Reaction of a tumour-associated trypsin inhibitor with serine proteinases associated with coagulation and tumour invasion

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The inhibition of six serine proteinases by a tumour-associated trypsin inhibitor (TATI) was studied using synthetic peptide substrates. Physiological concentrations of TATI inhibited the amidolytic activities of trypsin, plasmin, urokinase and tissue plasminogen activator (tPA). Chymotrypsin, kallikrein and thrombin were also inhibited, but by much higher concentrations of TATI. The ability of TATI to inhibit trypsin, plasmin, urokinase and tPA suggests that it has a role in proteolytic processes in vivo involving these enzymes.

INTRODUCTION

The Kazal-type inhibitor is a peptide of M, 6200 isolated first from pancreatic tissue (Kazal et al., 1948). This peptide is also called pancreatic secretory trypsin inhibitor (PSTI). Its role has been postulated to be the prevention of inadvertent proteolysis in the pancreas caused by premature activation of trypsinogen (Greene et al., 1966; Pubols et al., 1974). Its inhibitory specificity has been shown to be very narrow, with strong inhibitory capacity only against pancreatic trypsin (Fritz et al., 1967) and acrosin (Huhtala et al., 1984). A similar or identical inhibitor, called tumour-associated trypsin inhibitor (TATI), was recently identified (Stenman et al., 1982) and isolated from the urine of a patient with ovarian cancer (Huhtala et al., 1982). High concentrations of this inhibitor occur in ovarian tumour extracts, especially of the mucinous type (Halila et al., 1987) and in serum and urine of these patients (Halila et al., 1988). This suggests that Kazal-type inhibitors have a physiological function outside the pancreas. Further evidence for an extra-pancreatic role is the finding of TATI in pancreactectomy patients (Halila et al., 1985), as well as elevated urine and serum levels of TATI in connection with severe infections (Huhtala et al., 1983), after major surgery (Matsuda et al., 1985) and severe trauma (Ogawa et al., 1985).

Proteinases and their inhibitors occur together (Stråuli, 1980). Therefore, the elevation of TATI in cancer and other extrapancreatic diseases can be expected to be associated with the expression of extrapancreatic proteinases reacting with this Kazal-type inhibitor.

To elucidate mechanisms for elevation of TATI in cancer and inflammation we have investigated the interaction between TATI and some serine proteinases involved in tumour invasion, inflammation, coagulation and fibrinolysis. For comparison we also studied the pancreatic enzymes trypsin and chymotrypsin.

MATERIALS AND METHODS

TATI was isolated from human urine and its concentration was determined by radioimmunoassay (Huhtala et al., 1982). Bovine trypsin (EC 3.4.21.4) was from Worthington Biochemicals, Freehold, NJ, U.S.A.; human plasmin (EC 3.4.21.7) from KabiVitrum, Stockholm, Sweden; urokinase (EC 3.4.21.31) from Lövens Kemiske Fabrik, Ballerup, Denmark; tissue plasminogen activator (tPA) from KabiVitrum, Stockholm, Sweden; bovine chymotrypsin (EC 3.4.21.1) from Merck, Darmstadt, F.R.G.; human plasma kallikrein (EC 3.4.21.34) from Sigma Chemical Co., St. Louis, MO, U.S.A.; and bovine thrombin (EC 3.4.21.5) from Roche, Basel, Switzerland. Soya-bean trypsin inhibitor (SBTI) was from Merck, Darmstadt, F.R.G. and aprotinin (Apronin) from Medica, Helsinki, Finland. The synthetic peptide substrates were from KabiVitrum, Stockholm, Sweden: S-2222 (N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine p-nitroanilide) was used for the assay of trypsin and tPA; S-2251 (H-d-valyl-L-leucyl-L-lysine p-nitroanilide) for plasmin; S-2444 (L-pyroglutamyl-glycyl-L-arginine p-nitroanilide) for urokinase; S-2586 (carbomethoxypropionyl-L-arginyl-L-prolyl-L-tyrosine p-nitroanilide) for chymotrypsin; S-2302 (H-d-prolyl-L-phenylalanyl-L-arginine p-nitroanilide) for kallikrein; and S-2238 (H-dphenylalanyl-L-pipeocetyl-L-arginine p-nitroanilide) for thrombin.

Enzyme activities were determined by measuring the initial rate of hydrolysis by monitoring the increase of absorbance at 405 nm per min at 20 s intervals for 180 s in a Multistat III Plus Micro Centrifugal Analyzer (Instrumentation Laboratory, Lexington, MA, U.S.A.) at 30 °C. The conditions in the incubation mixture were as follows: 140 μl of substrate solution at a final concentration (S) of 70 μM and 5 μl of enzyme solution at final concentrations of 19-320 nM, all in 50 mM-Tris/HCl buffer, pH 7.0, containing 0.01 % Triton X-100. For the inhibition assays, 30 μl of inhibitor was added to 5 μl of enzyme solution. The final concentrations of inhibitor were from 0.6 nM to 16 μM. After preincubation of the enzyme and the inhibitor for 5 min the assay was started and the increase in absorbance recorded. The inhibition was calculated from the decrease in enzyme activity caused by various concentrations of inhibitors in comparison with a control containing buffer instead of inhibitor. The inhibition constant (K_i) was determined by

Abbreviations used: tPA, tissue plasminogen activator; TATI, tumour-associated trypsin inhibitor; SBTI, soya-bean trypsin inhibitor.
Table 1. Inhibition of various serine proteinases by TATI, SBTI and aprotinin

Table for inhibition of various serine proteinases by TATI, SBTI and aprotinin.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$S/K_m$</th>
<th>TATI</th>
<th>SBTI</th>
<th>Aprotinin</th>
<th>TATI $K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>1.3</td>
<td>0.47</td>
<td>0.5*</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Plasmin</td>
<td>0.3</td>
<td>5.0</td>
<td>0.94</td>
<td>0.53</td>
<td>9.7</td>
</tr>
<tr>
<td>Urokinase</td>
<td>0.8</td>
<td>17</td>
<td>no inhibition</td>
<td>no inhibition</td>
<td>300</td>
</tr>
<tr>
<td>tPA</td>
<td>2.3</td>
<td>52</td>
<td>no inhibition</td>
<td>no inhibition</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>1.4</td>
<td>88</td>
<td>1.0</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Kallikrein</td>
<td>0.4</td>
<td>108</td>
<td>0.62</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>7.8</td>
<td>138</td>
<td>no inhibition</td>
<td>no inhibition</td>
<td></td>
</tr>
</tbody>
</table>


the method of Henderson (1972), as modified by Bieth (1980). The $K_m$ value of the enzyme-substrate reaction given by the manufacturer of the substrate was checked by using various substrate concentrations.

RESULTS

All the serine proteinases studied were inhibited by TATI. However, large differences in the affinity of TATI for various proteinases were observed. Only trypsin, plasmin and urokinase had $K_i$ values indicating strong or moderate affinity (Table 1). The affinity for trypsin was about 160-fold that of plasmin, which had the highest affinity.

The comparison of TATI concentrations causing 50% inhibition of various enzymes gives some indication of which enzymes will be inhibited by physiological concentrations of TATI (Table 1). Nearly complete inhibition of the amidolytic activity of 19 nM-trypsin was obtained with 19 nM-TATI (Fig. 1), which corresponds to a 1:1 molar binding ratio. The concentrations of TATI required for 50% inhibition of plasmin, urokinase and tPA were 10–100-fold those required for trypsin (Table 1). All substrates were used at the same concentration of 70 μM. Because of different $K_m$ values for different enzyme-substrate combinations used, the $S/K_m$ ratios varied somewhat with various enzymes. Therefore, the inhibitor concentrations causing 50% inhibition are only roughly comparable. However, higher concentrations were clearly required for inhibition of chymotrypsin, kallikrein and thrombin. There was a linear correlation between the extent of inhibition of plasmin (Fig. 2a) and chymotrypsin (Fig. 2c) at TATI concentrations ranging from 0.45 to 6 μM and that of tPA from 8 to 15 μM (Fig. 2e). The results are summarized in Table 1.

Compared with TATI, SBTI and aprotinin were equally potent inhibitors of trypsin, but their inhibitory activity against plasmin (Fig. 2a), chymotrypsin (Fig. 2c) and kallikrein (Fig. 2d) was much higher than that of TATI. However, in contrast with TATI, SBTI and aprotinin did not inhibit urokinase, tPA and thrombin at all (Table 1).

DISCUSSION

Our results show a relatively strong inhibition of plasmin, urokinase and tPA by TATI. $K_i$ for plasmin and urokinase, 9.7 and 300 nM, respectively, are similar to the concentrations of TATI occurring in the serum and urine of cancer patients, e.g. 50–1400 μg/l, corresponding to 8–230 nM (Huhtala et al., 1983; Haglund et al., 1986; Halila et al., 1987).

The reaction between Kazal-type inhibitors and plasminogen activators has, to our knowledge, not been studied before. Earlier studies on the reaction between the pancreatic Kazal inhibitor (PSTI) and plasmin indicated that there is no inhibition (Fritz et al., 1967; Greene et al., 1976). The discrepancy between these results and those of the present study could possibly be explained by differences in the assay conditions, e.g. enzyme and substrate concentrations.

The physiological concentrations of TATI in serum are below 20 μg/l, corresponding to 3.2 nM (Stenman et al., 1982; Haglund et al., 1986). This is about 1000–10000-fold less than the concentrations (3.6–56 μM) of the major proteinase inhibitors, α1-proteinase inhibitor,
The inhibition of (a) plasmin (320 nM) by aprotinin (○), SBTI (□), and TATI (●) by using S-2251; (b) urokinase (90 nM; ●) and thrombin (17 nM; ○) by TATI with S-2444 and S-2238 respectively; (c) chymotrypsin (40 nM) by SBTI (□), aprotinin (○) and TATI (●) by using S-2586; (d) kallikrein (26 nM) by SBTI (□), aprotinin (○) and TATI (●) by using S-2302; and (e) tPA (215 nM) by TATI (●) with S-2222. All substrate concentrations were 70 μM in the assay mixture. The activity obtained without inhibitors was set at 100%.

\( \alpha_1 \)-antichymotrypsin and \( \alpha_2 \)-macroglobulin (Laskowski & Kato, 1980). Although the serum concentrations of TATI associated with cancer may be up to 230 nM (Haglund et al., 1986), this will hardly play a major role in proteinase inhibition in the circulation. Locally TATI might be of physiological importance in various tissues. Evidence for local production was provided by the finding of TATI immunoreactivity in tumour tissues (Stenman et al., 1982; Murata et al., 1983; Halila et al., 1987). The main sources of the Kazal inhibitors known so far are the pancreas (Matsuda et al., 1983) and mucinous ovarian tumours (Halila et al., 1987). Patients with mucinous ovarian cancer often have elevated serum levels of TATI (Halila et al., 1988), apparently as a result of production by the tumour. Hepatoma cells have also been shown to produce TATI (McKeehan et al., 1986), but production by normal liver has not been demonstrated so far.

Tumour invasion is associated with expression of plasminogen activators causing a local increase in plasmin levels (Danö et al., 1985). Our results show that both plasmin and urokinase are inhibited by TATI at concentrations occurring in vivo. Locally TATI could thus be involved in the control of their proteolytic activity, and activation of these proteinases could be a factor triggering the elevation of TATI.

Although activation of urokinase and plasmin could induce the expression of TATI in cancer patients, these enzymes do not need to be the only or even the major inducers. We have recently identified a proteinase which
reacts strongly to TATI in mucinous ovarian tumours (Stenman et al., 1988). So far we have observed this protease only in mucinous ovarian tumours, which also contain high levels of TATI. Therefore, other proteases, e.g. plasmin and urokinase may be responsible for the elevation of TATI in other forms of cancer.

REFERENCES


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