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Thrombospondin in Malignant and Non-malignant Breast Tissue

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Abstract—*Cytosols of malignant breast tissue contained significantly higher levels of thrombospondin (TSP) and von Willebrand factor (vWF) than non-malignant breast. TSP and vWF content of human breast were significantly correlated whereas there was no correlation between TSP and the platelet-specific protein β-thromboglobulin (βTG). Whilst TSP in pre-menopausal breast cancer was slightly lower than in post-menopausal breast cancer, it did not correlate with oestrogen receptors (ER) or progesterone receptors (PR), but was negatively correlated with tissue-type plasminogen activator (tPA), an oestradiol-inducible enzyme. Secretion of TSP by MCF-7 cells was low and refractory to hormones. High levels of TSP appeared to be associated with the centre of the tumour mass. It is suggested that activation of the endothelium may be responsible, at least in part, for the high levels of TSP found in malignant breast tissue and could be a factor in the growth and spread of breast cancer.*

INTRODUCTION

THROMBOSPONDIN (TSP), a glycoprotein first described in platelets and subsequently in a variety of cell types, is incorporated into the extracellular matrix of some cells in culture where it may function as an adhesive protein (for reviews see Refs. [1–3]). As a component of the extracellular matrix, TSP has been shown to have an autocrine function, augmenting the mitogenic response of smooth muscle cells to epidermal growth factor [4].

TSP has also been implicated in tumour cell metastasis. For example it promotes the attachment and spreading of several human cancer cells *in vitro* [5–7] and a recent report shows that TSP potentiates tumour cell metastasis in mice [8]. Specific receptor sites for TSP have also been identified on the membrane of platelets, endothelial cells and a variety of human tumour cells [9]. As a result, TSP could be involved at several points in the metastatic process through potential interactions of malignant cells with extracellular matrix, endothelium or circulating platelets.

TSP is present in high concentrations in milk, other breast secretions and in some types of cyst fluid [10]. We reported recently that TSP is a component of the aqueous phase of milk, that its level falls during the transition of colostrum to mature milk and also that malignant breast had a higher content of TSP than non-malignant breast [11]. The increased TSP levels observed in these studies did not reflect platelet activation since there were no parallel changes in the platelet-specific protein β-thromboglobulin (βTG). Tissue-type plasminogen activator (tPA), the major plasminogen activator (PA) in human milk, shows a similar pattern of secretion during early lactation as does TSP [12]. Increased levels of PA have also been reported in breast tumours [13] where they correlate with oestrogen receptors (ER) and progesterone receptors (PR) [14]. Moreover, production of PA by breast cells in culture is regulated by oestradiol and other steroid hormones [15–17].

Von Willebrand factor (vWF) is synthesized by endothelial cells and megakaryocytes and is contained in platelet α-granules. vWF functions as a carrier protein for coagulation factor VIII and is thought to be involved in platelet–vessel wall interactions [18]. Indeed, vWF is incorporated into the extracellular matrix of cultured endothelial cells [19] where it may function as an adhesive protein. Although not specific for endothelial cells, vWF has been used as a marker of endothelial perturbation *in vitro* [20–22] and as a criterion for endothelial cell identification [23].

We have undertaken a more comprehensive study

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of TSP in the human breast and the factors which may be responsible for the high levels which are associated with malignancy. Our findings suggest that endothelial activation may be responsible, at least in part, for the elevated TSP in breast cancer and this may have a role in regulating the growth and spread of the malignant epithelium.

MATERIALS AND METHODS

Samples

Breast tissue cytosols from patients attending the Edinburgh breast clinic (101 breast carcinoma, nine benign mammary dysplasia, five fibroadenoma, one gynaecomastia, one normal) were prepared by homogenization of tissue in 20 mM Tris–HCl, pH 7.6, containing 0.25 M sucrose, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM KCl at a dilution of either 1:5 (w/v) or 1:10 (w/v) using a Silverson homogenizer. They were centrifuged at 105,000 ***g*** at 4°C for 1 h and the supernatants stored at −20°C.

Eleven breast tissue cytosols from five patients with breast cancer were obtained at the time of mastectomy by snap-freezing of washed tissue samples followed by cryofragmentation using a mechanical dismembrator, and preparation of a supernatant from the tissue extract resuspended in buffered saline. They were classified according to whether the tissue had been taken from the centre of the tumour ($n = 5$) or the surrounding non-malignant tissue ($n = 6$).

Assays

TSP, βTG and vWF were measured by radioimmunoassays as previously described [24–26]. The sensitivity of the radioimmunoassay for vWF was 22 ng/ml but it was not possible to specify a detection limit due to the varying protein concentrations of individual cytosols. tPA was measured by enzyme-linked immunosorbant assay [27] by Dr.P. Gaffney and the results have been published elsewhere [28].

ER was measured in low speed supernatants of breast tissue homogenates as previously reported [29]. PR was determined in the same high speed supernatant used for TSP measurement as described elsewhere [30].

Protein assays were performed by a dye-binding method (Bio-Rad Laboratories Ltd., Watford, U.K.) using a bovine serum albumin standard.

Cell culture

Briefly, MCF-7 human breast cancer cells (sub-cultured from a stock obtained from Professor S.C. Brooks, Michigan Cancer Foundation) were grown on 10 cm^2 plastic culture wells (Costar, Cambridge, MA) in Eagle's MEM with Hank's BSS (Flow Laboratories, Irvine, U.K.) supplemented with NEAA, sodium pyruvate, L-glutamine, insulin, penicillin, streptomycin and 10% FCS (Gibco, Paisley, U.K.). At confluence, the monolayer was washed extensively with basal MEM to remove serum components and then incubated with 2 ml/well serum-free medium according to Barnes and Sato [31] with or without the test substance. Culture supernatants were decanted after 48 h, centrifuged to remove cell debris and stored at −40°C prior to assay.

Statistical analyses

Statistical analysis of differences between populations was performed by the Wilcoxon–Mann–Whitney rank sum test. Correlations between analytes were calculated by the Spearman rank correlation test.

RESULTS

TSP, vWF and βTG in breast cytosols

A wide range of TSP levels was found in breast cytosols (Fig. 1). Highest levels were observed in breast cancers ($n = 101$, median = 317 μg TSP/g cytosol protein, range = 17.4–23,400 μg TSP/g cytosol protein) and lowest in benign breast ($n = 15$, median 21.7, range = 1.78–155). The difference between cancer and benign samples was

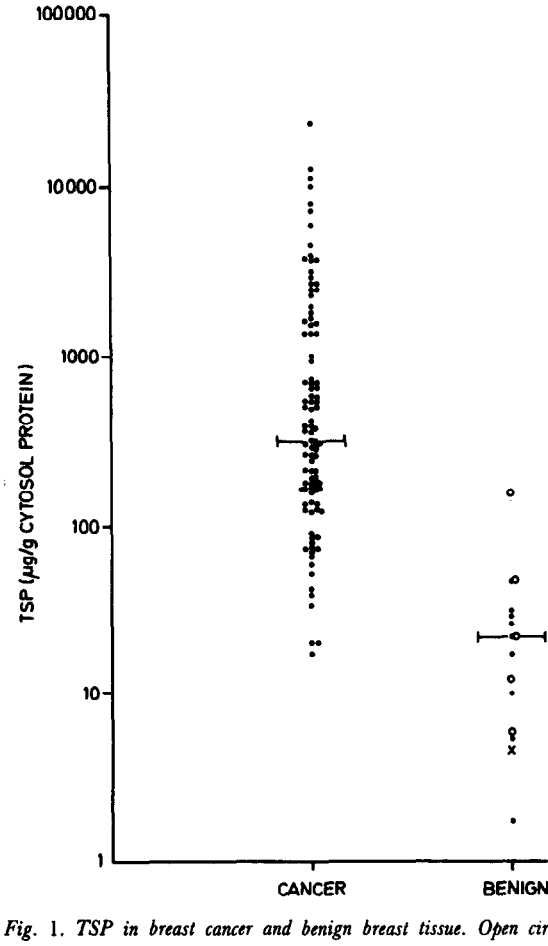

Fig. 1. TSP in breast cancer and benign breast tissue. Open circles represent fibroadenomas and one gynaecomastia is shown as a cross. Horizontal lines indicate median values. The difference between cancers and benigns was highly significant ($P < 10^{-6}$).

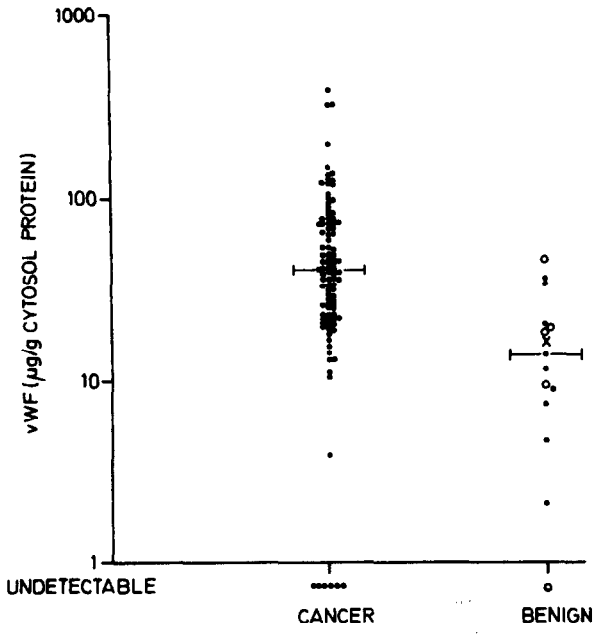

Fig. 2. vWF in breast cancer and benign breast tissue. Symbols as for Fig. 1. The difference between cancers and benigns was significant ($P < 10^{-4}$).

highly significant ($P < 10^{-6}$). TSP in fibroadenomas ($n = 5$, median = 21.7, range = 5.93–155) was not significantly different from other types of benign breast tissue ($n = 10$, median = 19.7, range = 1.78–47.6) and therefore all benigns were considered as a single group.

Measurement of vWF in breast cytosols revealed a narrower range of concentrations compared with TSP (Fig. 2), but higher levels of vWF were also found in cancers ($n = 101$, median = 40.8 μg vWF/g cytosol protein, range = undetectable–392 μg vWF/g cytosol protein) than in benigns ($n = 15$, median = 14.0, range = undetectable–46.0). The difference was significant ($P < 10^{-4}$) although less marked than for TSP. A plot of TSP against vWF (Fig. 3) showed that they were significantly correlated ($n = 116$, $r_s = 0.484$; $P < 0.001$).

βTG was measured together with TSP in 20 breast cytosols (18 cancer, one benign, one normal) and no correlation was observed between the two ($r_s = -0.365$; $P > 0.10$) (Fig. 4).

TSP and indicators of hormonal status in breast cancer cytosols

Breast cancer cytosol TSP levels were compared in a group of 92 patients classified according to menopausal status (Fig. 5). TSP in breast cancer cytosols from pre-menopausal women ($n = 32$, median = 213.5 μg TSP/g cytosol protein, range = 17.4–23400 μg TSP/g cytosol protein) was slightly lower than that in cancers from post-menopausal women ($n = 60$, median = 367, range = 41.8–12800), the difference just reaching statistical significance ($P = 0.043$).

TSP did not correlate with ER in breast cancers ($n = 98$, $r_s = -0.0075$; $P > 0.20$) nor with PR ($n = 33$, $r_s = -0.146$; $P > 0.20$) (Fig. 6a,b).

TSP secretion by MCF-7 human breast cancer cells

Levels of TSP in the supernatants from MCF-7 cells in serum-free culture for 48 h are shown in Table 1. Hormones (diluted into culture media from ethanolic solution) were added at concentrations previously shown to be maximally stimulatory for MCF-7 [32, 33]. No significant change in TSP secretion was observed (Wilcoxon–Mann–Whitney

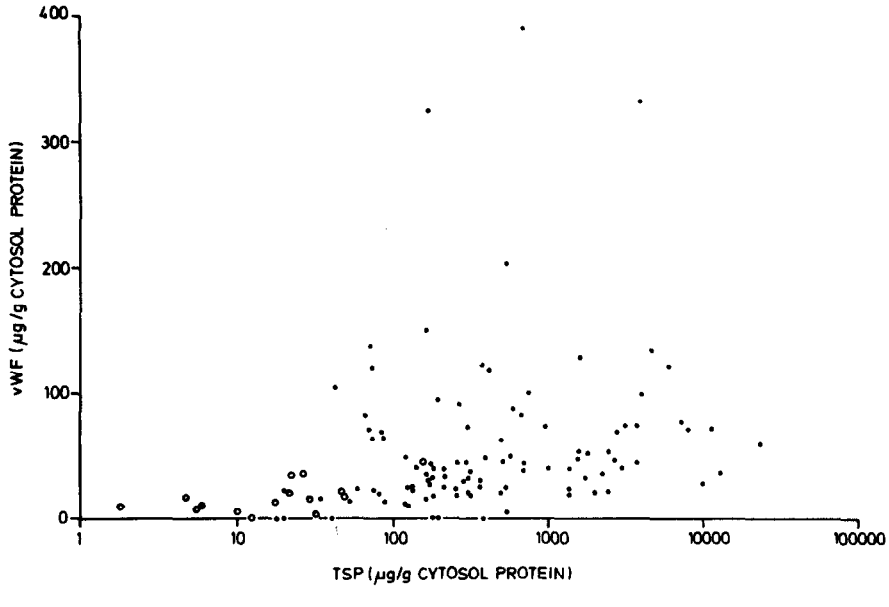

Fig. 3. Relationship between TSP and vWF in breast tissue. Closed circles represent cancers and benigns are shown as open circles. TSP and vWF were significantly correlated ($r_s = 0.484$; $P < 0.001$).

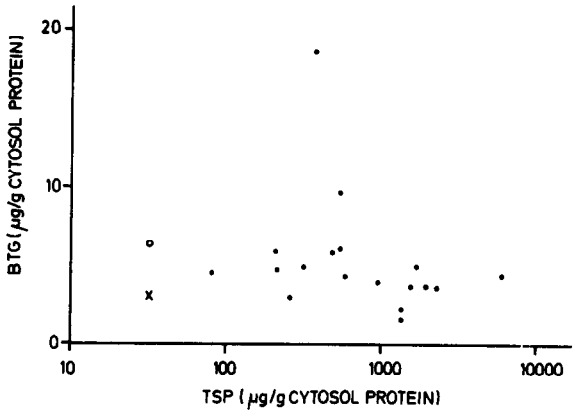

Fig. 4. Relationship between TSP and βTG in breast tissue. Symbols as for Fig. 3. One normal breast is shown as a cross. TSP and βTG were not correlated ($r_s = -0.365$; $P > 0.10$).

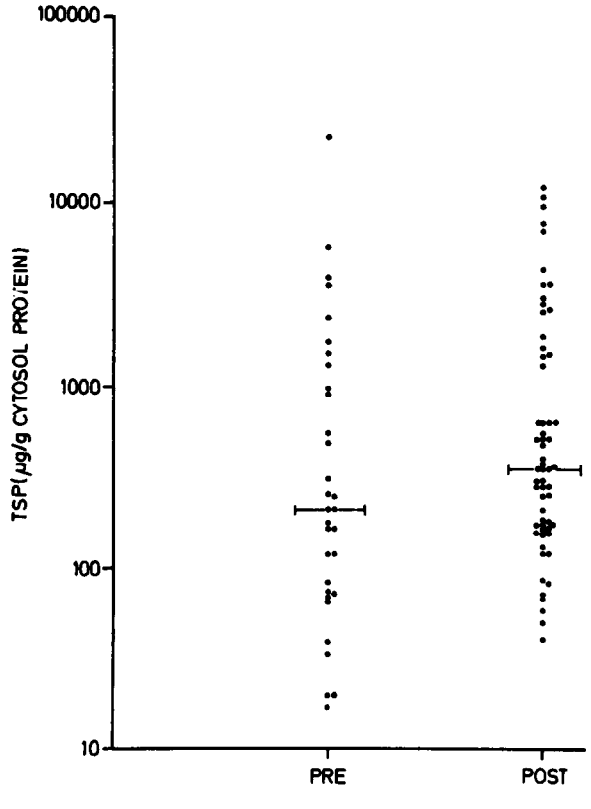

Fig. 5. Menopausal status and TSP in breast cancer tissue. Horizontal lines represent median values. The difference between pre- and post-menopausal cancers was marginally significant (P = 0.043).

rank sum test) in the presence of any of the subtances tested, and the rate of secretion was low in comparison with that reported for human endothelial cells [34].

TSP and tPA in cytosols of tissue taken from different locations within cancerous breast

Eleven breast tissue cytosols from five patients with breast cancer were measured for their TSP and tPA content. The tissue had been classified

Table 1a. Effect of hormones on TSP secretion by MCF-7 human breast cancer cells

	Secreted TSP 48 h
Control	88.0 (1.7)
17β-Oestradiol (10^{-8} M)	92.0 (6.9)
Testosterone (10^{-6} M)	95.7 (4.5)
5α-Dihydrotestosterone (10^{-6} M)	97.3 (4.6)

Table 1b. Effect of EGF on TSP secretion by MCF-7 human breast cancer cells

	Secreted TSP 48 h
Control	83.3 (7.0)
EGF (1 ng/ml)	92.7 (3.2)
EGF (10 ng/ml)	90.0 (6.9)

Values represent mean (S.D.) TSP levels in ng/ml from triplicate experiments.

according to whether it was taken from the centre of the tumour mass ('centre') or from the surrounding non-malignant tissue ('surround'). Comparison of the TSP concentration in corresponding samples from centre and surround (Fig. 7) showed that in four out of the five patients, the centre ($n = 5$, median = 1120 μg TSP/g cytosol protein, range =31.8–4480 μg TSP/g cytosol protein) contained much higher levels than the surround ($n = 6$, median = 50.9, range = 9.09–324). The difference between the two groups was significant ($P = 0.041$).

On the other hand, in every case tPA in corresponding samples was lower in the centre ($n = 5$, median = 1.86 μg tPA/g cytosol protein, range = 1.30–3.67 μg tPA/g cytosol protein) than in the surround ($n = 5$, median = 7.18, range = 3.54–11.99) (Fig. 8). Again, the difference between the two groups was significant ($P = 0.008$).

TSP and tPA were negatively correlated ($n = 10$, $r_s = -0.576$; $P < 0.05$) (not shown).

DISCUSSION

The elevated levels of TSP and vWF in malignant breast, and the correlation observed between these proteins in breast tissue suggest that they may have a common site of synthesis within the breast which is stimulated in malignant disease. vWF is synthesized by endothelial cells and megakaryocytes and is contained in platelet α-granules [18]. It is incorporated into the subendothelial matrix and thought to be involved in interactions between platelets and vessel walls. TSP is also synthesized by endothelial cells and megakaryocytes, and is stored in platelet

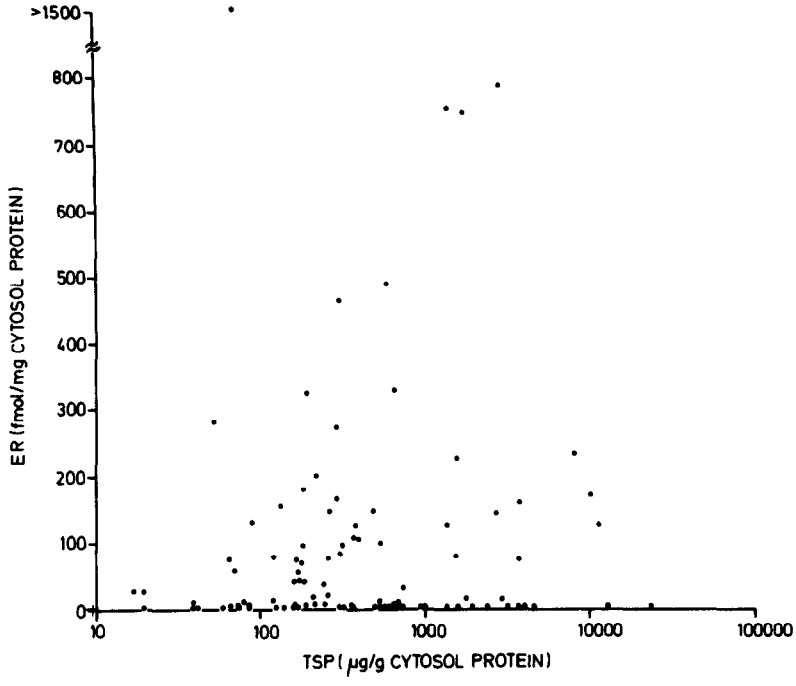

Fig. 6a. Relationship between TSP and ER in breast cancer tissue. TSP and ER were not correlated ($r_s = -0.0075$; $P > 0.20$).

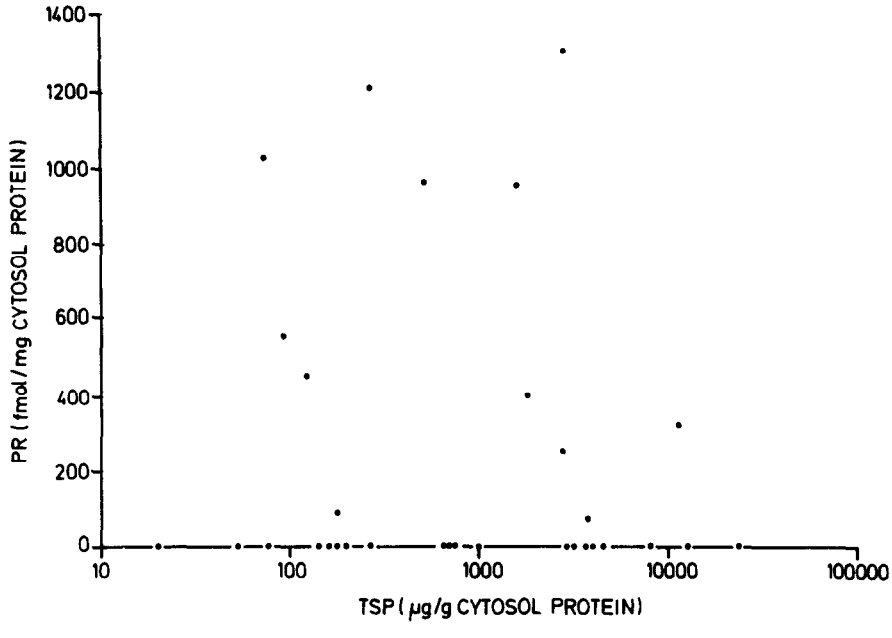

Fig. 6b. Relationship between TSP and PR in breast cancer tissue. TSP and PR were not correlated ($r_s = -0.146$; $P > 0.20$).

α-granules, but has also been described in a wide range of other cell types [1–3]. Since the platelet α-granule protein βTG did not correlate with TSP in breast cytosols, it is very unlikely that platelets are the source of the high levels of TSP and vWF in malignant breast. Increased vascularity and/or stimulation of the endothelium could explain the higher TSP and vWF content of cancer tissue despite our finding that the difference between cancer and benign samples was much greater for TSP than for vWF (the median cytosolic vWF of malignant breast was three times higher than non-malignant breast, whereas for TSP it was 15 times higher). TSP and vWF have been shown to be located in different subcellular compartments in endothelial cells where their secretion is separately controlled [35]. However, it is possible that another source within the breast, in addition to the endothelium, may be contributing to the very high level of TSP observed in some breast cancers.

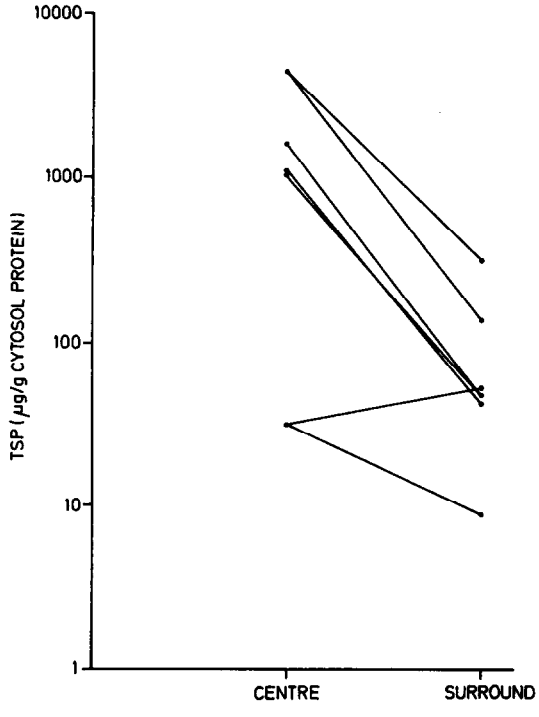

Fig. 7. TSP in breast tissue from different locations within cancerous breast. Lines connect corresponding samples taken from the centre of the tumour mass ('centre') and the surrounding non-malignant tissue ('surround') of the cancerous breasts of five patients. The difference between the two groups was significant (P = 0.041).

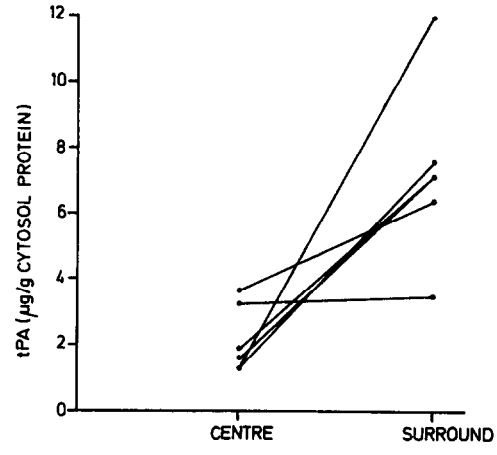

Fig. 8. tPA in breast tissue from different locations within cancerous breast. Lines connect corresponding samples as for Fig. 7. The difference between the two groups was significant (P = 0.008).

The growth of certain breast cancers is hormonally mediated. Approximately 30% of breast cancers respond to hormone treatment and most of these are ER positive [36]. Since oestrogens are so closely implicated in breast cancer, it was of interest to investigate whether the high levels of TSP in malignant breast were related to any of the known indicators of hormonal status. A slight difference was observed in the TSP content of breast cancers from pre- and post-menopausal women, but no correlation was found between TSP and ER or PR. TSP was secreted only in small amounts by cultured human breast cancer cells in a manner refractory to steroid hormones and epidermal growth factor, and therefore it seems unlikely that synthesis of TSP by malignant epithelial cells regulated by oestrogens is involved in causing the elevated levels found in breast cancer.

Further evidence for the specific elevation of TSP in malignant breast came from the measurement of TSP in tissue from different sites within the cancerous breasts of a group of patients. Very high levels of TSP (>500 μg/g cytosol protein) were only seen in tissue taken from the centre of the tumour mass; levels in tissue taken from elsewhere within the same breast were comparable to those found in benign breast.

It was interesting that measurement of tPA in these samples showed the opposite trend. Whilst elevated PA levels in breast cancer have been reported [13], some tumours contain predominantly urokinase-type plasminogen activator (UKPA) and others predominantly tPA [37] which makes early studies in which only total PA was measured difficult to interpret. However,it has been established that tPA, rather than UKPA, is induced by oestradiol [14, 38] and our finding that TSP is inversely correlated with tPA suggests that TSP and tPA are not coordinately expressed by the malignant epithelium.

In order to define the exact source and role of TSP in the breast further studies are required. To this end we are currently using immunocytochemical techniques for the localization of TSP *in situ* and attempting to characterize TSP isolated from human breast.

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