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SYNTHESIS OF A COLLAGENASE INHIBITOR BY GINGIVAL FIBROBLASTS IN CULTURE

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Summary

Human cllagenase was inhibited by test solutions of human gingival fibroblast culture media. The fibroblast-derived collagenase inhibitor was only slightly affected by 10 μg trypsin but was inactivated with 100 μg trypsin. The chaotropic agent KSCN (3 M) completely inactivated the inhibitor, whereas the thiol-blocking reagent, *p*-aminophenylmercuric acetate, partially inactivated the inhibitor. Inhibitory actlvity was retained at 60°C but was abolished at 100°C. Following ammonium sulfate fractionation, the fibroblast inhibitor was recovered in the supernatant at concentrations of at least 70% saturation. It is suggested that collagenase latency in soft connective tissues may derive from a collagenase-inhibitor complex formed by interaction of collagenase and a fibroblast-derived inhibitor.

Introduction

Extracellular degradation of collagen appears to be mediated by specific collagenase but the regulatory mechanisms whereby the protease activity is altered in response to physiologic or pathologic stimuli remain unclear [1—9]. Three modes for regulation of collagenase activity have been proposed. The first mode involves direct regulation at the level of de novo enzyme synthesis where physiologic or pathologic processes may have an inductive role [10,11]. The other two modes involve factors which regulate at the level of either activation or inhibition of preformed enzyme. Proteolytic activation may occur either via zymogen activation [12] or via inhibitor inactivation [13]. Alternatively, de novo synthesis of inhibitor may be a regulatory factor. The experimental evidence for each mode is insufficient to evaluate its relative impor-

tance in the regulation of collagenase catabolism. In particular, an evaluation of the in vivo role of collagenase-inhibitor complexes requires more precise definition of collagenase inhibitors, which have been identified in cultures of several mesenchymal tissues [14—19]. This study describes a collagenase inhibitor produced by human gingival fibroblasts.

Materials and Methods

Gingival fibroblast cell line cultures

Fibroblast cell lines were derived from gingival tissue specimens obtained from patients receiving surgical periodontal treatment. The fibroblast cell lines were obtained by standard procedures and were grown to confluence in RPMI *-fetal calf serum (10%). Once grown to confluence, the fibroblasts were washed free from serum-containing medium and were maintained in serum-free RPMI for 3 days. At the end of the culture period, the culture medium was decanted, cleared by centrifugation and dialyzed against assay buffer. The dialyzed culture medium was tested for collagenase inhibitory activity.

Collagenase

Gingival collagenase was generated from gingival explants obtained from patients receiving periodontal treatment. Gingival explants were obtained and cultured using procedures previously established [20]. After the first 24 h, the culture medium was decanted daily and fresh serum-free medium added to the explants. Collagenase from pooled media was partly purified by ammonium sulfate (30—60%) fractionation, desalted and lyophilized. The dry enzyme preparation was dissolved in assay buffer as needed at concentrations of 1 to 4 mg/ml.

Mononuclear cell collagenase was obtained from medium of cultured human peripheral blood mononuclear cells [21]. Mononuclear cells were separated from the peripheral whole blood of healthy donors by Ficoll (90%)/Hypaque (33%) gradient centrifugation; recovery of mononuclear cells by this method averaged 73 ± 9.3% (mean ± S.E.). Subpopulations of mononuclear cells were prepared by established procedures [22]. Collagenase was derived from medium after culturing either mixed or fractionated mononuclear cells for 3 days in 2.0 ml serum-free RPMI-1640 culture medium containing appropriate vitamins and antibiotics in 5% CO_2/95% air. At the end of the culture period the medium was cleared by centrifugation and the supernatant was dialyzed against assay buffer.

Collagenase assay

Viscometric assay of collagenase activity was performed using purified [23] rat-tendon collagen as substrate for the recation. Viscometric assays were carried out in Ostwald semi-microviscometers at 27°C using 0.1% collagen solutions in 0.23 M NaCl/0.05 M Tris-HCl (pH 7.5) containing 0.005 M $CaCl_2$ and 0.5 M arginine in a total volume of 1.0 ml. Alternatively, collagenase was

* RPMI, Grand Island Biological Co. New York.

assayed by the microgel lysis technique using purified guinea pig skin [^{14}C]-collagen or acetylated rat-tail tendon [^{14}C]collagen as substrate [24,25].

Electrophoresis

Samples of reaction mixtures to be electrophoresed were subjected to thermal denaturation at 45°C for 10 min. Polyacrylamide gel electrophoresis for basic protein was used to analyze reaction products [26,27].

Protein determination

Protein was determined by the Folin method of Lowry et al. [28] using serum albumin as standard.

Inhibitor characterization

p-Aminophenylmercuric acetate treatment. *p*-Aminophenylmercuric acetate was added at a final concentration of 1 mM to a solution containing the fibroblast inhibitor. After standing in an ice bath for 90 min, the fibroblast inhibitor solution was dialyzed against assay buffer.

KSCN treatment. A solution of collagenase plus fibroblast culture medium containing inhibitor was dialyzed against 3 M KSCN for 16 h at 4°C, and then dialyzed against assay buffer.

Trypsin treatment. In some experiments 10 μg crystalline trypsin was added to 0.5 ml fibroblast inhibitor solution containing 3.28 μg protein. The solution was incubated at 37°C for 5 min and 100 μg soybean trypsin inhibitor was added. In other experiments 100 μg trypsin was added and the solution was incubated at 37°C for 45 min. After the incubation 500 μg soybean trypsin inhibitor was added.

Temperature treatment. Solutions of fibroblast inhibitor were heated for 30 min at 60 or 100°C. The heat-treated solutions were cleared by centrifugation.

Dithiothreitol treatment. A solution of fibroblast inhibitor was dialyzed against 4 mM dithiothreitol for 4 h at 4°C. After dialysis the solution was adjusted so that 150 μg inhibitor-protein with dithiothreitol concentrations of 4 mM was used for inhibitor assays. Solutions containing 4 mM dithiothreitol with gingival collagenase and no inhibitor were included in the control group.

Molecular weight determination

Molecular weight estimations for the inhibitor were made on a calibrated 0.6 × 90 cm column of Sephadex G-75 equilibrated with 0.05 M Tris-HCl/5 mM $CaCl_2$ at pH 7.4. A 2.0 ml sample (5.4 mg protein) of fibroblast factor was applied to the column and eluted at a flow rate of 12 ml/h. Fractions (1.1 ml) were collected and analyzed for protein. Protein was measured by the method of Bradford [29], since ultraviolet absorbance of the culture media was low. Inhibition of gel lysis by collagenase (50 μg protein) under conditions where enzyme alone produced 70—80% gel digestion served to indicate the inhibitory activity.

Results

Human gingival fibroblast culture medium inhibited the activity of human collagenase as measured by viscometry (Fig. 1) or [^{14}C]collagen gel lysis

TABLE I

AMMONIUM SULFATE FRACTIONATION OF COLLAGENASE INHIBITOR

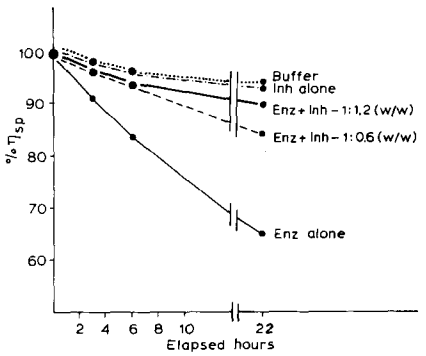
Test solution	Fibroblast solution (μg)	% [^{14}C]collagen gel digestion
Gingival collagenase	0.0	100
+ unfractionated fibroblast medium	27.5	38
+ 35% precipitate	33.0	93
+ 70% precipitate	26.3	96
+ 70% supernatant	46.5	25

(Table I). Collagen solutions incubated for 22 h with 0.2 mg partly purified collagenase showed viscosity reduction of 34%. However, when fibroblast culture medium was added to the reaction mixture containing collagenase, at ratios (w/w) of enzyme to inhibitor of 1 : 1.2 and 1 : 0.6, the viscosity reductions were diminished to 9 and 15%, respectively. Collagenase inhibition by the fibroblast factor was also observed in the [^{14}C]collagen-gel, lysis, assay. Collagenase activity measured by determining the percentage of gel lysis was inhibited 62% (Table I) when fibroblast culture medium was included in reaction mixtures. Thus, collagenase was effectively inhibited by a fibroblast factor, as determined by two established collagenase assay procedures.

Human fibroblast collagenase inhibitor was also tested for inhibitory activity against collagenase obtained from human peripheral blood mononuclear cells (Table II). Reaction mixtures containing culture medium from mixed mononuclear cells, T lymphocytes, non-T lymphocytes and macrophages showed collagen solution reduction of viscosities that ranged between 45 and 54% during a 24 h incubation. When fibroblast culture medium was included in reaction mixtures containing collagenase derived from either mixed mononuclear cells or purified mononuclear cell subpopulations, the collagenase-catalyzed viscosity reductions were inhibited and ranged between 9 and 17%. Thus, the fibroblast-derived inhibitor effectively inhibits activity of collagenase derived from human peripheral blood mononuclear cells.

Electrophoretic analysis of collagen denaturation products from the viscometric assay of collagenase in the presence or absence of fibroblast-derived inhibitor verified that collagenase cleavage of collagen was abolished by the inhibtor (Fig. 2). Electrophoretograms of reaction mixtures containing collagenase exhibited the characteristic 75% α^A fragment. However, where fibroblast culture medium was added to collagenase the 75% α^A fragment was absent from electrophoretograms. Therefore, the evidence is that fibroblast-derived factor inhibits specific collagenase.

Experiments were instituted to obtain preliminary chemical characterization of the inhibitor. In these experiments, reaction mixtures containing collagenase reduced the specific viscosity of a collagen solution by 50% and reaction mixtures containing collagenase and the fibroblast inhibitor showed a diminution of viscosity reduction to only 9%. The collagenase inhibitor was only partly inactivated by treatment with 1 mM *p*-aminophenylmercuric acetate, 10 μg trypsin or heating to 60°C; collagenase activities in the presence of inhibitor



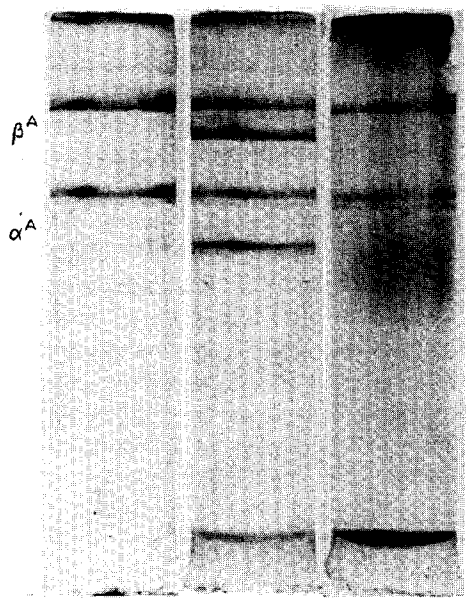


Fig. 1. Effect of fibroblast culture medium in gingival collagenase. Partial or complete inhibition of collagenase-catalyzed reduction in the viscosity of collagen solutions was effected by increasing amounts of fibroblast culture medium added to 0.2 mg collagenase. Fibroblast culture medium alone caused no change in collagen viscosity while 0.2 mg collagenase reduced the viscosity by 34%. Inh, inhibitor; Enz, enzyme.

Fig. 2. Collagen denaturation components from the assay of collagenase in the presence and absence of the fibroblast-derived inhibitor. From left to right: gel 1, buffer-treated collagen contains typical α and β components; gel 2, collagenase-treated collagen contains the faster migrating α^A and β^A fragments characteristic of the primary product of collagenase cleavage; gel 3, collagenase-treated collagen with fibroblast inhibitor contains only undegraded α and β components.

that had been treated with these agents were 28, 15 and 21%, respectively. However, treatment of the inhibitor with either 3 M KSCN, 4 mM dithiothreitol, 100 μg trypsin or heating to 100°C abolished the inhibitory activity; in the presence of treated inhibitor collagenase-catalyzed viscosity reductions were 48, 54, 51 and 57%, respectively.

TABLE II

EFFECT OF FIBROBLAST INHIBITOR ON COLLAGENASE FROM HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Viscometric assays were conducted for 24 h at 27°C. Protein concentrations: mixed mononuclear cells, 69.5 μg; T lymphocytes, 111.1 μg; Non-T lymphocytes, 117.0 μg; macrophages, 59.5 μg; fibroblast inhibitor, 5.2 mg.

Collagenase source	% η_{sp} reduction	
	No inhibitor	With inhibitor
Buffer-treated	8	9
Mixed mononuclear cells	48	9
T lymphocytes	45	12
Non-T lymphocytes	54	17
Macrophages	52	9

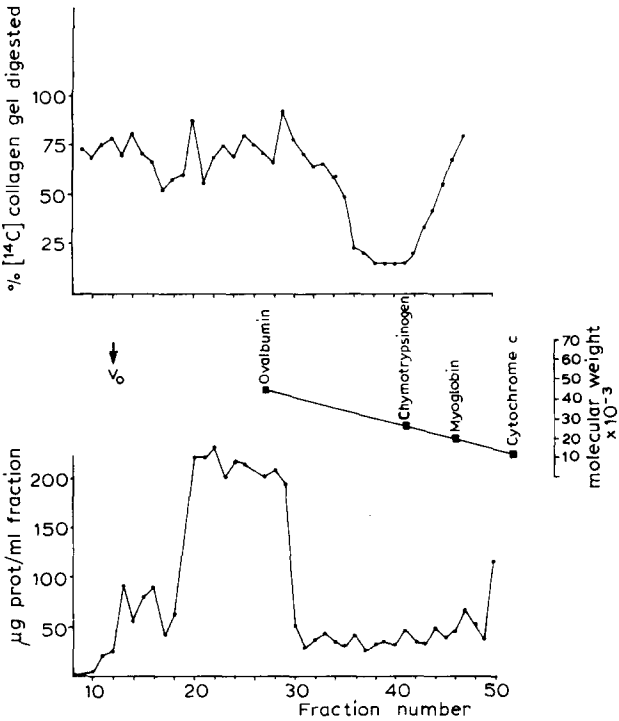


Fig. 3. Elution profile of fibroblast-derived collagenase inhibitor on Sephadex G-75. Gel filtration chromatography was performed on a calibrated 0.6 × 90 cm column of Sephadex G-75 equilibrated with 0.05 M Tris-HCl/0.005 M $CaCl_2$, pH 7.4. A 2 ml sample (5.4 mg) of fibroblast factor was applied to the column and eluted at a flow rate of 12 ml/h. Fractions (1.1 ml) were collected and analyzed for protein and for inhibitor activity. Inhibition of gel lysis by collagenase (5 µg) under conditions where enzyme alone produced 70—80% gel digestion served to indicate inhibitory activity.

An estimation of the molecular weight of the inhibitor was made by its elution profile after gel filtration chromatography using Sephadex G-75 (Fig. 3). The factor that inhibited collagenase corresponded to an elution position of 27 000—30 000 daltons.

Discussion

Human gingival fibroblasts during culture produce an inhibitor of human collagenase derived from gingiva and peripheral blood mononuclear cells. In this respect, gingival fibroblasts behave like skin fibroblasts in culture [15]. Production of a collagenase inhibitor may, therefore, be a general characteristic of fibroblasts. The gingival collagenase inhibitor has an apparent molecular weight ranging between 27 000—30 000 which is similar, but not identical, to the molecular weight of collagenase inhibitors from skin fibroblasts [30], rheumatoid synovial cells [17], tendon [18] and a variety of connective tissues [14].

The fibroblast inhibitor was relatively heat-resistant but was inactivated by a high concentration of trypsin. Chemically, the inhibitor was inactivated with cyanate and thiol-blocking reagents. These substances, as well as trypsin, have

been used to activate latent collagenase obtained from tissue and cell culture media [14,16,31]. Thus, it is suggested that latent collagenase in the tissues is formed by interaction of collagenase with an inhibitor.

A fibroblast-derived collagenase inhibitor could have an important regulatory function in normal connective tissue collagen metabolism. Alterations of the ratio of collagenase to inhibitor might account for increased collagenase activity during inflammation. However, human skin fibroblasts produce collagenase in a proenzyme form [32] and enhanced activation of proenzyme could also account for increased collagenase during disease. Thus, further work is necessary before determining the relative importance of proenzyme activation vis à vis perturbation of enzyme-inhibitor complexes in the regulation of collagenase.

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References

1 Gross, J. and Nagai, Y. (1965) Proc. Natl. Acad. Sci. U.S.A. 54, 1197—1204
2 Schroeder, H.E., Munzel-Pedrazzoli, S. and Page, R. (1973) Arch. Oral Biol. 18, 899—923
3 Fleider, D.E., Sun, L.N. and Schneider, B.C. (1966) Periodontics 4, 302—307
4 Krane, S.M. (1974) Arthritis Rheum. 17, 306—312
5 Beutner, E.H., Thrifthauser, C. and Hanzen, S.P. (1966) Proc. Soc. Exptl. Biol. Med. 121, 1082—1085
6 Fullmer, H.M., Baer, P.N., Driscoll, E.J. and Lightbody, P.M. (1970) in Proceedings of the First Pan-Pacific Congress of Dental Research Tokyo, Japan (The Lion Foundation for Dental Health ed.), pp. 161—171
7 Golub, L.M., Kenneth, S., McEwan, H., Curran, J.B. and Ramamurthy, N.S. (1976) J. Dent. Res. 55, 177—181
8 Golub, L.M., Kaplan, R., Mulvihill, J.E. and Ramamurthy, J. (1979) J. Dent. Res. 58, 2132—2136
9 Gross, J. (1976) in Biochemistry of Collagen (Ramachandran, G.N. and Reddi, A.H., eds.), pp. 275—317, Plenum Press, New York
10 Gross, J. (1974) The Harvey Lectures, 1972—1973, pp. 351—432, Academic Press, New York
11 Wahl, L.M., Wahl, S.M., Mergenhagen, S.E. and Martin, G.R. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3598—3601
12 Harper, E., Bloch, K.J. and Gross, J. (1971) Biochemistry 10, 3035—3041
13 Eeckhout, Y. and Vaes, G. (1977) Biochem. J. 166, 21—31
14 Reynolds, J.J., Sellers, A., Murphy, G. and Cartwright, E. (1977) Lancet (August 13), 333—335
15 Bauer, E.A., Striklin, G.P. and Jeffery, J.J. (1975) Biochem. Biophys. Res. Commun. 64, 232—240
16 Pettigrew, D.W., Sodek, J., Brunette, D.M. and Wang, H.M. (1979) J. Dent. Res. 58,)Special Issue A), A154
17 Vater, C.A., Mainardi, C.L. and Harris, E.D., Jr. (1978) J. Clin. Invest. 62, 987—992
18 Vater, C.A., Mainardi, D.L. and Harris, E.D., Jr. (1979) J. Biol. Chem. 254, 3045—3053
19 Nolan, J.C., Ridge, S., Oronsky, A.L., Slakey, L.L. and Kerwar, S.S. (1978) Biochem. Biophys. Res. Commun. 83, 1183—1190
20 Fullmer, H.M. Taylor, R.E. and Gutherie, R.W. (1972) J. Dent. Res. 51, 349—355
21 Simpson, J.W., Mackler, B.F., O'Neill, P.A. and Mailman, M.L. (1980) J. Dent. Res. 59, 2—10
22 Mackler, B.F., O'Neill, P.A. and Meistrich, M. (1977) Eur. J. Immunol. 1, 55—61
23 Gross, J. (1958) J. Exp. Med. 107, 247—263
24 Nagai, Y., Lapiere, C.M. and Gross, J. (1966) Biochemistry 5, 3123—3130
25 Gisslow, M.T. and McBride, B.C. (1975) Anal. Biochem. 68, 70—78
26 Resifeld, R.A., Lewis, U.J. and Williams, D.E. (1962) Nature 195, 281—283
27 Nagai, Y., Gross, J. and Piez, K.A. (1964) N.Y. Acad. Sci. 121, 494—500
28 Lowry. O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
29 Bradford, M.M. (1976) Anal. Biochem. 72, 248—254
30 Welgus, H.G., Striklin, G.P., Eisen, A.Z., Bauer, E.A., Cooney, V. and Jeffery, J.J. (1979) J. Biol. Chem. 254, 1938—1943
31 Robertson, P.B., Grup, H.E., Taylor, R.E., Shyu, K.W. and Fullmer, H.M. (1973) J. Oral Pathol. 2, 28—32
32 Valle, K.J. and Bauer, E.A. (1979) J. Biol. Chem. 254, 10115—10122