MULTIPLE MATRIX METALLOPROTEINASES IN TYPE II COLLAGEN INDUCED ARTHRITIS

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ABSTRACT

To study the relative contribution of various matrix degrading enzymes in the pathogenesis of arthritis, changes in the levels of various matrix metalloproteinases (MMPs) during the progression of collagen induced arthritis was studied in experimental animals. Arthritis was induced in male wistar rats by injecting an emulsion containing collagen type II and Freund's complete adjuvant. The duration of the experiment was 35 days. Synovial effusate was collected at regular intervals after induction. At the end of the experiment serum and cartilage were collected and analysed. Synovial fluid of osteoarthritic patients was also analyzed. Levels of MMP-2, MMP-3, MMP-9 and MT1-MMP were found to be high in synovial effusate and cartilage of experimental animals. In synovial effusate of arthritic animals the expression of MMP-3 was found to be high during the early stages while increase in MMP-2 and MMP-9 occurred at later stages. Synovial fluid of osteoarthritic patients also showed elevated levels of MMP-2, MMP-3 and MMP-9. Our results indicated that sequential action of MMPs such as MMP-3, MMP-2 and MMP-9 can cause degradation of articular cartilage extracellular matrix.

KEY WORDS

Matrix metalloproteinases, Osteoarthritis, Synovial effusate, Synovial fluid.

INTRODUCTION

Cartilage is a dynamic tissue, which under physiological conditions undergoes constant remodeling. Cells of the cartilage tissue respond to a number of cytokines and growth factors that stimulate the production of a variety of neutral proteinases that cleave the matrix macromolecules at the extracellular site and the progressive destruction of the joint is associated with arthritic disease (1-2). Although there are different types of proteinases, the enzymes most often implicated in joint destruction are matrix metalloproteinases (MMPs) (3-5). MMPs are a family of neutral Zn^{2+} dependent endopeptidases that cleave various components of the extracellular matrix (ECM) (6). At least three MMPs viz. MMP-1, MMP-8 and MMP-3 can cleave nonfibrillar collagen while

the gelatinases MMP-2 and MMP-9 degrade collagen type IV and V and denatured proteins (4). These two groups of MMPs work in concert so that collagen type I and II fragmented by MMP-1 and MMP-8 can unwind and become substrate for gelatinases (7). Apart from collagen, these MMPs cleave other macromolecular components of the cartilage such as proteoglycans and glycoproteins like fibronectin (8). MMPs are regulated at multiple levels including at the level of transcription (9). They are synthesized as latent proenzymes, which are cleaved to active form outside the cell. There are also endogenous inhibitors called the tissue inhibitor of metalloproteinases (TIMPs), which inhibit the MMPs (10, 11). As the MMPs are implicated in the pathological destruction of cartilage leading to arthritic disease, MMP inhibition has been the subject of intense research in an attempt to produce specific inhibitors (12).

In vitro studies have shown correlation between degradation of cartilage matrix components and the elevation of MMPs (13). Unlike *in vitro* studies, there is only indirect evidence in support of a role for MMPs in joint destruction *in vivo*. mRNA and the protein levels of various MMPs have been shown to be elevated in arthritic joints of both animals and humans

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(14,15). Although increase in the levels of MMP2 and MMP 9 in serum and synovial fluid of arthritic patients have been reported as markers of the disease, it is not clear how far these enzymes contribute to destruction of cartilage (16 , 17). In humans it is likely that all the different collagen cleaving enzymes are elevated at some stages in the process, but their regulation may not be coordinated (18, 19). Therefore we analysed different MMPs in type II collagen induced experimental arthritis as well as in the synovial fluid of osteoarthritic (OA) patients and the results are presented here.

MATERIALS AND METHODS

Chemicals: Acrylamide, bisacrylamide, gelatin, Freund's complete adjuvant, collagen type II, anti MMP-2, anti MMP-3, anti MMP-9 and anti MT1-MMP were from Sigma Chemical Co, USA.

Synovial fluid Samples: Synovial fluid from OA patients were collected from Medical College Hospital, Thiruvananthapuram. Synovial fluid from accident victims were collected after getting informed consent. Synovial fluid was aspirated from the knee joint, centrifuged and the supernatant kept at 4°C.

Induction of arthritis: Arthritis was induced in male wistar rats 8-10 week old with a mean body weight of 175-200g, by immunizing intradermally at the base of the tail with an emulsion containing type II collagen and Freund's complete adjuvant (300ml/100g body weight) (20). Synovial effusate was aspirated every week by injecting 200µl of saline into the femur joint. After 21 days, a booster dose was given and after one week, animals were sacrificed, synovial effusate, serum and cartilage were collected and various parameters studied. Animals injected with sterile saline without collagen and adjuvant served as control. Cartilage extract was prepared by homogenizing 20mg of cartilage in 1ml phosphate buffer (0.05M, pH 7.4) at 4°C, centrifuged and the supernatant was collected.

Assay of Lysosomal enzymes: Lysosomal enzymes such as β-glucuronidase and β-hexosaminidase in serum and cartilage were assayed as described earlier (21, 22).

Zymography: MMPs were assayed by Zymography using 7.5 % polyacrylamide copolymerized with gelatin (2mg/ml) as described earlier (23). After electrophoresis the gels were washed and incubated in substrate buffer containing 50mM Tris-HCl /5mM CaCl₂ pH 7.5. The gels were stained with coomassie blue and destained.

ELISA: Different concentrations of antigens in PBS (50µl) were coated in the wells of a multiwell plate and incubated for 3hrs at room temperature; the wells were washed with PBS and the free binding sites were blocked using 0.2% gelatin in 0.05% PBS-Tween. Primary antibody (1:500) in PBS-Tween was added to the wells and incubated for 2hrs at room temperature. After extensive washing with PBS-Tween, the wells were treated with HRP conjugated secondary antibody (1:1000) for 1hr at room temperature, o-Dianisidine reagent was added and absorbance measured at 400 nm (24).

Western blot analysis: After SDS-PAGE the gel was taken out and transferred to nitrocellulose membrane using semidry blotting apparatus. The membrane was blocked in 2% albumin, washed in PBS-Tween; primary antibody was added and incubated at room temperature for 3hrs. After extensive washing in 0.05%PBS-Tween it was treated with secondary antibody for 2hrs at room temperature. Then the membrane was washed with PBS-Tween, developing reagent containing 4-chloro naphthol and H₂O₂ was added and the band was visualized (25).

Succinylated gelatin assay: MMP-2 and MMP-9 were also assayed using succinylated gelatin as substrate. MMP-2 was assayed using 50mM borate buffer pH 7 containing 10 mM CaCl₂ and MMP-9 using 50mM borate buffer pH 8.5, 0.03% TNBSA was added and OD measured at 450nm (26).

Statistical analysis: Statistical analyses were done using one way ANOVA followed by Duncan's post-hoc test to identify the differences and Levenes't test using SPSS 10. Differences of p<0.05 are considered to be significant.

RESULTS

Assessment of induction of arthritis: Joint inflammation in experimental animals was observed. Clinical severity was assessed by measuring paw volume changes. An increase in paw volume was observed in arthritic group compared to normal control. Biochemical evaluation of experimental arthritis was done by assaying β-glucuronidase and β-hexosaminidase in serum and cartilage (Table 1). There was significant increase in the activity of β-glucuronidase and β-hexosaminidase in serum and cartilage of arthritic group compared to untreated control.

Changes in MMPs in synovial effusate with the progression of the disease: Synovial effusate was collected at regular intervals after treating with type II collagen to induce arthritis and MMPs were assayed by succinylated gelatin assay

Table 1: Changes in the activity of lysosomal enzymes on induction of arthritis

B Glucuronidase β Hexosaminidase (μ g pNP liberated/min/mg protein)				β Hexosaminidase (μ g pNP liberated/min/mg protein)			
cartilage		serum		cartilage		serum	
Control	Arthritic	Control	Arthritic	Control	Arthritic	Control	Arthritic
0.025 $\pm$ 0.001	0.09 $\pm$ 0.002*	0.03 $\pm$ 0.001	0.089 $\pm$ 0.001*	0.079 $\pm$ 0.001	0.12 $\pm$ 0.004*	0.03 $\pm$ 0.001	0.08 $\pm$ 0.001*

Cartilage extract and serum of control and arthritic animals were used for the assay of β -D glucuronidase using pNP- β -D-glucuronide and β -Hexosaminidase using pNP- β -D N-acetyl glucosaminide as substrates. Saline treated animals served as control. Values given are the average of five experiments $\pm$ SEM; *Statistically significant compared to control ($p < 0.05$)

(Table 2). The activities of MMP-2 and MMP-9 were low during the initial stages. On the 3rd week the activity of these enzymes were found to be significantly greater in arthritic group than those in control. After 4th week there was about 10-fold increase in MMP-2 and 6-fold increase in MMP-9 activity. Zymographic analysis of synovial effusate and cartilage also showed that the major enzymes were MMP-2 and MMP-9 (Fig 1).

Fig 1 : Assay of MMPs-Zymography: Experimental (T) and control (C) animals were sacrificed at the end of the experiment. Synovial effusate (A) and cartilage (B) were collected and was assayed using 7.5% polyacrylamide copolymerized with gelatin. After electrophoresis the gels were washed and incubated in substrate buffer containing 50mM Tris-HCl/5mM CaCl_2 pH 7.5. The gels were stained with coomassie brilliant blue and destained. Gelatin degrading activity appeared as white bands.

The protein level expression of MMPs in synovial effusate was studied by ELISA using specific antibodies against MMP-2, MMP-3 and MMP-9 (Fig 2A). The level of these proteinases was very low in untreated control. Synovial effusate of arthritis induced animals showed increase in the level of these enzymes. During the initial stages, as early as 8 days after treatment with collagen type II, there was 16-18 fold increase in MMP-3 while MMP-2 and MMP-9 did not show any significant change during this time. The level of MMP-3 progressively increased. But at later stages, two weeks after treatment, the level of MMP-2 also increased and that of MMP-9 showed no significant increase until 4th week. Assay of collagenase using azocollagen also showed a significant increase in the activity on 8th day after arthritis induction (data not shown). These results indicate that during the initial stages of arthritis, level of MMP-3 increased while that of MMP-2 and MMP-9 showed increase at later stages suggesting that MMP-3 is probably the first enzyme involved in the matrix remodeling during the progression of arthritis.

Changes in MMPs in experimental arthritic articular cartilage: Cartilage extract of experimental animals were subjected to succinylated gelatin assay, which also showed significant increase in MMP-2 and MMP-9 in arthritic group compared to normal control (Table 3). Cartilage extract of experimental animals were assayed by ELISA for different MMPs such as MMP-2, MMP-3, MMP-9 and MT1-MMP (Fig 2B). The protein level expression of MMP-2 did not show any significant change. Levels of MMP-3, MT1-MMP and MMP-9

Table 2: Changes in the activity of MMPs in synovial effusate on induction of arthritis

ODUnits/mgProtein	0 Day	7 Day	14 Day	21 Day	30 Day
MMP 2	0.25 $\pm$ 0.005 ⁱ	0.40 $\pm$ 0.008 ⁱ	1.3 $\pm$ 0.010 ⁱ	1.9 $\pm$ 0.015 ⁱ	2.44 $\pm$ 0.0.125 ⁱ
MMP 9	0.082 $\pm$ 0.007*	1.4 $\pm$ 0.011*	2.51 $\pm$ 0.008*	3.77 $\pm$ 0.331*	5.77 $\pm$ 0.051*

Synovial effusate was collected on each week after the induction of arthritis and was used for the assay of MMP-2 and MMP-9 using succinylated gelatin as substrate. MMP-2 was assayed using borate buffer pH 7.0 and MMP-9 was assayed using borate buffer pH 8.5. Values are the average of five experiments $\pm$ SEM; ⁱStatistically significant compared to 0 day MMP-2($p < 0.01$); *Statistically significant compared to 0 day MMP-9($p < 0.01$)

Fig 2: Changes in MMPs in synovial effusate and cartilage on induction of arthritis:- Synovial effusate (A) collected at different days after treatment with Col II for induction of arthritis and cartilage extract (B) were coated on a multi well ELISA plate as antigens, subjected to ELISA using specific antibodies and developed using o-dianisidine as substrate. OD measured at 400nm. Values given are the average of five experiments $\pm$ SEM; *Statistically significant compared to basal level (A); *Statistically significant compared to basal level (B)

Table 3: Changes in the activity of MMPs in cartilage

MMP 2(OD Units/mg protein)		MMP 9(OD Units/mg protein)	
Control	Arthritic	Control	Arthritic
0.0310 $\pm$ 0.001	0.0364 $\pm$ 0.002	0.781 $\pm$ 0.012	1.12 $\pm$ 0.011*

Cartilage was collected from control and arthritic animals and extracted with 0.1M phosphate buffer and used for the assay of MMPs using succinylated gelatin as substrate. MMP-2 was assayed using borate buffer pH 7.0 and MMP-9 was assayed using borate buffer pH 8.5. OD measured at 450nm and values are the average of five experiments $\pm$ SEM; *Statistically significant compared to Control ($p < 0.05$)

expression were significantly high in arthritic group compared to control.

MMPs in synovial fluid of Osteoarthritic patients:

Zymographic analysis of synovial fluid showed two distinct bands at 92kDa and 72kDa region. These correspond to MMP-9 and MMP-2 respectively (Fig 3A). The activities of these enzymes were found to be high in arthritic synovial fluid compared to non-arthritic. This was further confirmed by western blot analysis (Fig 3B). Succinylated gelatin assay of synovial fluid showed significantly high activity of MMP-2 and MMP-9 (Table 4). Protein level expression of different MMPs analyzed by ELISA (Fig 3C) showed that the expression of MMP-2, MMP-9 and MMP-3 was high in arthritic synovial fluid compared to non-arthritic synovial fluid, but the level of MT1-MMP was low in arthritic synovial fluid.

Changes in the level of TIMP-1 and TIMP-2: Levels of TIMP-1 and TIMP-2 were determined by ELISA (Fig 4). In cartilage

Fig 3: Activity of MMPs in Synovial Fluid:- MMPs in synovial fluid of non arthritic (n) and arthritic (a) subjects were assayed using 7.5% polyacrylamide copolymerized with gelatin. After electrophoresis the gels were washed and incubated in substrate buffer containing 50mM Tris-HCl/5mM CaCl_2 pH 7.5. The gels were stained with coomassie brilliant blue and destained. Gelatin degrading activity was seen as white bands (A). Synovial fluid of arthritic patients was subjected to PAGE in mini gel apparatus (7.5%). After electrophoresis and blotting on to NC membrane, proteins were probed using antibodies to MMP-2 and MMP-9 followed by treatment with biotinylated IgG and Streptavidin HRP (B). Arthritic (n=7) and non arthritic (n=3) synovial fluids were coated on a multi well ELISA plate as antigens, subjected to ELISA using specific antibodies against MMP-2, MMP-3, MMP-9 and MT1-MMP and developed using O-dianisidine as substrate. OD measured at 400nm (C); *Statistically significant compared to non arthritic ($p < 0.05$)

Table 4: Activity of MMPs in Synovial Fluid

MMP 2(OD Units/mg protein)		MMP 9(OD Units/mg protein)	
Non arthritic	Arthritic	Non arthritic	Arthritic
0.0310±0.001	0.0531±0.001*	2.1±0.016	2.35±0.003*

Arthritic (n=7) and non arthritic (n=3) Synovial fluid were used for the assay of MMPs using succinylated gelatin as substrate. MMP-2 was assayed using borate buffer pH 7.0 and MMP-9 was assayed using borate buffer pH 8.5. OD measured at 450nm; *Statistically significant compared to non arthritic ($p<0.05$).

Fig 4: Protein level expression of TIMPs: Serum (A) and cartilage extract (B) of experimental animals and synovial fluid of patients (C) were coated on a multi well plate as antigens, subjected to ELISA using specific antibodies against TIMP-1 and TIMP-2 and developed using O-dianisidine as substrate. OD measured at 400nm. Values given as OD units/mg protein are the average of five experiments; *Statistically significant compared to non arthritic ($p<0.05$)

of experimental animals a significantly high amount of TIMPs was observed in the case of arthritic group compared to normal. TIMP-1 and TIMP-2 levels in synovial fluid of osteoarthritic patients were high compared to non-arthritic synovial fluid.

DISCUSSION

The results presented here suggest that multiple matrix degrading enzymes belonging to MMP family are involved in the progression of arthritic disease. This conclusion is based on the results of investigations on the changes in the levels of multiple MMPs in the synovial effusate and articular cartilage in experimental animals and the synovial fluids of arthritic patients. Synovial effusate of arthritic rats showed a progressive increase in the level of MMP-3, MMP-2 and MMP-9. This suggested that the activated cells secrete MMPs during the progression of the disease. It is not clear whether these are produced by blood-borne inflammatory cells or cells of the cartilage tissue. Earlier reports have indicated that human rheumatoid synovial fibroblasts produce gelatinases, particularly MMP-2 (27). Correlation between MMP-2 and MMP-9 in synovial fluid and infiltration of polymorphonuclear leukocytes has also been demonstrated (5). Analysis of MMP level in cartilage from experimental arthritic animals also showed an increase in the activity of MMPs, particularly, MMP-3, MMP-2, MMP-9 and MT1-MMP, indicating alteration in the matrix degrading potential of the cartilage during the development of arthritis. This was evidenced by increase in the level of matrix components like fibronectin in synovial effusate and synovial fluid (SF) and membrane receptors for matrix proteins like integrins in soluble form in synovial fluid (data not shown). Although there are reports on the matrix degradation due to alteration in the activities of MMPs in osteoarthritis (28-31), our results indicates that there occurs a sequential change in the level of different MMPs. Our results on the differential appearance of MMPs in synovial effusate during the progression of the disease in experimental animals particularly the appearance of MMP-3 during the initial stages provide further insight into the molecular mechanisms involved in the degradation of cartilage during arthritis and suggest that probably MMP-3, is a key enzyme involved in the initiation of this event.

MMP-3 is the key enzyme involved in the initiation of the degradation of cartilage and can cause degradation of the non-fibrillar collagen and other components like proteoglycans of the ECM in cartilage. Gelatinases like MMP-2 and MMP-9, which act on Collagen IV and Collagen V, and denatured collagen fragments, appear at later stages in the progression

of the disease. MT1-MMP, which is a key cell surface membrane type MMP involved in altering cell matrix interaction and in the activation of other MMPs like MMP-2, increased along with MMP-3 during the early stages. Substrates for MT1-MMP include receptors and matrix proteins like proteoglycan and other components of the matrix. Increase in the level of MT1-MMP during early stages suggests that it may trigger alteration in cell-matrix interaction as well during the early stages.

Although MT1-MMP activity was high, corresponding increase in the activity of MMP-2 was not found (16). Further, during the early stages, action of MMP-2 and MMP-9 on the matrix components may not be significant as the endogenous inhibitors TIMP-1 and TIMP-2 are also present (32). In cartilage of arthritic animals, although TIMP-2 level showed about 10% increase over control, there was no significant difference in TIMP-1 level. Although the levels of TIMP-1 and TIMP-2 were not affected in cartilage, both increased in synovial fluid, indicating a greater production and secretion of these endogenous inhibitors of MMPs (33).

The level of MMP-1/TIMP-1 complex was reported to be less in OA patients compared to control (34). But we have analysed both TIMP-1 and TIMP-2 and found that along with increase in MMP-2 and MMP-9 there was increase in TIMPs as well, indicating possible variation in the production of these inhibitors with variation in the severity of the disease. The protein level expression of MMP-3, MMP-2, MMP-9 and MT1-MMP also found to be very high in OA patients. So these enzymes might have been released by the activated cells of the cartilage. Analysis of the cartilage of experimental animals showed a significantly high activity of these enzymes when compared to the control. It therefore appears that the sequential action of MMP-3, MMP-2 and MMP-9 can cause degradation of cartilage matrix, which is the hallmark of arthritic condition. As the matrix degrading potential of the cartilage increases, these MMPs particularly MMP-3 may be a potential target for drug action.

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