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OSTEOARTHRITIS and CARTILAGE

Increased secretion and activity of matrix metalloproteinase-3 in synovial tissues and chondrocytes from experimental osteoarthritis

By Fuad Mehraban*, Michael W. Lark†‡, Farah N. Ahmed*, Fong Xu* and Roland W. Moskowitz*
**Case Western Reserve University, Department of Medicine, 10900 Euclid Avenue, Cleveland, Ohio 44106-4946; and †Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, U.S.A.*

Summary

Objective: The aim of this study was to define the relative regulation of matrix metalloproteinase-3 (MMP-3), and tissue inhibitor of metalloproteinases-1 (TIMP-1), in chondrocytes and synovium in experimental osteoarthritis (EOA).

Methods: Partial-meniscectomized (PM) rabbits, surgical sham controls (SH), and normal non-surgical controls (N) were killed at times corresponding to early degenerative lesions (4 weeks) and increasingly progressive stages of EOA at 8 and 12 weeks post-PM. MMP-3 activity was measured in conditioned media from chondrocytes and synovium using a peptide cleavage assay with substance P (SP) as the substrate. TIMP-1 was quantitated using an enzyme-linked immunosorbent assay (ELISA).

Results: Early degenerative lesions (4 weeks post-PM) were characterized by inflammatory responses in the synovium accompanied by a significant rise of MMP-3 activity in synovial cultures ($P < 0.05$). At 8 weeks there was no discernible inflammation, and MMP-3 activity in EOA synovial cultures was comparable to that in the controls; this was followed by a second increase in MMP-3 activity in EOA samples at 12 weeks. MMP-3 activity was significantly elevated in EOA chondrocyte cultures at 8 weeks post-PM relative to N controls, corresponding to the most destructive phase of EOA, but not in the early phase (4 weeks) or 'late' degenerative phase (12 weeks). Medium derived from chondrocytes contained little or no TIMP-1. Synovia secreted relatively higher amounts of TIMP-1, and this was elevated at 8 weeks post-PM relative to the SH controls. The majority (approximately 90%) of MMP-3 activity could be inhibited using recombinant TIMP-1 or a hydroxamate MMP inhibitor. Complete inhibition was achieved with EDTA or 1,10 phenanthroline.

Conclusion: Together, these data indicate that in EOA, MMP-3 is initially upregulated in the synovium which may play a pivotal role in the pathogenesis of cartilage lesions. In contrast, chondrocyte-derived MMP-3 is upregulated in the later phases of EOA, contributing further to progression of cartilage lesions.

Key words: Osteoarthritis, Metalloproteinase, Cartilage, Synovium.

Introduction

The progressive destruction of articular cartilage is a salient feature of joint pathophysiology in osteoarthritis (OA). The destructive cartilage matrix alterations in OA are thought to be due to the action of matrix-degrading proteinases, some belonging to the matrix metalloproteinase (MMP) family [1–5], and some to other classes, such as the lysosomal cathepsins [6–8], and serine type proteinases such as plasmin [9, 10]. Several studies using human cartilage from OA patients, or tissue derived from animals with experimental OA (EOA) have suggested a major contribution to cartilage degradation by MMPs [3–5, 11–14]. Collagenase-1 (MMP-1) and collagenase-2 (MMP-8) are implicated in the initial proteolytic cleavage of native collagen followed by gelatinolytic activity that leads to complete degradation of collagen [15–18]. Stromelysin (MMP-3) can degrade aggrecan molecules at a defined site between the G1 and the G2 globular domains of the core protein corresponding to the sequence FMDIPEN↓FFGVG [19], although other members of the MMP family such as MMP-1 or MMP-8 can also produce this cleavage [20, 21]. MMP-3 can cleave a variety of other matrix components and has been suggested to play a role in the *in situ* degradation of cartilage link protein [22].

Recently, it was demonstrated that collagenase-3 (MMP-13), previously only known to be expressed in a carcinoma [23], was upregulated in human OA cartilage [14, 24]. MMP-13, like MMP-1, degrades type II collagen fibers, but appears to be more active than MMP-1 [24]. MMP-13 can also cleave

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Address correspondence to Fuad Mehraban, Ph.D., Case Western Reserve University, Department of Medicine, BRB 1022, 10900 Euclid Avenue, Cleveland, OH 44106-4946, U.S.A. Tel: 216 368 1371, FAX: 216 368 1332, E-Mail:fxm5@po.cwru.edu
‡Current address: SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, King of Prussia, Pennsylvania 19406.

cartilage aggrecan at the MMP cleavage site in the interglobular domain [25, 26].

Several studies have suggested that there may be an over-expression of MMPs relative to the tissue inhibitors of metalloproteinases (TIMPs) in OA joints [3, 27]. Measurement of MMP-1, MMP-3 and TIMP in synovial fluid (SF) samples of OA patients revealed elevated concentrations of MMPs in the SF relative to TIMP [13]. The higher concentrations of MMPs relative to TIMP would favor cartilage matrix catabolism.

It has been shown that some aggrecan fragments isolated from human SF contain an N-terminus corresponding to a cleavage other than that created by the typical MMP cleavage in the G1–G2 interglobular domain of aggrecan. An enzyme termed aggrecanase, was proposed to be responsible for the cleavage leading to creation of these aggrecan fragments with N-termini ending in the sequence ARGSV [28]; however, MMP-8 was shown to be capable of producing this cleavage also [29].

We have previously reported that pro-stromelysin and pro-collagenase genes are upregulated in chondrocytes from the knees of rabbits with EOA; pro-stromelysin mRNA was upregulated early in experimental disease and increased significantly as EOA pathology progressed. Pro-collagenase mRNA upregulation followed a similar pattern but the level of expression was not as high as pro-stromelysin mRNA [4]. In the present study we use a peptide substrate, substance P [SP], to quantitate the MMP-3 activity secreted from EOA chondrocytes and synovium at time points in EOA representing early degenerative lesions [4 weeks post partial menisectomy (PM)], and progressive stages of disease (8 weeks and 12 weeks). The temporal sequence of the upregulation of MMP-3 activity and of TIMP-1 in chondrocytes and synovium of EOA rabbits is presented and its significance discussed in relation to possible mechanisms of pathogenesis.

Methods

INDUCTION OF EOA

EOA was induced in both knees of female New Zealand white rabbits (specific pathogen-free, 6–8 weeks old, 1.8–2.3 kg; Hazelton Laboratories, Denver, PA, U.S.A.) using the technique of PM as described [30]. Sham operated (SH) and non-operated (N) rabbits served as controls. A total of 87 rabbits were used in the study over a period of 2 years. These were divided up into groups that were killed at 4 weeks ($N = 9$ each of N, SH, PM), 8 weeks ($N = 14$ each group), and 12 weeks ($N = 6$ each group) post-surgery. Joint pathology was recorded at the time of death. Typical EOA cartilage pathology at 4 weeks post-surgery consisted mainly of small osteophytes and moderate pitting. At 8 weeks, full-thickness cartilage erosions were present with larger marginal osteophytes; progression to 12 weeks was accompanied by increasing ulceration. The pathological changes encountered in this EOA model have been previously described in detail [30].

ASSESSMENT OF SYNOVIAL PATHOLOGY

Synovia were fixed in 4% paraformaldehyde overnight and embedded in paraffin by standard procedures. Six micron sections were cut, deparaffinized, stained with hematoxylin, examined under the light microscope and photographed.

TISSUE CULTURE METHODS

Reagents and media

All enzymes used for cell dissociation were from Worthington Biochemical Corporation, Freehold, NJ, U.S.A.; tissue culture media and serum were obtained from Gibco BRL, Grand Island, NY, U.S.A.

Cartilage was shaved under sterile conditions and pooled from both legs of each rabbit. Chondrocytes were liberated from matrix by enzymatic digestion with trypsin and collagenase. All details pertaining to the cell dissociation were as previously described [8]. Liberated chondrocytes were cultured in suspension in Ham's F12 medium containing antibiotics and 0.2% lactalbumin hydrolysate. Synovia were pooled from both legs of each rabbit and cultured without cell dissociation. Details regarding tissue culture methods were as described [8].

MEASUREMENT OF MMP ACTIVITY IN CULTURE SUPERNATANTS

Metalloproteinase activity was quantitated using the SP assay [31, 32]. In this assay, SP is used as substrate followed by separation of proteolytic cleavage products by high-pressure liquid chromatography (HPLC) on a reverse-phase column (C-18) using acetonitrile gradient with detection of the peptide fragments at 215 nm. Various quantities of SP (1–10 μg) were applied to the HPLC column and eluted. A standard curve was constructed by plotting the area under the SP peak versus the quantity of SP applied to the column. The data was

fitted to a first-order regression plot ($r^2 = 0.9318$). The MMP-3 activity in the test samples were calculated from the area under the peak of the SP remaining after the incubation, by reference to the standard curve and converted into micrograms of SP cleaved per hour at 37°C. One unit of enzyme activity is defined as that which degrades 1 μg of SP per hour at 37°C, producing the peptide fragment SP^{7-11}.

We modified the original method for culture medium as follows. To avoid proteolysis of SP by nonspecific proteases that may be present in the medium, we performed the assays in the presence of a cocktail of protease inhibitors that would inhibit all classes of proteolytic activity except metal-dependent enzymes. The medium to be assayed (50 µl) was diluted in 2× assay buffer (50 mM Tris–HCl pH 7.5, 10 mM $CaCl_2$, 0.04% sodium azide, 0.1% Brij-35, 2 mM phenylmethyl sulfonyl fluoride, 2 mM iodoacetic acid, 5 µg/ml pepstatin, 5 µM E64). Five micrograms of SP was added and the sample incubated for 18 h at 37°C. The reaction was stopped with addition of ethylene glycol-bis (β-aminoethyl ether) N,N,N'N'-tetra-acetic acid (EGTA) to final concentration of 20 mM and 50 µl aliquot of the sample was analyzed by reverse-phase HPLC. Total enzyme (active + latent) was assayed after activation with 0.1 mM p-aminophenyl mercuric acetate (APMA) for 4 h at 37°C. Control samples were incubated with buffer containing 10 mM EDTA instead of $CaCl_2$. When recombinant human MMP-3 (rMMP-3) was added into medium it produced the expected cleavage products in this system.

MEASUREMENT OF TIMP-1

TIMP-1 was measured with an enzyme-linked immunosorbent assay (ELISA) as described [33, 34]. This ELISA measured only free TIMP-1 and not TIMP-1 complexed to MMPs.

ENZYME INHIBITION STUDIES

Inhibition of metalloproteinase activity was assessed using the following inhibitors. Recombinant rabbit TIMP (rTIMP), and a hydroxamic acid MMP inhibitor with broad specificity against all members of MMP family, BB2116. This inhibitor was a gift from Dr Allen Drummond of British Biotech Pharmaceuticals Limited, Oxford, U.K. The IC_{50} of BB2116 versus MMP-3 is 30 nM (data from British Biotech).

For quantitation of percent inhibition produced, the culture media were diluted with 2× assay buffer followed by preincubation with the inhibitor under study for 15 min at 23°C before the addition of SP. The percent inhibition was calculated relative to that produced with 10 mM EDTA (100%). The final concentrations of the inhibitors were, BB2116, 2 µM; rTIMP, 10 µg/ml.

RECOMBINANT PROTEINS AND REAGENTS

Recombinant rabbit TIMP (rTIMP) was produced in by Celltech Ltd, as described [35]. Recombinant human MMP-3 (rMMP-3) was expressed in a mammalian expression system and purified as previously reported [36]. SP and SP^{7-11} were purchased from Sigma (St Louis, MO, U.S.A.). Tissue culture media and solutions were obtained from Gibco (Grand Island, NY, U.S.A.). All other chemicals and HPLC solvents were of the highest purity available obtained from Sigma, or Fisher Scientific (Pittsburgh, PA, U.S.A.). Other synthetic peptides were custom made by Genemed Laboratories, San Francisco, CA, U.S.A.

STATISTICAL ANALYSES

The significance of the data was determined using Student's two-tailed t-test with a significance level at $P < 0.05$. Statistical parameters of kurtosis and skewness were used in order to determine whether the data were normally distributed. For normal distribution the values of kurtosis and skewness were between -2 to $+2$. Sample size analysis was performed to determine the adequacy of group size. The parameters used for sample size determination were, $P = 0.05$ and power = 0.9.

Results

Fig. 1 shows light microscope photographs of 6 µm stained synovial sections taken at 4 weeks post-PM [Fig. 1(a)], similar sections from SH [Fig. 1(b)] and N [Fig. 1(c)] rabbits are shown. For comparison, a synovial section from an 8-week post-PM rabbit is shown (d). Examination of the sections revealed a low degree of cellular infiltration at 4-weeks post-PM; no evidence of inflammation could be discerned in the controls at 4 weeks, or in the EOA synovia sampled at 8 weeks (d) or 12 weeks (not shown).

The SP degradation HPLC assay was previously used for measurement of activity of rMMP-3. We modified the HPLC procedure so that the peptides were separated with a gradient of acetonitrile rather than under isocratic elution; this led to the separation of the SP peak and the main cleavage product produced by MMP-3 so as to minimize interference from other medium components.

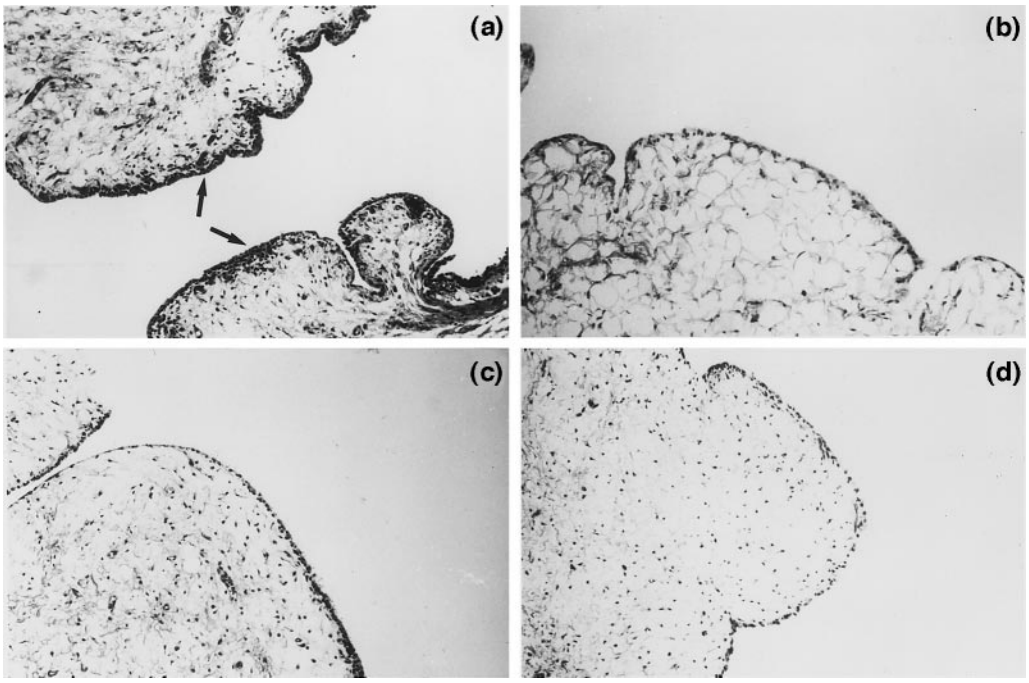

FIG. 1. Six micron paraffin sections of synovium from (a) 4 weeks post-partial meniscectomized rabbit (PM), (b) 4 weeks sham-operated (SH), (c) 4 weeks normal (N), and (d) 8 weeks post-PM EOA rabbits were stained with hematoxylin and photographed. Original magnification, ×100).

Under these conditions SP had a retention time of approximately 17.1 min [Fig. 2(a)], and $SP^{7–11}$ eluted with a retention time of 18.6 min [Fig. 2(b)]. The fragment $SP^{1–6}$ had a low retention, and a very low absorbance relative to SP or $SP^{7–11}$. The production of fragment $SP^{7–11}$ was confirmed with a synthetic peptide corresponding to $SP^{7–11}$ sequence which eluted at the same position in the profile.

In order to validate the SP cleavage assay for crude culture medium we first determined the SP degrading activity of conditioned media in the presence and absence of divalent metal ions. We confirmed that SP degrading activity was only detectable in the presence of calcium ions. Addition of metal chelators EDTA, EGTA, or 1,10-phenanthroline to the conditioned medium abolished all SP degrading activity. When rMMP-3 was added to the medium that had no detectable TIMP, full activity was recovered relative to buffer control. The fragment $SP^{7–11}$ could be seen in the HPLC profile. Fragment $SP^{1–6}$ had a low retention time it was not detected in medium because of co-elution with other medium components; however, it was detectable in buffer.

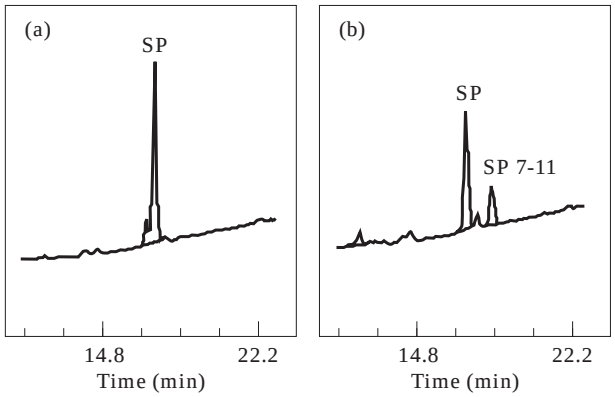

FIG. 2. An HPLC chromatogram showing the elution profile of SP before (a), and after (b) cleavage by recombinant human MMP-3.

We also used a peptide with the sequence MDIPENFFGVG containing the putative MMP recognition sequence found in aggrecan. As expected, this peptide was cleaved by rMMP-3 into two fragments, and also by the MMP activity in the media. The medium activity towards this peptide was also inhibited with metal chelators (data not shown). Because MMP-1 also cleaves the aggrecan peptide [26], we used SP so as to minimize interference of MMP-1 activity in the assay.

Fig. 3 shows the means ($\pm$ S.D.) of MMP-3 activity expressed as SP cleavage units, in media derived from synovia obtained from rabbits of the three experimental groups, at three sequential time points (4 weeks, 8 weeks and 12 weeks post-PM) in EOA. The mean activity in the PM group was significantly higher compared with the N or SH group at 4 weeks post-PM. At 8 weeks,

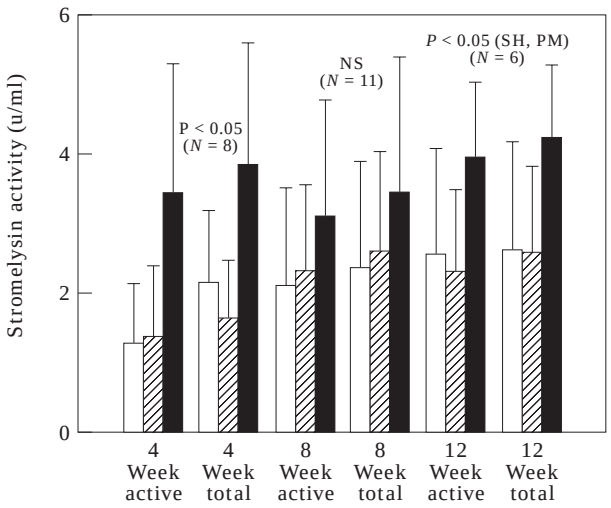

FIG. 3. MMP-3 activity (active and total) was measured in synovial conditioned media (SCM) from non-operated controls (N□), sham-surgical controls (SH▨), and partial-meniscectomized (PM■) rabbits at various time points in EOA. Group means (error bar = S.D.) are shown; n is the number of rabbits in each group. NS = not statistically significant.

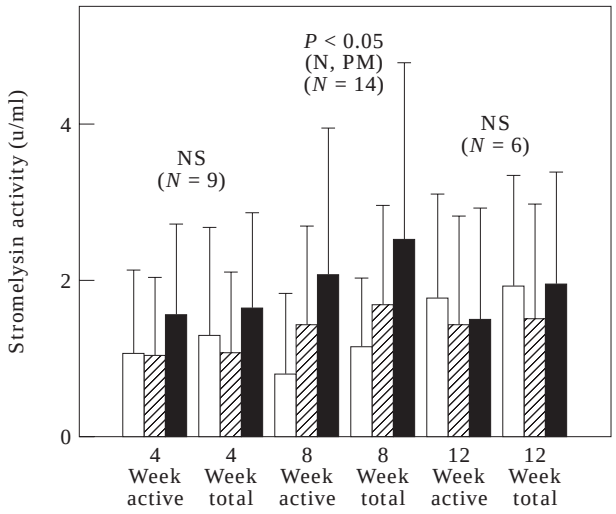

FIG. 4. MMP-3 activity (active and total) was measured in chondrocyte conditioned media (CCM) from non-operated controls (N□), sham-surgical controls (SH▨), and partial-meniscectomized (PM■) rabbits at various time points in EOA. Group means (error bar = S.D.) are shown; n is the number of rabbits in each group. NS = not statistically significant.

activity levels in the PM group declined so that there was not a significant difference in the three groups at this time point. At 12 weeks post-surgery, the mean activity in OA-derived synovial-conditioned media (SCM) was significantly higher than in SH group, but not compared with N group. Activation of latent enzyme in the media produced small increments in the activities measured in all cases and did not impact the statistical significance of the data.

Fig. 4 shows the mean and standard deviations ($\pm$ S.D.) of MMP-3 activity, in conditioned media from EOA chondrocytes, at the same time points as above, before and after activation of the latent MMP-3. Comparison of the means by Student's two-tailed *t*-test revealed that there was no significant difference in active or total metallo-proteinase activity in the media at 4 weeks post-PM when compared with either N or SH chondrocyte-derived media. However, at 8 weeks post-PM there was a significantly higher active and total SP degrading activity in the PM chondrocyte-conditioned (CCM) media compared with media obtained from age-matched normals (N) ($P < 0.05$). The mean activity in sham-derived CCM was not significantly lower than PM. At 12 weeks post-PM the mean enzyme levels (active and total) were not significantly different between the groups. It is of note, however, that at 12 weeks N chondrocytes produced higher amounts of enzyme than at 4 or 8 weeks.

A few CCMs contained free detectable TIMP-1 at very low levels. By comparison SCMs contained relatively high concentrations of free immuno-reactive TIMP (Fig. 5). The mean concentration of free TIMP in the media was not significantly different in the three groups at either 4 or 12 weeks post-PM. At 8 weeks, TIMP was significantly higher in EOA-derived media relative to SH media only. Interestingly, there was an age-related

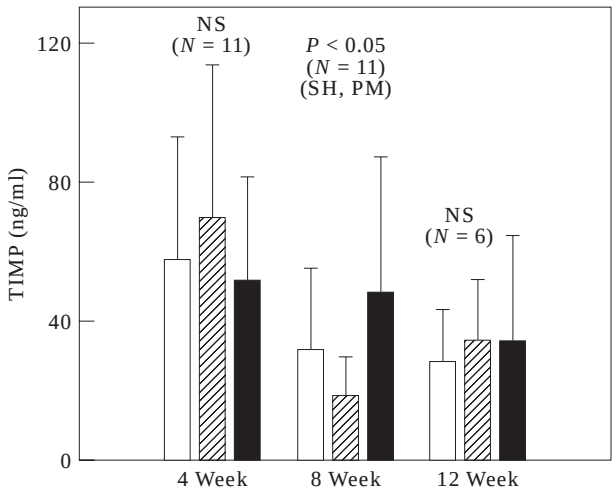

FIG. 5. TIMP-1 was quantitated in synovial conditioned media from non-operated controls (N□), sham-surgical controls (SH▨), and partial-meniscectomized (PM■) rabbits at various time points in EOA. Group means (error bar = S.D.) are shown; n is the number of rabbits in each group. NS = not statistically significant.

Table I
Inhibition of metalloproteinase activity in chondrocyte-conditioned media (CCM) and synovial-conditioned media (SCM) by various MMP inhibitors

	EDTA (10 mM)	BB2116 (2 μM)	rTIMP (10 μg/ml)
rMMP-3 ($N = 3$)	100	94.4 ± 11.2	95.6 ± 5.1
CCM ($N = 4$)	100	86.9 ± 6.6	85.3 ± 6.2
SCM ($N = 4$)	100	99.9 ± 9.3	86.0 ± 11.5

Four different media obtained at 8 week post-PM were evaluated with MMP inhibitors. The average percent inhibition (± S.D.) is shown.

decline in TIMP secretion noted in the normal control group. TIMP was significantly higher in the younger rabbits (at 4 weeks) relative to 8 weeks ($P < 0.05$). The decrease in secretion leveled off between the 8th and the 12th weeks such that no further difference was noted between 8 and 12 weeks. A similar significant decline in TIMP was seen in the SH group between the 4 and 8 week time periods, but in the latter there was higher TIMP secretion at 12 weeks. The TIMP secreted by the PM group did not decline significantly with time.

Table I shows the mean of percentage inhibition of MMP-3 activity in conditioned media of chondrocytes and synovia from EOA rabbits produced by specific MMP inhibitors. Inhibition was 100% with metal chelator EDTA. A broad-spectrum MMP inhibitor BB2116, produced 86.9% inhibition in CCMs and 99.9% inhibition in SCMs; rTIMP produced 85.3% inhibition of activity in CCMs and 86% inhibition in SCMs. The inhibition achieved with either BB2116 or rTIMP with rMMP-3 was close to 95%.

Discussion

In OA, the degradation of cartilage leading to the formation of lesions is likely brought about by the action of multiple matrix degrading proteases. These proteases may be synthesized and activated/deactivated at different times in the emergence of OA pathology according to the environmental signals and stimuli in the pathogenesis mechanism.

In a previous study we demonstrated up-regulation of both pro-collagenase and pro-stromelysin mRNAs in rabbit OA chondrocytes [4]. The expression of both pro-MMP-3 and pro-MMP-1 mRNAs in the synovium was later confirmed (unpublished data). Accordingly, in order to exclude MMP-1 activity in this investigation we evaluated recombinant human MMP-1 (a gift from G.D. Searle and Company, St Louis, MO, U.S.A.) and demonstrated that it did not cleave SP (data not shown).

In the present study we showed that conditioned media from chondrocytes and synovial tissue obtained from EOA rabbit knees contains elevated levels of an MMP that degrades SP. The activity was inhibited with rTIMP, and also with a hydroxamic acid MMP inhibitor BB2116, this being consistent with MMP-3 activity. However, recombinant MMP-13 can degrade SP (data not shown); it is therefore possible that some of the activity detected may be due to the presence of MMP-13 in the media. It is not possible with the present techniques and substrates to ascertain what, if any, the contribution to SP degradation in the assay due to MMP-13 may be. Purification and quantitation of each MMP activity in the medium is necessary in order to answer this question. In this respect it is worth noting that the total MMP SP degrading activity is likely to be a reliable measure of aggrecan degrading enzymes in the medium since all MMPs including MMP-3, and MMP-13 degrade aggrecan at the MDIPEN↓FFGVG locus [5, 19, 25].

The finding (Table I) that a small percentage of the SP degrading MMP activity was not inhibited with rTIMP, or BB2116 for CCM, but was totally inhibited with metal chelators such as EDTA or 1,10-phenanthroline may be due to competitive interactions of the rTIMP and BB2116 with other components in the crude medium. It is also conceivable that a small percentage of the calcium dependent enzyme activity may be due to other metal-dependent proteinases for example a soluble membrane-type MMP [37].

We also considered the possibility of aggrecanase activity contributing to SP cleavage. When a peptide with the aggrecanase cleavage sequence (NITEGE↓ARGSVI) was used in the assay, there was no cleavage of this peptide (data not shown). Therefore, if aggrecanase is present in the media from EOA tissue, its activity is not detectable under these conditions.

An interesting finding in this study was that the MMP-3 upregulation first appeared in the synovium at an early stage (4 weeks post-PM) coincident with a mild transient inflammatory response in this tissue and the appearance of

superficial cartilage lesions, suggesting that synovial tissue has a major role in the pathogenesis of EOA in this model. This early-stage upregulation may be due to the local production of cytokines such as interleukin-1 (IL-1) in the synovium. The production of MMP-3 in the synovium at 4 weeks post-PM is likely to contribute to cartilage degradation characterized mainly by superficial erosions and pitting at this time point.

In contrast, at 8 weeks when advanced cartilage lesions characterized by increased ulcerations are encountered, the synovial MMP-3 expression in the controls is similar to that found in the medium from EOA synovia. At the 8-week time-point the chondrocyte-derived MMP activity was significantly upregulated relative to the N controls; it did not reach statistical significance relative to SH controls. The reason for the latter is not clear; it may be due to differences existing between surgical controls and non-surgical controls because of the effects of surgery. Thus sham-surgery appears to elicit certain tissue responses due to the release of cytokines/factors from soft tissues following surgical procedures. Although the release of tissue enzymes in SH knees is not enough to cause cartilage degradation, it blunts the comparison with PM rabbits, thus making the levels statistically insignificant.

Of note was the finding that most of the enzyme in both the CCMs and the SCMs were in an activated form since further activation with APMA resulted in only a marginal increase in activity (if any). Therefore the enzyme is activated in the medium prior to assay by activators also synthesized and released by the chondrocytes and the synovium.

The majority of the CCM did not contain detectable levels of TIMP-1; a few contained very low levels. This suggests that in this animal model the synovium has a profound influence on the pathologic degradation of cartilage by MMPs by possibly regulating MMP and TIMP-1 levels. In this regard it is interesting that TIMP-1 was upregulated significantly only at 8 weeks post-PM, a time point that corresponds to the progressive stage of EOA, coincidental with upregulation of MMP-3 in the cartilage. TIMP was not found to be significantly elevated at 4 weeks post-PM when the synovial enzyme pathway is upregulated. However, since the ELISA assay used only measures free TIMP, there may be MMP-TIMP complexes in SCM at the 4 week time-point. Likewise, it is possible that chondrocytes secrete small amounts of TIMP that is bound by activated MMPs in the medium, and thus not detected.

The finding that there was free TIMP-1 in the SCMs together with active MMP-3 in the same media is somewhat surprising. The reason for this is not presently clear; it may be that the TIMP in the medium had lost biological activity even though it retained its antigenicity. An alternative explanation could be that there may be an inhibitory factor in the media that interferes with binding of TIMP to the MMP. MMP-3 activity was not fully inhibited even in the presence of high concentrations (10 μg/ml) of exogenously added TIMP suggesting that a portion of the MMP in the medium is refractory to TIMP binding.

An interesting finding was a significant decrease in the mean TIMP secretion by the N and the SH synovium between the 4- and 8-week time-points which is likely related to the maturation of the animals. This curtailment in TIMP secretion may be a contributory factor in the overall disease pathophysiology, since by contrast, PM synovia did not show a significant decrease in TIMP output. This is expected since TIMP secretion by EOA synovia is increased at 8 weeks post-PM, and is in agreement with the differential upregulation of TIMP relative to MMPs in OA [3, 27, 38].

Because the degradation of articular cartilage in OA is a multi-component process with interplay of multiple enzyme pathways each with its related inhibition/activation mechanisms, it is difficult if not impossible to dissect the processes in such a way to study the behavior of one enzyme alone *in vivo* or in tissue culture. Any substrate used may be degraded by a group or family of related enzyme activities such as the MMPs. Likewise, the use of inhibitors such as TIMP or hydroxamate type compounds to inhibit a certain MMP enzyme pathway is not usually achievable because of the broad specificity of such inhibitors towards all MMPs. Also, it is possible that cultured chondrocytes or synovium may show alterations in MMP and TIMP synthesis due to culturing. Relative to this, we confirmed the presence of MMP-3 in fresh frozen cartilage buffer extracts (data not shown), suggesting that MMP-3 is synthesized *in vivo*. Likewise, the amount of TIMP present in cultures also may not best reflect the true concentrations in vivo. To completely address the *in vivo* expression of enzyme and TIMP, well-defined antibodies would be required using quantitative immunohistochemical techniques.

Given the above caveats, we showed here that metal-dependent proteinase activity with characteristics of MMP-3 on the basis of substrate specificity and inhibition with specific inhibitors is secreted by EOA chondrocytes and synovium and that its activity is upregulated at different time

points in the progression of cartilage pathology. A better understanding of cartilage degradation in EOA as it pertains to human disease will be achieved by purification and quantitation of different MMPs from the media.

Acknowledgments

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