A Thiol Protease Inhibitor Released from Cultured Human Malignant Melanoma Cells

Yasuhiro Nishida,1 Hiroyuki Sumi, and Hisashi Mihara

ABSTRACT

A thiol protease inhibitor (TPI) was found in culture media of human malignant melanoma cells (Bowes) at 1.5 to 2.3 units/day/flask (full sheet, 75 sq cm). This amount well exceeded that for cultured nonmalignant cells (human fetal lung fibroblasts). In the intracellular region of the melanoma cells, TPI activity was localized mainly in the cytosol fraction. The difference in specific activities between the intracellular and extracellular TPI and the TPI accumulation in the culture media indicated that cultured melanoma cells release TPI. Partial purification and characterization of the TPI by column chromatography using Sephadex G-150, papain-Sepharose, and Sephadex G-50, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, revealed two distinct TPIs with molecular weights of 56,000 and 9,800 to 10,800. The latter (main) TPI had a high specificity for thiol proteases and was heat stable (60° for 60 min), like previously reported normal human TPIs. The inhibitor, however, differed from normal human TPIs in that it had a lower molecular weight than any normal TPI, was unable to inhibit bromelain, and exhibited a mosaic pattern; namely, the low-molecular-weight TPI resembled liver-type TPI but the pH stability curve resembled serum-type TPI. The thiol protease, cathepsin B, was not detected in culture media of this human melanoma cell line.

INTRODUCTION

Many kinds of TPIs2 have been separated from microorganisms (leupeptin, antipain, chymostatin, and E-64) and from various specimens of several animals [hen (4, 9), rat (6, 7, 10), rabbit (38), pig (11, 13, 16), bovine (24)] as well as human beings. Human TPIs have been partially purified or from normal human TPIs in that it had a lower molecular weight than any normal TPI, was unable to inhibit bromelain, and exhibited a mosaic pattern; namely, the low-molecular-weight TPI resembled liver-type TPI but the pH stability curve resembled serum-type TPI. The thiol protease, cathepsin B, was not detected in culture media of this human melanoma cell line.

On the other hand, Poole et al. (19) reported that an excess amount of a cathepsin B-like thiol protease was released from human breast carcinomas and that the thiol protease activity was highest in the region where tumor invasion had occurred. Sloane et al. (30, 31) indicated that cathepsin B activity was higher in high-metastatic murine melanoma than in low-metastatic melanoma. Thus, cathepsin B-like activity has been suggested to be correlated with tumor invasion or metastasis.

In the present study, using one particular established cell line of human malignant melanoma, we examined the TPI activity in the culture media and compared it with that of nonmalignant fibroblasts. The melanoma cell line was found to release far more TPI than did the fibroblasts. We therefore investigated the released TPI and cathepsin B activity in the culture media of this cell line and attempted to evaluate the possible pathophysiological significance of the TPI.

MATERIALS AND METHODS

Congo red-elastin (200 to 400 mesh) was purchased from United States Biochemical Corporation, Cleveland, OH. BAPA was obtained from the Protein Research Foundation, Osaka, Japan. H-o-Valylleucyl-lysyl-p-nitroanilide was from AB Kabi Diagnostica, Sweden. BANA was from Koch-Light Laboratories, Colnbrook, Berkshire, England. Steam bromelain (EC 3.4.22.4) was further purified from a commercial bromelain (Wako Pure Chemical Company) with the use of the alternative purification method of Murachi (15). Cathepsin B (EC 3.4.22.1) was purified from human liver by the modified method of Towatari et al. (37). Instead of acetone treatment, lyophilization was performed. Elution on carboxymethyl-Sephadex C-50 was carried out with 0.2 M NaCl in the buffer. Ficin (EC 3.4.22.3), bovine trypsin (EC 3.4.21.1), and porcine stomach pepsin (EC 3.4.23.1) were products of Sigma Chemical Company, St. Louis, MO. Papain (EC 3.4.22.2) was obtained from P-L Biochemicals Inc., Milwaukee, WI, porcine elastase (EC 3.4.21.11) was from Boehringer/Mannheim GmbH, Mannheim, West Germany, and human plasmin (EC 3.4.21.7) was from Green Cross Corporation, Osaka, Japan. All other chemicals were obtained from commercial sources and were of the best grade available.

Cell Culture, Cell Extraction, and Subcellular Fractionation. An established human malignant melanoma cell line (Bowes) and a human fetal lung fibroblasts line were kindly supplied by Professor O. Matsuo, Kinki University, Osaka, Japan. These cells were cultured in Corning’s tissue culture flasks (75 sq cm) as described by Rijkin and Collen (23). To collect large pools of conditioned media, confluent flasks were rinsed once with DBPS and twice with serum-free Eagle’s minimum essential medium (Nissui 2; Nissui Seiyaku, Co., Ltd., Tokyo, Japan), replenished with 7.5 ml of the serum-free medium, and incubated under the same conditions. The collected cell culture supernatant (serum free) was centrifuged at 2800 x g for 10 min, and the supernatant was stored at −20° until use.

Monolayer cultures were washed twice with ice-cold DBPS and removed from the surface with 1 mw EDTA in Ca2+- and Mg2+-free DBPS by pipeting gently. The cell volume was determined after centrifugation (800 x g for 5 min). The cell pellets were frozen (−20°) and rewarmed at 37° for 10 min in an equal volume of 0.5% Triton X-100, sonicated (10 to 20 KHz for 1 min) at 4°, and centrifuged (1200 x g for 5 min). The resultant supernatant was used as a cell extract. Subcellular fractionation

1 To whom requests for reprints should be addressed.
2 The abbreviations used are: TPI, thiol protease inhibitor; LTPI, low-molecular-weight thiol protease inhibitor; BAPA, N-o-benzoyl-L-arginine-p-nitroanilide; BANA, N-o-benzoyl-L-arginine-p-naphthylamide; DBPS, Dulbecco’s phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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was carried out as described by De Duve et al. (3) and Quigley (21). The removed and centrifuged cell pellets were washed again by centrifugation (800 x g for 5 min) in ice-cold homogenizing medium and were homogenized until 90 to 98% of the cells appeared broken. The homogenates were then subjected to differential centrifugation to provide a nuclear pellet (600 x g for 10 min), a heavy mitochondrial pellet (10,000 x g for 90 min), and a soluble fraction (supernatant from the 100,000 x g step). The pellets were finally resuspended in the homogenizing medium.

**Inhibition and Enzyme Assay.** Ficin, papain, bromelain, trypsin, chymotrypsin, and plasmin activities were assayed by the caseinolytic (casein; Hammarsten) technique described previously (32, 33), except that cysteine (base free) was included at 5 mM in the cases of ficin, papain, and bromelain. Cathepsin B activity was assayed by the method of Hammarsten (base free) was included at 5 mM in the cases of ficin, papain, and bromelain. Cathepsin B activity was assayed by the method of De Duve et al. (3) and Quigley (21). The thiol protease inhibitory activities were subsequently performed to confirm the presence of TPI in the melanoma cells.

**RESULTS**

**Presence and Accumulation of Human Melanoma TPI**

The thiol protease inhibitory activity in the melanoma cell-conditioned media (serum free) was examined by the general method using ficin. As shown in Chart 1, the conditioned media inhibited ficin under both assay conditions of caseinolysis (Chart 1a) and amidolysis (Chart 1b). These results indicated that the culture supernatant of melanoma cells contained TPI.

We investigated the accumulation of TPI in the culture media of human melanoma cells as compared to nonmalignant cells (human fetal lung fibroblasts). Each group of melanoma cells and fibroblasts formed a confluent monolayer on the 75-cm² surface in the culture flask. In the case of the human melanoma cells, the TPI activity in the culture supernatant increased progressively with increasing incubation time (Chart 2). On the other hand, the TPI activity in the culture supernatant of the human fetal lung fibroblasts was barely detectable and scarcely increased. The fibroblasts could not be stably cultured with serum-free medium; therefore, we were unable to investigate them after 24 hr. We clearly recognized that human melanoma cells accumulated TPI activity in the culture supernatant, and the mean specific activities of TPI in the 6-, 12-, 24-, and 48-hr-incubated media were 1.60, 1.90, 2.79, and 1.64 units/mg protein, respectively (data not shown in Chart 2). The specific activity of TPI in the culture media thus increased up to 24 hr incubation.

Cell extraction and fractionation studies were then performed to confirm the presence of TPI in the cells and the localization of the TPI in the subcellular components. These techniques were carried out twice, and the 2 sets of results were found to be similar. One set is shown in Table 1. TPI activity was also

![Chart 1. Inhibitory effect of human melanoma cell-conditioned media on ficin activity. Concentrated media were prepared by lyophilization of crude conditioned media for 24 hr. a, caseinolysis with 30-fold-concentrated media; b, amidolysis with 6-fold-concentrated media (the reaction mixture was 0.6 ml with 0.1 mg ficin, 0.5 mM H-o-valyl-leucyl-lysyl-p-nitroanilide, and 5 mM cysteine, pH 7.4).](image)

![Chart 2. Accumulation of TPI in culture media of human malignant melanoma cells and human fetal lung fibroblasts. @, melanoma cells; O, fibroblasts. Points, mean accumulated TPI activity; bars, S.D. (N = 4). Each group of melanoma cells and fibroblasts formed a confluent monolayer on the 75-cm² surface of the culture flask. U, units.](image)
Intracellular activity and subcellular distribution of human melanoma TPI

<table>
<thead>
<tr>
<th>Subcellular fractionation</th>
<th>Cell extraction</th>
<th>TPI activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear fraction (800 x g for 10 min)</td>
<td>9.35</td>
<td>1.5 14.0</td>
</tr>
<tr>
<td>Heavy mitochondrial fraction (10,000 x g for 5 min)</td>
<td>8.15</td>
<td>0 0</td>
</tr>
<tr>
<td>Light particulate fraction (100,000 x g for 90 min)</td>
<td>6.91</td>
<td>0 0</td>
</tr>
<tr>
<td>Soluble fraction (100,000 x g supernatant)</td>
<td>12.15</td>
<td>9.2 86.0</td>
</tr>
<tr>
<td>Total</td>
<td>0.50</td>
<td>36.56 10.7 100</td>
</tr>
</tbody>
</table>

a Total amount.

b Casein analysis.

The molecular distribution of melanoma TPI was investigated by Sephadex G-150 (fine) gel filtration of crude conditioned media in order to separate the main peak. We searched all fractions by passage through a Sephadex G-150 column for several activities: neutral thiol protease activity; cathepsin B activity; trypsin-inhibitory activity; and trypsin-like activity. However, none of these 4 activities could be detected in any fraction in spite of 114-fold concentration of the applied sample under such assay conditions (Chart 3). The results indicated that both types of TPI were unable to inhibit trypsin and revealed no other biochemical functions.

Partial Purification of Human Melanoma TPI

The whole-purification procedure was carried out at 4°C. A volume of 12,664 ml of serum-free melanoma cell-conditioned medium for 24 hr was used as starting material. This contained 1,242 mg of protein and 3,604 units of TPI activity. The specific activity of the crude TPI was thus 2.9 units/mg protein.

Papain-Sepharose Affinity Chromatography. As shown in Chart 4, TPI activity was detected in only 20 mM NaPO₄, pH 12.1, containing 0.1 mM NaCl elution fractions but was partially separated from the main protein peak. These active fractions were collected and dialyzed against 0.15 M NH₄HCO₃ and then lyophilized. The same procedures were repeated several times, and the lyophilized samples were pooled at ~80°C.

We also examined the cathepsin B activity and neutral thiol protease activity in other fractions in which TPI could not be eluted. Conditioned media contained TPI, but such fractions did not contain the inhibitor. The 2 thiol protease activities should thus be more detectable in these fractions than in the crude media. However, we failed to detect cathepsin B activity and neutral thiol protease activity. We further concentrated these fractions to about 10-fold using solid polyethylene glycol 20,000 in order to enhance the detectability of the 2 activities. However, still no cathepsin B activity or neutral thiol protease activity was detected.

Sephadex G-50 Chromatography. Next, pooled TPI obtained from papain-Sepharose affinity chromatography was gel filtered on Sephadex G-50 (superfine) in order to separate the main double peaks, corresponding to molecular weights of about 56,000 and less than 12,500, respectively. However, the LTPI activity formed the main peak. We searched all fractions by passage through a Sephadex G-150 column for several activities: neutral thiol protease activity; cathepsin B activity; trypsin-inhibitory activity; and trypsin-like activity. However, none of these 4 activities could be detected in any fraction in spite of 114-fold concentration of the applied sample under such assay conditions (Chart 3). The results indicated that both types of TPI were unable to inhibit trypsin and revealed no other biochemical functions.
Chart 5. Gel filtration of papain-Sepharose eluate on Sephadex G-50. Pooled TPI obtained from a papain-Sepharose column was dialyzed, lyophilized, and then dissolved in 5 ml of the equilibration buffer and centrifuged at 1500 x g for 10 min. The resultant supernatant (1.0 ml) was applied to a Sephadex G-50 column (1.6 x 40 cm) and equilibrated with 10 mw phosphate buffer, pH 7.4, containing 0.2 M NaCl. Fractions of 1.0 ml were collected at a flow rate of 21 ml/hr. O, TPI activity; •, absorbance at 280 nm; I, sample collected (Fractions 41 to 49). Arrows, elution positions of the following standard proteins: 1, Blue Dextran; 2, chymotrypsinogen A (M, 25,000); 3, cytochrome c (M, 12,500); 4, aprotinin (M, 6,512); 5, bacitracin (M, 1,450). U, units.

Characterization of Melanoma TPI

Inhibition Spectrum. The inhibition spectrum of human melanoma TPI was investigated using partially purified LTPI. The amount of each enzyme used is indicated in µg in Chart 6. The incubation times were all 1 hr, except in the case of the ficin inhibition assay where it was 45 min. As shown in Chart 6, melanoma TPI strongly inhibited human cathepsin B, ficin, and papain but not bromelain. The TPI did not inhibit the serine protease group (trypsin, chymotrypsin, and plasmin), pepsin, or elastase using the standard assay methods. Human melanoma LTPI was found to have a high specificity for thiol proteases, although among the thiol proteases bromelain was not inhibited by melanoma LTPI.
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Stability. Three stability tests were performed using the partially purified melanoma TPI. After 5 freezings and thawings, about 96% of the activity remained. At neutral and alkaline pH values, the TPI was stable, but it was labile below pH 5.0 (Chart 7). As shown in Chart 8, it was relatively stable below 60° but was gradually inactivated above 70°.

DISCUSSION

TPI activity was first detected in the culture media of one particular established cell line (Bowes) of malignant melanoma. We then confirmed the presence of TPI activity also in the intracellular parts of the melanoma cells. Extracellular TPI activity was accumulated progressively in the culture media as the incubation time increased (Chart 2). The specific activity of TPI in the culture media also increased until 24 hr of incubation. However, melanoma cells cultured for 48 hr without fetal calf serum became more broken, so that abundant intracellular proteins flowed out into the culture media and the extracellular proteins increased. The specific activity of TPI in the 48-hr cultured media thus decreased in spite of the TPI activity increase. The specific activity of TPI in the 24-hr-incubated media was about 10-fold higher than that of the intracellular TPI or about 3.7-fold higher than that of the cytosol TPI. These results demonstrated that the cultured human melanoma cells were releasing TPI. Approximately 1.5 to 2.3 units of TPI per day was released per culture flask (full sheet, 75 sq cm; 1.0 ± 0.3 × 10⁷ (S.D.) cells; about 0.067 ml of cell volume). On the other hand, in culture media of nonmalignant cells (human fetal lung fibroblasts), TPI activity was neither accumulated nor detected.

Preliminary examinations by Sephadex G-150 column chromatography and partial purification of human melanoma TPI were carried out. The latter involved 2 column chromatographic steps, with papain-Sepharose and with Sephadex G-50. The human melanoma TPI consisted of 2 types, high-molecular-weight TPI and LTPI, of which LTPI was the main type. LTPI was therefore partially purified, and the LTPI thus obtained had a specific activity of 3215 units/mg protein.

Based on our investigations of the properties of the partially purified melanoma LTPI, the inhibitor was found to have a high specificity for thiol proteases and to be heat stable (60° for 60 min) like normal human TPIs. However, it displayed 3 distinct characteristics. (a) The melanoma LTPI had a lower molecular weight than did any of the normal human TPIs previously reported. Normal human serum TPI is reported to consist of 2 broad types. One is full-component TPI with a molecular weight of about 170,000, and the other is its subunit-like groups with molecular weights of approximately 90,000 and 95,000 (25, 26).

Urinary TPIs have been reported to have molecular weights of 76,000 and 22,500 (35). Epidermal TPI has a small molecular weight (from 12,100 to 13,100) (8). The present melanoma LTPI revealed molecular weights of 10,800 (gel filtration) or 9,800 (SDS-PAGE). (b) The melanoma LTPI was unable to inhibit bromelain, although normal human TPIs have been reported to inhibit bromelain with slightly lower activity than in their inhibition of other thiol proteases. (c) Concerning the combination of individual properties, recently, Wakamatsu et al. (41) reported that TPIs could be divided into 2 types, the serum type and the liver type, based on differences in their molecular weights, antigenicity, inhibition spectrum, and affinity to concanavalin A-Sepharose. The serum type had a large molecular weight (more than 90,000) (26, 41), and its inhibitory activity was stable at neutral pH but unstable at acid pH (26). The liver type had a small molecular weight (about 12,500) (8, 10, 41), and its inhibitory activity was unstable at neutral pH but stable at acid pH (10, 13). The present melanoma LTPI, however, cannot be assigned to either type. It is similar to the liver type from the viewpoint of its molecular weight but resembles the serum type from the viewpoint of its pH stability. This distinction from normal human TPIs is significant. Clearly, it is necessary to determine the detailed properties of melanoma TPI in order to assess its usefulness as a possible diagnostic tool for human melanomas. We plan to undertake immunological and enzymological investigations in the future.

Poole and Recklies et al. (20, 22) reported that human breast carcinoma secreted a thiol protease, cathepsin B, at significantly higher levels than found in nonmalignant tissue. Sloane et al. found that the cathepsin B activity was 3-fold higher in homogenates of B16 F10 tumor (a murine melanoma cell line of high
metastatic potential) than in those of B16 F1 tumor (the same with a low metastatic potential) (30) and indicated that cathepsin B activity could represent a possible marker for metastatic potential (31). Pietras et al. (18) reported that the serum cathepsin B level was high in women with clear cell adenocarcinoma of the genital tract. Thus, increase in cathepsin B activity has been correlated with the ability of primary malignant neoplasms to invade or metastasize normal tissues. In the present study, however, human melanoma cells appeared not to secrete cathepsin B and neutral thiol protease under the conditions of tissue culture used. Human melanoma cells were found to release dominantly TPI which strongly inhibits cathepsin B activity.

We cannot make a comparison with TPI release from other human melanoma cell lines or other cell lines, since there are as yet no further reports of such cells releasing TPI. We need to investigate the TPI activity of other malignant cell lines and also of tissues removed from patients with cancer. Further, it is clear that we need to consider the inhibitor as well as cathepsin B when discussing the mechanisms of invasion and metastasis of human melanoma cells and other malignant tumor cells in terms of thiol proteases.

There are several reports suggesting that TPI may play a significant role in regulating the inflammatory process (5, 16, 38). Moreover, Toki et al. (36) reported that purified urinary TPI suppressed the inflammatory reaction in Arthus skin lesions in rats. Based on these facts, it could also be speculated that the released TPI may in some way be related to the inflammatory reaction of hosts with a melanoma such as that used in the present experiment.

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