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Urokinase and Tissue-type Plasminogen Activators are Present in Breast Cyst Fluids

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Abstract—*Breast cyst fluids obtained by needle aspiration from 20 patients were analysed for the presence of urokinase-like (u-PA) and tissue-type (t-PA) plasminogen activators. Plasminogen, the natural substrate for these PAs, was also measured. u-PA activity was detected in 18 samples (mean ± S.D. = 1.11 ± 1.08 IU ml^{-1}) and t-PA activity in 16 samples (0.35 ± 0.28 IU ml^{-1}). Plasminogen was detected in 10 samples (mean = 37 μg ml^{-1}), range 28–85 μg ml^{-1}) and was below the assay detection limit in the other samples. While both u-PA and t-PA specific activities were greater in the 10 cyst fluids categorized as Group A (Na$^+$ to K$^+$ concentration ratio < 4) than in the 10 samples categorized in Group B (Na$^+$ to K$^+$ concentration ratio > 4), the differences were not statistically significant. Nevertheless, this is the first evidence that u-PA and t-PA are present in active forms in breast cyst fluids. The significance of such activities must be hypothetical, but it is possible that a study of factors influencing the levels of u-PA and t-PA within the breast may yield information about pathophysiology of cystic and other diseases of the breast.*

INTRODUCTION

CONVERSION of the zymogen plasminogen to the active serine protease plasmin is catalysed by plasminogen activators (PAs) which are themselves serine proteases. Two structurally different types have been purifed and characterized. These are urokinase (u-PA) and tissue-type PA (t-PA). While both types cleave the same single peptide bond in plasminogen thereby converting it to plasmin, t-PA is distinguished by its high affinity for fibrin which greatly enhances its activity. Plasmin's main substrate in blood plasma is fibrin but there is evidence that this broadly specific protease can also degrade extracellular matrix components such as fibronectin, laminin and proteoglycans. It can also activate collagenases and lead to further plasmin generation by activating a zymogenic form of u-PA [1]. Such plasmin-mediated extracellular proteolysis may have a role in the degradation of normal tissue and PAs released from cancer cells could thereby promote invasive growth and metastases (for reviews see [1, 2]).

Recently PAs have been characterized in human breast tumours [3, 4]. However, there are no published data on the presence of PAs in breast cysts which represent the most common benign lesion of the breast [5] and which may be associated with an increased risk of subsequent breast cancer [6]. Here, we have investigated the presence of u-PA and t-PA in fluids from breast cysts which have been classified according to electrolyte composition [7].

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MATERIALS AND METHODS

Clinical material

Twenty breast cyst fluids were obtained from 20 patients by needle aspiration and centrifuged at 5000 ***g*** for 5 min to sediment cellular debris. The supernatants were retained and part of each was acidified by addition of an equal volume of 1 M sodium acetate buffer, pH 3.9. Acidified and untreated supernatants were then stored at −40°C until assay.

The fluids were classified on the basis of Na$^+$ to K$^+$ concentration ratio as determined by flame photometry with an EEL model 150 flame photometer [7]. Fluids with a concentration ratio of less than 4 were classified as group A while those with a ratio of greater than 4 were designated as group B.

Materials

Plasminogen was purified from freshly frozen

citrated human plasma by chromatography on lysine–agarose (Pharmacia, U.K.) [8] and depleted of plasmin by treatment with aprotinin–agarose (Trasylol, Bayer Pharmaceuticals, F.R.G.). Fibrin monomer I was prepared by treatment of human fibrinogen (Grade L, Kabi Vitrum, Sweden) with Reptilase venom (Pentapharm via Payne and Byrne, U.K.) as described by Ranby *et al.* [9]. Chromogenic substrate for plasmin, S-2251, was obtained from Kabi Vitrum via Flow Laboratories, U.K. An immunoglobulin fraction of monospecific goat antiserum raised against the 55,000 Dalton form of human u-PA was a gift from Dr. G. Murano, National Institute of Health, Maryland, U.S.A. A protein A-purified mouse monoclonal antibody againt human melanoma t-PA, ESP2, which completely quenches biological activity of t-PA by binding to its active site was produced as described previously [10]. International Standard Preparations of u-PA and t-PA were obtained from Dr. P. Gaffney at the National Institute of Biological Standards and Control, London, U.K.

PA assays

PA activity was measured by a spectrophotometric assay [11] in which the generation of plasmin from plasminogen, catalysed by PA, was detected with a tripeptide chromogenic substrate for plasmin, S-2251. The assay was run in multiwell plates and generated colour was measured at 405 nm in an eight channel photometer (Titertek Multiskan, Flow Laboratories, U.K.). Absorbance due to sample was accounted for by subtracting A_{492} from A_{405} value for each determination and by running appropriate control wells without chromogenic substrate.

Before assay, acidified breast cyst fluid samples were thawed and held at 37°C for 15 min to inactivate any plasmin inhibitors [11] prior to dilution 1:24 v:v with 0.05 M Tris buffer at pH 8.8 containing 0.1 M NaCl and 0.1% w/v Tween 80. Plasminogen-independent hydrolysis of the chromogenic peptide substrate was determined by omission of plasminogen from the assay reactants. t-PA-like activity was assayed in the presence of fibrin monomer I, a stimulator of t-PA but not u-PA activity, against a standard curve based on activity of known concentrations of the International Standard Preparation of t-PA. The difference between activity in the absence and presence of excess quenching monoclonal antibody ESP2 (final concentration of 20 μg/ml) against t-PA was used to quantify the total t-PA activity. Likewise u-PA activity was assayed against the International Standard Preparation of u-PA, in the absence of fibrin monomer I and the difference in activity in the presence and absence of excess quenching anti u-PA immunoglobulin (final concentration 20 μg/ml) was used to quantify the total u-PA activity. Mouse and goat non-immune immunoglobulin was used as a control for non-specific quenching of activity in the respective assays.

t-PA antigen was determined in untreated breast cyst fluid supernatants using a competition radioimmunoassay as described by MacGregor and Prowse [12]. The assay buffer was 0.05 M sodium phosphate, 0.3 M NaCl, 0.01 M ethylenediaminetetraacetic acid, disodium salt, 3% v/v bovine serum albumin, 0.05% w/v Tween 80, 0.05% w/v sodium azide, pH 7.6. A monospecific antiserum against human melanoma t-PA was kindly provided by Dr. D. Collen, University of Leuven, Belgium. Purified human melanoma t-PA (>90% single chain form) was generously given by Dr. M. Einarsson, Kabi Vitrum, Sweden. It was used to contruct standard curves having been standardized against the International Standard preparation of t-PA.

Other methods

Plasminogen content of the breast cyst fluids was assayed by radial immunodiffusion using a monospecific antiserum (Sigma, U.K.). Total protein was measured by the method of Bradford [13].

The Mann–Whitney test was used to determine differences between the Group A and Group B breast cyst fluids.

RESULTS

Plasminogen antigen was detectable in 10 of the 20 breast cyst fluids with a mean value of 37 μg ml^{-1} and a range of 25–85 μg ml^{-1}. Levels were below the detection limit of 25 μg ml^{-1} in the other samples. These values can be compared with the normal range in blood plasma of 60–250 μg ml^{-1}, mean = 120 μg ml^{-1}. Following the 1/50 dilution of the breast cyst fluids prior to assay for PA, the concentrations of endogenous plasminogen did not influence the sensitivity of the assay towards t-PA or u-PA, since the final concentration of purified plasminogen in the reaction well was 1 μM or ≃100 μg ml^{-1} with a contribution of only ≃1% by breast cyst fluid plasminogen.

The breast cyst fluids caused little hydrolysis of chromogenic substrate in the PA rate assay in the absence of added plasminogen and in all samples the predominant portion of measured activity was plasminogen dependent (92 ± 4%). PA activity was detectable in 18 of the 20 samples and was quantified by comparison with activity of standard samples containing known concentrations of International Standard preparations of t-PA and u-PA and the use of monospecific quenching antibodies, as described earlier. A combination of both anti-t-PA and anti-u-PA antibodies effectively inhibited total PA activity in all the breast cyst fluids.

Table 1. Plasminogen activators in breast cyst fluids

	Activity (IU ml^{-1})		Specific activity* (IU mg^{-1}) × 10^2		t-PA antigen* (ng mg^{-1})
	u-PA	t-PA	u-PA	t-PA	
Group A					
Mean (n = 10)	1.27	0.41	6.5	2.1	0.48
S.D.	1.24	0.36	7.6	1.8	0.14
Group B					
Mean (n = 10)	0.95	0.29	4.4	1.3	0.76
S.D.	0.93	0.17	4.2	0.8	0.61
All samples					
Mean (n = 20)	1.11	0.35	5.4	1.7	0.62
S.D.	1.08	0.28	6.1	1.4	0.45

*Specific activities were determined by expressing measured activity or antigen as a proportion of the protein concentration of each sample.

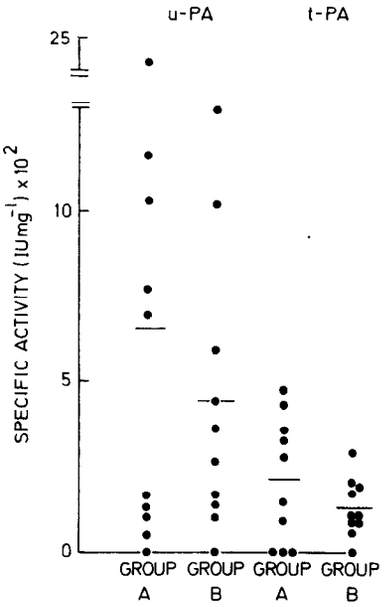

Fig. 1. Specific activity, expressed as PA activity per mg *total protein, of u-PA and t-PA in 20 breast cyst fluids. Bars indicate mean values.*

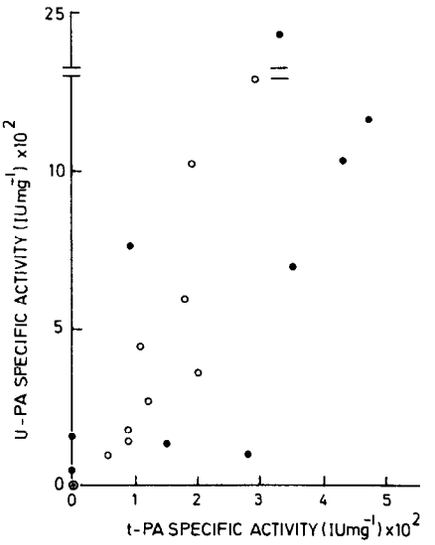

Fig. 2. Scattergram showing relationships between specific activities of u-PA and t-PA in 20 breast cyst fluids. Group A cyst fluids are denoted by closed circles and group B cyst fluids by open circles. A correlation coefficient of r = 0.621 (r = 0.632 *for* P = 0.05) *and* r = 0.886 (P < 0.001) *was obtained for Groups A and B respectively. The correlation coefficient for all samples was* r = 0.684 *with* P < 0.001.

The mean values for t-PA and u-PA activity, specific activity and t-PA antigen are shown in Table 1. The data are presented for the two groups of breast cyst fluids classified on the basis of electrolyte composition as well as for all the samples together. Specific activity values for individual samples are shown in Fig. 1. Both u-PA and t-PA activity was detectable in most of the breast cyst fluids (u-PA in 18 and t-PA in 16 of the 20 samples). As t-PA antigen was present in all 20 samples and the activity assay had a lower detection limit than the t-PA antigen assay it is concluded that inactive t-PA antigen was present in at least some samples. The mean u-PA and t-PA activities were higher in Group A than Group B but the difference was not statistically significant.

The specific activities of u-PA and t-PA were calculated by dividing the PA activity by protein concentration for each sample (Table 1 and Fig. 1). Although the mean specific activities for u-PA and t-PA were higher in Group A compared with Group B breast cyst fluids at 1.7 and 1.9 times respectively, the differences were not statistically significant. The specific activities for u-PA and t-PA covered a wide range (Fig. 1) and correlation coefficients were determined to ascertain if u-PA and t-PA specific activities were inter-related (Fig. 2). The correlation coefficient for all pairs of data was $r = 0.684$, $\nu = 18$ with a probability value of $P < 0.001$. The correlation coefficient for Group A samples was $r = 0.621$ ($r = 0.632$ for $P = 0.05$) and for Group B samples it was $r = 0.886$ ($P < 0.001$).

When t-PA activity was expressed as a ratio of

activity:t-PA antigen a mean value of 27,400 IU mg^{-1} was obtained compared with the fully active International Standard t-PA which is assigned a potency of 500,000 IU mg^{-1}.

DISCUSSION

The development of sensitive functional assays for PA coupled with the production of specific quenching antibodies against u-PA and t-PA has permitted the quantitation and characterization of PAs in various cancer cells including those from the breast [1, 2]. Here we provide the first evidence that both u-PA and t-PA are present in breast cyst fluids and have shown that plasminogen is present in at least some of the samples examined. Failure to detect plasminogen in the other samples may be a reflection of the relatively insensitive assay used. Equivalent units of the International Standard preparations of u-PA and t-PA are not equiactive in a standard fibrin clot assay system and so it is not possible to deduce whether one or other of the two types of PA confers the major portion of PA activity. Moreover, the relative activities will differ in the presence and absence of fibrin since while t-PA activity is stimulated several hundred-fold by fibrin, it is a relatively poor activator of plasminogen in the absence of fibrin [9]. In contrast the activity of u-PA is not influenced by fibrin or fibrin(ogen) degradation products.

The presence of t-PA in the breast cyst fluids was confirmed in two ways. Quenching of activity was achieved with a murine monoclonal antibody shown by ourselves and others to be directed against an epitope at the active site of t-PA and which can completely block the ability of t-PA to convert plasminogen to plasmin [10, 14]. The monoclonal antibody shows no cross-reactivity with 35,000 or 55,000 Dalton forms of u-PA [10]. In addition we were also able to detect t-PA antigen in all samples tested. Expressing t-PA activity per mg of t-PA antigen produced a mean value of 27,400 IU mg^{-1}; since the International Standard for t-PA has an assigned value of 500,000 IU mg^{-1}, a major portion of the t-PA appears to be present in an inactive form. Whether this is the result of degradation of active t-PA or formation of t-PA inhibitor complexes is unknown. Attempts to clarify this point by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis followed by fibrin zymography using the technique of Booth *et al.* [15] was unsuccessful due to the low amounts of t-PA activity present in the samples.

t-PA and plasminogen are found in blood plasma. t-PA antigen is present at 7 ng ml^{-1} [12] but active t-PA is only found after a fibrinolytic stimulus such as venous occlusion. u-PA antigen has also been detected in blood plasma at about 2–3 ng ml^{-1}, although it is in an inactive form and is not increased by fibrinolytic stimuli [16]. Thus, while it is possible that plasma is the source of these proteins it seems unlikely for the following reasons. Plasma constituents may appear in breast cyst fluid but they occur at lower concentrations than in plasma and appear to be particularly associated with Group B cysts [17, 18]. In contrast t-PA antigen concentrations in the breast cyst fluids were greater than those found in plasma while plasminogen concentrations were considerably lower. Also, active u-PA was present in the breast cyst fluids but is not detectable in plasma [16].

The question therefore arises as to the source of the PAs and plasminogen and their significance in breast cyst fluids. Plasminogen is synthesized in the liver and no other sites of synthesis have been demonstrated [19]. However, about 40% is located in extravascular compartments and it has been detected in numerous locations including human saliva and basal layers of the epidermis [1]. Vascular endothelium is the major source of t-PA in the blood but it has also been detected in apocrine secretions such as saliva and tears [20]. In this respect, it may be pertinent that levels of t-PA were not significantly higher in Group A cyst fluid compared with Group B, in view of Group A cyst fluids having a more apocrine-like composition (high concentrations of intracellular cation as K^+ and androgen conjugates). However, although the mean level of t-PA was higher in Group A cysts the difference from Group B was not significant.

t-PA has been shown to be the major active PA in human milk [21] and with regard to other breast fluids and tissue, u-PA has been detected immunochemically in involuting mouse mammary glands [22]. The significance of t-PA and u-PA activity in breast cyst fluids can only be hypothetical at this stage. Overexpression in cancer has been suggested to be associated with dissemination of the disease. However, breast cysts are benign lesions and although they may be associated with increased risk of developing breast cancer, there is little evidence to suggest that cysts are pre-malignant *per se*. Levels of other enzymes have also been reported to be elevated in breast cysts [23] and again the significance of this is unknown. It is possible that the spectrum of activity reflects the cellular activity within the breast and if so factors which influence levels may yet yield useful information concerning the pathophysiology of cystic and other diseases of the breast.

REFERENCES

1. Dano K, Andreasen A, Grondahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. Plasminogen activators, tissue degradation and cancer. In: Klein G, Weinhouse S, eds. *Advances in Cancer Research*. London, Academic Press, 1985, Vol. 44, 140–266.
2. Duffy MJ, O'Grady P. Plasminogen activator and cancer. *Eur J Cancer Clin Oncol* 1984, **20**, 577–582.
3. O'Grady , Lijnen HR, Duffy MJ. Multiple forms of plasminogen activator in human breast tumors. *Cancer Res* 1985, **45**, 6216–6218.
4. Evers JL, Patel J, Madeja JM *et al.* Plasminogen activator activity and composition in human breast cancer. *Cancer Res* 1982, **42**, 219–226.
5. Haagensen CD, Bodian C, Haagensen DE. Gross cystic disease of the breast. In: Hagensen CD, Bodian C, Haagensen DE, eds. *Breast Carcinoma: Risk and Detection*. Philadelphia, WB Saunders, 1981, 55–77.
6. Harrington E, Lestuck G. The association between gross cysts of the breast and breast cancer. *Breast* 1981, **7**. 13–17.
7. Miller WR, Dixon JM, Scott WN, Forrest APM. Classification of human breast cysts according to electrolyte and androgen conjugate composition. *Clin Oncol* 1983, **9**, 227–232.
8. Deutsch DG, Mertz ET. Plasminogen: purification from human plasma by affinity chromatography. *Science* 1970, **170**, 1095–1096.
9. Ranby M, Norrman B, Wallen P. A sensitive assay for tissue plasminogen activator. *Thromb Res* 1982, **27**, 743–749.
10. MacGregor IR, Micklem L, James K, Pepper DS. Characterisation of epitopes on human tissue plasminogen activator recognised by a group of monoclonal antibodies. *Throm Haemostas* 1985, **53**, 45–50.
11. Wiman B, Mellbring G, Ranby M. Plasminogen activator release during venous stasis and exercise as determined by a new specific assay. *Clin Chim Acta* 1983, **127**, 279–288.
12. MacGregor IR, Prowse CV. Tissue plasminogen activator in human plasma measured by radioimmunoassay. *Thromb Res* 1983, **31**, 461–474.
13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* 1976, **72**, 248–254.
14. Van Zonneveld A-J, Veerman H, Brakenhoff JPJ, Aarden LA, Cajot J-F, Pannekoek H. Mapping of epitopes on human tissue type plasminogen activator with recombinant deletion mutant proteins. *Thromb Haemostas* 1987, **57**, 82–86.
15. Booth NA, Anderson JA, Bennett B. Plasminogen activators in alcoholic cirrhosis: demonstration of increased tissue type and urokinase type activator. *J Clin Pathol* 1984, **37**, 772–777.
16. Huber K, Kircheimer J, Binder BR. Characterisation of a specific anti-human urokinase antibody: development of a sensitive competition radioimmunoassay for urokinase antigen. *J Lab Clin Med* 1984, **103**, 684–694.
17. Yap PL, Miller WR, Roberts MM *et al.* Protein concentrations in fluid from gross cystic disease of the breast. *Clin Oncol* 1984, **10**, 35–43.
18. Miller WR, Dixon JM, Forrest APM. Hormonal correlates of apocrine secretion in the breast. *Ann NY Acad Sci* 1986, **464**, 275–287.
19. Saito H, Hamilton SM, Tavill AS, Louis L, Ratnoff OD. Production and release of plasminogen by isolated perfused rat liver. *Proc Natl Acad Sci* 1980, **77**, 6837–6840.
20. Rijken DC, Wijngaards G, Webergen J. Immunological characterisation of plasminogen activation activities in human tissues and body fluids. *J Lab Clin Med* 1981, **97**, 477–486.
21. Marshall JM, Rees MCP, Cederholm-Williams SA. Identification of t-PA as the major active plasminogen activator in human milk. *Thromb Haemostas* 1986, **55**, 279–281.
22. Larsson L, Skriver L, Nielsen LS, Grondahl- Hansen J, Kristensen P, Dano K. Distribution of urokinase-type plasminogen activator immunoreactivity in the mouse. *J Cell Biol* 1984, **98**, 894–903.
23. Biagioni S, Stella F, Mannello F, Cerroni L, Stella C, Troccoli R. α_1-Antitrypsin, transferrin, alkaline phosphatase, phosphohexoseisomerase and r-glutamyltransferase in breast cyst fluid. *Tumori* 1985, **71**, 135–140.