Biochem. J. (1984) **218**, 277–280
Printed in Great Britain

Extracts of human articular cartilage contain an inhibitor of tissue metalloproteinases

David D. DEAN and J. Frederick WOESSNER, JR.
Departments of Biochemistry and Medicine, University of Miami School of Medicine, R-127, P.O. Box 016960, Miami, FL 33101, U.S.A.

(*Received 1 December 1983/Accepted 19 December 1983*)

When human articular cartilage is extracted with 2M-guanidinium hydrochloride at pH7.5, an inhibitor is obtained that blocks the activity of three metalloproteinases, including collagenase. Molecular-sieve chromatography of the inhibitor gives an M_r value for the inhibitor of 28500. The inhibitor is stable to heat (60°C, 1h) and acid (pH2, 24°C, 10min). It is destroyed by trypsin and by reduction and alkylation. It is slowly inactivated by aminophenylmercuric acetate. It binds to concanavalin A–Sepharose and is eluted with α-D-1-*O*-methyl glucopyranoside. Complexes of enzyme and inhibitor are not re-activated by aminophenylmercuric acetate and only partially so by high levels of trypsin. These properties indicate that this inhibitor is a member of the TIMP (tissue inhibitor of metalloproteinases) class. Such an inhibitor, previously found in tissue culture and amniotic fluid, is now shown to be directly extractable from tissue.

Various cells of mesenchymal origin produce metalloproteinases that are secreted from the cell and digest extracellular-matrix components such as collagen and proteoglycan. It is important to regulate the activity of such proteinases once they are released from the cell; one mechanism is by the production of inhibitors. A large variety of cells and tissues have been shown, upon growth in tissue culture, to release inhibitors of metalloproteinases such as collagenase, gelatinase and proteoglycanase (reviewed in the Discussion section). These inhibitors are typically heat-stable cationic glycoproteins of M_r about 30000. A generic term, TIMP (tissue inhibitor of metalloproteinases), has been applied to such inhibitors (Cawston *et al.*, 1981).

TIMP has not yet been directly extracted from any tissue source, although it has been recovered from amniotic fluid (Murphy *et al.*, 1981*a*). There have been reports of collagenase inhibitors in cartilage extracts (Kuettner *et al.*, 1976; Roughley *et al.*, 1978), but these have not included sufficient characterization to assign the inhibitor to the TIMP class. In the present paper we describe an inhibitor in extracts of human articular cartilage that has properties typical of the TIMP group and is active against several metalloproteinases, including collagenase.

Abbreviations used: APMA, aminophenylmercuric acetate; GdmCl, guanidinium chloride; TIMP, tissue inhibitor of metalloproteinases.

Experimental

Human patellas were obtained at autopsy or under sterile conditions at surgery. They were frozen at −20°C, then thawed as needed. Cartilage was dissected, minced and homogenized in a Polytron (Brinkmann, Westbury, NY, U.S.A.) homogenizer. A 2g sample of tissue was homogenized in 20ml of buffer containing 2M-GdmCl. The buffer contained 50mM-Tris/HCl, pH7.5, 10mM-$CaCl_2$ and 0.02% NaN_3. This same buffer, with the addition of 0.05% Brij-35 and 0.2M-NaCl, was used for all experiments. The cartilage extracts were clarified by centrifugation, made 0.05% with respect to Brij and 1M with respect to NaCl, concentrated on a PM-10 membrane (Amicon, Lexington, MA, U.S.A.) to 6ml, and applied to a column (1.6cm × 90cm) of Sepharose-6B (Pharmacia, Piscataway, NJ, U.S.A.) equilibrated with buffer containing 1M-NaCl at 4°C. Fractions were dialysed against buffer and assayed.

Assay methods

Assays were conducted with three metalloproteinases. A low-M_r metalloproteinase was partially purified (150-fold) from rat uterus (Sellers & Woessner, 1980) by chromatography on Ultrogel AcA-54 (LKB, Rockville, MD, U.S.A.) and CM-Bio-Gel (Bio-Rad, Richmond, CA, U.S.A.) columns. The enzyme was activated by incubation

for 1h at 37°C with 0.5mM-APMA, and then dialysed against buffer. Enzyme (40μl), inhibitor (0–360μl) and buffer to a total of 400μl were mixed and incubated at 37°C for 1h. The assay was then conducted by adding 0.85ml of buffer containing 1mg of Azocoll (Calbiochem, San Diego, CA, U.S.A.) and incubating for 18h at 37°C. The released colour was quantified at 520nm. Collagenase was also extracted from rat uterus (Woessner, 1979) and partially purified (60-fold) by passage through Ultrogel AcA-54, DEAE-cellulose (Bio-Rad) and heparin–Sepharose (Pharmacia) columns. It was similarly activated by APMA before use and dialysed. A second collagenase was tested in the form of a crude extract of epiphyseal growth-plate cartilage from rachitic rats. It was similarly activated. Collagenase (10–50μl) and inhibitor (0–10μl) were made to 100μl with buffer and incubated for 1h at 37°C. Then 20μl of buffer containing 4μg of labelled collagen (15000c.p.m./μg) were added. The collagen was telopeptide-free rat skin collagen prepared by the method of Ryhanen *et al.* (1982) and labelled with [^{3}H]acetic anhydride (Montelaro & Reuckert, 1975). After 18h incubation at 30°C, the enzyme was assayed by the method of Ryhanen *et al.* (1982).

Inhibitor properties

The properties of the inhibitor were tested by treating the inhibitor in various ways. The activity of the inhibitor towards the uterus metalloproteinase was then assayed as follows. Inhibitor (250μl) was allowed to react with 40μl of metalloproteinase for 1h. Azocoll was added and the incubation continued for 18h with a total volume of 1.25ml. The amount of inhibitor was adjusted to give 70–75% inhibition. Inhibitor was treated with trypsin at a final concentration of 50μg/ml for 1h at 37°C. The trypsin was then blocked with a 4-fold (by weight) excess of soya-bean trypsin inhibitor. In experiments with APMA, the reagent was not removed, but was diluted about 4-fold in the final assay. Acidification was accomplished with 0.1M-HCl, followed by neutralization with 0.5M-Tris. The inhibitor was reduced with 2mM-dithiothreitol for 60min at 37°C and then alkylated with 5mM-iodoacetamide for 15min. The reagents were then removed by dialysis. In testing the stability of the enzyme–inhibitor complex, the was first allowed to form for 1h at 37°C before treatment.

Results

Extracts were prepared from specimens of 55–75-year-old cartilage which was moderately osteoarthritic. The extracts were chromatographed on Sepharose-6B; a typical elution pattern is illustrated in Fig. 1. Most of the proteoglycan and protein elutes near the void volume. Inhibitory activity against uterine metalloproteinase and collagenase elutes in a broad peak centered on fractions 30–31. Cartilage metalloproteinase activity (not illustrated) emerges at tube 27. The apparent M_r of the inhibitor, on the basis of six separations, is approx. 28000. There is an uncertainty of ±0.6 fraction in the position of the peak, which gives an uncertainty in M_r of ±6000 due to the limited resolving power of Sepharose-6B in this size range. A better estimate, M_r 28500, was obtained by re-chromatography of the pooled inhibitor fractions 29–31 from the Sepharose column on a column (0.9cm × 100cm) of Ultrogel AcA-54 in the same buffer (results not shown).

Three different rat metalloproteinases were titrated with the same preparation of inhibitor, which had been purified approx. 15-fold by passage through the Sepharose-6B column. As shown in Fig. 2, each enzyme was inhibited in a linear fashion up to about 70% inhibition. If we assume that the inhibitor binds stoichiometrically to the enzymes (Cawston *et al.*, 1981), then it can be seen that the relatively insensitive Azocoll assay requires more enzyme, and hence more inhibitor. Maximum inhibition is obtained if the enzyme and inhibitor are allowed to react in the absence of

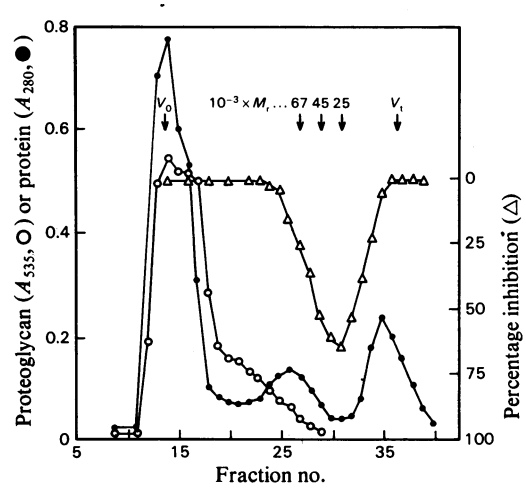

Fig. 1. *Chromatography of a 2M-GdmCl extract of human articular cartilage on Sepharose-6B*

A 1.6cm × 90cm column was equilibrated in, and eluted with, buffer + 1M-NaCl. The extract of 2g of tissue was concentrated to 6ml and applied to the column. Fractions (4ml) were collected. Protein was determined by absorption at 280nm, proteoglycan by reaction with dimethyl-Methylene Blue (Farndale *et al.*, 1982), and inhibitor by assay of 75μl portions with uterine metalloproteinase sufficient to digest Azocoll in 18h to yield an A_{520} of 0.220.

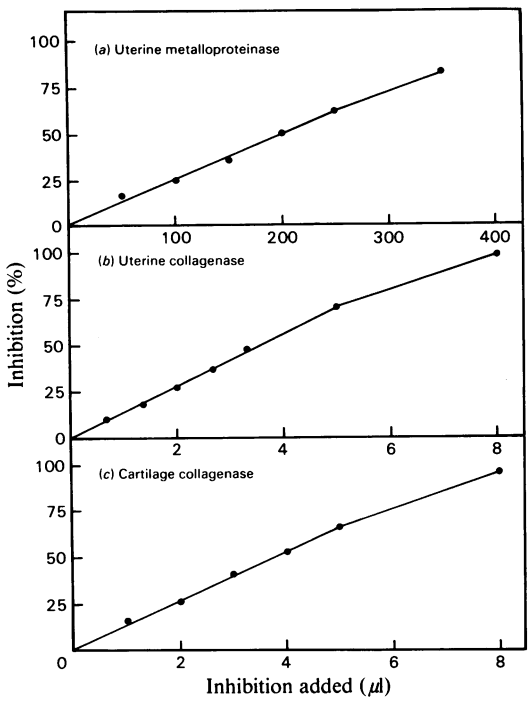

Fig. 2. *Inhibition of various metalloproteinases of the rat by a single preparation of human cartilage inhibitor*
Details of the assays are given in the Experimental section. Each point represents a triplicate determination. (*a*) Rat uterus metalloproteinase (40 μl) was incubated with inhibitor for 1 h, then assayed with Azocoll. Uninhibited enzyme produced a ΔA_{520} of 0.246 in 18 h. (*b*) Rat uterus collagenase (50 μl) was incubated with inhibitor for 1 h, then 4 μg of labelled collagen was added for the assay. Uninhibited collagenase digested 31% of the collagen in 18 h. (*c*) Collagenase from rat growth-plate cartilage (10 μl) was incubated with inhibitor for 1 h. Collagen was added as above; 33% was digested by the uninhibited enzyme.

substrate for 2 h. However, the reaction is 90% complete in 1 h and that time interval was used for the experiments described here. This point was established for each enzyme at both high and low levels of inhibitor. Inhibitor was also found in a single specimen of young (24-year) cartilage. However, quantitative comparisons of cartilage of various ages and disease states have not yet been attempted.

Properties of the inhibitor were tested as shown in Table 1. The inhibitor was sensitive to trypsin, but reacted only slowly with APMA. It was stable to acid and heat, but was destroyed by reduction and alkylation. Reversal of the enzyme–inhibitor complex could not be accomplished by APMA. There was partial reversal by trypsin, possibly due

Table 1. *Properties of metalloproteinase inhibitor from human articular cartilage*
An amount of inhibitor was chosen that gave 70–75% inhibition of the uterine metalloproteinase, to ensure that there would be a linear response of inhibition to the amount of inhibitor. This extent of inhibition was then set equal to 100. Full details of the assay and treatments are given in the Experimental section.

Treatment	Relative inhibition
(*a*) Treatment of inhibitor before enzyme addition:	
None	100
Trypsin (50 μg/ml, 37°C)	
3 min	38
60 min	0
APMA (1 mM, 37°C)	
15 min	89
120 min	63
Adjust pH to 2.0, 10 min, 24°C	97
Heat to 60°C, 60 min	97
Reduce and alkylate	0
(*b*) Treatment of enzyme–inhibitor complex before assay:	
None	100
Trypsin (50 μg/ml), 3 min; trypsin inhibitor (200 μg/ml)	76
APMA (1 mM, 60 min, 37°C)	96

to digestion of the inhibitor. In a further experiment it was shown that the inhibitor bound to concanavalin A–Sepharose (Fig. 3) and could be eluted with 0.2 M-α-D-1-*O*-methyl glucopyranoside in this buffer. This step produced a 4-fold increase in the specific activity of the inhibitor over that of the Sepharose-6B preparation. A rough calculation indicates that articular cartilage contains about 2 μg of inhibitor/g of wet tissue. This calculation is based on the assumption that purified uterine collagenase digests 500 μg of collagen/min per mg of enzyme at 30°C, that TIMP binds in a 1:1 molar ratio, and that the extraction and chromatography recovered all of the inhibitor.

Discussion

The inhibitor of metalloproteinases extracted from human articular cartilage displays properties that permit its assignment to the TIMP class of inhibitors (Murphy *et al.*, 1981*a*). The inhibitor has M_r 28 500, which is typical of this class. It is stable to heat (cf. Nolan *et al.*, 1980; Yasui *et al.*, 1981; Murphy *et al.*, 1981*a*), but is destroyed by reduction and alkylation. The cartilage inhibitor is digested by trypsin (cf. Murphy *et al.*, 1977; Vater *et al.*, 1978; Sakamoto *et al.*, 1981 etc.) and binds to

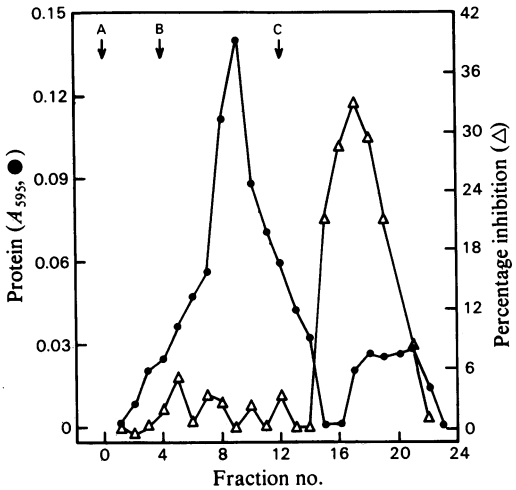

Fig. 3. *Chromatography of inhibitor on concanavalin A–Sepharose*

The 'peak inhibitor' tube from the Sepharose-6B column (Fig. 1) was dialysed against assay buffer and applied (A) to 1.5ml of gel equilibrated in 20mM-Tris/HCl/0.5M-NaCl/0.05% Brij 35, pH7.4. The column was washed (B) with 6.0ml of equilibrating buffer and then eluted (C) with 6.0ml of this buffer containing 0.2M-α-D-methyl glucopyranoside. Fractions (0.7ml) were collected. Inhibitor (350 μl) was assayed for its activity towards uterine metalloproteinase (40 μl).

concanavalin A (cf. Aggeler *et al.*, 1981; Cawston *et al.*, 1981). Complexes of the enzyme and inhibitor are not dissociated by APMA (cf. Nolan *et al.*, 1980; Aggeler *et al.*, 1981; Cawston *et al.*, 1981, 1983). In all of these properties, the cartilage inhibitor matches the inhibitor produced in culture by bovine chondrocytes (Morales *et al.*, 1983). The cartilage inhibitor is somewhat more sensitive to trypsin than some of the other inhibitors, and this may account for a partial dissociation of the complex that has not been seen with other preparations (Aggeler *et al.*, 1981; Morales *et al.*, 1983). The finding that APMA does not reverse the inhibition of uterine metalloproteinase and collagenase, although it does activate the latent forms of these enzymes (Sellers & Woessner, 1980), indicates that the latent forms are unlikely to be enzyme–inhibitor complexes. The inhibitor agrees in heat stability with the inhibitor isolated from bovine nasal cartilage (Roughley *et al.*, 1978); however, that inhibitor has a smaller apparent M_r of 22000.

We believe that the inhibitor found in human cartilage is produced by the chondrocytes. This is based on the observation that bovine articular chondrocytes produce an inhibitor in culture under conditions in which the cell phenotype is preserved (Morales *et al.*, 1983). Moreover, human cartilage in culture can synthesize the inhibitor (Murphy *et al.*, 1981*b*). Presumably the inhibitor is of critical importance in the regulation of the metabolism of both collagen and proteoglycan, components which together constitute about 95% of the cartilage matrix.

This research was supported by National Institutes of Health grants HD-06773 and AM-16940. D. D. D. is a Fellow of the Arthritis Foundation. We thank Ms. Marie Selzer and Ms. Carolyn Taplin for their excellent technical assistance. Collagenase from growth plate cartilage was generously given by Dr. Julio Pita and Ms. Ofelia Muniz of this University.

References

Aggeler, J., Engvall, E. & Werb, Z. (1981) *Biochem. Biophys. Res. Commun.* **100**, 1195–1201

Cawston, T. E., Galloway, W. A., Mercer, E., Murphy, G. & Reynolds, J. J. (1981) *Biochem. J.* **195**, 159–165

Cawston, T. E., Murphy, G., Mercer, E., Galloway, W. A., Hazelman, B. L. & Reynolds, J. J. (1983) *Biochem. J.* **211**, 313–318

Farndale, R. W., Sayers, C. A. & Barrett, A. J. (1982) *Connect. Tissue Res.* **9**, 247–248

Kuettner, K. E., Hiti, J., Eisenstein, R. & Harper, E. (1976) *Biochem. Biophys. Res. Commun.* **72**, 40–46

Montelaro, R. C. & Reuckert, R. R. (1975) *J. Biol. Chem.* **250**, 1413–1421

Morales, T. I., Kuettner, K. E., Howell, D. S. & Woessner, J. F. (1983) *Biochim. Biophys. Acta* **760**, 221–229

Murphy, G., Cartwright, E. C., Sellers, A. & Reynolds, J. J. (1977) *Biochim. Biophys. Acta* **483**, 493–498

Murphy, G., Cawston, T. E. & Reynolds, J. J. (1981*a*) *Biochem. J.* **195**, 167–170

Murphy, G., McGuire, M. B., Russell, R. G. G. & Reynolds, J. J. (1981*b*) *Clin. Sci.* **61**, 711–716

Nolan, J. C., Ridge, S. C., Oronsky, A. L. & Kerwar, S. S. (1980) *Atherosclerosis* **35**, 93–102

Roughley, P. J., Murphy, G. & Barrett, A. J. (1978) *Biochem. J.* **169**, 721–724

Ryhanen, L., Rantala-Ryhanen, S., Tan, E. M. L. & Uitto, J. (1982) *Collagen Relat. Res.* **2**, 117–130

Sakamoto, S., Sakamoto, M., Matsumoto, A., Nagayama, M. & Glimcher, M. J. (1981) *Biochem. Biophys. Res. Commun.* **103**, 339–346

Sellers, A. & Woessner, J. F., Jr. (1980) *Biochem. J.* **189**, 521–531

Vater, C. A., Mainardi, C. L. & Harris, E. D., Jr. (1978) *J. Biol. Chem.* **254**, 3045–3053

Woessner, J. F., Jr. (1979) *Biochem. J.* **180**, 95–102

Yasui, N., Hori, H. & Nagai, Y. (1981) *Collagen Relat. Res.* **1**, 59–72