APMA or trypsin, which activates both proenzymes. This process is now explained by the 'stepwise activation' mechanism (see below).

Rutgers medical school (1983–1986)

In 1983 Ted Harris moved to at Rutgers Medical School (now University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School) as Chairman of Medicine. I followed him and looked after his lab. It also gave me an opportunity to learn molecular cloning techniques, as the Biochemistry Department headed by Darwin Prockop had a number of expert molecular biologists who were cloning new collagen genes. Yoshifumi Ninomiya in Bjorn Ohlsen's lab. gave me a good introduction and Makku Kurkinen guided my hands-on experiments on molecular cloning.

Cloning of MMP cDNAs and transcriptional regulation of the MMP gene

Our objective was to clone the human collagenase cDNA, but we were not the winner in this competition. In 1994 Connie Brinckerhoff in Dartmouth published the rabbit collagenase cDNA clone, but without the sequence information (Gross et al., 1984). In May 1986, Greg Goldberg in Washington University published the full-length cDNA clone of human collagenase (MMP-1) (Goldberg et al., 1986). It revealed homology with the protein (later called 'transin') that Lynn Matrisian cloned from rat fibroblasts treated with epidermal growth factor or oncongenic viral transformation, but its function was not known when cloned (Matrisian et al., 1985). In November 1986, Celltech, Strangeways and the Karlsruhe collaboration reported cDNA sequences for human collagenase (MMP-1) and human stromelysin (MMP-3) (Whitham et al., 1986). This work identified transin as the rat ortholog of stromelysin. This series of work brought considerable excitement, as it revealed that MMP-1 and MMP-3 gene expression was regulated by many factors such as growth factors, oncogenic transformation, phorbol ester and UV irradiation. Prior to those discoveries, Jean-Michel Dayer and Steve Krane reported that soluble factors secreted from activated macrophages induced the production of collagenase (MMP-1) in rheumatoid synovial cells (Dayer et al., 1977), and the factors were later identified as interleukin 1 and tumor necrosis factor α. Thus, transcriptional regulation of MMP-1 and MMP-3 became considerable interests for molecular biologists. Transcription factors such as AP1, Ets, and the suppressive regulatory mechanism of MMP-1 gene by glucocorticoids were subsequently reported (see Woessner, 1991 for review). Tissue inhibitor of metalloproteinases 1 (TIMP-1) was cloned in 1985 by Andy Docherty at Celltech and Gill Murphy at Strangeways (Docherty et al., 1985).

MMP-1, MMP-2 and MMP-3

We made some progress in the biochemistry of MMPs at Rutgers working with Yasunori Okada, a pathologist from

Kanazawa University, who came to Ted Harris' lab from Kanazawa University as an International Fogarty Fellow. Okada found that rheumatoid fibroblasts secrete three metalloproteinases: collagenase, gelatinase, and the third metalloproteinase that digests many ECM components. We called it 'matrix metalloproteinase 3 (MMP-3)' referring to the collagenase and gelatinase as MMP-1 and MMP-2, respectively (Okada et al., 1986). We suspected that MMP-3 was the collagenase activator, but it did not activate procollagenase under the conditions of our study. Meanwhile, the Murphy and Docherty team reported that the collagenase activator was stromelysin 1 (MMP-3) using recombinant enzymes (Murphy et al., 1987). The activation results were similar to those reported with rabbit enzymes (Vater et al., 1983), the unconventional activation mechanism of procollagenase was not solved.

Kansas city (1986–1999)

Starting my own laboratory

In November 1986, I took up a post in the Department of Biochemistry at the University of Kansas Medical Center in Kansas city, Kansas, USA. It was my first 'own' laboratory. I started with a very small group, but soon I was supported by many collaborators.

Our main contributions to the field from Kansas city were: the elucidation of the stepwise activation mechanism of proMMPs; elucidation of the inhibition mechanism of TIMP; and the demonstration collagenase unwinding of triple helical collagen.

Stepwise activation mechanism of proMMPs

Ko Suzuki and I worked together on proMMP-1 and proMMP-3 in parallel as the two enzymes have similar activation processes. The N-terminal sequencing of activated forms essential for this study and it was carried out by Jan Enghild and Guy Salvesen, who were then at Duke University.

The 57-kDa proMMP-3 was activated by various proteases with different specificities and also by APMA to the 45-kDa active form via short-lived intermediates. All 45-kDa forms resulted from the cleavage of the His^{82}-Phe^{83} bond, which was processed by bimolecular reactions of the intermediates. The initial protease cleavage sites were located in the middle of the propeptide ('bait' region) and the intermediates generated by APMA resulted from intramolecular cleavage of proMMP-3. How APMA triggers proMMPs is explained by the 'cysteine switch' mechanism proposed by Harold Van Wart and Henning Birkedal-Hansen (Van Wart and Birkedal-Hansen, 1990). However, active MMP-3 could not activate proMMP-3; presumably the His^{82}-Phe^{83} bond is not exposed unless a part of the propeptide is removed. MMP-3 could not cleave the bait region. We called this process 'stepwise activation' (Nagase et al., 1990).

This basic principle applies to proMMP-1 activation, except trypsin-like proteases such as plasmin and kallikrein activate and generate initial intermediates. APMA also generates an intermediate. Those intermediates however cannot cleave at the full-activation site Gln^{80}-Phe^{81} bond, which is cleaved by MMP-3 (Suzuki et al., 1990). Thus, MMP-1 activated without MMP-3 expresses only 10–25% of its full collagenolytic activity. Furthermore, the Gln^{80}-Phe^{81} bond in the proform of MMP-1 was not readily cleaved by MMP-3. Those results explained the earlier observation that procollagenase (proMMP-1) and procollagenase activator (proMMP-3) needed to be activated together for full collagenase activity.

Among 23 human MMPs, seven MMPs conform to the stepwise activation mechanism. Such an apparently cumbersome activation mechanism might have evolved to regulate the ECM degradation more precisely. A rapid full activation of MMPs may not compatible with the sustaining of the tissue structure, particularly during tissue remodeling or repair processes. This mechanism may be contrasted with the cascade activation of blood clotting enzymes, where each activation step amplifies the clotting event.

The inhibition mechanism of TIMPs

The first three-dimensional structure of TIMP was solved for the N-terminal MMP-inhibitory domain of TIMP-2 by NMR by Richard Williamson in 1994 (Williamson et al., 1994). However, it was difficult to suggest how TIMP would inhibit MMPs. We noticed a clue for it when neutrophil elastase inactivated TIMP-1 by a single cleavage, but this cleavage was protected when TIMP-1 was complexed with MMP-3. The N-terminal sequencing of the cleaved fragment showed that Val69-Cys70 bond was cleaved, where Cys70 and Cys1 are disulfide bonded. In Williamson's TIMP-2 structure, this region forms a ridge-like surface, which we proposed is a part of MMP interaction site (Nagase et al., 1997).

Soon after, the co-crystallization of TIMP-1 and the MMP-3 catalytic domain was successful through

collaboration among Wolfram Bode and his colleagues, Keith Brew and us (Gomis-Rüth et al., 1997). TIMP-1 is a wedge-like structure and the four N-terminal residues and Ser68-Val69 that are connected by the Cys1-Cys79 disulfide bond slot either side of the catalytic zinc ion. The N-terminal NH_2-group and the carbonyl group of Cys1 chelate the catalytic zinc bidentately (Figure 2). This formed the basis for the design of TIMP variants that selectively inhibit MMPs, ADAMs and ADAMTSs.

Moving to London

My journey continued on. I moved from Kansas city to London in 1999 to take up the post of Professor and Head of Matrix Biology at the Kennedy Institute of Rheumatology in Imperial College London. This was instigated by Jerry Saklatvala, a good old friend from the Strangeways time.

In London while I continued MMP biochemistry and TIMP mutagenesis studies, I also initiated, or rather re-activated my PhD project in Miami, research in cartilage matrix degradation during the progression of osteoarthritis (OA). OA is the most prevalent joint disorder mainly affecting elderly people, but there is no disease modifying drug available. Our research goals were, and still are, to identify target enzymes and understand the mechanisms of action of these enzymes on natural substrates, so that we may develop ways to block disease progression.

Exosite inhibitors of aggrecanases

We focused on aggrecanases, members of the ADAMTS family, and collagenases (MMP-1 and MMP-13). They are multi-domain proteins and their non-catalytic ancillary domains play important roles in recognition of natural substrates. It is therefore attractive to find molecules that bind to these domains as potential exosite inhibitors.

Linda Troeberg found that calcium pentosan polysulfate (CaPPS), a chemically sulfated xylanopyranose, is such an example (Troeberg et al., 2008). Salvo Santamaria (PhD student) screened a phage-displayed single chain antibody library and isolated inhibitory antibodies that recognize non-catalytic domains of ADAMTS-5 (Santamaria et al., 2015). The hemopexin domains of collagenase are also candidates for exosite inhibitors. As the space of this article is limited, I will just discuss our recent work on collagenase (MMP-1).

How does collagenase cleave triple helical collagen?

I have been interested in this question for long time. When the crystal structures of collagenases (MMP-1 and MMP-8) were solved, it became apparent that the active site of the enzyme of 5 Å width is too narrow to accommodate the triple helical collagens of 15 Å diameter (Bode, 1995). While several theoretical models were proposed

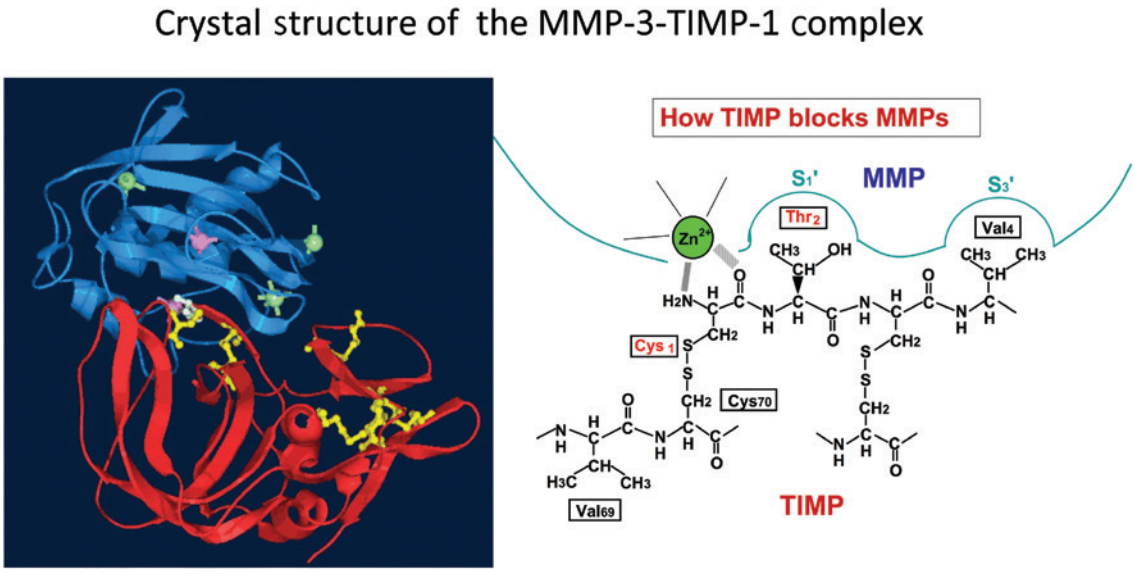

Figure 2: Crystal structure of the MMP-3-TIMP-1 complex.
Left, Ribbon structure of the complex of MMP-3 (blue) and TIMP-1 (red). Zinc ions and calcium ions in MMP-3 are shown in pink and light green, respectively. Disulfide bonds in TIMP-1 are in yellow. The model was made by Rob Visse based on the atomic coordinates (PDB ID code: IUEA). Right, The schematic representation of how TIMP-1 blocks MMP-3. See the text for details.

how collagenase might cleave collagen, we demonstrated that collagenase locally unwinds triple helical collagen before hydrolyzing the peptide bond using the catalytically incapable mutant MMP-1 [MMP-1(E200A)](Chung et al., 2004) (Figure 3A). Szymon Manka (my PhD student) then mapped the contact sites of the two molecules by hydrogen/deuterium exchange mass spectrometry and screening of a collagen peptide library. This allowed us to design the appropriate collagen peptide and co-crystalize it with MMP-1(E200A), which was carried out in collaboration with Richard Farndale (peptide chemistry) in Cambridge and Erhard Hohenester (X-ray crystallography) at Imperial College. The structure revealed that all three collagen chains interact extensively with the two domains of MMP-1 (Figure 3B) (Manka et al., 2012). It however did not show unwound collagen. This was demonstrated by the NMR study by Ivano Bertini and Gregg Fields reported in the same year (Bertini et al., 2012). Our study emphasizes that the S10′ exosite in the hemopexin domain is critical for collagenolysis, which represents a novel target site for inhibitor development for the collagenolytic specific activity of MMP-1.

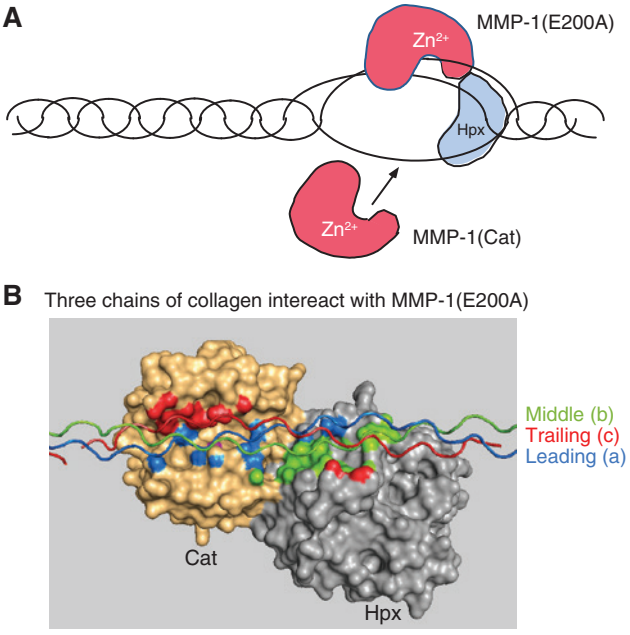

Figure 3: Interaction of collagenase and triple helical collagen. (A) Schematic representation of unwinding of triple helical collagen when bound to the catalytically inactive MMP-1(E200A) mutant. MMP-1(E200A) binds to collagen, but is unable to cleave peptide bonds. Collagen bound to MMP-1(E200A) is then cleaved by the catalytic domain of MMP-1 [MMP-1(Cat)] that does not have the collagenolytic activity. (B) Crystal structure of the complex of triple helical collagen peptide and MMP-1(E200A). Three chains form triple helical collagen in a one-residue stagger arrangement of leading (a), middle (b), and trailing (c) chains. All three chains interact with MMP-1(E200A) in specific sites indicated in blue, green and red with leading, middle, and tailing chains, respectively. Cat, catalytic domain; Hpx, hemopexin domain. The model was made by Rob Visse based on the atomic coordinates (PDB ID code: 4AUO).

LRP1 regulates extracellular trafficking of matrix-degrading metalloproteinases and TIMP-3 in cartilage

ADAMTS-5 is considered to be a key aggrecanase in OA, but its mRNA levels are not significantly elevated in OA cartilage (Bau et al., 2002). This apparent discrepancy is explained by Kazuhiro Yamamoto's finding that ADAMTS-5 is endocytosed by the chondrocytes in healthy cartilage via the endocytic receptor low-density lipoprotein receptor-related protein 1 (LRP1), but that this endocytic pathway is impaired in OA cartilage (Yamamoto et al., 2013). This impairment is due to an increased proteolytic shedding of LRP1 and he has recently identified ADAM17 and membrane-type MMP (MMP-14) as LRP1 sheddases in human cartilage. Shed LRP1 (sLRP1) behaved as a decoy receptor and the sLRP1-ADAMTS-5 complex retained aggrecanase activity (Figure 4). Yamamoto also found that ADAMTS-1, ADAMTS-4, ADAMTS-15 and MMP-13 are endocytosed via LRP1. This in part explains the increased aggrecan and collagen degradation in OA cartilage. TIMP-3 is also endocytosed by LRP1 (Troeberg et al., 2008). Therefore, the level of TIMP-3 was expected to be increased upon LRP1 shedding, but this increase was not sufficient to block the overall increased degradation of aggrecan.

Blocking of ADAMTS17 and MMP-14 activities in OA cartilage by specific inhibitory antibodies (kindly provided by Gill Murphy) reduced the degradation of aggrecan and collagen, suggesting that LRP1 is an important regulator, maintaining cartilage homeostasis. In cartilage, besides major matrix degrading metalloproteinases and TIMP-3, LRP1 controls the extracellular levels of connective tissue growth factor (CCN2) involved in endochondral ossification and articular cartilage regeneration (Kawata et al., 2012). It also down-regulates the canonical WNT-β-catenin signaling pathway by interacting with frizzled-1 (Zilberberg et al., 2004), further emphasizing that LRP1 is an important regulator of skeletal development and the maintenance of healthy cartilage.

Thoughts for the future

The ECM molecules exist in the tissue as large insoluble polymers interacting with other ECM molecules.

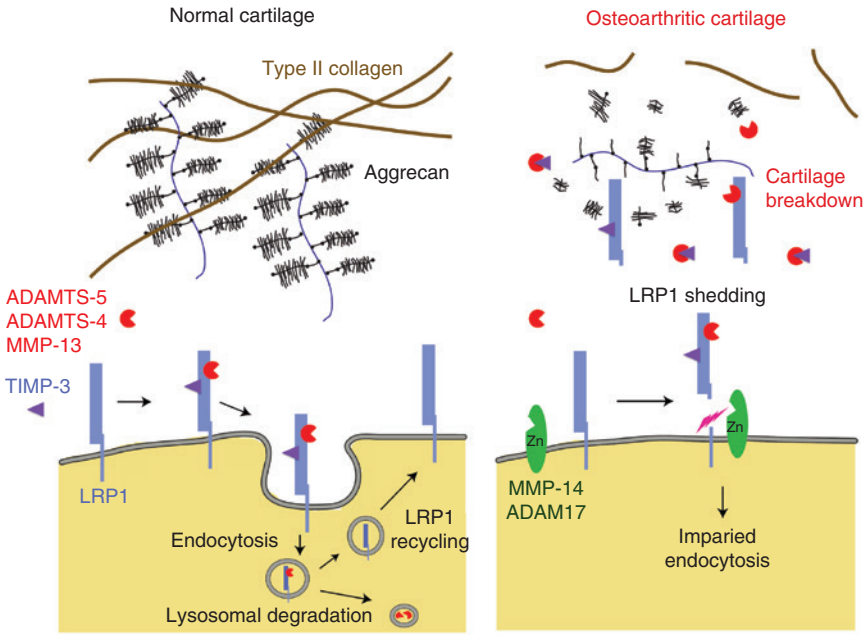

Figure 4: LRP1-mediated endocytic pathways of ECM-degrading metalloproteinases and TIMP-3 in cartilage.
In normal cartilage ADAMTS-4 and -5, MMP-13 and TIMP-3 are endocytosed via the endocytic receptor LRP1. In OA cartilage, ADAM17 and MT1-MMP are activated and shed the ectodomain of LRP1, resulting in impairment of endocytosis of ECM-degrading enzymes and TIMP-3, and an increase in cartilage ECM degradation. Released LRP1 ectodomain also acts as a decoy receptor of these metalloproteinases and TIMP-3, leaving them in the extracellular space. This image was prepared by Kazu Yamamoto.

Biochemical proteolysis studies with isolated ECM molecules as substrates thus provide only limited information, as proteinase cleavage sites in the tissue may be hindered. Is there a hierarchical order of proteolysis? Aggrecan cleavage is considered to be necessary for collagenases to cleave collagen II fibrils in the cartilage (Pratta et al., 2003). In interstitial collagen fibrils, the collagenase cleavage site may be protected unless the telopeptide regions of collagen are cleaved (Perumal et al., 2008), or collagen fibrils are mechanically damaged. Thus, the rates of cleavage of the matrix molecules in the tissue are likely to be reduced compared with those for isolated molecules in solution, and it is even more so when the ECM components are crosslinked upon aging (Mott et al., 1997). Conversely, newly synthesized collagens, whose synthesis is increased upon injury, are much more readily cleaved as they form thinner fibrils. Such young collagens may be the ones that interact with integrins more readily and affect the cellular signaling and behavior. Proteolysis of ECM molecules may release growth factors bound to matrix and increase their bioavailability for cell signaling, and proteolytic fragments of matrix molecules can exhibit new biological function. Many interesting biological paradigms associated with ECM degradation have been described (see Nagase et al., 2006 for review), but many of those biological activities observed *in vitro* are yet to be investigated in *in vivo* experiments. Overall impact of increased LRP1 shedding may cause changes in cellular homeostasis, aberrant ECM turnover, cell signaling, and cellular metabolism, thus contributing to the progression of many diseases.

We have a lot more to learn. The journey with MMPs does not end.

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