A Specific Inhibitor of Vertebrate Collagenase Produced by Human Skin Fibroblasts*

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Normal human skin fibroblasts which synthesize procollagenase were shown also to produce a specific inhibitor of the active form of this proenzyme. The inhibitor was derived from the fibroblasts themselves, as demonstrated by the following criteria: 1) 3H-labeled inhibitor was produced when fibroblasts were exposed to a 3H-amino-acid mixture in serum-free culture medium; 2) fetal calf serum, when processed through the basic purification steps employed for the inhibitor, displayed no collagenase inhibitory activity.

Inhibitor was purified extensively from serum-free medium by a combination of cation exchange and gel filtration chromatography. Spectrophotometric scanning of polyacrylamide gels indicated that the inhibitor was greater than 95% pure. It had an apparent molecular weight of 31,000 as determined by sodium dodecyl sulfate-gel electrophoresis, and was remarkably heat-stable, retaining more than 90% of its activity after 20 min at 90°C. Inhibition was stoichiometric, a 1:1 molar ratio of inhibitor to enzyme being required for complete inhibition of collagenase activity. Attempts to demonstrate an enzyme-inhibitor complex were not successful; in fact, enzyme and inhibitor behaved independently of each other in a variety of chromatographic systems. Tight binding between active enzyme and inhibitor occurred only in the presence of the collagen substrate. It is therefore suggested that the inactive species is the ternary complex E·I·S.

Human fibroblast inhibitor was effective against all vertebrate collagenases tested. Noncollagenolytic proteases and collagenases of nonvertebrate origin were not inhibited. Procollagenase, which does not bind to collagen, also failed to bind the inhibitor. However, inhibitor itself was capable of binding to collagen.

The vertebrate collagenases form a class of enzymes capable of initiating the specific degradation of native collagen in the animal organism. Although specific collagenolytic enzymes have now been identified in numerous tissues from a wide variety of species, the nature of the processes which regulate vertebrate collagen degradation have not been clearly defined. The existence of naturally occurring inhibitors of collagenase activity in vivo has been recognized for some time, and it has been speculated that such inhibitors are involved in the regulation of collagenase activity in vivo.

The major collagenase inhibitors studied thus far have been identified in serum, and it is unknown whether they can exist and function within the interstices of an organized connective tissue. α2-Macroglobulin, the principal serum anti-collagenase, is a potent inhibitor of all vertebrate collagenases investigated to date (1, 2). Yet its large molecular weight (780,000) and irreversible mechanism of inhibition (2) raise doubt concerning its actual in vivo role in the regulation of collagen degradation at the tissue level. α1-Antitrypsin, although a major serum protease inhibitor, is relatively ineffective as an anti-collagenase (3). Furthermore, both α2-macroglobulin and α1-antitrypsin are nonspecific antiproteases, since they are capable of inhibiting a broad range of proteolytic enzymes of varying specificity and chemical characteristics. Recently, Woolley et al. have identified a β1-serum protein which appears to display the properties of a specific collagenase inhibitor (4, 5).

Tissue-derived collagenase inhibitors are more likely to be of functional physiologic significance in the regulation of connective tissue structure than inhibitors found principally in serum. Thus far, several such tissue-derived inhibitors have been reported, including an inhibitor extracted from rabbit tumor (6) and several small proteins of molecular weight less than 15,000 (7-12).

In this laboratory, cultures of normal human skin fibroblasts have been employed for the production, purification, and characterization of human procollagenase. This proenzyme can be converted to fully active enzyme by either incubation with trypsin, and resultant loss of a 10,000-dalton peptide, or via an autoactivation process without detectable change in molecular weight (13, 14). In addition to synthesizing a collagenasezymogen, the human fibroblasts were found to produce an inhibitor of this enzyme simultaneously (15). The present report describes studies on the purification, properties, and mechanism of action of human skin fibroblast collagenase inhibitor.

MATERIALS AND METHODS

Reagents—Acrylamide and bisacrylamide were purchased from Eastman. Sodium dodecyl sulfate, 99% pure, was obtained from Gallard-Schlesinger. Tris base, bovine pancreatic trypsin, bovine serum albumin, ovalbumin, and soybean trypsin inhibitor were procured from Sigma. Pepsin was purchased from Worthington. All other chemicals used were reagent grade.

Methods—Normal human skin fibroblasts (CRl 1157) were purchased from American Type Culture Collection. The cells were grown in glass roller bottles (1585 cm²), Belco) at 37°C in 50 ml of Dulbecco's Modified Eagle's Medium-HG + glutamine (Microbiological Associates), 0.05 μM Hepes* buffer (pH 7.6), 20% fetal calf serum,
and 200 units/ml of penicillin + 200 μg/ml of streptomycin. At visual confluence, the cells were washed with Hanks’ Balanced Salt Solution (Grand Island Biological Co.) and put through several cycles of serum-free medium for 45 to 47 h, as described previously by Bauer et al. (15). The harvested serum-free medium was made 0.05 M with Tris-HCl, pH 7.5, and concentrated 10-fold by vacuum dialysis using a hollow fiber device (MDA Scientific). The resulting concentrated medium was stored at -30°C.

Sources of Other Collagenases—Rat skin and rat uterus collagenases were obtained from tissue culture medium and partially purified by use of techniques previously described (16, 17). Pure collagenase from the hepatopancreas of the fiddler crab, Uca pugilator, was prepared by methods described by Eisen et al. (18). Bacterial collagenase from Clostridium histolyticum was purchased from Advanced Biofactors.

Activation of Procollagenases—The activation of procollagenase was accomplished protelytically at 25°C by the addition of trypsin for 10 min. A 4-fold molar excess of SBTI was then added to prevent any further trypsinic activity. A range of trypsin concentrations, usually from 0.1 to 5.0 μg/50-μl sample was used to ensure that maximal collagenase activity was achieved (15).

Assay Procedures—Collagenase activity and inhibitor activity were measured at 37°C using native reconstituted ['Clglycine-labeled collagen. The incorporation of 6.25 g/ml of dithiothreitol into the sample buffer. Fractions collected were added to active collagenase, then dialyzed, lyophilized, and stored at -70°C for approximately 24 h.

RESULTS

Human skin collagenase inhibitor could be highly purified from serum-free fibroblast culture medium. The use of cation exchange chromatography was the essential step in this purification process. As can be seen in Fig. 1, phosphocellulose bound both collagenase and its specific inhibitor. The inhibitor was eluted at approximately 0.1 M (NH₄)₂SO₄. Collagenase, a more cationic protein, was eluted at 0.2 M (NH₄)₂SO₄. The inhibitor-containing fractions (tubes 45 to 66) were pooled, dialyzed, lyophilized, and the product was applied to an Ultrogel AcA 44 column as shown in Fig. 2. On AcA 44, two major protein peaks remained, with the second peak containing all of the inhibitory activity. When the pooled inhibitor from the AcA-44 column (tubes 49 to 60) was concentrated and then subjected to gel filtration on Sephadex G-100, the inhibitor eluted as a nearly symmetrical protein peak (Fig. 3).

Fig. 4 illustrates the electrophoresis of highly purified human fibroblast inhibitor (Slot C) on SDS-polyacrylamide gels together with a number of protein standards of known molecular weight. The gel revealed an apparent molecular weight for the inhibitor of 31,000. Interestingly, this apparent molecular weight appeared to increase by approximately 1500 upon

![Fig. 1. Phosphocellulose chromatography. Serum-free fibroblast culture medium was prepared as described under “Materials and Methods” and applied at a flow rate of 60 ml/h to a column (2.5 x 20 cm) of phosphocellulose equilibrated with 0.05 M Tris-HCl, pH 7.5. The bound proteins were then eluted with a 800-ml linear gradient of 0.0 to 0.4 M (NH₄)₂SO₄ in 0.05 M Tris buffer. 7-ml fractions were collected and assayed for the presence of both collagenase and inhibitor activity on ¹⁴C-labeled collagen fibrils at 37°C. Auba, collagenase inhibitor; , collagenase. G represents initiation of the gradient.](http://www.jbc.org/)

![Fig. 2. Ultrogel AcA-44 chromatography. The inhibitor fractions from phosphocellulose (tubes 45 to 66) were pooled, dialyzed, lyophilized, and the product was then reconstituted in 0.05 M Tris-HCl, pH 7.4, and applied to an AcA-44 column equilibrated with this same buffer. Fractions collected were applied to active collagenase, then assayed on ¹⁴C-labeled collagen gels at 37°C to detect inhibitory activity. , Auba, collagenase inhibitor.](http://www.jbc.org/)
fibrils at 37°C to determine inhibitory activity. M, were added to active collagenase and assayed on [C-labeled collagen fractions from AcA-44 (tubes 49 to 60) were lyophilized, the product was resuspended in 0.05
0---0, collagenase inhibitor.

Column fractions were then assayed for inhibitor and none was found. Second, *H-labeled collagenase proteins were prepared by adding a *H-amino-acid mixture (New England Nuclear, NET-249) to serum-free culture medium. The *H-proteins produced were partially purified through phosphocellulose and compared electrophoretically with highly purified inhibitor. The slab gel was then applied to a photographic film. The autoradiographic results shown in Fig. 5 clearly establish that the protein band corresponding to the purified inhibitor had indeed been labeled during exposure of the cells to *H-amino-acids. Finally, when anti-whole bovine serum was reacted against crude, concentrated serum-free fibroblast medium by Ouchterlony gel diffusion, no reaction was noted (not shown). Therefore, we conclude that this inhibitor is a fibroblast cell product.

The specificity of human fibroblast inhibitor was investigated against a number of collagenases and noncollagenolytic proteases (Table I). Significant inhibition of human skin, rat skin, and rat uterus collagenases was seen, but little or no inhibition of either the clostridial or crustacean collagenase was noted. Furthermore, no inhibition was observed when trypsin or chymotrypsin were employed as enzyme sources. The inhibitor, therefore, appears to be specific for and effective against vertebrate collagenases only.

The relationship between collagenase and its inhibitor is complex. When either inhibitor was titrated with increasing enzyme (Fig. 6) or enzyme titrated with increasing inhibitor (Fig. 7), a sigmoidal relationship was seen. In Fig. 7, 2.0 pg of pure procollagenase, having a molecular weight of 57,500 (14), was trypsin-activated and then titrated with increasing amounts of highly purified inhibitor, apparent molecular weight 31,000. Complete inhibition of the fibroblast collagenase was attained at a molar ratio of inhibitor to enzyme of approximately 1:1. Similar calculations at 50% inhibition showed an inhibitor to enzyme ratio of 0.5:1.0, indicating that at 50% inhibition, all inhibitor present was bound to enzyme and none remained free in solution. Therefore, the enzyme-inhibitor interaction appeared to be characterized by tight binding.

However, when a fully inhibited pure enzyme-inhibitor mixture was subjected to gel filtration or ion exchange chromatography, using the same conditions as described under "Materials and Methods" for purification of the inhibitor, no evidence of a higher molecular weight complex was seen. This suggested that to produce the tight binding characteristics evidenced by the above titration curves, all three proteins, enzyme, inhibitor, and the substrate collagen, were required to be present.

In order to assess the possibility that the inhibitor does indeed bind to collagen, a constant amount of inhibitor was incubated with increasing amounts of fibrillar collagen (Fig. 8). Following centrifugation, both supernatants and collagen precipitates were assayed separately for inhibitor. As the
amount of collagen was increased in each sample, increasing inhibitor was bound to the collagen substrate and decreased inhibitory activity remained in the supernatant.

The relationship of collagenase proenzyme to inhibitor was investigated, and the results are shown in Fig. 9. The solid lines represent solutions prepared containing identical concentrations of total enzyme protein but varying percentages of active enzyme (trypsin activated or autoactivated). The remainder of each solution was proenzyme. The dashed line shows a separate solution of fully active enzyme, but containing only one-half of the total enzyme protein of the other samples. These solutions were then titrated with increasing inhibitor. As can be seen, the amount of inhibitor required

| Collagenase | Inhibitor | Counts per min (above blank) | Per cent inhibition |
|-------------|-----------|------------------------------|---------------------|
| Human fibroblast | -         | 760                          | 73                  |
|             | +         | 205                          |                     |
| Rat skin    | -         | 138                          | 59                  |
|             | +         | 57                           |                     |
| Rat uterus  | -         | 873                          | 41                  |
|             | +         | 519                          |                     |
| Crab hepatopancreas | -   | 1206                         | 7                   |
|             | +         | 1118                         |                     |
| Cl. histolyticum | -     | 243                          | 0                   |
|             | +         | 273                          |                     |

**Fig. 6.** Inhibitor versus increasing enzyme. 100-μl aliquots of inhibitor were titrated with increasing amounts of partially purified collagenase on 14C-labeled collagen gels at 37°C.

**Fig. 7.** Pure enzyme versus increasing purified inhibitor. Two micrograms of pure procollagenase was trypsin-activated as described under “Materials and Methods” and then titrated with increasing amounts of highly purified inhibitor on 14C-labeled collagen fibrils at 37°C. The arrows indicate the points of 50% and 95% (complete) inhibition. Complete inhibition occurred at a molar ratio of inhibitor to enzyme of 1:1.
was a function only of the amount of active enzyme in the sample. Thus, the curve produced by an enzyme solution containing 57% active enzyme and 43% proenzyme was similar to the inhibition curve for the enzyme solution which was fully active but contained only 50% of the total enzyme protein of the other samples. Therefore, proenzyme forms do not appear to compete with the active enzyme for inhibitor.

**DISCUSSION**

Human skin fibroblast collagenase inhibitor is a tissue-derived protein which is specific for vertebrate collagens. Noncollagenolytic proteases failed to be inhibited, as were two collagenases of non-vertebrate origin (Table 1). The inhibitor has been purified extensively from serum-free culture medium, and is greater than 95% pure, as assessed by spectrophotometric scanning of polyacrylamide gels. It has a molecular weight of approximately 31,000 and is remarkably heat-stable, retaining more than 60% of its activity after 20 min at 90°C.

Of particular interest is the nature of the interaction of fibroblast inhibitor with human skin collagenase. Our results suggest that inhibition is stoichiometric, approximately a 1:1 molar ratio being required for complete inhibition of collagenase activity. The results of enzyme-inhibitor titrations are indicative of a tight binding of enzyme to inhibitor (24); yet attempts to demonstrate the existence of a complex between inhibitor and collagenase have been unsuccessful. Inhibitor and enzyme appear to behave as independent entities in the presence of each other. Hence, it is reasonable to assume that the tight binding required by kinetics occurs only in the presence of the collagen substrate, and that the inactive species is the ternary complex E-I-S. To our knowledge, this is the only example of a protease-antiprotease system in which a stable enzyme-inhibitor complex does not form in the absence of substrate. It is clear from the experiment depicted in Fig. 8 that the inhibitor itself is capable of binding to collagen, and it is possible that formation of the inactive complex is mediated by the precise, specific binding of both enzyme and inhibitor to the substrate. The ultimate fate of these enzyme-inhibitor-collagen complexes is unknown. It is interesting in this regard that both trypsin-activated and autoactivated enzyme compete effectively for inhibitor in the presence of the collagen substrate, but procollagenase, which does not bind to collagen (14), also fails to bind the inhibitor.

It should be noted that Reynolds and co-workers (25) have postulated a mechanism for the physiologic activation of vertebrate collagenases which involves the removal of an inhibitor from a stable, inactive enzyme-inhibitor complex. As discussed above, no evidence exists for such a complex between human skin fibroblast collagenase and the inhibitor described in these studies. The inhibitor produced by the fibroblasts appears to be destined to participate in the inactivation of collagenase, rather than its activation.

The results presented in this communication provide the first evidence for the existence of an inhibitor of a collagenolytic enzyme synthesized by the same cell type that produces the collagenase itself, and indicate that certain tissues may well have the ability to regulate their own collagenase activity. It is not known at this time whether the same cell can simultaneously produce both collagenase and its inhibitor, or whether different populations of cells exist within a culture, some synthesizing enzyme, others producing the inhibitor. In typical fibroblast cultures, collagenase levels appear to be considerably higher than levels of inhibitor, as activatable collagenase can always be observed in crude, serum-free culture medium, even though inhibitor is present. All fibroblast cultures examined to date have yielded detectable amounts of inhibitor, but different cell lines, all derived from normal human skin, vary markedly in the amount of inhibitor produced. In contrast, explants of human skin in culture have consistently failed to yield detectable levels of an inhibitor of the kind described here. The reason for the absence of inhibitor in this organized tissue is unknown, but may be related to the observation that the explants undergo massive collagen resorption in culture. It is possible that under these conditions production of inhibitor does not occur. Delineation of the pathways by which cells and tissues may regulate levels of collag enase inhibitor will be of great value in our attempts to understand the mechanisms whereby an organized connective tissue maintains its three-dimensional structure.

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**REFERENCES**

1. Eisen, A. Z., Bloch, K. J., and Sakai, T. (1970) J. Lab. Clin. Med. 75, 258-263

2. T. J. Koob, A. Z. Eisen, and J. J. Jeffrey, unpublished.
2. Werb, Z., Burleigh, M. C., Barrett, A. J., and Starkey, P. M. (1974) Biochem. J. 139, 359–368
3. Eisen, A. Z., Bauer, E. A., Stricklin, G. P., Seltzer, J. L., Koob, T. J., and Jeffrey, J. J. (1977) in Cholesteatoma: First International Conference (McCabe, B. F., ed) pp. 115–123, Aesculapius Publishing Co., Birmingham, AL
4. Woolley, D. E., Roberts, D. R., and Evanson, J. M. (1975) Biochem. Biophys. Res. Commun. 66, 747–754
5. Woolley, D. E., Roberts, D. R., and Evanson, J. M. (1976) Nature 261, 325–327
6. McCroskery, P. A., Richards, J. F., and Harris, E. D., Jr. (1975) Biochem. J. 152, 131–142
7. Kuettnern, K. E., Hiti, J., Eisenstein, R., and Harper, E. (1976) Biochem. Biophys. Res. Commun. 72, 40–46
8. Horton, J. E., Wezeman, F. H., and Kuettnern, K. E. (1978) Science 199, 1342–1344
9. Hiti, J., Wohl, H., and Harper, E. (1978) Science 199, 991–992
10. Shinkai, H., Kawamoto, T., Hori, H., and Nagai, Y. (1977) J. Biochem. (Tokyo) 81, 261–263
11. Shinkai, H., and Nagai, Y. (1977) J. Biochem. (Tokyo) 81, 1261–1268
12. Nagai, Y., Shinkai, H., and Ninomiya, Y. (1978) Proc. Jpn. Acad. 54B, 140–144
13. Stricklin, G. P., Bauer, E. A., Jeffrey, J. J., and Eisen, A. Z. (1977) Biochemistry 16, 1607–1619
14. Stricklin, G. P., Eisen, A. Z., Bauer, E. A., and Jeffrey, J. J. (1978) Biochemistry 17, 2331–2337
15. Bauer, E. A., Stricklin, G. P., Jeffrey, J. J., and Eisen, A. Z. (1975) Biochem. Biophys. Res. Commun. 64, 232–240
16. Jeffrey, J. J., and Gross, J. (1970) Biochemistry 9, 268–273
17. Tokoro, Y., Eisen, A. Z., and Jeffrey, J. J. (1972) Biochim. Biophys. Acta 238, 289–302
18. Eisen, A. Z., Henderson, K. O., Jeffrey, J. J., and Bradaishaw, R. A. (1973) Biochemistry 12, 1814–1822
19. Nagai, Y., Lapiere, C. M., and Gross, J. (1966) Biochemistry 5, 3123–3130
20. Groves, W. E., Davis, F. C., Jr., and Sells, B. (1968) Anal. Biochem. 22, 195–210
21. Kunz, M. (1947) J. Gen. Physiol. 30, 291–320
22. Fairbanks, G., Strock, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2566–2617
23. Bonner, M. W., and Lasky, R. A. (1974) Eur. J. Biochem. 46, 83–88
24. Bieth, J. (1974) Proteinase Inhibitors: Bayer-Symposium V, pp. 463–490, Springer-Verlag, Berlin
25. Sellers, A., Cartwright, E., Murphy, G., and Reynolds, J. J. (1977) Biochem. J. 163, 303–307
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J. Biol. Chem. 1979, 254:1938-1943.