Identification of a new metalloproteinase inhibitor that forms tight-binding complexes with collagenase

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Connective-tissue cells produce a family of metalloproteinases which, once activated, can degrade all the components of the extracellular matrix. These potent enzymes are all inhibited by the tissue inhibitor of metalloproteinases (TIMP), and it was thought that the levels of this inhibitor controlled the extracellular activity of these enzymes. We recently detected a new metalloproteinase inhibitor present in culture media of WI-38 fibroblasts. The inhibitor, named 'large inhibitor of metalloproteinases' (LIMP), can be separated from TIMP by gel filtration on Ultrogel AcA 44, where it is eluted with an apparent M_r of 76000. A portion of this inhibitor-containing peak binds to concanavalin A-Sepharose, indicating that at least some of the inhibitor contains carbohydrate. LIMP inhibits collagenase (MMP-1), stromelysin (MMP-3) and gelatinase (MMP-2) in a dose-dependent fashion. Collagenase forms tight-binding complexes with LIMP, which can be separated from free collagenase on gel-filtration columns. The complex is eluted with M_r 81 600 (AcA 44) or M_r 60000 (Superose 12). This complex is larger than that formed between collagenase and TIMP, which has M_r 52 800 (AcA 44) or 41 000 (Superose 12). Polyclonal antibody to TIMP does not recognize LIMP by immunoblotting, and will not block the inhibition of collagenase by LIMP, showing that LIMP is not a multimeric form of TIMP. The role of this new inhibitor in connective-tissue breakdown studies and its relationship to previously described inhibitors of metalloproteinases is discussed.

INTRODUCTION

The metalloproteinases are a family of enzymes which can degrade all the components of the extracellular matrix [1]. Consequently, a number of control mechanisms exist to regulate their activities. These mechanisms operate at different levels, and include the control of the synthesis and secretion of the enzymes, the secretion of the enzyme in a pro-form that requires activation before substrate is digested, and the presence of metalloproteinase inhibitors.

The naturally occurring metalloproteinase inhibitors are likely to have important physiological roles in a wide variety of situations where connective-tissue resorption is taking place, and they can be divided into three or four separate groups.

The most widely studied of these is α_2 -macroglobulin, the inhibitor of most endopeptidases, which accounts for over 95 % of the anti-metalloproteinase activity of human plasma [2]. The second group consists of tissue-derived glycoproteins that are found in body fluids, culture media conditioned by cells and tissues, and have been identified in a variety of tissues from human, pig, mouse, cow, rabbit and chicken. These inhibitors (M_r ~ 28000) are stable to changes in temperature and pH, are specific for mammalian metalloproteinases, and have been named the 'tissue inhibitor of metalloproteinases' (TIMP) [3].

A third group of biologically active collagenase inhibitors are of lower M_r (10000–20000), and are present in extracts of bone, aorta and cartilage. This group may be related to substances extracted from connective tissues that have been reported to inhibit human vascularization, bone resorption and tumour invasion. Recent reviews have been published [4,5].

Other inhibitors have been described which are apparently unrelated to these three groups. McCroskery *et al.* [6] described a collagenase inhibitor of M_r 40000–50000 that was present in extracts of a rabbit VX2 carcinoma, but no further characterization was reported. Borth *et al.* [7] fractionated human serum by preparative flat-bed isoelectric focusing into three anti-

collagenolytic bands designed α , β and γ . The last band had a pI of 8.7–9.4, but the M_r was not reported.

In 1984 we discovered that human rheumatoid synovial fluid contained a M_r -76000 collagenase inhibitor in addition to TIMP and α_2 -macroglobulin, but this was unstable in dilute solution and could not be further characterized [8]. In 1986 De Clerck & Laug [9] described collagenase inhibitors present in bovine endothelial-cell culture medium. Two peaks of collagenase inhibitor, separated by gel-filtration chromatography, were present. One inhibitor appeared identical with TIMP, whereas the other closely resembled the M_r -76000 inhibitor that we had described in synovial fluid. The inhibitor was sensitive to heat but resistant to trypsin, reduction and alkylation and acid treatment. However, no further purification or characterization of this inhibitor was reported.

Recently Morris [10] has described a similar inhibitor (M_r 68000), which is sometimes present in rabbit chondrocyte culture medium, and which inhibits collagenase. Some preliminary characterization was attempted: the inhibitor was destroyed by reduction and alkylation, was stable to 60 °C, and it did not bind to concanavalin A-Sepharose; no further purification or characterization was performed.

In our recent work purifying TIMP from the media of human WI38 and MRC5 fibroblasts, we have detected a collagenase inhibitor of M_r 76000 on gel filtration. This inhibitor binds to heparin-Sepharose, but not to zinc-chelate columns. In this study we have partially purified the new collagenase inhibitor from human WI-38 fibroblast culture medium and studied its interaction with the matrix metalloproteinases.

MATERIALS AND METHODS

Chemicals were obtained from the following suppliers: concanavalin A-Sepharose from Pharmacia, Milton Keynes, U.K.; *N*-succinimidyl 3-(4-hydroxy-5-[¹²⁵I]iodophenyl)propion-

Abbreviations used: TIMP, tissue inhibitor of metalloproteinases; LIMP, large inhibitor of metalloproteinases; MMP, matrix metalloproteinase.
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ate (1M 861, Bolton & Hunter reagent) from The Radiochemical Centre, Amersham, Bucks., U.K.; Coomassie Brilliant Blue G250 from Raymond A. Lamb, London NW10 5JL, U.K.; heparin-Sepharose and the zinc-chelate column were prepared as previously described [11]. All other chemicals and biochemicals were commercially available analytical-grade reagents or have been previously described [3,11,12].

Enzyme assays

[^3H]Acetylated collagen was used to measure collagenolytic activity by the diffuse-fibril assay [13]. For collagenase-inhibitor assays, a known amount of collagenase (0.06 unit) was added and assayed in the presence of inhibitor samples. A unit of collagenase degrades 1 μg of collagen/min at 37 °C, and 1 unit of inhibitor is defined as that giving 50% inhibition of 2 units of collagenase. Gelatin-degrading activity was assayed with ^3H -labelled gelatin at 37 °C as described previously [1]. Casein-degrading activity (stromelysin) was assayed with ^3H -labelled casein prepared by acetylating casein with [^3H]acetic anhydride. Casein (20 mg/ml) was treated with [^3H]acetic anhydride in the same way as collagen [13], except that the CaCl_2 in the borate buffer was omitted. One unit of caseinase activity degrades 1 μg of casein/min at 37 °C to peptides soluble in 3% (w/v) trichloroacetic acid [3].

Protein determination

Column eluates were monitored for protein by measuring A_{280} .

Iodination of proteins with ^{125}I

Protein samples (80 μl) were added to 0.1 M-sodium borate buffer, pH 8.5 (20 μl), and added to tubes containing 50–100 μCi of dried *N*-succinimidyl 3-(4-hydroxy-5- ^{125}I)iodophenyl)propionate and agitated for 15 min at 0 °C.

SDS/polyacrylamide-gel electrophoresis

Protein samples were mixed with 50 mM-Tris/HCl, pH 6.8, containing 1% (w/v) SDS, 1% (v/v) glycerol and 0.0002% Bromophenol Blue, and heated at 100 °C for 5–10 min. Polyacrylamide slab gels (10.2 cm $\times$ 7.2 cm $\times$ 0.1 cm) were poured, the lower gel containing 12.5% (w/v) total acrylamides and 2.5% (w/w) bisacrylamide and the spacer gel containing 4.5% (w/v) total acrylamides and 2.5% (w/w) bisacrylamide. Polymerization and all other procedures were as previously described

[11], and the gels were run in a Bio-Rad Mini-Protean II system.

Gel slabs were fixed for 5 min in 40% trichloroacetic acid, washed in water and stained for 30 min in a solution of 0.25% Coomassie Brilliant Blue G250 in methanol/acetic acid/water (9:1:11, by vol.), and destained in methanol/acetic acid/water (9:1:11, by vol.). The polyacrylamide gels were photographed, dried and radioautographed as described previously [11]. Western blotting was as described by Clark & Cawston [14].

Column chromatography

All columns were run at 4 °C, and all buffers contained 0.05% Brij 35, with 0.02% NaN_3 as a preservative; 10 litres of crude culture medium containing 270000 units of collagenase-inhibitory activity were concentrated by heparin-Sepharose chromatography. The peak of inhibitory activity eluted from the column with 1 M-NaCl was concentrated to 40 ml by ultrafiltration with a PM10 membrane and used as the starting material for the gel-filtration column. A total of 200000 units of collagenase inhibitor were loaded on the gel-filtration column. Interaction of collagenase inhibitors with collagenase was performed as described by Cawston *et al.* [15].

RESULTS

Gel filtration on Ultrogel AcA 44

A 4.4 cm $\times$ 88 cm column of AcA 44 was equilibrated with 25 mM-sodium cacodylate buffer, pH 7.2, containing 10 mM- CaCl_2 , 1 mM-NaCl, 0.05% Brij 35 and 0.02% NaN_3 . The inhibitory peak from the heparin-Sepharose column (40 ml) was loaded and the column eluted with the above buffer at a flow rate of 24 ml/h. Fractions (10 ml) were collected and monitored for protein and collagenase-inhibitor activity. The elution profile is shown in Fig. 1. Two peaks of inhibitory activity were eluted: a major peak (I) eluted at M_r 30000 was TIMP, and a second peak at M_r 70000–80000 was also present (II). This second peak, which we have named 'large inhibitor of metalloproteinase' (LIMP), was not always present in concentrated culture medium, and we investigated it further.

The LIMP-containing fractions from the Ultrogel AcA 44 column were combined and applied to a column (22 ml bed volume) of concanavalin A-Sepharose equilibrated with column buffer containing 1 mM- MnCl_2 and 1 mM- MgCl_2 , and then eluted with this buffer until the A_{280} of the fractions was equal to that

Fig. 1. Separation of collagenase inhibitor from TIMP by gel filtration

Concentrated human fibroblast medium (40 ml) was loaded on a column of Ultrogel AcA 44 (4.4 cm $\times$ 88 cm) equilibrated with 25 mM-sodium cacodylate buffer, pH 7.2, containing 10 mM- CaCl_2 , 1 M-NaCl, 0.05% Brij 35 and 0.02% NaN_3 and eluted with this buffer at a flow rate of 24 ml/h. Fractions (10 ml) were collected and monitored for protein (A_{280} ; ●) and collagenase inhibitor (□).

Fig. 2. Inhibition of collagenase (MMP-1) by increasing amounts of LIMP

Increasing amounts of crude LIMP were added to assays containing a known amount of collagenase digesting approx. 60% of the collagen present during the assay period. The collagenase was inhibited linearly up to 80% inhibition.

Fig. 3. Inhibition of stromelysin (MMP-3) by increasing amounts of LIMP

Increasing amounts of crude LIMP were added to assays containing a known amount of stromelysin (MMP-3) digesting approx. 60% of the casein present. The stromelysin was inhibited linearly up to 80% inhibition.

Fig. 4. Inhibition of gelatinase (MMP-2) by increasing amounts of LIMP

Increasing amounts of crude LIMP were added to assay tubes containing a known amount of gelatinase (MMP-2) digesting approx. 60% of the gelatin present. The gelatinase was inhibited linearly up to 80% inhibition.

Fig. 5. Interaction of LIMP with collagenase to form a high- M_r complex studied by Aca 44 gel-filtration chromatography

Fractions (0.5 ml) of 125 I-labelled collagenase were chromatographed in the presence (●—●) and absence (○---○) of LIMP on an Aca 44 column (1.6 cm $\times$ 88 cm). The column was eluted with column buffer at a flow rate of 12 ml/h, and fractions (1.7 ml) were collected and counted for 125 I radioactivity. The elution positions of M_r markers are shown.

of the buffer. Adsorbed glycoproteins were eluted from the column by washing with column buffer containing 2% (w/v) 1-*O*-methyl α -D-glucopyranoside (results not shown). Most of the protein passed straight through the column, and with it some of the collagenase-inhibitory activity. A further peak of collagenase-inhibitory activity was eluted with the pyranoside. This result suggests that at least some of the high- M_r inhibitor contains carbohydrate, but some may be unglycosylated.

Interaction of LIMP with metalloproteinases

Increasing amounts of the partially purified inhibitor were added to a known amount of purified collagenase in the collagenase assay, and the percentage inhibition was calculated at each concentration of inhibitor. The results are shown in Fig. 2. Collagenase was inhibited essentially linearly up to approx. 80% inhibition, and then reached a plateau between 90 and 100% inhibition. Figs. 3 and 4 illustrate similar experiments

where increasing amounts of LIMP were added to a known amount of either stromelysin (MMP-3) or gelatinase (MMP-2). The percentage inhibition of each enzyme is shown. Both enzymes were essentially linearly inhibited up to approx. 80% inhibition. These results show that the inhibitor is a true metalloproteinase inhibitor, in that it inhibits three enzymes of the matrix metalloproteinase (MMP) family. LIMP did not inhibit the bacterial metalloproteinases clostridial collagenase or thermolysin, or the serine proteinases trypsin or elastase (results not shown).

Interaction of LIMP with collagenase: formation of complex

A sample of 125 I-labelled collagenase was separated on an Aca 44 gel-filtration column (1.6 cm $\times$ 88 cm) into two peaks. The high- M_r peak eluted at the void volume was discarded, as it contained aggregated collagenase that was inactive. The lower-

Fig. 6. Interaction of LIMP with collagenase to form a high- M_r complex, studied by gel-filtration f.p.l.c.

Fractions (0.2 ml) of ^{125}I -labelled collagenase were chromatographed in the presence (●—●) and absence (×---×) of LIMP on a Superose 12 column (1.0 cm $\times$ 30 cm). The column was eluted with column buffer at a flow rate of 30 ml/h, and fractions (0.3 ml) were collected and counted for ^{125}I radioactivity. The elution positions of M_r markers are shown.

Fig. 7. Immunoblotting of LIMP-containing fractions using polyclonal antibody to TIMP

Fractions containing LIMP from the Aca 44 gel-filtration column (Fig. 1) were concentrated and loaded in duplicate on to an SDS/polyacrylamide gel with TIMP and standards. After electrophoresis, one half of the gel was blotted on to nitrocellulose and probed with a polyclonal antibody to human TIMP, while the other half was fixed and stained. Lanes: 1, standards; 2, TIMP control; 3, LIMP-containing fraction.

M_r peak containing active enzyme was pooled, and 0.5 ml samples were rechromatographed in either the presence or the absence of LIMP. The column was eluted with column buffer, and the fractions were collected and counted for ^{125}I radioactivity. Collagenase was eluted from the column with an apparent M_r of 30170 ± 1570 , but in the presence of LIMP a complex was formed that was eluted at M_r 81600 ± 2200 (Fig. 5). A similar experiment using Superose 12 gel-filtration f.p.l.c. is shown in Fig. 6. Collagenase alone was eluted at M_r 24500 ± 850 , whereas in combination with LIMP a complex of M_r 60000 was formed. These results contrast with similar experiments performed in the presence of TIMP, where complexes of M_r 52800 ± 150 are formed on Aca 44, or of M_r 41000 on the Superose 12 f.p.l.c. column.

Is LIMP a form of TIMP?

The apparent M_r of LIMP on Aca 44 gel-filtration columns (70000–76000) was approximately double that of TIMP. As our previous studies have indicated that higher- M_r aggregates do form in purified samples of TIMP [16,17], we considered the possibility that the M_r -76000 peak could be a TIMP dimer that retained inhibitory activity against collagenase. Fractions containing LIMP (peak II) from the Aca 44 gel-filtration column (Fig. 1) were concentrated and loaded in duplicate on to an SDS/polyacrylamide gel along with a TIMP control. After separation, one half of the gel was blotted on to nitrocellulose and probed with a polyclonal antibody to human TIMP [14], while the other half was fixed and stained.

Fig. 7 illustrates the results obtained. The stained gel showed the pure TIMP to be present in lane 2, with the multiple stained proteins present in the sample of LIMP in lane 3. Although the purified TIMP is recognized by the antiserum on the blot in lane 2, no band was detected at either M_r 28000 or any higher M_r in the sample of LIMP loaded on to lane 3.

We also tried to block the inhibition of collagenase by LIMP by the addition of a polyclonal anti-TIMP antibody, but no decrease in inhibition was seen. The anti-TIMP antibody completely blocked the TIMP inhibition of collagenase in control experiments (results not shown).

DISCUSSION

Our previous purification of TIMP from plasma [16] and synovial fluid [17] demonstrated that higher- M_r aggregates of this protein existed. However, although these were undoubtedly present after SDS/polyacrylamide-gel electrophoresis, we had no evidence that the multimer forms were biologically active. Initially we thought that the M_r -76000 inhibitor could be an active dimer form of TIMP. Subsequent studies showing that polyclonal anti-TIMP antibodies would not cross-react with this protein indicated that it was not identical with TIMP. Therefore this represents a new collagenase inhibitor produced by connective-tissue cells in culture.

It is likely that this is the same inhibitor that we originally described to be present in some rheumatoid synovial fluids [8]. Although we tried to purify this inhibitor from pooled synovial fluids [17], very low levels of inhibitor protein were present, and it was unstable after initial purification steps.

De Clerck & Laug [9] described a similar inhibitor in endothelial-cell culture medium. Bovine endothelial cells secreted two inhibitors (one of which was TIMP) that inhibited tumour-mediated collagenolysis. The M_r -75000 inhibitor was separated from TIMP on gel-filtration columns and was resistant to trypsin, acid, and reduction and alkylation, but was destroyed by heat.

Morris [10] described an M_r -66000 inhibitor of collagenase which accounted for 20% of the collagenase-inhibiting activity secreted by rabbit chondrocytes cultured in medium with acid-treated foetal-bovine serum. The inhibitor was stable to heat, but sensitive to reduction and alkylation, and it did not bind to concanavalin A-Sepharose. The inhibitor was partially purified, but it was not tested to see if it inhibited other metalloproteinases.

The inhibitor that we have detected in tissue-culture medium appears to have inhibitory activity against collagenase (MMP-1), gelatinase (MMP-2) and stromelysin (MMP-3). However, it did not completely abolish the activity of these enzymes, which suggests that it binds less tightly than TIMP. It could be that TIMP is produced by cells as a first line of defence to control connective-tissue breakdown; the production of the second inhibitor may act as a second line of control, which is more selective. This could mean that other inhibitors are produced

which selectively block the activity of each of the matrix metalloproteinases.

It is not clear if the inhibitor that we describe is related to other low- M_r collagenase inhibitors which appear to be similar in size to that purified by Bunning *et al.* [18] from bovine cartilage and body fluids. Recently De Clerck *et al.* [19], Howard *et al.* [20], Moore *et al.* [21] and Stetler-Stevenson *et al.* [22] have purified and characterized collagenase inhibitors of $M_r \sim 20000$. Some of these inhibitors [20,21] have been found to bind specifically to gelatinase with 1:1 stoichiometry, and in some cases show significant similarity to TIMP, including conservation of 12 cysteine residues and 3 of 4 tryptophan residues [19,22].

All these reports suggest that there may be a family of matrix metalloproteinase inhibitors, and have suggested different names for the M_r -20000 inhibitors which include TIMP-2 [22] and IMP [20]. Some authors suggest different affinities of the inhibitors for the matrix metalloproteinase family, with M_r -20000 inhibitor preferentially inhibiting gelatinase and TIMP binding tightly to collagenase. No results are available for the relative inhibition constants, but it is obvious that the precise control of connective-tissue degradation involves more than one metalloproteinase inhibitor.

In this study a further metalloproteinase inhibitor has been detected which forms tight-binding complexes with the matrix metalloproteinases. Purification and sequence analysis will show if it too is related to the other two inhibitors that have been characterized.

The identification of additional inhibitors to TIMP present in tissue-culture medium introduces substantial uncertainty into previous studies measuring collagenase inhibitors in tissue-culture medium. The conventional bioassays measure the total inhibitory activity against collagenase and do not distinguish between different inhibitors. Consequently any investigation of the control of the synthesis and secretion of TIMP in cell- or organ-culture systems must in future rely on immunoassays in addition to measuring the total biological activity. Immunoassays therefore need to be developed to measure accurately the levels of this new inhibitor. As we and others [23,24] have previously pointed out, the existing bioassays for inhibitory activity are also inaccurate, as they do not account for the present of enzyme-complex inhibitor.

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