COLLAGENASE INHIBITION BY CATIONIC PROTEINS DERIVED FROM CARTILAGE AND AORTA

Klaus E. Kuettner, Judith Hiti, Reuben Eisenstein and Elvin Harper
Depts. of Orthopedic Surgery and Biochemistry and Pathology, Rush Medical College, Chicago, IL 60612 & Dept. of Chemistry, University of California at San Diego, La Jolla, CA 92093

Received July 1,1976

SUMMARY

Collagenolytic activity from normal human skin and human hypertrophic scar tissue are inhibited by protease inhibitors isolated by immobilized trypsin affinity chromatography from bovine cartilage and bovine aorta. The collagenase inhibitors are of low molecular weight (approximately 11,000) and are cationic proteins. Other trypsin inhibitors such as the bovine basic pancreatic trypsin inhibitor (Kunitz) as Trasylol^{R} and soybean trypsin inhibitor show only minimal effects on collagenase activities. Human skin collagenase is inhibited by a protein (protease-inhibitor) obtained from human cartilage using human skin collagen as substrate. The inhibitors described may function in the physiological regulation of collagenase activity in connective tissues.

Little is known of collagenase regulation in terms of inhibition. The only "regulator" studied extensively, α2-macroglobulin, is an irreversible inhibitor of collagenase (1,2), so regulation may be mediated by other factors. It is well known that collagen turnover takes place in most tissues (3-7) and recent work has demonstrated cationic protease inhibitors in connective tissues (8). The question arose as to the role of these inhibitors in the in vivo regulation of connective tissue turnover in general and in collagen metabolism specifically. We present here preliminary data demonstrating that protease inhibitors isolated and purified from cartilage and aorta are capable of inhibiting human collagenases.

MATERIALS

^{14}C-glycine labeled guinea pig skin collagen was isolated as previously described (9). In brief, ^{14}C uniformly labeled glycine (25uCi) was injected into growing 150-200 gram guinea pigs at zero time. Another dose was given after 3 hours and the animals sacrificed by sodium pentobarbital administration 3 hours later. The collagen was immediately extracted with 0.05 M Tris-1.0M NaCl pH 7.4, at 4° and purified by repeated salt precipitation. The final precipitate was dialyzed against 0.01M acetic acid and lyophilized.

Human skin collagen was prepared by extraction with 0.5M acetic acid for 7 days without pepsin and subsequently precipitated with 7% NaCl according to the methods of Miller (10) and Epstein (11).

Human skin collagenases from normal skin and hypertrophic scar tissue were isolated by the method of Eisen and Gross (12). Bacterial collagenase (CLS-III) and trypsin (2x recrystalized) were purchased from Worthington.

INHIBITORS

Bovine cartilage protease inhibitor was prepared by extracting 1mm slices of bovine scapula cartilage with 1M guanidinium hydrochloride (GuHCl) for 24 hours at 4° (1g/10ml). The extract was decanted from the tissue and the GuHCl concentration raised to dissociative conditions (3M) by adding solid GuHCl. The extract was then ultrafiltered on an Amicon XM-50 membrane (MW cut-off 50,000) and the filtrate desalted in a Spectrapor #3 membrane (3,000 MW exclusion). The low molecular weight material underwent affinity chromatography on insoluble trypsin as previously described (13) yielding a single protein as examined on SDS polyacrylamide gel electrophoresis. Chromatography on Sephadex G-75 in 3M GuHCl yielded a lower molecular weight material than lysozyme which has a molecular weight of approximately 14,000 (14). Our current data indicate a molecular weight of approximately 11,000 for the inhibitor. It has been previously shown that this material acts as a trypsin inhibitor (13). On cellulose acetate electrophoresis (pH 8.4) this protein migrates in the electrophoretic field as a cationic protein similar to lysozyme which has an isoelectric point of about 11 (15).

Identical extraction methods were applied to human distal femoral articular cartilage obtained after therapeutic surgery of a 32 year old male. An inhibitor with similar properties was isolated from bovine aorta under the same conditions. Bovine basic pancreatic trypsin inhibitor (Kunitz) as Trasylol^{R}, was kindly provided by Dr. Ernst Truscheit, Bayer Pharmaceutical Company, Germany. Soybean trypsin inhibitor was purchased from Mann. Bovine cartilage lysozyme was isolated and purified as previously described (16).

METHODS

MEASUREMENT OF COLLAGENASE ACTIVITY

The ^{14}C-gycine peptide release assay was employed (17). In brief, a 100ul aliquot of ^{14}C-glycine guinea pig skin collagen (1200-1600 cpm) in 0.4 M NaCl was placed in a plastic microtube (400ul maximum capacity) and incubated for 16-24 hours at 37°. The reaction mixtures (300ul) were pre-incubated at 22° for 30 minutes and subsequently 200ul pipetted into the pre-formed gels. Each mixture was suspended with the aid of a Vortex Mixer and incubated at 37° for 4 hours. The reaction was terminated by centrifugation in a Beckman Spinco 152 Microfuge (5min, 1500 rpm). A 200ul aliquot of the supernate was added to 10ml of Bray's Solution containing 4% Cab-O-Sil and counted in an automatic liquid scintillation counter.

Polyacrylamide gel electrophoresis

Using the method of Nagai et al. (18), quantitative determinations of the collagenase reaction products separated by polyacrylamide gel electrophoresis were made by densitometric evaluation. Protein was determined by the method of Warburg and Christian (19).

RESULTS

Preliminary experiments showed that tadpole and human skin collagenases behaved similarly with regard to their susceptibility to inhibitors. Cartilage lysozyme had no effect as a collagenase inhibitor, whereas, Trasylol and soybean trypsin inhibitor were slightly inhibitory. Substantial collagenase inhibition, however, was observed with the two inhibitors isolated from bovine cartilage and bovine aorta.

Quantitative determination of the inhibitory effect of these proteins was carried out by the use of the ^{14}C-glycine peptide release assay and polyacrylamide gel electrophoresis. As can be seen from Table 1, the preparations isolated from bovine cartilage and bovine aorta are effective inhibitors of human skin collagenase. To determine the lower limitations on cartilage and aortic inhibitor we observed a 35% inhibition with 5ug cartilage inhibitor and a 24% inhibition with 8ug of the aortic inhibitor in the system described. Trasylol, which acts as a potent trypsin inhibitor, showed little effect on the collagenase system examined.

Table 1. HUMAN SKIN COLLAGENASE* ACTIVITY IN THE PRESENCE OF PROTEASE INHIBITORS

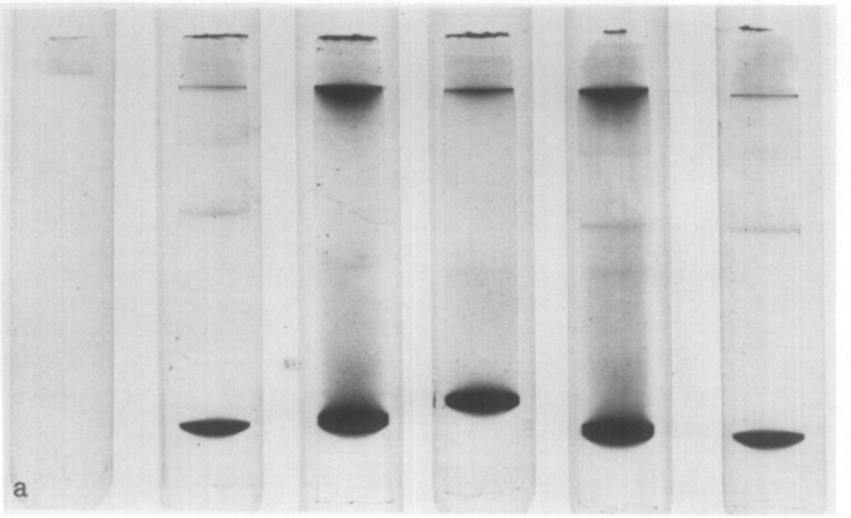
REACTION MIXTURE[a,b]	ACTIVITY IN CPM [^{14}C][c]	PER CENT INHIBITION
ENZYME WITH BUFFER, 0.05 M TRIS-HCl, 0.005 M $CaCl_2$, pH 7.4, (CONTROL)	654	—
ENZYME WITH 25µg BOVINE CARTILAGE INHIBITOR	0	100.0
ENZYME WITH 5 µg BOVINE CARTILAGE INHIBITOR	415	34.8
ENZYME WITH 20 µg BOVINE AORTA INHIBITOR	3	99.5
ENZYME WITH 8 µg BOVINE AORTA INHIBITOR	497	24.4
ENZYME WITH 188 µg TRASYLOL	559	15.5
ENZYME WITH 100 µg SOYBEAN TRYPSIN INHIBITOR (5 TIMES RECRYSTALLIZED)	536	18.0

a) REACTION MIXTURE CONSISTED OF A TOTAL VOLUME OF 300 MICROLITERS. 200 MICROLITERS OF ENZYME PLUS BUFFER AND/OR INHIBITOR WAS ADDED TO 100 MICROLITERS OF A PREFORMED RADIOACTIVE COLLAGEN GEL.

b) ALL PROTEIN CONCENTRATIONS WERE DETERMINED BY THE WARBURG AND CHRISTIAN OD_{280}/OD_{260} METHOD.

c) EXPRESSED AS CPM MINUS TRYPSIN CONTROL; 100 µg OF BACTERIAL COLLAGENASE (Cl. histolyticum) RELEASED 948 CPM OF A TOTAL 1070 CPM PER ASSAY TUBE.

*) PREPARED FROM HUMAN HYPERTROPHIC SCAR TISSUE, 160 µg PER ASSAY.



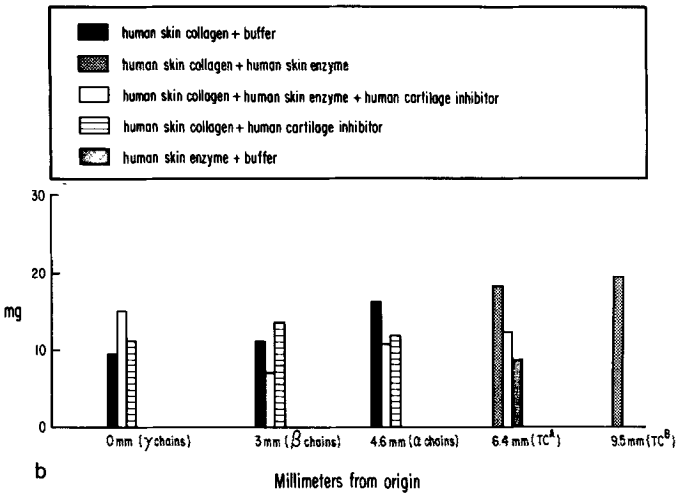


FIG. 1.a. Polyacrylamide gel electrophoresis data illustrating the effect of human cartilage inhibitor on collagenolytic activity from normal human skin on human skin collagen.

From left to right
1. Buffer alone
2. Human Skin Collagen + Buffer
3. Human Skin Collagen + Buffer + Human Skin Enzyme: Reaction products
4. Human Skin Enzyme + Buffer
5. Human Skin Enzyme + Human Skin Collagen + Buffer + Human Cartilage Inhibitor
6. Human Cartilage Inhibitor + Buffer + Human Skin Collagen

FIG. 1b. Quantitative densitometric evaluation of gels pictured in Fig. 1.

As seen in Figure 1, the polyacrylamide gel electrophoresis data illustrate the degradation of human abdominal skin collagen by normal human collagenase yielding typical reaction products. The addition of the inhibitor derived from human cartilage prevents collagenolysis, preserving the characteristic collagen banding pattern, as observed in Figure 1 and as shown in the quantitative densitometric evaluation of the gels (Figure 1b). The inhibitor itself does not possess collagenolytic activity as can be seen from the gel pattern obtained.

DISCUSSION

Collagenase activity may be documented by the following criteria: release of ^{14}C-glycine peptides from undenatured collagen and characterization of the resulting reaction products by polyacrylamide gel electrophoresis. The studies described above demonstrate that two connective tissues, cartilage and aorta, contain low molecular weight cationic proteins which, according to the isolation procedure, act as a trypsin inhibitor and are also capable of inhibiting collagenase. Our data suggest that these connective tissues contain potent collagenase inhibitors which differ in their physical characteristics from the serum derived collagenase inhibitor, α_2-macroglobulin. This concept is supported by our observation that human cartilage also contains an inhibitor which acts on the human skin enzyme employing human skin collagen as a substrate.

The presence of collagenase inhibitors in connective tissues such as cartilage and aortic walls may be of physiological significance. These tissues are composed of a dense network of structural proteins interposed by high molecular weight proteoglycans. This matrix functions as a gel filtration medium by steric exclusion. Small ions or proteins of small molecular weight, however, can diffuse through the matrix of cartilage (20). A small molecular weight protein which might act as a collagenase inhibitor may, therefore, be physiologically important.

Our observation that cartilage lysozyme, another cationic protein present within the extracellular matrix, does not act as a collagenase inhibitor is not in accord with the findings of Sakomoto et al. (21). The semi-purified cartilage lysozyme used in their studies was provided by our laboratory and may have contained the cartilage inhibitor as an impurity. Commercially available egg white lysozyme may have been contaminated by ovomucoid, a trypsin inhibitor, suggesting the reason for their data.

Our observations may also lead to a re-interpretation of the results of Hook et al. (22) who demonstrated that if proteoglycans were extracted from cartilage with 4M GuHCl and then added to collagen before re-constituting fibrils to use as substrate, collagenolysis was inhibited. GuHCl extracts not only proteoglycans from cartilage but also the inhibitor. This cationic protein interacts with the highly anionic proteoglycans. The retardation of collagenolysis may therefore be due to the inhibitor rather than the "stabilization effect" of proteoglycan on the collagen structure.

Our findings noting the presence of a collagenase inhibitor in cartilage may also explain the fact that no cartilage collagenase has been detected so far. The turnover or anabolism of collagen in cartilage may therefore be under a strict control via an enzyme-inhibitor-complex, and a deficiency of inhibitor may be implicated in cartilage collagen degradation in disease processes such as rheumatoid arthritis.

ACKNOWLEDGEMENTS

We wish to thank Mrs. Alice Croxen, Miss Susannne Sagartz and Mr. Richard Croxen for their skilled technical assistance, and Miss Verhonda Hearon and Ms. Margaret Brunette for preparation of the manuscript. This work was supported by the Hulbert Fund, Rush-Presbyterian-St. Luke's Medical Center, a grant of the American Heart Association, and in part by the General Research Support Grants RR-05477 and RR-08135 and by grant AM-16020 from the National Institutes of Health.

REFERENCES

1. Werb, Z., Burleigh, M. C., Barrett, A. J., and Starkey, P. M. (1974) Biochem. J., 142, 359-368.
2. Harpel, P. C. (1973) J. Exp. Med., 138, 508-521.
3. LaCroix, P. (1971) The Biochemistry and Physiology of Bone, Vol. 3, Second ed., editor G. H. Bourne, pp. 119-129, Academic Press, New York.

4. Numni, M. E., de Guia, E. and Bavetta, L. A. (1967) Biochem. J., 102, 143-147.
5. Woessner, J. F. Jr. (1962) Biochem. J., 83, 304-400.
6. Fullmer, H. M., Lazarus, G., and Gibson, W. A. (1966) Lancet, 1, 1007-1009.
7. Grillo, H. C. and Gross, J. (1967) Dev. Biol., 15, 300-317.
8. Kuettner, K. E., Croxen, R. L. Eisenstein, R. and Sorgente, N. (1974) Experientia, 30, 595-597.
9. Nagai, Y. Lapiere, C. M. and Gross, J. (1966) Biochemistry, 5, 3123-3130.
10. Epstein, E. H., Scott, R. D., Miller, E. J. and Piez, K. A. (1971) J. Biol. Chem., 246, 1718-1724.
11. Epstein, E. H. (1974) J. Biol. Chem., 249, 3225-3231.
12. Eisen, A. Z., Jeffrey, J. J. and Gross, J. (1968) Biochem. Biophys. Acta, 151, 637-645.
13. Sorgente, N. Kuettner, K. E., and Eisenstein, R. (1976) in Protides of the Biological Fluids, editor Peeters, H., Vol. 23, Pergamon Press, Oxford; in press.
14. Jolles, P. (1964) Angew. Chem. Int. Ed. Engl., 3, 28-36.
15. Jolles, P. (1969) Angew. Chem. Int. Ed. Engl.,8, 227-239.
16. Guenther, H. L., Sorgente, N., Guenther, H. E., Eisenstein, R. and Kuettner, K. E., (1974) Biochim. Biophys. Acta, 372, 321-334.
17. Harper, E., Bloch, K. J. and Gross, J. (1971) Biochemistry, 10, 3035-3041.
18. Nagai, Y., Gross, J. and Piez, K. A. (1964) Ann. N. Y. Acad. Sci., 121, 494-500.
19. Warburg, O. and Christian, W. (1941) Biochem. Z, 310, 384-421.
20. Laurent, T. C. (1970) Chemistry and Molecular Biology of the Intercellular Matrix, Vol. 2., pp. 703-732, Academic Press, New York.
21. Sakamoto, S., Sakamoto, and M. Goldhaber, P. (1974) Calcif. Tiss. Res., 14, 291-299.
22. Hook, C. W., Brown, S. I. and Ewanij, W. (1971) Invest. Ophtalmol., 10, 496-503.