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_r 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, 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 extrapancreatic role is the finding of TATI in pancreatectomized 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 (Sträuli, 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 (Aprolin) 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-D-phenylalanyl-L-pipecolyl-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 μ l of substrate solution at a final concentration (S) of 70 μ M and 5 μ M 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 μ l of inhibitor was added to 5 μ l of enzyme solution. The final concentrations of inhibitor were from 0.6 nM to 16 μ M. 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

Table 1. Inhibition of various serine proteinases by TATI, SBTI and aprotinin

Ratio of inhibitor to enzyme (mol/mol) causing 50 % inhibition at enzyme concentrations of 19–320 nM (see the Materials and methods section) is given. The S/K_m ratios for the various enzymes and their substrates are indicated. The K_i for the reaction between TATI and trypsin, plasmin and urokinase is also shown.

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

* According to Laskowski & Kato (1980).

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 next 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 μ M. 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 μ M and that of tPA from 8 to 15 μ M (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).

Fig. 1. Inhibition of trypsin by TATI

Trypsin (19 nM) was incubated with various concentrations of TATI followed by the addition of 70 μ M substrate solution of S-2222.

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 μ g/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 μ g/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 μ M) of the major proteinase inhibitors, α_1 -proteinase inhibitor,

Fig. 2. Comparison of enzyme inhibition by TATI, aprotinin and SBTI

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 μ M in the assay mixture. The activity obtained without inhibitors was set at 100%.

α_1 -antichymotrypsin and α_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 proteinase only in mucinous ovarian tumours, which also contain high levels of TATI. Therefore, other proteinases, e.g. plasmin and urokinase may be responsible for the elevation of TATI in other forms of cancer.

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