A novel TIMP-insensitive type IV collagen-degrading metalloproteinase from murine metastatic sarcoma cells

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A novel type IV collagen-degrading metalloproteinase was purified from the conditioned media of a murine metastatic sarcoma cell line. The molecular weight of the purified enzyme was determined to be 100 kDa by SDS-PAGE, while 700 kDa by gel filtration suggesting that the enzyme has a multimer structure. This enzyme degrades type IV collagen, but neither type I collagen nor casein. The failure of trypsin treatment to enhance the enzyme activity suggested that the purified enzyme did not require activation. Although the enzyme seems to be classified as a matrix metalloproteinase, it was inhibited by neither tissue inhibitor of metalloproteinases (TIMP) nor TIMP-2 and thus represents a novel type IV collagen-degrading metalloproteinase.

Type IV collagenase; Metastasis; TIMP (tissue inhibitor of metalloproteinase); Basement membrane; Metalloproteinase; Mouse T241 sarcoma cell

1. INTRODUCTION

One of the important steps of cancer metastasis is the traversion of basement membranes by tumor cells. A neutral metalloproteinase, which degrades type IV collagen, a major structural component of basement membrane, has been identified in a number of cultured tumor cell lines [1,2]. Gelatinase/type IV collagenase, either 72 kDa [3] or 92 kDa [4] enzyme, and stromelysin [5–7] have been reported to degrade type IV collagen as one of their substrates. These enzymes are generally secreted as proenzyme and activated by organomercurial compounds or some proteinases such as trypsin and plasmin [8], and are inhibited by TIMP [9,10]. In the present study, we report the purification and characterization of a novel type IV collagen-degrading metalloproteinase from murine sarcoma cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Materials were obtained from the following sources: TSK DEAE-5PW column, Toyo Soda Manufacturing Co. Ltd, Tokyo, Japan; Shodex WS-803F and 804F column, Showa Denko K.K., Tokyo, Japan; L-[2,3,4,5-^{3}H]proline and [^{14}C]methylated δ-casein, Amersham, Bucks, UK. All other chemicals used were reagent grade materials from various commercial sources.

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Abbreviations: TIMP, tissue inhibitor of metalloproteinases; APMA, 4-aminophenylmercuric acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography

2.2. *Preparation of substrates*

Engelbreth-Holmes-Schwarm (EHS) were maintained C57BL/6J mice. [^{3}H]Proline-labeled type IV collagen (300 dpm/μg) was prepared from EHS mouse tumor according to Salo et al. [11]. [^{14}C]Glycine labeled type I collagen (20 dpm/μg) was prepared from guinea-pig skin according to Terato et al. [12]. [^{14}C]Gelatin was prepared from [^{14}C]type I collagen by heating at 60°C for 30 min.

2.3. *Enzyme assay*

Type I collagen, gelatin and casein degrading activities were all assayed according to Murphy et al. [13] using [^{14}C]type I collagen, [^{14}C]gelatin (1000 dpm/200 μl/tube) and [^{14}C]casein (2000 dpm/250 μl/tube). Type IV collagen degrading activity was assayed according to Salo et al. [11] using [^{3}H]type IV collagen (1500 dpm/650 μl/tube) with or without trypsin treatment. The effects of inhibitors were assessed by addition in the assay. One unit (U) of collagenase was defined as the amount of enzyme which degrades 1 μg of substrate per min at 37°C. One unit (U) of collagenase inhibitor is defined as the amount of protein required for 50% inhibition of 2 U of collagenase.

2.4. *Purification of type IV collagen-degrading enzyme*

Conditioned media from cultures of the highly metastatic murine T241 sarcoma cells grown under serum-free conditions was harvested according to Liotta et al. [14]. All the following procedures were performed at 4°C. The media proteins were precipitated with ammonium sulfate from saturation of 25–80%, and the precipitate was dissolved in and dialyzed against buffer A (50 mM Tris-HCl, pH 7.5). The crude enzyme preparation (100 ml) was concentrated to 10 ml by ultrafiltration using an Amicon PM-10 membrane. The sample was applied to a DEAE-5PW (HPLC) column (2 × 12 cm) equilibrated with buffer A. The column was eluted with a 0–0.5 M NaCl gradient. The majority of the enzyme activity was eluted with 0.25 M NaCl. Also, several minor peaks of type IV collagen-degrading activity was

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Table I

Purification of a new type IV collagen-degrading metalloproteinase from mouse metastatic sarcoma cells

Purification steps	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg protein)	Purification (fold)
Conditioned medium	1120	32 300	29	1.0
$(NH_4)_2SO_4$ ppt.	378	15 600	41	1.4
DEAE-5PW (HPLC)	6.1	9770	1590	55
Gel filtration (Shodex WS-803F, HPLC)	0.11	220	2000	69

detected. These minor peaks may correspond to 72 kDa or 92 kDa gelatinase/type IV collagenases and stromelysin. The main peak of activity was concentrated to 1 ml, followed by gel-filtration (Shodex WS-803F, HPLC). The active fractions were collected and pooled.

2.5. *Partial purification of HT1080 gelatinase/type IV collagenase*

Serum-free culture medium of HT1080 cells was passed through a DEAE-5PW (HPLC) column equilibrated with buffer A and washed with same buffer containing 0.20 M NaCl, followed by elution with 0.25 M NaCl. The enzyme preparation was activated by APMA. The gelatin zymographic analysis suggested this enzyme corresponded to the 72 kDa gelatinase/type IV collagenase.

2.6. *Purification of TIMP and TIMP-2*

TIMPs were purified as reported previously from bovine aortic medial tissue [15], bovine dental pulp [16], human amniotic fluid [17], human gingival fibroblast [18].

TIMP-2 was purified more than 2000-fold from the culture medium of mouse colon 26 carcinoma cells using DF-52 Cellulose, CM-52 Cellulose, Ultrogel AcA 54, Con A-Sepharose and Sephadex G-50 Superfine columns (Kishi, J., Ogawa, K., Yamamoto, S. and Hayakawa, T., submitted).

3. RESULTS AND DISCUSSION

A new type IV collagen-degrading enzyme was purified from the culture media of mouse T241 sarcoma cells by ammonium sulfate precipitation, anion exchange (HPLC) and gel filtration (HPLC) column chromatographies (Table I). The molecular mass of the new enzyme was determined to be 100 kDa by SDS-PAGE in either reducing (Fig. 1A) or nonreducing conditions (data not shown), while it was estimated to be 700 kDa by gel filtration (Fig. 1B). These results suggest that the new enzyme has a multimer structure, and disulfide covalent bonds are not required for multimerization.

The purified enzyme did not degrade type I collagen and casein at all. Gelatin was a poor substrate compared to type IV collagen (data not shown). Furthermore, this enzyme did not require activation. Treatment of the enzyme with trypsin (5 μg/ml) at 37°C for various incubation times did not increase enzyme activity (data not shown).

Recently a high-molecular-mass type IV collagen-specific metalloproteinase having a molecular mass of 1000 kDa was purified from human carcinoma tissue [19]. The 1000 kDa enzyme was essentially inactive and activated by trypsin. The enzyme we described here seems to resemble the 1000 kDa enzyme in respect to molecular mass and substrate specificity. Both enzymes, however, are quite different in respect to their latency, since the 1000 kDa enzyme is latent and requires activation.

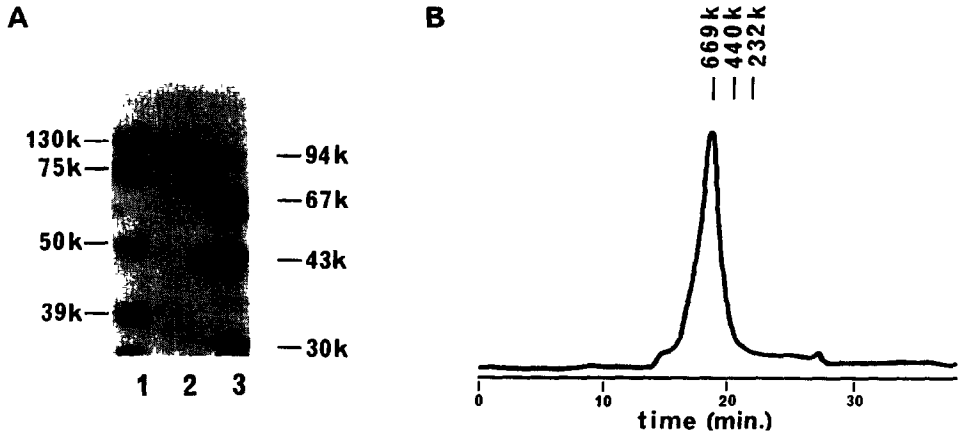

Fig. 1. Molecular mass of the T241 sarcoma type IV collagen-degrading metalloproteinase. (A) 7.5% SDS-PAGE in the presence of β-mercaptoethanol. Gels were stained with Coomassie brilliant blue. (Lanes 1 and 3) molecular mass standards; (lane 2) purified type IV collagen-degrading enzyme. (B) Elution profile of the purified enzyme on Showdex WS-804F gel filtration (HPLC), detected by absorbance at 280 nm. Column was equilibrated with 50 mM Tris-HCl/0.2 M NaCl (pH 7.5) and the flow rata was 0.5 ml/min. Fractions were collected and subjected to the analysis of type IV collagen-degrading activity. Enzyme activity peak coincided with the elution peak of protein. Molecular mass standards used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa).

Table II

Inhibitory effects of TIMP and TIMP-2 on a new type IV collagen-degrading metalloproteinase and HT1080 gelatinase/type IV collagenase

Inhibitors (sources)	Inhibitor/enzyme ratio (U/U)	Inhibition (%)	
		A new type IV collagen-degrading enzyme	HT1080 gelatinase/ type IV collagenase
TIMP (human gingival fibroblast)	1	ND	30.9
	10	ND	91.3
	50	0	ND
	250	27.8	ND
TIMP-2 (mouse colon 26 cells)	1	ND	0
	10	ND	80.3
	50	0	ND
	250	19.0	ND

ND = not determined.

The purified new enzyme was inhibited about 80% by 7.5 mM EDTA, but was insensitive to the thiol protease inhibitor *N*-ethylmaleimide, and the serine protease inhibitor, aprotinin (data not shown), indicating that the new enzyme is a metalloproteinase. It has been shown that most of type IV collagen-degrading enzymes such as gelatinase/type IV collagenase, either 72 kDa or 92 kDa enzyme, and stromelysin are metalloproteinases and are inhibited by TIMP [20]. The high-molecular-mass type IV collagen-specific metalloproteinase is also inhibited by TIMP [21]. The new enzyme, however, was not inhibited by any TIMP derived from bovine aortic cells, bovine dental pulps, human gingival fibroblasts and human amniotic fluid which are all known to be potent inhibitors of interstitial collagenase [15–18]. All of these TIMPs were confirmed to inhibit gelatinase/type IV collagenase from human HT1080 fibrosarcoma cells in this study. Inhibitory effects of one of the TIMPs, derived from human gingival fibroblasts, are summarized in Table II.

Recently a new TIMP, called TIMP-2, has been reported from several laboratories [22–24]. TIMP-2 shows about 41% similarity to the TIMP and inhibits interstitial collagenase and gelatinase/type IV collagenase at almost the same concentrations. Having recently purified TIMP-2 from the culture media of mouse colon 26 cells (Kishi, J., Ogawa, K., Yamamoto, S. and Hayakawa, T., submitted), we checked whether it inhibits the new enzyme and HT1080 gelatinase/type IV collagenase. As shown in Table II, the new enzyme resisted inhibition by TIMP-2, while the HT1080 type IV degrading activity was completely inhibited. Mikuni-Takagaki and Cheng found neutral metalloproteinases which degraded proteoglycan and/or gelatin in developing chick endochondral bone [27]. These enzymes are distinct from our enzyme in respect to the substrate specificity; however, it is interesting that these enzymes are also insensitive to TIMP and probably to TIMP-2.

In conclusion, neither TIMP nor TIMP-2 inhibited the new enzyme. As TIMP is known to be widely distributed in tissues and body fluids [25–26], the new TIMP-insensitive enzyme may play an important role in tumor invasion and metastasis.

REFERENCES

[1] Liotta, L.A., Rao, C.N. and Wewer, U.M. (1986) Annu. Rev. Biochem. 55, 1037–1057.
[2] Tryggvason, K., Höyhtyä, M. and Salo, T. (1987) Biochim. Biophys. Acta 907, 191–217.
[3] Collier, I.E., Wilhelm, S.M., Eisen, A.Z., Marmer, B.L., Grant, G.A., Seltzer, J.L., Kronberger, A., He, C., Bauer, E.A. and Goldberg, G.I. (1988) J. Biol. Chem. 263, 6579–6587.
[4] Wilhelm, S.M., Collier, I.E., Marmer, B.L., Eisen, A.Z., Grant, G.A. and Goldberg, G.I. (1989) J. Biol. Chem. 264, 17213–17221.
[5] Murphy, G., McAlpine, C.G., Poll, C.T. and Reynolds, J.J. (1985) Biochim. Biophys. Acta 831, 49–58.
[6] Okada, Y., Nagase, H. and Harris, E.D. Jr. (1986) J. Biol. Chem. 261, 14245–14255.
[7] Wilhelm, S.M., Collier, I.E., Kronberger, A., Eisen, A.Z., Marmer, B.L., Grant, G.A., Bauer, E.A. and Goldberg, G.I. (1987) Proc. Natl. Acad. Sci. USA 84, 6725–6729.
[8] Stetler-Stevenson, W.G., Krutsch, H.C., Wacher, M.P., Margulies, I.M.K. and Liotta, L.A. (1989) J. Biol. Chem. 264, 1353–1356.
[9] Murphy, G., Ward, R., Hembry, R.M., Reynolds, J.J., Kühn, K. and Tryggvason, K. (1989) Biochem. J. 258, 463–472.
[10] Wilhelm, S.M., Collier, I.E., Marmer, B.L., Eisen, A.Z., Grant, G.A. and Goldberg, G.I. (1989) J. Biol. Chem. 264, 1713–17221.
[11] Salo, T., Liotta, L.A. and Tryggvason, K. (1983) J. Biol. Chem. 258, 3058–3063.
[12] Terato, K., Nagai, Y., Kawanishi, K. and Yamamoto, S. (1976) Biochim. Biophys. Acta, 445, 753–762.
[13] Murphy, G., Reynolds, J.J., Bretz, U. and Baggiolini, M. (1982) Biochem. J. 203, 209–221.
[14] Liotta, L.A.., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C.M. and Shafie, S. (1980) Nature (Lond.) 284, 67–68.

[15] Kishi, J. and Hayakawa, T. (1989) Connect. Tiss. Res. 19, 63–76.
[16] Kishi, J. and Hayakawa, T. (1984) J. Biochem. 96, 395–404.
[17] Murphy, G., Cawston, T.E., and Reynolds, J.J. (1981) Biochem. J. 195. 167–170.
[18] Simpson, J.W. and Mailman, M.L. (1981) Biochim. Biophys. Acta 673, 279–285.
[19] Tsuda, M., Yamagishi, Y. and Katsunuma, T. (1988) FEBS Lett. 232, 140–144.
[20] Cawston, T.E. (1986) in: Proteinase Inhibitors (Barnett, A.J. and Salvesen, G. eds) vol. 12, pp. 589–610. Research Monographs in Cell and Tissue Physiology.
[21] Yamagishi, Y., Tsuda, M., Tsuda, T. and Yamamura, M. (1989) The Proceedings of the Matrix Metalloproteinases Conference (Birkedal-Hansen, H. ed), in press.
[22] DeClerck, Y.A., Yean, T.D., Ratzkin, B.J., Lu, H.S. and Langley, K.E. (1989) J. Biol. Chem. 264, 17445–17453.
[23] Stetler-Stevenson, W.G., Krutzsch, H.C. and Liotta, L.A. (1989) J. Biol. Chem. 264, 17374–17378.
[24] Goldberg, G.I., Mamer, B.L., Grant, G.A., Eisen, A.Z., Wilhelm, S. and He, C. (1989) Proc. Natl. Acad. Sci. USA 86, 8207–8211.
[25] Welgus, H.G. and Stricklin, G.P. (1983) J. Biol. Chem. 258, 12259–12264.
[26] Kodama, S., Yamashita, K., Kishi, J., Iwata, K. and Hayakawa, T. (1989) Matrix 9, 1–6.
[27] Mikuni-Takagaki, Y. and Cheng, Y. (1987) Arch. Biochem. Biophys. 259, 576–588.