Vol. 98, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1981 Pages 184-190

IDENTIFICATION OF A TYPE V COLLAGENOLYTIC ENZYME

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Received November 12, 1980

SUMMARY

A neutral metal protease has been identified which cleaves native type V collagen under conditions where pepsinized type IV collagen or the interstitial collagens are not significantly degraded. The enzyme is secreted into the media of cultured M50-76 reticulum cell sarcoma (malignant macrophages) and leiomyosarcoma tumor cells. Biosynthetically labeled type V collagen prepared from organ cultures of human amnion membrane is used for a routine assay of type V collagenolytic activity. The partially purified enzyme a) exists in a latent form requiring trypsin activation for maximum activity; b) has a molecular weight estimated by molecular sieve chromatography of approximately 80,000 daltons; c) is inhibited by EDTA but not phenylmethylsulfonyl fluoride; and d) produces specific cleavage products of both A and B collagen chains.

INTRODUCTION

The genetically distinct collagen types differ in their susceptibility to collagenase. Interstitial collagens type I, II and III are cleaved at a specific locus by the "classic" mammalian collagenase obtained from such sources as skin, rheumatoid synovium and leukocytes, under conditions where type IV (basement membrane) and type V collagen are not cleaved (1-3). Based on this finding and the identification of a collagenolytic activity specific for type IV but not the other types of collagen, it was proposed that a separate protease might also exist for type V (A B C) collagen (1,4).

Collagenases are often identified in tissues that show histologic evidence of collagen breakdown or cellular invasion of connective tissue. Such pathologic situations occur in inflammation and tumor invasion (5). Since some tumor cells are known to secrete elevated levels of proteases compared to their

0006-291X/81/010184-07$01.00/0

normal cell counterparts (6,7), we have utilized malignant cells as a source of connective tissue degrading enzymes (1,4). After surveying a number of tumor cell lines for proteases, we have now identified a neutral type V collagen degrading metal protease produced by reticulum cell sarcoma and leiomyosarcoma tumor cells. Enzyme assays utilize biosynthetically labeled type V collagen from organ cultures of human amnion. The enzyme is extracted from the culture media and partially purified by molecular sieve chromatography. The substrate specificity of the enzyme is studied using native collagens type I, II, III, pro type IV, pepsinized type IV, and human placenta type V collagen.

MATERIALS AND METHODS

Preparation of substrates. Biosynthetically [^{14}C]-proline labeled type V collagen was prepared from organ cultures of human amnion. Fetal membranes were obtained from fresh human placentas obtained at the time of delivery. The amnion was dissected away from the chorion and minced in 20 ml of DMEM containing glutamine, 10% dialyzed fetal calf serum, 10 µg/ml of ascorbate and 10 µg/ml of beta-aminopropionitrile. After preincubation for 30 min at 37°C in 5% CO_2, [^{14}C]-proline (Amersham) in 0.85 N NaCl was added to a final concentration of 50 µCi/ml and the tissue was incubated for 10 additional hours. The tissue fragments were collected by centrifugation at 2,000 g for 10 min and resuspended in 0.5 M acetic acid, 10 mM EDTA and 10 mM N-ethyl maleimide (NEM) and then extracted at 4°C for 48 hours. Type V collagen was isolated after pepsinization (1% pepsin to tissue ratio at 4°C for 24 h) using established salt precipitation methods (8). The 4.5 M NaCl precipitate contained type V collagen with a specific activity of approximately 10^5 cpm/mg.

Type V collagen standards were kindly supplied by Dr. J. Bateman and Dr. P. Fietzek.

Type I collagen was prepared from guinea pig skin by the method of Nagai et al. (9) and type III collagen was purified from fetal calf skin by the method of Epstein (10). Pro-type IV collagen was prepared according to Tryggvason et al. (11). Pepsinized bovine lens capsule collagen was kindly supplied by Dr. E. Miller (Birmingham, Alabama). Type II collagen was isolated from a human chondrosarcoma serially transplanted in nude mice (12).

Tumor cell culture. M50-76 murine reticulum cell sarcoma cells were supplied by Dr. Ian R. Hart, Fredrick Cancer Research Center, Fredrick, Maryland. These tumor cells were first obtained from an ovarian tumor and metastasize preferentially to the abdominal organs regardless of the route of tumor cell injection. By surface markers and histologic studies this tumor is histiocytic (macrophages) in origin (Talmage et al., manuscript submitted). The tumor cells are cultured in RPMI 1640 with 10% equine serum (Flow Labs) 2 mM glutamine and 50 µg gentamycin/ml, 37°C, 5% CO_2 in air.

PMT murine sarcoma cells, B16-BL6 melanoma cells and MCF-7 human breast carcinoma cells are cultured as described previously (7). Human leiomyosarcoma cells were established in vitro from tumor tissue taken at the time of surgery and cultured in a similar fashion to the above cells.

Enzyme assays. For the assay of type V and type IV collagenase activity, 200 µl of the enzyme solution was activated with 50 µl of trypsin (10 µg/ml, 3 x crystallized, Sigma) at 37°C for 4 min followed by addition of 50 µl of soybean trypsin inhibitor (50 µg/ml, Sigma). The substrate was added in 50 µl of reaction buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.4) and the reaction was carried out for 6 hrs at 37°C. The undigested substrate was precipitated by trichloroacetic acid/tannic acid (4) and the supernatant radioactivity was counted.

Samples positive for type V degrading activity were examined by slab polyacrylamide gel electrophoresis. Type I collagenolytic activity was assayed by the method of Johnson-Wint (13). Elastase (plasminogen independent) activity was assayed according to Goldfarb and Quigley (14).

Partial purification of type V collagenolytic activity. Ammonium sulfate fractionation: serum-free media from cultures of the highly metastatic M50-76 murine reticulum cell sarcoma (malignant macrophages) were harvested as described previously for the PMT sarcoma (4). The ammonium sulfate (25-50%) precipitate of media was dialyzed against the enzyme reaction buffer. The sample (2 ml) was then passed over a 1.6 x 95 cm Sephacryl S-200 Superfine column (Pharmacia) equilibrated with the enzyme buffer at 4°C with a flow rate of 0.4 ml/min. Fractions of 2 ml were collected and those containing enzyme activity were pooled and precipitated with 50% ammonium sulfate as above. The precipitate was dissolved in the collagenase assay buffer and dialyzed against the same buffer.

RESULTS

Log phase tumor cell cultures were incubated for 2 days in serum-free media. All cell types contained some degradative activity for type IV collagen.

Table I

Relative type IV, type I and type V collagen degrading protease activity per 1 x 10^5 cells from selected tumors.

Enzyme	Substrate		
	type IV	type I	type V
M50-76 mouse reticulum cell sarcoma	625 ± 29	3003 ± 89	3122 ± 140
MCF-7 human breast carcinoma	594 ± 60	290 ± 45	none detected
B16-BL mouse melanoma	1140 ± 64	944 ± 70	none detected
PMT mouse sarcoma	1815 ± 90	410 ± 38	none detected
LY-80 human leiomyosarcoma	711 ± 44	383 ± 47	2024 ± 215
Bacterial collagenase (50 U)	1980	4014	3808

Digestion was performed at 37°C for 6 hrs, pH 7.6 (trypsin activated enzyme, mean±range, appropriate controls subtracted).

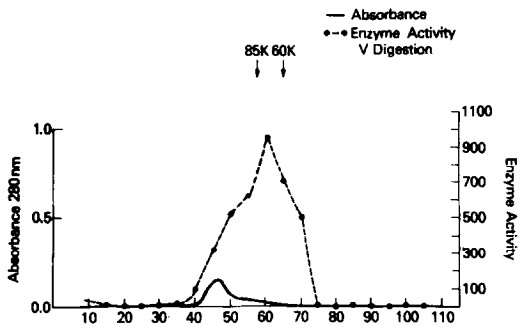

Fig. 1. Gel filtration of M50-76 cell type V collagenolytic activity on 1.6 x 95 cm. Sephacryl S-200 column. Fractions of 2 ml. were collected and 0.4 ml. was assayed for type V collagenolytic activity at 37°C, pH 7.4. The enzyme activity eluted as a single peak of M_r approx. 80K, and was inactive at pH 5.0. Activity is expressed in cpm substrate radioactivity solubilized with a maximum of 1600 cpm released by bacterial collagenase and a background of 50-80 cpm. Column front= fraction 38. Pooled fractions 55-65 contained 72 µg protein/ml with enzyme purification of at least 50 fold over the crude M50-76 media. Pooled fractions 75-90 contained type I collagenolytic activity (20 µg protein/ml.). Both collagenolytic activities were enhanced by trypsin activation.

The M50-76 cells and the leiomyosarcoma cells contained significant degradative activity for type V collagen. The M50-76 and B16-BL6 tumor cells contained degradative activity for type I collagen (Table I). All collagenolytic activities were enhanced by trypsin activation.

The M50-76 type V collagenolytic activity was further purified by ammonium sulfate precipitation and molecular sieve chromatography. The column fractions were pooled and studied for elastase and type I collagen degrading activity. A peak of latent type V collagen degrading activity was noted in column fractions 50-65 (M_r approximately 80,000) (Fig. 1). Type 1 collagenase and elastase eluted in later fractions (M_r 20-50,000) (Table II).

The degradation products and substrate specificity of the type V collagenolytic enzyme was studied by gel electrophoresis (Fig. 2). Under native digestion conditions in the presence of soybean trypsin inhibitor and phenylmethylsulfonyl fluoride (PMSF) the enzyme cleaved both A and B chains producing two specific high molecular weight cleavage products and smaller products which migrated with the gel front. EDTA abolished the enzyme activity. When digestion was conducted at 25°C for 28 hours, 90% of the type V collagen was cleaved and no

Table II

Protease activity of molecular sieve chromatography fractions of M50-76 cell media.*

Fractions	enzyme (max. cpm/assay)		
	type V collagen protease (1600)	type I collagen protease (4000)	elastase (4280)
50-54	642	none detected	90
55-69	1082	185	210
70-80	390	2433	434
81-90	none detected	1016	1873

*Assays were performed on 50 ul aliquots of pooled column fractions (Front = fraction 38). Values shown are means of duplicate assays with range less than 20% of mean, appropriate controls subtracted. The type V activity was trypsin activated and such treatment enhanced the activity two fold. Pooled fractions concentrated 10 fold by ammonium sulfate precipitation.

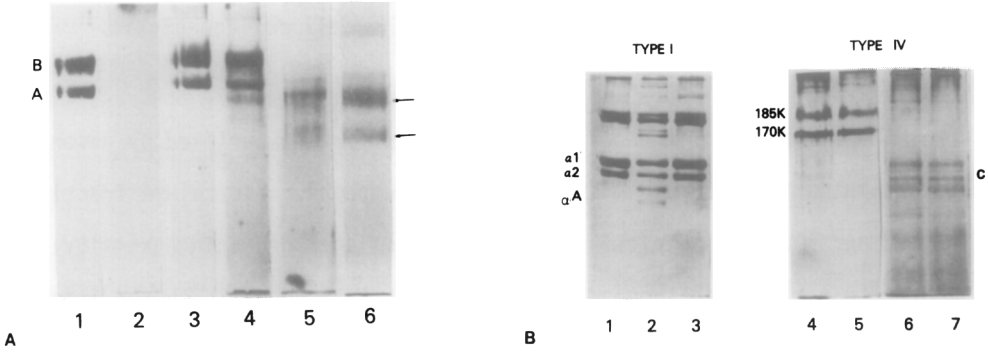

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (ref. 19) of collagen degradation products (50μg substrate/2μg enzyme, pH 7.4).

A. Trypsin activated enzyme from pooled fractions. 50-65 (Fig. 1) were incubated with unlabeled placenta type V (AB) collagen. All digestions contained .01% soybean trypsin inhibitor. Substrate alone(1), enzyme alone(2), substrate + enzyme + EDTA 30mM 25°C 28hours(3), substrate + enzyme 25°C 8 hours (4), substrate + enzyme 25°C 28 hours(5) substrate + enzyme + PMSF 30mM 25°C 28 hours(6). Arrows indicate reaction products. 6% gel.

B. Comparison digestion with collagen types I and IV, conditions identical to lane 6 Fig. 2A. Type I collagen alone (1), substrate + enzyme fractions 75-90 (2), substrate + enzyme fractions 50-65 (3) 7.5% gel, Pro IV collagen + enzyme fractions 50-65 (4), Pro IV alone (5), pepsinized bovine lens capsule collagen alone (6), substrate + enzyme fractions 50-65 (7), 5% gel. Collagens type I and type IV, type II and type III (data not shown) were less than 15% digested by the type V collagenolytic activity.

significant reaction products were produced for collagens I, pro-IV, and pepsinized type IV. The type V digestion products were different from those produced by trypsin which consist of three high molecular weight doublets (1). The type I collagenolytic activity from column fractions 75-90 produced typical TC^A fragments for type I collagen (Fig. 2B lane 2).

DISCUSSION

A metal protease that degrades type V collagen has been partially purified from murine tumor cell cultures. Under usual digestion conditions this enzyme is selective for this collagen and produces specific large molecular weight cleavage products for both A and B chains. By molecular sieve column chromatography the enzyme is purified 50 fold and elutes at a position different from type I collagenase and elastase. The type V protease activity is activated by trypsin and has a molecular weight slightly larger than other collagenases (15). Whether or not other substrates exist for this enzyme remains to be studied. Leukocyte elastase does not degrade type V collagen (16,17) and the physiologic enzymes which participate in its turnover have not been identified. The existence of the present enzyme, the identification of a type IV collagenolytic activity (1,4,16,17) and the failure of leukocyte collagenase to degrade type III collagen (18), all suggest that a family of metal proteases exist with different collagen substrate specificities.

Tumor cells, which can be cultured as homogeneous populations in large numbers, may be a good source of connective tissue degrading enzymes. Similar enzymes are probably produced by normal cells. Mainardi and colleagues have identified a protease from alveolar macrophages that degrades type V collagen (personal communication). The histologic distribution and function of type V collagen is unclear at this time. This collagen may be pericellular in distribution and cell migration may be associated with type V collagen turnover. The unusual ability of the M50-76 reticulum cell sarcoma tumor cells to rapidly invade all abdominal organs may in part be related to their ability to degrade type V

collagen. It is possible that the relative abundance of tumor proteases selective for the different types of genetically distinct collagens may influence metastases organ distribution.

ACKNOWLEDGEMENTS

We thank Dr. George R. Martin and Dr. Hynda Kleinman for helpful discussions.

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