Tumor-associated Trypsin Participates in Cancer Cell-mediated Degradation of Extracellular Matrix

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ABSTRACT

We have recently demonstrated that many cancer cell lines produce a novel trypsinogen isoenzyme called tumor-associated trypsinogen 2 (TAT-2). It was found during a search of the target protease for tumor-associated trypsin inhibitor (TATI). We now show that degradation of subendothelial cell extracellular matrix (ECM) by four different cell lines (COLO 205 colon carcinoma, K-562 erythroleukemia, CAPAN-1 pancreatic carcinoma, and HT 1080 fibrosarcoma) can be partially inhibited by TATI or neutralizing trypsin antibodies. When cells were cultured in serum-free medium on ECM, TATI and trypsin antibodies inhibited the release of immunoreactive fibronectin fragments from ECM by 47–54 and 40%, respectively. Degradation of isotopically labeled [3H]serine, [3H]proline, and [35S]sulfate ECM was also significantly prevented by TATI. At its maximum, it exerted a 57% inhibition on the degradation of [3H]serine-labeled ECM. Plasminogen added exogenously to the culture medium further potentiated the proteolysis of ECM. Interestingly, addition of enteropeptidase, an activator of TAT-2, also enhanced cell-mediated proteolysis as assessed by degradation of purified fibronectin coated onto the surface of wells. Immunoblot analysis showed that enteropeptidase-mediated proteolysis generated a pattern of fibronectin fragments similar to that obtained by digestion of purified fibronectin by TAT-2. These results demonstrate the existence of a proteolytic system in tumor cells which is dependent on the activation of TAT-2. We suggest that TAT-2 is involved in a protease cascade-stimulating tumor cell invasion and degradation of extracellular matrix.

INTRODUCTION

Production of proteolytic enzymes by malignant tumor cells is believed to be essential to the ability of the tumor to invade and degrade extracellular matrix. Of the proteases secreted by tumor cells, collagenases (1), plasminogen activators (2), transin/stromelysin (3), and cathepsins (4–6) have been the most thoroughly studied. We have recently characterized a novel tumor-associated protease, TAT-I (7, 8). It was initially identified in the cyst fluid of mucinous ovarian tumors during a search of the target protease for TATI, which is a marker for mucinous ovarian cancer (9). TAT-I occurs as two isoenzymes, TAT-I and TAT-2. They are identical to the corresponding pancreatic trypsins with respect to amino-terminal amino acid sequence, molecular weight, and immunoreactivity, but they differ with respect to substrate specificity, enzyme stability, and elution pattern in ion-exchange chromatography. At present it is not known whether these differences are explained by tissue-specific modification of trypsin or whether distinct genes code for trypsinogenes derived from tumors and pancreas.

The levels of type IV collagenase (10), plasminogen activators (11), and cathepsin B (4) have been found to correlate with invasiveness and the metastatic ability of tumor cell lines. In extracts of tumor tissues the levels of urokinase-type plasminogen activator (u-PA) generally correlate with the degree of malignancy of the tumors (12). It is interesting that TAT-2, the major TAT isoenzyme, also shows close association with malignancy; the levels are higher in malignant and borderline ovarian tumor cyst fluids than in benign ones (13). In addition, purified TAT-1 and TAT-2 activate pro-u-PA in vitro (8). These results suggest that TAT-2 may promote the dissemination of tumor cells.

TAT-2 zymogen is produced by many cancer cell lines, and it has been purified from the culture medium of COLO 205 colon adenocarcinoma and HT 1080 fibrosarcoma cell lines (14). In the present study we have examined the role of TAT-2 in cell-mediated degradation of extracellular matrix. We developed a method for determining degradation of fibronectin based on analysis of immunoreactive fibronectin fragments liberated by tumor cells growing on protein-coated surfaces. We also analyzed the degradation of isotopically labeled ECM. The results show that tumor cell-mediated proteolysis can be significantly abrogated by inhibiting TAT-2.

MATERIALS AND METHODS

Chemicals. Reagents were obtained as follows: aprotinin from Sigma Chemical Co. (St. Louis, MO); EACA from Fluka AG (Buchs, Switzerland); RPMI 1640, Medium 199, PBS (without magnesium and calcium), and fetal bovine serum from Flow Laboratories (Irvine, Scotland); pooled human serum from the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland); L-glutamine and antibiotics from GIBCO Laboratories (Grand Island, NY); and gelatin from Merck (Darmstadt, Germany).

Proteinases. Human plasminogen and plasmin were obtained from Kabivitrum (Stockholm, Sweden). Porcine enteropeptidase and bovine trypsin were obtained from Sigma. Enteropeptidase was dissolved in PBS and passed through an affinity column of TATI-Sepharose 4B to remove contaminating trypsin-like enzymes. TAT-2 was purified by a monoclonal antibody affinity column from serum-free culture medium of COLO 205 cells as described (14). TAT-2 was obtained inzymogen form and autoactivated by neutralizing the pH.

Purification of TATI. TATI was purified by a novel affinity chromatography method from the urine of cancer patients. The pH of the urine was adjusted to 7.4, and it was dialyzed against water with a hollow fiber dialyzer (15) until the conductivity corresponded to that of 50 mM Tris, pH 7.4. Dialyzed urine was then applied at a flow rate of 60 ml/h at 4°C to a Q Sepharose anion-exchange column (Pharmacia, Uppsala, Sweden) and an affinity column of bovine trypsin-Sepharose 4B previously equilibrated with Tris buffer. Most urine proteins were removed by the first anion-exchange column. After the trypsin-Sepharose column was washed with Tris buffer containing 1 M NaCl, 0.1% Brij 35, and 1% 2-propanol, TATI was eluted with 0.1% trifluoroacetic acid. TATI was finally purified by a reversed-phase chromatography on a C18 column with a linear acetonitrile gradient (from 10 to 60% in 20 min) in 0.1% trifluoroacetic acid. By this method, 2 mg of TATI were obtained from 1 liter of urine.

Anticytolytic Antibodies. The IgG fraction was prepared from a rabbit preimmune serum and antiserum against human pancreatic trypsin I.
(8) by sequential affinity chromatography on Protein G-Sepharose and protein A-Sepharose (Pharmacia). Anti-u-PA IgG was prepared from goat antiserum against human low-molecular-weight u-PA (Biopool AB, Umeå, Sweden) by using protein A-Sepharose. Anti-trypsin IgG and anti-u-PA IgG were anticycatalytic, because they inhibited the amido
dolysis activity of TAT-2 and u-PA, respectively.

Cell Culture. Human cancer cell lines COLO 205 colon adenocarcinoma (ATCC CCL 222), HT 1080 fibrosarcoma (ATCC CCL 121), K-562 erythroleukemia (ATCC CCL 243), and CAPAN-1 pancreatic adenocarcinoma (ATCC HTB 79) were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in 800-
mM Nunclon flasks (Roskilde, Denmark) and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 100 µg/ml streptomycin. Treatment with trypsin was not required for detachment of the adherent cells, possibly because the cells produce their own trypsin. After serum removal, and the flasks were washed twice with PBS, cells were incubated for 10–15 min in PBS at room temperature. The flasks were vigorously shaken until all cells detached.

Preparation of Wells Coated with ECM and Fibronectin. Endothelial cells were isolated from human umbilical cord veins by the method originally described by Jaffe et al. (16), and ECM was prepared essentially as described (17, 18). Endothelial cells were seeded in Nunclon 24-well multidishes, which were previously coated with gelatin, and cultured in Medium 199 containing 20% human serum. Labeling medium containing 5 µCi/ml [3H]serine, 5 µCi/ml [3H]proline, or 20 µCi/ml [35S]sulfate was supplemented with 20 µg/ml fresh ascorable added daily. Labeling medium was replaced after 3 days of culture. After culturing for 5 to 7 days, cells were lysed by addition of 30 mM NH4OH for 30 min. The wells were washed with PBS, H2O, and 70% ethanol. To prepare wells coated with fibronectin, Nunclon 24-well multidishes were incubated with fibronectin (2 µg/ml in PBS, 1 ml/well) overnight at 37°C. Before use, the wells were washed twice with PBS. Fibronectin was purified from human plasma as described (19).

Degradation of Fibronectin and Labeled ECM. Cells were plated at varying densities in coated wells as described in the text and cultured in serum-free RPMI. In some experiments plasminogen and enteropeptidase were added at final concentrations of 6 and 1 µg/ml, respectively. Immune and preimmune IgG were used at concentrations of 10–25 µg/ml. The concentrations of TAT1 and aprotinin were 1–10 µg/ml, and that of EACA was 330 µg/ml. Aliquots of 50 and 200 µl were collected at various time points, centrifuged at 9000 rpm for 5 min, and analyzed by fibronectin immunoassay and β-scintillation counting, respectively.

Detection of Fibronectin Immunoreactivity. Polyclonal antibodies were prepared from rabbit antiserum against fibronectin (20) by Na2SO4 precipitation and used to develop a time-resolved immunofluorometric assay according to methods previously described (21). In a typical assay of cell culture media, the samples were incubated for 60 min at 25°C in microtiter wells coated with the fibronectin antibodies. After washing, the wells were incubated for 30 min with fibronectin antibodies that had been labeled with a europium chelate (Wallac Oy, Turku, Finland) (22). After washing of the wells and addition of an “enhancement solution” (22), the bound europium was measured with an Arcus 1230 time-resolved fluorometer (Wallac). Using a 50-µl sample volume, the linear measuring range of the assay was 0.5–250 ng/ml of fibronectin, and the coefficient of variation was below 10% in this concentration range.

Characterization of Fibronectin Fragments. The culture media were dialyzed against 5 mM NH4HCO3 for 2 h, lyophilized, and analyzed by gel filtration and immunoblotting. Gel filtration was performed on a Superose 12 column (Pharmacia) at a flow rate of 30 ml/h, using 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl and 0.02% NaN3. Fractions of 400 µl were collected and analyzed by fibronectin immunoassay. Sodium dodecyl sulfate gel electrophoresis was performed on 7.5% polyacrylamide gels under nonreducing conditions (23). Proteins were electrotransferred to nitrocellulose membranes (24), and fibronectin fragments were detected by incubating with polyclonal fibronectin antibodies followed by secondary peroxidase-conjugated swine anti-rabbit antibodies (Dakopatts, Glostrup, Denmark). Peroxidase staining was performed with 3,3'-diaminobenzine tetrahydrochloride (Fluka).

RESULTS

The adherent tumor cell lines used in this study can easily be detached from culture dishes by removing the serum and incubating the cells in PBS. When COLO 205 cells collected this way were plated in serum-free medium in fibronectin-coated wells, immunoreactive fibronectin was released into the serum-free medium as detected by fibronectin immunoassay (Fig. 1). Control experiments showed that fibronectin immunoreactivity was not released into the medium in the absence of cells. In addition, COLO 205 cells themselves were not found to secrete fibronectin.

The cell-mediated release of coated fibronectin could be significantly increased by adding plasminogen zymogen or active enteropeptidase to the culture medium (Fig. 1). Fibronectin immunoreactivity was readily detectable after a 4-h culture and increased further over 65 h. Control experiments indicated that plasminogen and enteropeptidase alone at the low concentrations used (6 and 1 µg/ml, respectively) did not cause degradation of coated fibronectin during a 24-h incubation. However, fibronectin appears to be sensitive to higher concentrations of enteropeptidase (20 µg/ml), as demonstrated by release of fibronectin immunoreactivity from the wells (not shown).

The fibronectin immunoreactivity released into the culture medium during 24-h culture was analyzed by gel filtration and immunoblotting. Gel filtration experiments indicated that proteolysis mediated by both untreated cells and cells treated with plasminogen or enteropeptidase yielded a major immunoreactive fragment with a molecular mass of about 200 kDa (not shown). In addition, several minor fragments of smaller molecular weight were detected. Immunoblot analysis showed differences in the patterns of fibronectin fragments generated by treatment with enteropeptidase and plasminogen, respectively (Fig. 2). Plasminogen-mediated proteolysis generated larger fibronectin fragments (>100 kDa) than those mediated by enteropeptidase (Fig. 2, Lanes 4 and 5). A similar difference in fragment pattern was found when purified fibronectin was digested by plasmin and TAT-2, respectively (Fig. 2, Lanes 1 and 2). In addition, enteropeptidase treatment and TAT-2 digestion yielded a characteristic fragment migrating at 85 kDa. Proteolysis mediated by untreated cells predominantly generated large (>100 kDa) fibronectin fragments (Fig. 2, Lane 6).

The plasminogen-mediated proteolysis of fibronectin was
inhibited by aprotinin, EACA, and anticalytic polyclonal u-PA IgG, but not by TATI or anticalytic polyclonal trypsin IgG (Table 1). By contrast, TATI and anti-trypsin IgG inhibited both the enteropeptidase-mediated proteolysis and the proteolysis mediated by untreated cells (Table 1). TATI and anti-trypsin IgG caused 81 and 92% inhibition on the enteropeptidase-mediated proteolysis, respectively, and 75 and 32% inhibition on the proteolysis mediated by untreated cells, respectively. Preimmune IgG had no effect.

When COLO 205 cells were plated on ECM deposited by human umbilical vein endothelial cells, proteolysis of fibronectin was very rapid (Fig. 3). Fibronectin immunoreactivity was detected in the medium after a 1-h culture and further increased for 24 h. After a 6-h culture, large (>200 kDa) fibronectin fragments predominated in the culture medium, as detected by immunoblotting (not shown). TATI and anti-trypsin IgG inhibited degradation by 51 and 40%, respectively (Figs. 3 and 4). The proteolysis caused by two other cell lines which produce trypsin antibodies. Cells were plated on ECM-coated wells at a density of 2 x 10^5/well and incubated in 1 ml serum-free medium in the absence (C) or presence of anti-trypsin IgG (●) and preimmune IgG (□). Control wells were incubated without cells (X). The concentrations of immune and preimmune IgG were 25 μg/ml. Aliquots of culture media were collected for analysis by fibronectin immunoassay at 2-h intervals. Points, means for quadruplicate measurements; bars, SD.

![Fig. 2. Immunoblot analysis of fibronectin fragments released by cells from coated wells or generated by digestion of purified fibronectin with TAT-2 and plasmin. Lane 1, fibronectin digested for 1 h with plasmin (0.025 casein unit); Lane 2, fibronectin digested for 1 h with TAT-2 (10 ng); Lane 3, untreated fibronectin; Lane 4, culture medium of plasminogen-treated cells; Lane 5, culture medium of enteropeptidase-treated cells; Lane 6, culture medium of untreated cells. Asterisks, 85-kDa fragment characteristic of enteropeptidase- and TAT-2-mediated proteolysis.](https://example.com/fig2)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% of inhibition with following additions to serum-free culture medium</th>
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<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>TATI</td>
<td>75 ± 6.2</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>80 ± 8.4</td>
</tr>
<tr>
<td>EACA</td>
<td>ND</td>
</tr>
<tr>
<td>Anti-trypsin IgG</td>
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<tr>
<td>Preimmune IgG</td>
<td>3 ± 1.2</td>
</tr>
<tr>
<td>Anti-u-PA IgG</td>
<td>0 ± 8.0</td>
</tr>
</tbody>
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* TATI and aprotinin were used at a concentration of 1 μg/ml, EACA at a concentration of 330 μg/ml, and preimmune and immune IgG at a concentration of 10 μg/ml.

* Fibronectin immunoreactivity released into the culture medium was determined by the fibronectin immunoassay after culturing for 16-24 h. The inhibition is given as the decrease of the immunoreactivity compared with that observed in the absence of inhibitors. Cells (2 x 10^5) were plated on fibronectin-coated wells in the presence or absence of plasminogen and enteropeptidase in serum-free medium.

* Mean ± SD for at least 2 experiments with 2 wells/experiment.

* ND, not determined.

![Fig. 3. Inhibition of COLO 205 cell-mediated degradation of ECM fibronectin by trypsin antibodies. Cells were plated on ECM-coated wells at a density of 2 x 10^5/well and incubated in 1 ml serum-free medium in the absence (C) or presence of anti-trypsin IgG (●) and preimmune IgG (□). Control wells were incubated without cells (X). The concentrations of immune and preimmune IgG were 25 μg/ml. Aliquots of culture media were collected for analysis by fibronectin immunoassay at 2-h intervals. Points, means for quadruplicate measurements; bars, SD.](https://example.com/fig3)

![Fig. 4. Prevention of cancer cell-mediated degradation of ECM fibronectin by TATI. COLO 205, K-562, and CAPAN-1 cells were seeded at densities of 2 x 10^5, 5 x 10^4, and 2.5 x 10^4/well, respectively, in 0.5 ml serum-free medium. Cells were cultured in the absence (○) or presence (□) of TATI, and the culture media were analyzed for fibronectin immunoreactivity. COLO 205 cells were incubated for 4 h, and K-562 and CAPAN-1 cells were incubated overnight. The concentration of TATI was 10 μg/ml. Columns, means from triplicate wells; bars, SD. Untreated control wells did not release fibronectin immunoreactivity.](https://example.com/fig4)
serum-free RPMI medium. Cells were cultured for 24-48 h in the absence (•) or presence (□) of 5 × 10⁴/well, and CAPAN-1 cells at a density of 2.5 × 10⁴/well in 0.5 ml [35S]sulfate-labeled ECM. COLO 205 and HT 1080 cells were seeded at a density of 10000 cells/well in COLO 205 cells was 63%. TATI in COLO 205 cells was 63%.

As in the case of fibronectin proteolysis, addition of plasminogen to the culture medium substantiated the degradation of all three isotopically labeled matrices by COLO 205 cells. In contrast to the proteolysis of fibronectin, TATI weakly but significantly prevented proteolysis in the presence of plasminogen (Fig. 6). The TATI-mediated inhibition (expressed in cpm) corresponded to the radioactivity released in the plasminogen-independent proteolysis. Therefore, TATI may have inhibited the portion of proteolysis that was exerted by TAT-2 but not by plasmin. Active TAT-2 purified from COLO 205 culture medium (100 ng) hydrolyzed all three labeled matrices, as demonstrated by release of label into the medium (not shown).

DISCUSSION

These studies demonstrate that the novel trypsinogen iso-enzyme TAT-2 produced by cancer cells contributes to the cell-mediated degradation of ECM. To demonstrate TAT-2 activity in vitro, it was essential to culture cells in serum-free medium to avoid the presence of serum trypsin inhibitors and plasminogen. Under these conditions, the specific inhibitor TATI prevented the proteolysis of fibronectin or isotopically labeled ECM by tumor cell lines that produce TAT-2. Further evidence for involvement of TAT-2 in proteolysis was the finding that anticalytic trypsin antibodies were inhibitory but preimmune antibodies had no effect. In addition, the cell-mediated degradation of fibronectin was augmented by adding exogenous enteropeptidase, an activator of trypsinogen, to the culture medium. Analysis of fibronectin fragments generated indicated that enteropeptidase-induced proteolysis yielded fragments similar to those formed by digestion of purified fibronectin with TAT-2.

Previous studies have indicated that cell-mediated degradation of ECM or protein substrates is enhanced by addition of plasminogen (17, 18, 25-28). This was also found in the present study. Addition of plasminogen to serum-free medium increased up to 10-fold the degradation of labeled ECM and fibronectin by COLO 205 cells. Plasminogen-dependent tumor cell-mediated proteolysis can be inhibited by protease nexin I (29), plasminogen activator inhibitor 1 (30), or plasminogen activator inhibitor 2 (18), indicating an important role for u-PA in the protease cascade. However, several investigators have found that there is also a basal cell-mediated proteolytic activity in the absence of plasminogen (25-28). Our results suggest that in the cell lines studied a major portion of this is due to the activity of TAT-2. In addition to degrading ECM by itself, TAT-2 can also participate in the activation of pro-u-PA (8). This could be an explanation for the rapid activation of plasminogen exogenously added to cell culture.

Perhaps surprisingly, TATI prevented the degradation of [3H]proline-labeled ECM by all three tumor cell lines studied: COLO 205; CAPAN-1; and HT 1080. As the [3H]proline label is assumed to be biosynthetically incorporated primarily into the collagen component of newly synthesized subendothelial matrix (26), our results suggest that TAT-2 could mediate the degradation of collagenous material. This is also supported by the finding that purified TAT-2 released [3H]proline label from the matrix. It remains to be shown whether TAT-2 can degrade native collagens in ECM or whether it degrades other trypsin-sensitive proline-containing matrix components. It is also possible that TAT-2 can activate latent collagenase, because trypsin is a known activator of procollagenase (1).

TATI inhibited the degradation of [3H]serine-labeled ECM by COLO 205 and CAPAN-1 cells and the degradation of [35S]sulfate-labeled ECM by COLO 205 cells. The [3H]serine label would be biosynthetically incorporated into most matrix proteins (18), and [35S]sulfate label into sulfated proteoglycans and proteins (25). These results suggest that depending on tumor cell line, TAT-2 contributes to the degradation of matrix (glyco)proteins and proteoglycans. Because TATI did not inhibit the destruction of all labeled matrices by CAPAN-1 and HT 1080 cells, other proteinases not characterized in this study are also involved in the proteolysis. When proteolysis by COLO 205 cells was studied in plasminogen-supplemented medium, TATI caused an inhibition similar to that observed in the absence of plasminogen, apparently that portion that was due to TAT-2. These results suggest that ECM degradation is accomplished by the concerted action of plasmin, plasminogen activators, TAT-2, and other proteases. In COLO 205 cells plasminogen was activated by u-PA, inasmuch as the proteolysis
of fibronectin could be partially inhibited by anticalcine urokinase antibodies. Recently, COLO 205 cells have been shown to express u-PA and its mRNA (18, 31).

Because TAT-2 is secreted in trypsinogen form from the cells (14), an obvious question is how it is activated. Part of TAT-2 is in active form in cell culture as indicated by inhibition of proteolysis by TATI and trypsin antibodies. Enteropeptidase (32) and cathepsin B (33) are activators of pancreatic trypsinogen. Purified TAT-2 is activated by enteropeptidase (14), and we show here that it potentiates COLO 205 cell-mediated degradation of fibronectin. The potentiation is due to activation of TAT-2, as the proteolysis can be inhibited by TATI and trypsin antibodies. It remains to be shown whether an enteropeptidase-like protease-activating TAT-2 exists in tumor cells.

This study demonstrates that TATI, also known previously as pancreatic secretory trypsin inhibitor (34), is a potential inhibitor of tumor cell-mediated degradation of ECM. Even though the plasminogen-dependent proteolysis was poorly or not at all inhibited by TATI, apparently because it is only a weak inhibitor of plasmin with a Kᵢ of 9.7 nm (35), TATI may play an important matrix-stabilizing role by controlling the activation of TAT-2 zymogen. Coexpression of TATI with TAT-2 in the cyst fluids of ovarian tumors (13) and cancer cell lines COLO 205 (14) and CAPAN-1 (36) further suggests a specific role for TATI in controlling TAT-2. TATI was initially identified as a tumor marker for ovarian cancer (37, 38), but the levels in serum and urine are also elevated in patients with other types of advanced cancer (9, 37, 39). We suggest that the elevation of TATI is a reaction to TAT-2 expression with the n.

REFERENCES


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