Cyst Fluid of Ovarian Cancer Patients Contains High Concentrations of Trypsinogen-2

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ABSTRACT

We have determined the concentrations of two tumor-associated trypsinogen (TAT) isoenzymes, called TAT-1 and TAT-2, in human ovarian tumor cyst fluid using monoclonal antibody-based immunofluorometric assays specific for each isoenzyme. TAT-1 and TAT-2 are immunologically indistinguishable from the two pancreatic trypsinogen isoenzymes, cationic trypsinogen (-1) and anionic trypsinogen (-2). Our results show that the two isoenzymes TAT-2 is the predominant form in cyst fluid and its concentrations are significantly higher than the levels of the cationic trypsinogen (-1) and anionic trypsinogen (-2). Our results show that the two isoenzymes TAT-2 is the predominant form in cyst fluid and its concentrations are significantly higher than the levels of the cationic trypsinogen (-1) and anionic trypsinogen (-2). The increased levels of TAT-2 were also observed (median 62 µg/liter). In fluids from other types of malignant ovarian carcinomas slightly elevated levels of TAT-2 were also observed (median 62 µg/liter). The identity of the trypsinogens was verified by isolating them by immunofinity chromatography using monoclonal antibodies. The increased levels in association with malignancy suggest that TAT is involved in ovarian tumor dissemination and breakage of tissue barriers.

INTRODUCTION

A critical step in cancer progression is the dissolution of basement membrane and extracellular matrix surrounding the tumor and the subsequent dissemination of malignant cells (1, 2). The conversion of the tumor from benign to invasive and malignant is believed to involve increased production of matrix-degrading enzymes that may form a protease cascade. A correlation between production of certain proteases and metastasis has been observed for some transformed cell lines (3-5), and specific inhibitors of urokinase-type plasminogen activator, collagenase, and cathepsins are able to block cellular invasion and/or matrix degradation (6-9). In addition, increased levels of urokinase-type plasminogen activator extracted from tumors have been found to correlate with invasiveness and grade of malignancy in various cancers, e.g., human colon and urinary bladder cancers (10, 11).

Increased levels of protease inhibitors in association with cancer have also been observed (12). This may reflect a defense mechanism of the host against tumor invasion. We have studied the clinical behavior and biology of a M, 6000 peptide called TAT, which was originally identified as a tumor marker for ovarian cancer (13, 14). It is identical to the Kazal-type pancreatic secretory trypsin inhibitor that controls trypsinogen activation in pancreatic secretions (15). Cyst fluid of ovarian tumors contains high levels of TATI (16), and elevated serum and urine levels occur in cancer patients (17, 18) but also in connection with severe infections (17) and after major surgery (19). These results suggest an association of TATI/pancreatic secretory trypsin inhibitor, not only with cancer but also with the acute phase reaction.

A protease similar to pancreatic trypsin, called TAT, occurs in mucinous ovarian tumor cyst fluids which also contain high levels of TATI (20, 21). The two isoenzymes of TAT, called TAT-1 and TAT-2, are similar to pancreatic trypsinogen-1 and -2, respectively, with respect to aminoterminal amino acid sequence, molecular weight, and immunoreactivity, but the tumor-derived enzymes differ slightly from their pancreatic counterparts with respect to substrate specificity. We have produced monoclonal antibodies against TAT and developed immunoassays for the measurement of each isoezyme (22). We have now determined the concentrations of the isoenzymes in ovarian tumor cyst fluid and used affinity chromatography with these antibodies to isolate the enzymes. TAT-2 was found to be the predominant isoenzyme, and its levels were remarkably higher in malignant than in benign tumors.

MATERIALS AND METHODS

Samples. Cyst fluid was obtained from patients undergoing surgery for removal of the tumor during the years 1985-1989 at Helsinki University Central Hospital. Their ages ranged from 22 to 81 years. The samples were classified by histopathology into three main groups: mucinous, serous, and other types of ovarian tumors. The last mentioned group comprised 5 benign and 7 malignant cases. The benign tumors included a follicle cyst, a corpus luteum cyst, a dermoid cyst, a struma ovari, and a cystic teratoma. The malignant cases included 3 mesonephroid carcinomas, 2 unclassified carcinomas, one endometroid carcinoma, and one metastatic carcinoma of unknown origin. The volume of cyst fluid obtained ranged from 2 to 4000 ml. The fluids were centrifuged at 10,000 rpm for 10 min and the aliquots stored at -20°C until assayed. Preovulatory follicular fluid was obtained from patients participating in an in vitro fertilization program and stored at -20°C until examined. All samples were from hyperstimulated cycles.

Immunonaassays. Two sandwich immunofluorometric assays were used, one recognizing TAT-1 and the other one TAT-2. Each assay is based on a combination of two MAb, one immobilized onto the walls of microtiter strips and the other one used as a tracer. The combinations of MAb and establishment of the immunoassays have been described (22). The sensitivity of the TAT-1 assay was 0.1 µg/liter and that of the TAT-2 assay was 0.3 µg/liter. The assays for each isoezyme showed <1% cross-reaction with the other isoezyme. In the assay for TAT-1 pancreatic trypsin-1 inhibited with phenylmethylsulfonyl fluoride was used as the standard covering the concentration range 0.2-245 µg/liter. In the TAT-2 assay thezymogen form of TAT-2 was used as standard, and the tne range was 0.96-495 µg/liter. Radioimmunoassay of TATI was performed as described (13).

Purification of TAT-1 and TAT-2. Purification of TAT-1 and TAT-2 was performed essentially as described (21) except that in immunofinity chromatography MAb were used instead of polyclonal antibodies. Briefly, pooled cyst fluid from serous and mucinous tumors was centrifuged, dialyzed, and subjected to batchwise anion exchange chromatography on Q Sepharose (Pharmacia). The fraction eluting with 14FIO in the second affinity column. These antibodies recognize TAT-1 and TAT-2, respectively (22). After washing, the columns were...
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dissociated and eluted with 0.1% trifluoroacetic acid containing 1
mM CaCl₂ at a flow rate of 12 ml/h. Fractions of 2 ml volume were
collected. The eluate was neutralized with 1 M Tris-HCl containing 10
mM benzamidine (pH 9.0), and the immunoaffinity chromatography
was repeated to remove nonspecifically bound proteins. TAT-2 was
finally purified by reversed-phase high performance liquid chromatog-
raphy using an acetonitrile gradient (21).

Analytical Methods. SDS-gel electrophoresis was performed accord-
ing to the method of Laemmli (23) under nonreducing conditions on
thin 20% polyacrylamide gels using the Pharmacia Phastsystem (Phar-
macia, Uppsala, Sweden). Proteins were stained with silver according
to the instructions of the manufacturer. Gel filtration was performed
on a Superose 12 column (Pharmacia) in 50 mM Tris buffer (pH 7.4)
containing 0.1 M NaCl and 0.02% NaN₃ at a flow rate of 0.5 ml/min.
The sample volume was 200 μl and fraction size 400 μl. Before appli-
cation, cyst fluid was filtered through 0.22-μm pore filters (Millipore)
to remove viscous mucus.

Determination of Trypsin Activity. The eluate after the second round
of immunoaffinity purification was neutralized with 1 M Tris-HCl
containing 20 mM CaCl₂ (pH 9.0), and the zymogens were autoactivated
by incubation at 37°C for 2 h. Trypsin activity was determined using
S-2222 (Kabi, Stockholm, Sweden) as a substrate. The concentrations
of active trypsin were determined by active-site titration with the specific
inhibitor TATI (21).

Statistical Analysis. Student's unpaired t test was used for compar-
ison of TAT and TATI levels between groups. The data were log-
transformed before evaluation. P <0.05 was considered statistically
significant.

RESULTS

Concentrations of TAT in Ovarian Follicular Fluid. In hyper-
stimulated follicular fluid the median levels (± SD) of TAT-1 and
TAT-2 were 22 ± 6 and 15 ± 5 μg/liter, respectively, which were
similar to those in normal serum. The range of TAT-1 and TAT-2 levels in normal serum have been found to be 2–65
μg/liter and 3–38 μg/liter, respectively (22). As in serum, type 1 tryp-
sinogen was also the predominant form in follicular fluid (Fig. 1).

Concentrations of TAT in Mucinous Cyst Fluids. Mucinous
fluids contained the highest levels of TAT-2, and the two
samples of malignant and two of three samples of borderline
cyst fluids studied contained very high concentrations of TAT-
2, i.e., 100–1000-fold those in follicular fluid (Fig. 1B; Table
1). Some benign cases contained levels similar to those in
malignant fluids. However, the difference in the TAT-2 level
between the benign cases on one hand and the malignant and
borderline cases on the other hand was statistically significant
(P = 0.028) (Fig. 1B).

In contrast to TAT-2, the concentrations of TAT-1 in mucin-
ous cyst fluids were generally similar to those in follicular
fluid and serum (Table 1), but in one sample of malignant fluid
the level was clearly elevated (1260 μg/liter) and in three
samples of benign fluids it was moderately elevated (107, 111,
and 206 μg/liter) (Fig. 1A). There was no statistically significant
difference between benign cases on one hand and borderline
and malignant ones on the other hand.

Concentrations of TAT in Serous Cyst Fluids. In serous cyst
fluids, the TAT-2 levels were elevated in all borderline and
malignant cases, but the levels were not as high as in the
malignant mucinous tumors. In the benign cyst fluids the levels
were similar to those in serum (Table 1), and they were signifi-
cantly lower than those in malignant and borderline cases (P
= 0.000) (Fig. 1A).

The levels of TAT-1 were higher than those in serum (>65
μg/liter) in one borderline and two malignant but not in any of
the benign cases, and there was a significant difference in the
level between the borderline and malignant versus benign cases
(P = 0.008) (Fig. 1A).

Concentrations of TAT in Other Tumors. In cyst fluids from
other malignant ovarian tumors the median TAT-2 concentra-
tion was 3-fold that in follicular fluid (Table 1), and in 4 of the
7 cases the concentrations exceeded the serum levels (>60 μg/
liter) (Fig. 1B). Slightly elevated TAT-2 levels were also de-
tected in 2 of 5 benign ovarian tumors, in a follicle cyst, and in
a struma ovarii (Fig. 1B). The difference in TAT-2 levels
between malignant and benign cases was not significant. The
levels of TAT-1 were similar to those in serum (Table 1). Only
one carcinoma had a TAT-1 level (111 μg/liter) higher than in
normal serum (Fig. 1A).

Concentrations of TATI in Cyst Fluids. Very high concentra-
tions of the trypsin inhibitor TATI occurred in mucinous fluids,
both in benign and malignant ones (Table 1). In contrast, with
the exception of one malignant adenocarcinoma, serous fluids
contained TATI at levels similar to those in serum (3–21 μg/
liter, Ref. 13). TATI levels were not elevated in other benign
ovarian tumors, but 4 malignant ones exhibited high levels (69–
240 μg/liter).

Characterization of TAT in Cyst Fluid by Gel Filtration. Gel
filtration of cyst fluid indicated that the immunofluorometric
assays recognize components eluting at molecular weights of
about 25,000–28,000, which corresponds to the molecular
weight of trypsinogen (Fig. 2). In addition, a minor immuno-
reactive peak eluted ahead of the M, 25,000–28,000 weight
components. There was a small difference in the elution volume
of the two TAT isoenzymes in that TAT-2 eluted slightly earlier
than TAT-1. This indicates a difference in relative molecular

Fig. 1. Concentrations of TAT-1 (A) and
TAT-2 (B) in normal (C) follicular (Follic.)
fluid and cyst fluid from benign (B), borderline
(A), and malignant (M) ovarian tumors. Bars,
median levels.
The column was calibrated with albumin (M, 67,000), ovalbumin (M, 43,000), a Superose 12 column, and the fractions were assayed for TAT-1 and TAT-2, soybean trypsin inhibitor (M, 20,000), and aprotinin (M, 6,000). Because of the low level in the starting material and because of weight that has also been found by sodium dodecyl sulfate-gel electrophoresis, which showed molecular weights of 28,000 and 25,000 for TAT-2 and TAT-1, respectively (21). This suggests that TAT measured by the immunosays predominantly is in the zymogen form. The minor peak of TAT-2 immunoreactivity eluting before the main peak could represent a complex of the enzyme with an inhibitor. The elution pattern of TAT-1 and TAT-2 corresponded to about 10% of the protein content determined on the basis of the absorbance at 280 nm.

**DISCUSSION**

This study shows that cyst fluid of ovarian cancer patients contains high levels of two trypsinogen isoenzymes. TAT-2 is the predominant isoenzyme and its concentrations may be more than 1000-fold those in normal serum and hyperstimulated ovarian follicular fluid. These results suggest that ovarian tumors and especially the mucinous ones produce considerable amounts of the very potent trypsinogen isoenzyme TAT-2. The levels found in fluids of many malignant tumors (several mg/liter) are actually close to the levels of trypsinogen in duodenal juice (24). An interesting finding in this study was that the levels of TAT-2 and TAT-1 were higher in borderline and malignant than in benign cyst fluids of both mucinous and serous tumors. In addition, the levels in malignant cyst fluids were higher than those in borderline tumors (see Fig. 1). The TAT level thus appears to correlate with degree of malignancy. It is also notable that many benign mucinous tumors contained high levels of TAT-2, but this was not the case with benign serous tumors.

Whereas trypsinogen-2 was the major isoenzyme in tumor fluids, type-1 trypsinogen was the principal isoenzyme in preovulatory follicular fluid, which was studied for comparison. The concentrations of the two trypsinogens in follicular fluid were similar or slightly lower than the serum levels, and it is thus possible that the trypsinogens are derived from peripheral plasma. It is notable that follicular fluid proteins are mainly produced by the theca and granulosa cells, whereas most ovarian tumors arise from epithelial cells (25). At present it is not known whether trypsinogen isoenzymes are produced by nor-

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**Table 1** Concentrations of TAT-1, TAT-2, and TATI in mucinous, serous, and other types of ovarian tumor cyst fluids

<table>
<thead>
<tr>
<th>Type of cyst fluid</th>
<th>n</th>
<th>TAT-2 (µg/liter) Median</th>
<th>Range</th>
<th>TAT-1 (µg/liter) Median</th>
<th>Range</th>
<th>TATI (µg/liter) Median</th>
<th>Range</th>
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<tbody>
<tr>
<td>Mucinous</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Benign</td>
<td>22</td>
<td>84</td>
<td>11-10,400</td>
<td>16</td>
<td>1-206</td>
<td>2,360</td>
<td>7-9,840</td>
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<td>Borderline or malignant</td>
<td>5</td>
<td>2,640</td>
<td>6-31,200</td>
<td>42</td>
<td>2-1,260</td>
<td>6,640</td>
<td>1,880-15,000</td>
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<td></td>
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<tr>
<td>Benign</td>
<td>6</td>
<td>18</td>
<td>8-44</td>
<td>8</td>
<td>1-31</td>
<td>13</td>
<td>3-18</td>
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<tr>
<td>Borderline or malignant</td>
<td>5</td>
<td>345</td>
<td>201-1,790</td>
<td>86</td>
<td>18-127</td>
<td>15</td>
<td>11-1,200</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign</td>
<td>5</td>
<td>32</td>
<td>5-95</td>
<td>17</td>
<td>2-25</td>
<td>10</td>
<td>3-14</td>
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<tr>
<td>Malignant</td>
<td>7</td>
<td>62</td>
<td>15-563</td>
<td>31</td>
<td>3-111</td>
<td>69</td>
<td>6-240</td>
</tr>
</tbody>
</table>

* P < 0.05, significance of the difference of borderline or malignant cases vs. benign cases.

* P < 0.01, significance of the difference of borderline or malignant cases vs. benign cases.

Fig. 1. Gel filtration of cyst fluid. Two hundred µl cyst fluid was separated on a Superose 12 column, and the fractions were assayed for TAT-1 and TAT-2. The column was calibrated with albumin (M, 67,000), ovalbumin (M, 43,000), soybean trypsin inhibitor (M, 20,000), and aprotinin (M, 6,000).

Table 2 Extraction of trypsin activity from cyst fluid by immunoaffinity chromatography

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Trypsin activity (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material</td>
<td>1,200</td>
<td>26,000</td>
<td></td>
</tr>
<tr>
<td>Q Sepharose</td>
<td>300</td>
<td>21,000</td>
<td></td>
</tr>
<tr>
<td>MAb[14F10]-Sepharose</td>
<td>4</td>
<td>1.9</td>
<td>9</td>
</tr>
<tr>
<td>MAb[14E8]-Sepharose</td>
<td>6</td>
<td>3.2</td>
<td>300</td>
</tr>
</tbody>
</table>

Fig. 2. Gel filtration of cyst fluid. Two hundred µl cyst fluid was separated on a Superose 12 column, and the fractions were assayed for TAT-1 and TAT-2. The column was calibrated with albumin (M, 67,000), ovalbumin (M, 43,000), soybean trypsin inhibitor (M, 20,000), and aprotinin (M, 6,000).

nonspecific binding of cyst fluid proteins to the immunoaffinity matrix (not shown).

DISCUSSION

This study shows that cyst fluid of ovarian cancer patients contains high levels of two trypsinogen isoenzymes. TAT-2 is the predominant isoenzyme and its concentrations may be more than 1000-fold those in normal serum and hyperstimulated ovarian follicular fluid. These results suggest that ovarian tumors and especially the mucinous ones produce considerable amounts of the very potent trypsinogen isoenzyme TAT-2. The levels found in fluids of many malignant tumors (several mg/liter) are actually close to the levels of trypsinogen in duodenal juice (24). An interesting finding in this study was that the levels of TAT-2 and TAT-1 were higher in borderline and malignant than in benign cyst fluids of both mucinous and serous tumors. In addition, the levels in malignant cyst fluids were higher than those in borderline tumors (see Fig. 1). The TAT level thus appears to correlate with degree of malignancy. It is also notable that many benign mucinous tumors contained high levels of TAT-2, but this was not the case with benign serous tumors. Whereas trypsinogen-2 was the major isoenzyme in tumor fluids, type-1 trypsinogen was the principal isoenzyme in preovulatory follicular fluid, which was studied for comparison. The concentrations of the two trypsinogens in follicular fluid were similar or slightly lower than the serum levels, and it is thus possible that the trypsinogens are derived from peripheral plasma. It is notable that follicular fluid proteins are mainly produced by the theca and granulosa cells, whereas most ovarian tumors arise from epithelial cells (25). At present it is not known whether trypsinogen isoenzymes are produced by nor-

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mal ovarian tissue and whether they participate in the proteolytic rupture of the follicle during ovulation. The degradation of the follicular wall during ovulation is believed to be due to the hormone-dependent induction of plasminogen activators and collagenase (26, 27).

To confirm the identity of the immunoreactivity measured, we purified TAT using monoclonal antibody affinity chromatography. The isolated trypsinogens could be activated and the corresponding pancreatic enzyme, anionic trypsin, has also been shown to be sensitive to autolysis after activation (28).

This study confirms earlier observations (16) showing that the specific TAT inhibitor TATI is expressed at high levels in cyst fluid from both benign and malignant mucinous cystadenomas but not in the benign serous tumors and only in some malignant ones. Interestingly, comparison of the levels of TAT and TATI in serous cyst fluid from borderline and malignant tumors revealed 4 cases in which the concentrations of both TAT isoenzymes were remarkably elevated but the levels of TATI were not. This might reflect a disturbance in proteolytic balance, which may contribute to the invasive properties of malignant cells.

Expression of proteolytic enzymes is typical of many invasive tumors (1–6). The levels of TAT-2 in malignant mucinous tumors were high enough to exert a considerable proteolytic activity as such, if activated. In addition, we have previously shown that both TAT isoenzymes are efficient activators of the pro-urokinase-type plasminogen activator in vitro (21). TAT could thus promote cellular invasion by participating in the tumor-associated protease cascade (cf. Ref. 6) as well as by degrading trypsin-sensitive matrix components. Clarification of this issue requires investigation of the effects of trypsin inhibitors and function-blocking antibodies on tumor invasion and metastasis.

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