Identification of tissue inhibitor of metalloproteinase-2 (TIMP-2)-progelatinase complex as the third metalloproteinase inhibitor peak in rheumatoid synovial fluid

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Abstract

The metalloproteinases are a family of enzymes that can degrade all the components of the extracellular matrix. These potent enzymes are often found in proenzyme forms and require activation before the substrate can be digested. To prevent unlimited connective tissue destruction a number of inhibitors exist to limit their activity. In a previous study it was found that metalloproteinases in proenzyme form and metalloproteinase inhibitors were often present in rheumatoid synovial fluids. Two of these inhibitors were identified in rheumatoid synovial fluid as α_2 macroglobulin and tissue inhibitor of metalloproteinase (TIMP), the specific metalloproteinase inhibitor. A third inhibitory peak was unidentified. In the study reported here it was shown that this third inhibitor can be purified using gelatin-Sepharose chromatography and consists of TIMP-2 bound to progelatinase (relative molecular weight 72000) in a similar way to that found in concentrated connective tissue culture medium. The importance of these proteinase inhibitors in synovial fluid is discussed.

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The metalloproteinases are a family of enzymes that can degrade all the components of the extracellular matrix.[1] A number of control mechanisms exist to regulate these potent enzymes and these include the control of their synthesis and secretion, the presence of proenzyme forms that require activation, and the presence of specific metalloproteinase inhibitors.[2]

The naturally occurring metalloproteinase inhibitors have important physiological roles in a wide variety of situations where connective tissue resorption is taking place.[3] In serum and synovial fluid most of the collagenase inhibitor activity (>95%) is due to α_2 macroglobulin,[4] the large plasma inhibitor that inhibits all four classes of proteinases[5] and is thought to be important for the inhibition of the metalloproteinases in the fluid phase.[6]

A glycoprotein of relative molecular weight (Mr) 28000, tissue inhibitor of metalloproteinase (TIMP), has been identified in a variety of different connective tissues from a large variety of species.[3] It interacts with the active forms of the metalloproteinases to form a non-covalent but tight binding complex.[7] Tissue inhibitor of metalloproteinase has been sequenced[8 9] and shown to contain six disulphide bonds[10] which hold the protein in two major domains. The N terminal domain has been identified as the site of interaction with the active forms of the metalloproteinases,[11] whereas the C terminal domain is thought to bind the proform of MMP9, the neutrophil gelatinase.[11 12]

Another related inhibitor has been described called TIMP-2,[13–15] which, although sharing only 40% sequence identity with TIMP, is likely to have a similar tertiary structure as the six disulphide bonds are retained and so the two domain structure is probably conserved.

We were interested to examine the role of metalloproteinase inhibitors in diseases where connective tissue breakdown occurs such as rheumatoid arthritis. We discovered that rheumatoid synovial fluid contains three inhibitors of metalloproteinases[16] and identified the smallest of these as TIMP[17] and the largest as α_2 macroglobulin.[13]

In this study we identified the third metalloproteinase inhibitor present in synovial fluid as being TIMP-2 bound to 72000 Mr progelatinase (MMP2). This observation fits with descriptions of a similar complex that is present in concentrated connective tissue culture medium from bovine endothelial cells,[18] human chondrocytes,[19] and human fetal lung fibroblasts.[20 21]

Materials and methods

Chemicals were obtained from the following suppliers: gelatin-Sepharose from Pharmacia (Milton Keynes, United Kingdom); hyaluronidase (bovine testes) type 1S from Sigma Chemical Co (Poole, United Kingdom); and Ultrogel AcA44 from Jones Chromatography (Hendon, United Kingdom). All other chemicals and biochemicals were of analytical reagent grade and obtained from BDH (Poole, United Kingdom) or Fisons (Loughborough, United Kingdom), or have been described previously.[16 20] Synovial fluid, aspirated as part of treatment from the knee joints of patients with rheumatoid arthritis, was put into sterile plastic containers and centrifuged at 12000 *g* for 10 minutes at 4°C. The cell free

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supernatant synovial fluid was stored at −20°C until required. Fluids were assayed for metalloproteinase inhibitor as described previously.[16]

ENZYME AND INHIBITOR ASSAYS

^{3}H-Labelled collagen was used to measure collagenase in the diffuse fibril assay.[22] Collagenase inhibitor was assayed as described previously.[23] One unit of collagenase activity degrades 1 μg of collagen per minute at 37°C and one unit of collagenase inhibitor inhibits two units of collagenase by 50%.

COLUMN CHROMATOGRAPHY

All columns were run at 4°C and all buffers contained 0·05% Brij 35 and 0·02% NaN_3 as a preservative. Column eluates were monitored for protein by measuring the absorbance at 280 nm.

SODIUM DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein samples were mixed with 50 mM TRIS-HCl (pH 6·8) containing 1% sodium dodecylsulphate, 1% glycerol, and 0.0002% bromophenol blue, and were heated at 100°C for three minutes. Polyacrylamide slab gels (10·2 × 7·2 × 0·1 cm) were poured, the lower gel containing 12·5% total acrylamides and 2·5% bisacrylamide, and the spacer gel containing 4·5% total acrylamides and 2·5% bisacrylamide. Polymerisation and all other procedures were as described previously[24] and the gels were electrophoresed in a Bio-Rad Mini-Protean II system.

Gel slabs were fixed for five minutes in 40% trichloroacetic acid, washed in water and stained for 30 minutes in a solution of 0·25% Coomassie Brilliant Blue G250 in methanol-acetic-water (9:1:11, v/v). The polyacrylamide gels were photographed, dried, and autoradiographed as described previously.[24]

GELATIN-ACRYLAMIDE ZYMOGRAPHY

Acrylamide gels containing gelatin (1 mg/ml) were prepared as described by Clark and Cawston.[25] Samples were treated with sodium dodecylsulphate and incubated at room temperature for 10 minutes. After electrophoresis proteins were renatured by shaking the gel in 2·5% Triton X100.

IMMUNOBLOTTING

Proteins were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose paper. This was then incubated with rabbit anti(bovine TIMP-2) IgG, followed by horseradish peroxidase conjugated pig antirabbit IgG antibody (Dako). Colour was developed with 4-chloro-1-naphthol.

Results

Rheumatoid synovial fluids from 10 patients with rheumatoid arthritis were screened to determine if they contained a high molecular weight metalloproteinase inhibitor intermediate in size between α_2 macroglobulin and TIMP as described previously.[16] Two positive fluids were identified and pooled to a total volume of 40 ml. This combined synovial fluid preparation was treated with hyaluronidase (100 μg/ml for one hour at 22°C) before gel filtration to reduce the fluid viscosity. Methylamine was then added to a concentration of 200 mmol/l and incubated for a further two hours at 20°C to destroy α_2 macroglobulin. This hyaluronidase and methylamine treated pool was then applied to an AcA44 gel filtration column (88 × 4·4 cm) and eluted with 25 mM sodium cacodylate (pH 7·2) containing 1 M NaCl, 0·05% Brij 35, 0·02% NaN_3, and 10 mM $CaCl_2$. Fractions (10 ml) were eluted at a flow rate of 40 ml/hour and assayed for protein and inhibitory activity against collagenase. Figure 1 illustates that two peaks of inhibitory activity were separated from the column, one with an approximate Mr 80000 and the other with Mr 30000, which was obviously TIMP as previously characterised.[17]

The fractions in the 80000 pool were combined and dialysed against 25 mM sodium cacodylate buffer (pH 7·2) containing 10 mM $CaCl_2$, 0·5 M NaCl, 0·05% Brij 35, and 0·02% NaN_3, and then applied to a gelatin-Sepharose column (2·5 × 1·0 cm) equilibrated with the same buffer. After loading the column was washed with this same buffer until the absorbance at 280 nm of the fractions decreased to background levels and then the column eluted with the same buffer containing 10% dimethylsulphoxide (DMSO). All the inhibitory activity bound to the column and was eluted from the column with DMSO (fig 2). Inhibitory fractions were pooled and concentrated using a Centriprep 10

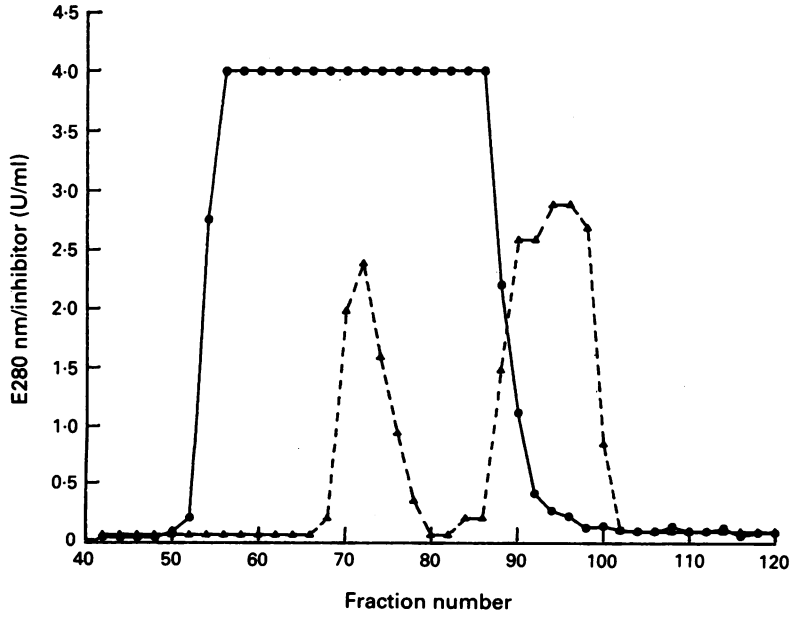

Figure 1 Gel filtration chromatography of methylamine treated pooled rheumatoid synovial fluid. See text for experimental details. (●——●) Protein content; (▲—▲) inhibitory activity against collagenase.

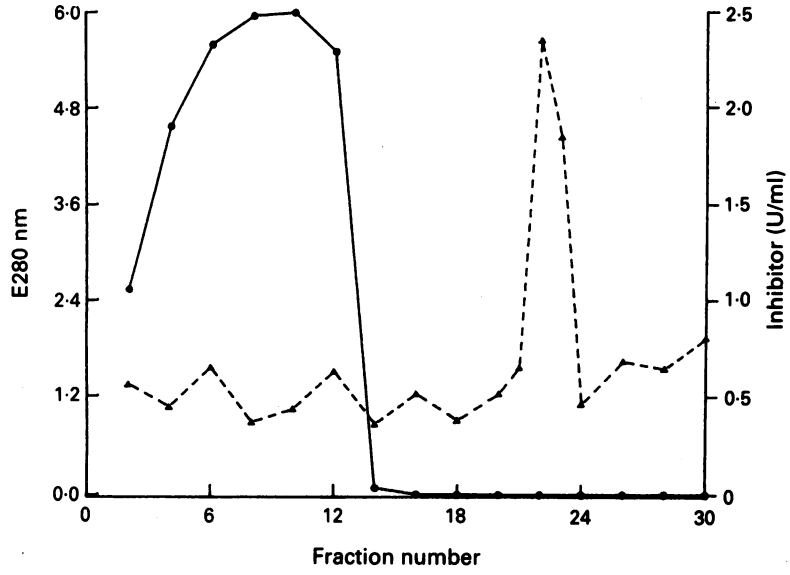

Figure 2 Gelatin-Sepharose chromatography of high molecular weight synovial fluid inhibitor. See text for experimental details. (●——●) Protein content; (▲---▲) inhibitory activity against collagenase.

concentrator (Amicon), reduced with dithiothreitol (4 mg/ml), and then applied to a sodium dodecylsulphate polyacrylamide gel (12·5%). Two proteins were visible after fixing and staining of approximately Mr 72000 and Mr 21000, suggesting that the gelatin-Sepharose binding peak consisted of Mr 72000 progelatinase with TIMP-2 attached (fig 3), as previously found for the human fibroblast inhibitor.[21]

Portions of this peak were diluted 500-fold (fig 4, lane a) and 2500-fold (fig 4, lane b) and applied to a sodium dodecylsulphate acrylamide gel containing 1 mg/ml gelatin (type I) and after separation the gel was equilibrated in a neutral pH buffer containing high levels of Triton X100 to displace sodium dodecylsulphate from the proteins. After staining it could be seen that a proteinase was present digesting the gelatin at approximately Mr 72000 and 66000 (fig 4), indicating that this protein was Mr 72000 progelatinase (the bands correspond to pro and active forms of gelatinase). Incubation of the zymogram in buffer containing EDTA prevented any digestion, showing that this protein was a metalloproteinase (data not shown). A further portion of the binding peak was separated by sodium dodecylsulphate gel electrophoresis, blotted onto nitrocellulose, and then probed with TIMP-2 antibody. Figure 5 illustrates that the compound of Mr 21000 was recognised by the TIMP-2 antibody. In addition, a 67000 band was also recognised, which is presumably a high molecular weight complex of TIMP-2 bound to a proteinase that binds to gelatin.

The Mr 72000 progelatinase-TIMP-2 complex was inhibitory towards collagenase. We investigated whether the complex was able to form a tertiary complex with collagenase (MMP1). Addition of excess Mr 72000 progelatinase-TIMP-2 complex to ^{125}I-labelled collagenase on a Superdex 75 column (120 ml) and elution at 12 ml/hour showed clearly that a tertiary complex was formed (fig 6). Fractions (1·8 ml) were collected and counted for ^{125}I radioactivity. Free collagenase elutes from the column with an apparent Mr of 42000 but addition of the synovial fluid complex to the collagenase resulted in the formation of a tertiary complex that eluted from the column at Mr 82000, leaving a portion of the collagenase peak eluting at Mr 42000. The formation of this tertiary complex was dose dependent, though complete association of the collagenase was not achieved and this is being investigated further. Addition of TIMP to the collagenase resulted in the formation of a complex at 55500 (data not shown).

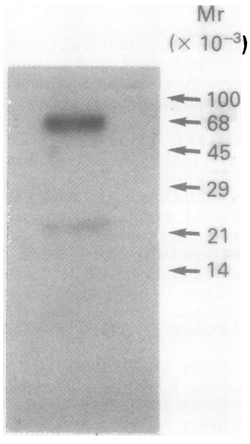

Figure 3 Sodium dodecylsulphate polacrylamide gel electrophoresis of gelatin-Sepharose binding inhibitor. See text for experimental details. The position of the Mr markers is shown (phosphorylase 100000, bovine serum albumin 68000, ovalbumin 45000, carbonic anhydrase 29000, soybean trypsin inhibitor 21000, α-lactalbumin 14000).

Discussion

A number of studies have reported that high Mr metalloproteinase inhibitors are present in concentrated connective tissue culture medium from chondrocytes,[19] bovine endothelial cells,[18] rheumatoid synovial fluid,[16] and fetal lung fibroblasts.[20] De Clerck purified the high Mr of inhibitor from bovine endothelial cells[26] but reported that the pure protein had an Mr of 20400, although the original Mr in concentrated crude culture medium was more than 70000. This work suggested that the purified inhibitor existed as a complex with another protein. Subsequent work has cloned and sequenced the bovine and human inhibitors.[14 15 27] Stetler-Stevenson[14] purified the human melanoma cell inhibitor TIMP-2 by gelatin-Sepharose chromatography; this inhibitor was tightly bound to Mr 72000 progelatinase. Goldberg *et al*[15] also reported that Mr 72000 progelatinase was complexed with TIMP-2 in culture medium from human epithelial cells and lung and skin fibroblasts. A number of studies have purified this protein from mouse tumour cells,[28] human hepatoma cells,[29] human rheumatoid synovial cells,[30] and human transformed fibroblasts.[31 32]

In our previous study we detected a high molecular mass collagenase inhibitor in rheumatoid synovial fluid that was distinct from either α_2 macroglobulin or TIMP.[16] Our subsequent attempts to purify this inhibitor were unsuccessful as the inhibitory activity was lost during purification. In this study we showed that this inhibitor consists of TIMP-2 bound to Mr 72000 progelatinase.

Osthues *et al*[33] have reported that TIMP and TIMP-2 can be purified from rheumatoid synovial fluid after gelatin-Sepharose chromatography. The TIMP and TIMP-2 were then separated from gelatin binding proteins by dissociative gel filtration in the presence of sodium dodecylsulphate. The TIMP was therefore purified from rheumatoid synovial fluid bound to active gelatinases. Our previous studies showed that high molecular weight complexes of TIMP existed in septic synovial fluids. These complexes were thought to be active gelatinase-TIMP complexes as no inhibitory activity was measured in these fluids.[34] Tissue inhibitor of metalloproteinase can also bind to Mr 92000 progelatinase. If TIMP is present in rheumatoid synovial fluid

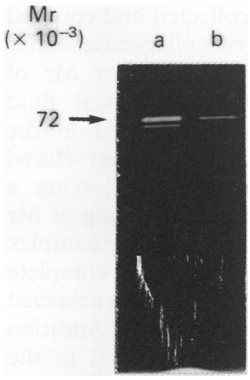

Figure 4 Sodium dodecylsulphate gel electrophoresis on gelatin-acrylamide gel. See text for experimental details.

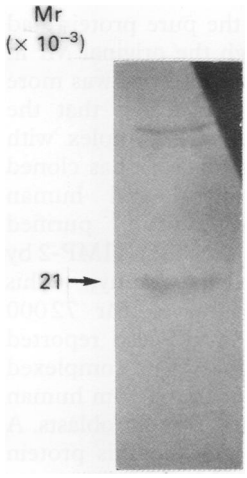

Figure 5 Identification of TIMP-2 after separation from progelatinase by sodium dodecylsulphate gel electrophoresis and immunoblotting. See text for experimental details.

bound to Mr 92000 progelatinase then an inhibitory peak would be expected to be present at high molecular mass in the gel filtration fractions after treatment with methylamine to destroy α_2 macroglobulin. No such complexes were evident in the present study although the Mr 72000 progelatinase-TIMP-2 complex was clearly identified.

The physiological or pathological role for this inhibitory complex in rheumatoid synovial fluid is not clear. Not all rheumatoid synovial fluids contain detectable levels of the TIMP-2-progelatinase complex and it is not clear if this complex is only present at certain stages of the disease or degrees of disease severity. Our own studies have shown that it is also present in a proportion of osteoarthritic fluids (data not shown).

We know that the major inhibitor in the fluid phase for the matrix metalloproteinases is α_2 macroglobulin as our previous studies have shown that the addition of purified collagenase to such fluids results in binding to α_2 macroglobulin rather than to TIMP or the TIMP-2 complex.[6] The inhibitors of the MMPs that are present in synovial fluid are unlikely to play a part in preventing cartilage resorption within the tissue because of their restricted access. Activated neutrophil proteinases released into the synovial fluid however, would rapidly be inhibited by α_2 macroglobulin and the TIMPs. The degradation that occurs in the arthritides is unlikely to occur from the fluid phase but may be caused by enzymes released from within the cartilage by the chondrocytes or from the overlying synovium. Indeed, addition of TIMP to resorbing cartilage does not prevent interleukin 1 stimulated cartilage resorption, as the inhibitor is unable to penetrate into the cartilage. To prevent matrix resorption deep within cartilage, agents such as transforming growth factor β that can stimulate the production of TIMP by chondrocytes within the cartilage matrix may be an alternative form of treatment. Locally produced inhibitors of the MMPs can hence be delivered in the area around the chondrocyte where connective tissue resorption is taking place.[35] Conversely, the addition of low Mr metalloproteinase inhibitors able to penetrate into the cartilage matrix completely prevents matrix degradation[36]; these low Mr inhibitors show great potential in preventing unlimited connective tissue breakdown and more specific inhibitors should be available once the crystal structure of the MMPs is determined.[37]

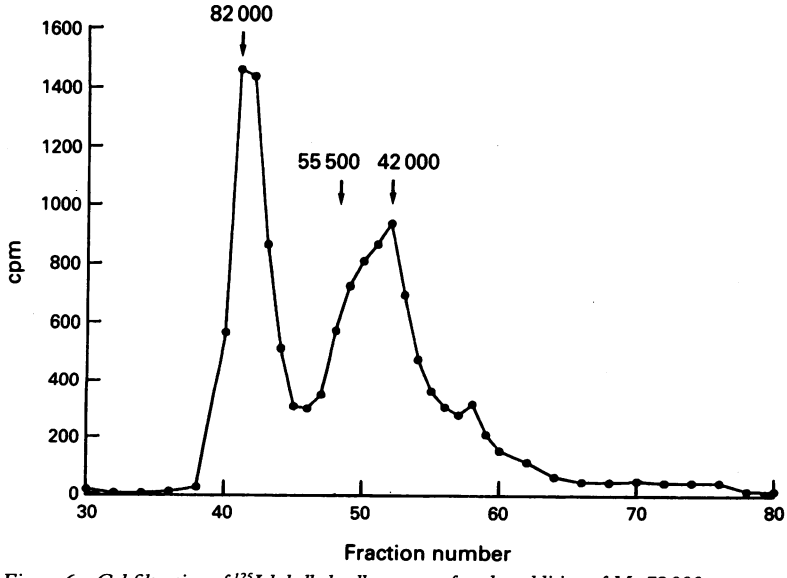

Figure 6 Gel filtration of ^{125}I-labelled collagenase after the addition of Mr 72000 progelatinase-TIMP-2 complex. See text for experimental details.

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