COLLAGENASE PRODUCTION BY HUMAN SKIN FIBROBLASTS

Eugene A. Bauer, George P. Stricklin, John J. Jeffrey

and Arthur Z. Eisen

Division of Dermatology, Department of Medicine
Washington University School of Medicine
St. Louis, Missouri 63110

Received March 18,1975

SUMMARY

Normal human skin fibroblasts, when cultured in serum free medium, produce collagenase in an inactive form. The enzyme in the crude medium can be activated by a brief preincubation with trypsin or by autoactivation. Once activated, the fibroblast collagenase is identical in its mechanism of action to human skin collagenase obtained from organ cultures. In addition, an inhibitor of collagenase is also present in the medium of fibroblast cultures. The inhibitor appears to be produced by the cells and its molecular weight is slightly higher than that of the enzyme. The presence of this inhibitor may account for previous inability to detect collagenase in human skin fibroblast cultures. It is also possible that some of the inactive enzyme exists in the medium in the form of a pro-enzyme.

INTRODUCTION

The definition of the exact mechanisms by which human and animal cells regulate the production of collagenase is an ultimate goal in the study of connective tissue dynamics. Collagenase is an enzyme of crucial importance to the function of mammalian organ systems (1,2) in that it stands at a unique point in collagen metabolism; it is the specific enzyme required for the initiation of collagen degradation.

Human skin in organ culture has served previously as an important source of collagenase in short term experiments (3). However a system, which can be maintained for longer periods of time and be subjected to well defined changes, is essential if the various parameters involved in the control of collagenase biosynthesis are to be adequately examined in both

---

Abbreviations: Tris-$CaCl_2$ buffer, 0.05 M Tris-HCl (pH 7.5) with 0.005 M $CaCl_2$; SBTI, soybean trypsin inhibitor.

normal and diseased states. Cell cultures have proven to be an ideal system for studying a variety of other enzyme systems (4) and initial studies in this laboratory employing fibroblasts in culture succeeded in localizing collagenase in human skin fibroblasts (5,6) using immunohistochemical techniques.

Despite the immunologic evidence for the presence of collagenase, we were unable to detect enzymatic activity in the fibroblast culture medium. This was attributed to the fact that the culture medium was rich in whole serum, a potent inhibitor of human skin collagenase (7,8). Indeed, Werb and Burleigh (9), by briefly withdrawing serum from the culture medium of rabbit synovial fibroblasts, were able to demonstrate collagenase activity in the culture medium of those cells. More recently, Werb and Reynolds (10) obtained increased production of both collagenase and a neutral protease by rabbit synovial fibroblasts by stimulating the cells with latex particles, concluding that it was unlikely that conversion of a precursor to active enzyme was responsible for the increased activity.[a] The failure to detect collagenase in human skin fibroblast cultures, even in the absence of serum, could be due to the fact that extremely small amounts of the enzyme are secreted from the cells or that the enzyme is secreted into the medium in an inactive form.

This report describes the detection of collagenase in cultures of normal human skin fibroblasts. The enzyme exists in an inactive form in the medium and when activated is identical to the collagenase isolated from short term organ cultures of human skin (3).

METHODS

Human skin fibroblasts. Primary cell lines were initiated from a 3 mm skin punch biopsy obtained from healthy volunteers (11) or were purchased from the American Type Culture Collection (Rockville, Md.). Cells were passed into glass roller bottles (1585 cm^2, Bellco) and grown in

---

[a]Latex stimulation has also been employed to induce collagenase production by human cells (W.S. Sly and Z. Werb, personal communication).

Dulbecco's Modified Eagle's Medium - HG + glutamine (GIBCO) with 0.03 M HEPES buffer (pH 7.6), 20% fetal calf serum and 200 units of penicillin and 200 μg of streptomycin/ml at 36°C. At confluence (5-10 x 10^7 cells) serum-containing medium was removed, cells were washed 4 times with 100 ml of Hank's Balanced Salt Solution (GIBCO) and cultures were maintained in 100 ml of the serum-free medium for 48-96 hr.

Preparation of fibroblast collagenase. Crude, serum-free culture medium, concentrated about 20-fold by a vacuum dialysis at 4°, was dialyzed against Tris-$CaCl_2$ buffer and was assayed for collagenase activity either alone or after preincubation with trypsin to detect latent activity. The optimum conditions of preincubation were determined using 0.1-20 μg of trypsin (Sigma Chemical) for time periods of 0.5-20 min at both 25° and 37°. In each case, preincubation was followed by the addition of at least a 5-fold molar excess of SBTI (Sigma Chemical) and the entire mixture was assayed on radioactive collagen fibrils as described below. Gel filtration was carried out on a column (1 x 40 cm) of Sephadex G-150 equilibrated with Tris-$CaCl_2$ buffer with 0.1 M NaCl at 4°. Column calibration for determining molecular weight was done according to Andrews (12,13).

Assays. Collagenase activity was measured at 37° by using native, reconstituted ^{14}C-glycine labeled collagen fibrils as a substrate (14) or at 27° by determining the specific viscosity of collagen in solution as a function of time in Ostwald viscometers having flow times for water of 26-35 sec (3). Thermally denatured collagen-enzyme reaction mixtures were subjected to electrophoresis in polyacrylamide gels (15). Trypsin activity was determined by measuring the rate of hydrolysis of Tos-Arg-OMe at 25° (16). Protein was determined by the method of Lowry et al. (17). Purified alpha$_1$-antitrypsin (18) was the gift of Dr. Jo Seltzer.

RESULTS AND DISCUSSION

Crude culture medium from human skin fibroblasts contained almost undetectable levels of active collagenase during a 6-hr assay period. However, brief preincubation with trypsin markedly increased enzyme activity. The optimum conditions for the activation of one such preparation of culture medium are presented in Table I. Concentrated culture medium (43 μg) was maximally activated by preincubation for 5 min with 2 μg of trypsin at 37° and under these conditions collagenase activity was increased approximately 220-fold. Higher concentrations of trypsin or longer preincubation times destroyed some of the enzyme activity.

In some cultures, unactivated crude medium, which displayed no collagenase activity after a 6-hr incubation at 37°, completely degraded the collagen substrate in the ensuing 12 hr, indicating that an apparent autoactivation process was occurring. Once activated, fibroblast collagenase was identical in its mechanism of action to the enzyme produced by

Table I

Effect of Preincubation Conditions on Collagenase Activity

Preincubation Temperature	Trypsin (μg)	Preincubation Time (5 min)	(10 min)	(20 min)
		Collagenase Activity[a]		
25°	0	37	4	20
	0.5	14	25	32
	1	16	255	34
	2	ND[b]	898	1338
	5	583	1416	1301
	10	858	1708	1286
	20	1301	1653	1263
37°	0	11	20	36
	0.5	18	ND	0
	1	0	0	17
	2	2431	2291	1864
	5	1481	ND	1647
	10	1630	1815	1404
	20	1683	2078	1229

Crude culture medium (43 μg) was preincubated with varying concentrations of trypsin for 5, 10 or 20 min at either 25° or 37° after which the trypsin was inhibited by a 5-fold molar excess of SBTI for 10 min. This reaction mixture was then incubated with 200 μg native ^{14}C-glycine labeled collagen fibrils (3932 cpm/substrate) for 45 min at 37°. A trypsin blank (20 μg) released 10.1% of the total counts.
[a]Collagenase activity, cpm above trypsin blank
[b]ND, assay not done

human skin explants (3) as assessed by examining the products of the reaction of the enzyme and collagen in solution at 27° (Fig. 1). These results indicate that normal human skin fibroblasts secrete collagenase into serum free culture medium and that the enzyme is a normal gene product of these cells.

The kinetics of trypsin-dependent collagenase activation are shown in Fig. 2. Activation was dependent on trypsin concentration as well as time and temperature of preincubation. For any given preincubation time there was a critical concentration of trypsin below which virtually no

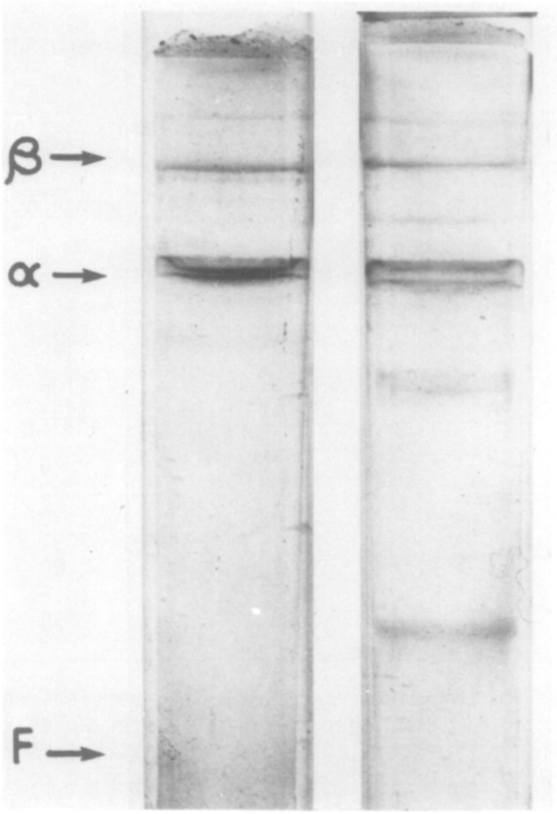

Fig. 1. Acrylamide gel electrophoresis of fibroblast collagenase-collagen reaction mixture after thermal denaturation. On the left is the 0 time reaction mixture; on the right, after a 41% reduction in specific viscosity, note the appearance of newly formed α^A and α^B bands.

activation occurred (Fig. 2A). In addition, at any given trypsin concentration, there was typically a lag period before activation began, which could be shortened by preincubation with higher trypsin concentration (Fig. 2B) or by increasing the preincubation temperature to 37° (Table I). These kinetics could be consistent either with conversion of proenzyme to enzyme (19,20) or with a time-dependent degradation of an inhibitor in the medium (20).

In an effort to elucidate the activation process, inactive crude fibroblast culture medium was subjected to gel filtration on Sephadex G-150

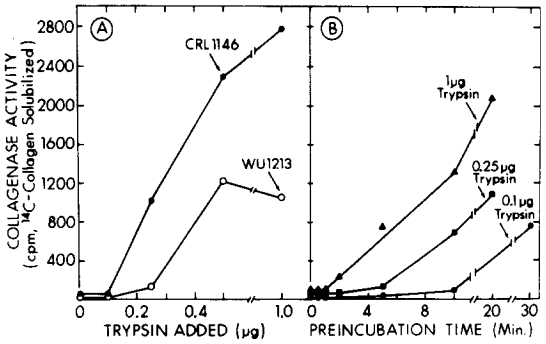

Fig. 2. Kinetics of trypsin-dependent activation of fibroblast collagenase. (A) Preincubation was carried out at 25° for 5 min using varying amounts of trypsin and 25 μg (CRL 1146) or 17 μg (WU 1213) of crude culture medium. After the addition of 10 μg SBTI, the entire mixture was assayed with 200 μg ^{14}C-labeled collagen fibrils (3506 cpm/substrate) for 90 min at 37°. (B) 17 μg crude medium (WU 1213) was preincubated with varying amounts of trypsin for varying time at 25° after which collagenase activity was assessed as in "A".

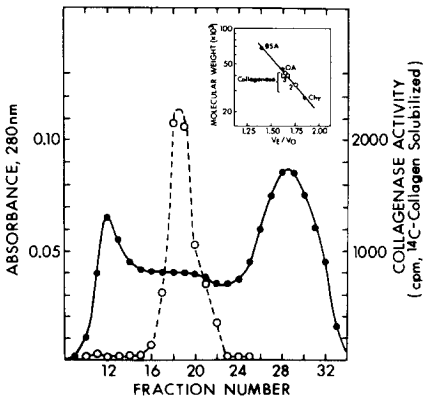

Fig. 3. Gel filtration of fibroblast culture medium on Sephadex G-150. A sample containing 0.44 mg/ml protein was applied to a column (1 x 40 cm) at 4° and effluent fractions of 1.3 ml were collected at a rate of 3.9 ml/hr. ●—●, absorbance at 280 nm; O---O, collagenase activity. (Insert) Calibration of Sephadex column for molecular weight determination. BSA, bovine serum albumin; OA, ovalbumin; Chy, chymotrypsinogen. 1,2 and 3 refer to 3 different filtration procedures to determine the approximate molecular weight of fibroblast collagenase.

(Fig. 3). Fractions were assayed for trypsin-dependent collagenase activity and for inhibitory activity. A single peak of enzyme activity was detected at a position corresponding to a molecular weight of approx-

imately 40,000 daltons (Fig. 3, insert). The enzyme detected on the column retained its ability to autoactivate and was trypsin activatable. Of particular interest was the detection of an inhibitor of collagenase which emerged from the column at a slightly higher molecular weight than the enzyme (Fig. 3, fractions 15-17). The inhibitor abolished over 90% of the collagenase activity, while displaying virtually no inhibition of trypsin (Table II). Conversely, purified alpha$_1$-antitrypsin, a serum inhibitor of approximately the same size, in a 2-fold excess by weight, completely failed to inhibit fibroblast collagenase (Table II, Exp. IV).

Table II

Comparison of Enzyme-Inhibitory Effects of the Medium-derived Inhibitor and Alpha$_1$-antitrypsin

Exp.	Inhibitor Protein	(μg)	Enzyme Protein	(μg)	Enzyme Activity	Inhibition (%)
I	G-150 Fractions 15-17	0	Collagenase	8.7	1333[a]	0
		3.0		8.7	168	87.4
		7.5		8.7	78	94.1
II	G-150 Fractions 15-17	0	Collagenase	16.0	547[a]	0
		3.0		16.0	166	69.7
		6.0		16.0	60	89.7
III	G-150 Fractions 15-17	0	Trypsin	3.0	18625[b]	0
		3.0		6.0	17500	5.5
		3.0		3.0	16319	12.4
		6.0		3.0	17290	7.2
IV	Alpha$_1$-at	0	Collagenase	9.9	1529[a]	0
		3.0		9.9	1647	0
		6.0		9.9	1601	0
		9.7		9.9	1479	3.3
		19.3		9.9	1601	0
V	Alpha$_1$-at	0	Trypsin	3.0	16000[b]	0
		3.0		6.0	10000	37.4
		3.0		3.0	300	98.1

Aliquots of previously activated fibroblast collagenase (CRL 1146) or trypsin were incubated with increasing quantities of either medium-derived inhibitor (Sephadex G-150 fractions 15-17) or alpha$_1$-antitrypsin (alpha$_1$-at). These mixtures were then assayed for collagenase activity as described in Fig. 2 or for trypsin activity using Tos-Arg-OMe.

[a]Collagenase activity, cpm above blank
[b]Trypsin activity, units x 10^3

Additionally, the fractions containing inhibitory activity were devoid of material reacting with anti-bovine whole serum in gel diffusion. These results suggest strongly that the inhibitor is derived from the fibroblasts rather than from residual serum in the medium.

The major features, therefore, of the fibroblast culture system are that the collagenase exists in an inactive form and that a potent inhibitor of the enzyme is also present in the medium. The inhibitor may explain the inability to detect collagenase activity in human fibroblast cultures (6). The presence of trypsin-activatable material after partial separation of the enzyme from the inhibitor by gel filtration indicates that a zymogen may also be present, as suggested for other vertebrate collagenases (21-25).

ACKNOWLEDGMENTS

This was supported by USPHS Grants AM 12129, AM 05611 and HD 05291. Dr. Bauer is the recipient of Research Career Development Award 1 K04 AM 00077 from the National Institutes of Health. Our appreciation goes to Mr. William Fiehler for his excellent technical assistance.

REFERENCES

1. Eisen, A.Z., Bauer, E.A. and Jeffrey, J.J. (1970) J. Invest. Derm. 55, 359-373.
2. Harris, E.D. and Krane, S.M. (1974) New Eng. J. Med. 291, 557-563, 605-609, 652-661.
3. Eisen, A.Z., Jeffrey, J.J. and Gross, J. (1968) Biochim. Biophys. Acta 151, 637-645.
4. Pollack, R., Ed. (1973) Readings in Mammalian Cell Culture, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
5. Reddick, M.E., Bauer, E.A. and Eisen, A.Z. (1973) Clin. Res. 21, 481A.
6. Reddick, M.E., Bauer, E.A. and Eisen, A.Z. (1974) J. Invest. Derm. 62, 361-366.
7. Eisen, A.Z., Bloch, K.J. and Sakai, T. (1970) J. Lab. Clin. Med. 75, 258-263.
8. Eisen, A.Z., Bauer, E.A. and Jeffrey, J.J. (1971) Proc. Nat. Acad. Sci. 68, 248-251.
9. Werb, Z. and Burleigh, M.C. (1974) Biochem. J. 137, 373-385.
10. Werb, Z. and Reynolds, J.J. (1974) J. Exp. Med. 140, 1481-1497.
11. Boyle, J.A. and Seegmiller, J.E. (1971) in Meth. Enzymol., W.B. Jakoby, Ed., Vol. 22, pp. 161-165, Academic Press, New York.
12. Andrews, P. (1964) Biochem. J. 91, 222-233.
13. Andrews, P. (1965) Biochem. J. 96, 595-606.
14. Nagai, Y., Lapiere, C.M. and Gross, J. (1966) Biochemistry 5, 3123-3130.

15. Nagai, Y., Gross, J. and Piez, K.A. (1964) Ann. N.Y. Acad. Sci. 121, 494-500.
16. Hummel, B.C. (1959) Can. J. Biochem. Physiol. 37, 1393-1399.
17. Lowry, O., Rosebrough, N., Farr, A. and Randall, R. (1951) J. Biol. Chem. 193, 265-275.
18. Pannell, R., Johnson, D. and Travis, J. (1974) Biochemistry 13, 5439-5445.
19. Kay, J. and Kassell, B. (1971) J. Biol. Chem. 246, 6661-6665.
20. Kunitz, M. and Northrop, J.H. (1948) Crystalline Enzymes, Ed. 2, Columbia University Press, New York.
21. Vaes, G. (1972) Biochem. J. 126, 275-289.
22. Vaes, G. (1972) FEBS Lett. 28, 198-200.
23. Harper, E., Bloch, K.J. and Gross, J. (1971) Biochemistry 10, 3035-3041.
24. Kruze, D. and Wojtecka, E. (1972) Biochim. Biophys. Acta 285, 436-446.
25. Oronsky, A.L., Perper, R.J. and Schroder, H.C. (1973) Nature 246, 417-419.