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Serum and Tissue Proteinase-like Peptidase Activities in Women Undergoing Total Mastectomy for Breast Cancer*

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Abstracts—*Serum proteinase-like peptidases and proteinase inhibitor activities have been determined in 40 women with breast cancer at presentation and following total mastectomy. Activities of these enzymes have also been determined in homogenates of malignant (n = 13) and non-malignant (n = 11) breast tissue and benign breast lesions (n = 10). Following surgical treatment, the serum collagenase-like, cathepsin B-like and cathepsin H-like peptidase activities were significantly reduced. In addition, the activities of collagenase-like, cathepsin B-like and elastase-like peptidases were significantly higher in malignant breast tissue than in either non-malignant tissue from the same breast, or benign breast lesions. These findings are consistent with the suggestion that proteinases may have a role in tumour invasion.*

INTRODUCTION

THERE is increasing evidence that proteinases may play an important role in tumour invasion. The ability of tumour cells in culture to degrade extracellular proteins has led to suggestions that breakdown of connective tissue may be an essential requirement of tumour invasion [1]. This process is likely to be mediated by the extracellular activity of collagenases [2] and by both extracellular and intracellular action of lysosomal cysteine proteinases [3]. The latter have been implicated as a means of both local invasion and metastatic spread of tumour cells [4]. More recently, cultures of human breast cancer tissue have been shown to synthesise and secrete large amounts of cathepsin B-like activity compared with non-malignant breast tissue [5]. This finding is consistent with the reported significant increase in cathepsin B-like activities in serum of women with gynaecological cancers compared with healthy women, and which is followed by a decrease in enzyme activities after tumour resection [6]. In fact these workers have suggested that measurement of serum cathepsin B-like activities might serve as an indicator of the extent of malignant spread and response to therapy. In a large study of women with breast cancer, we did not find any significant differences in several proteinase-like peptidase activities in the serum prior to treatment compared with women with benign disease or healthy controls of cancer bearing age [7]. However, serum proteinase-like peptidase activities were altered following total mastectomy and we now report these findings, together with studies on homogenates of malignant and non-malignant breast tissue and benign breast lesions.

MATERIALS AND METHODS

Patients

Serum studies have been performed on 40 women (mean age 59 ± 2 (S.E.M.), range 32–78 yr) with histologically confirmed breast cancer on presentation at the Breast Clinic at Ninewells Hospital, Dundee, and 7–16 months (mean 10.6 months) following total mastectomy. These women were among the 102 patients with breast cancer in whom pre-treatment serum proteinase-like peptidase activities were studied [7]. The primary tumour was ≤20 mm in 11 women and >20 mm but <60 mm in 29 women and none of

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the women had evidence of tumour fixation or distant metastases as revealed by metastatic survey (radiology, isotope scans and blood enzyme tests). Histological examination of axillary nodes following axillary node clearance was made in all patients and nodal involvement was observed in 13 women. Approval for the study was obtained from the Ethical Committee of the Tayside Health Board and informed consent was obtained from all the women in the study.

In a separate series of women histologically confirmed malignant breast tissue was obtained at mastectomy in 12 women and from an excisional biopsy in one patient. Non-malignant breast tissue was obtained from sites distant to the tumour shortly after removal of the breast in 11 women with breast cancer. Tissue from benign lesions was obtained following excisional biopsy in 10 women with benign breast disease. In 9 patients malignant and non-malignant tissue was obtained from the same breast. Within a short-time of surgical excision the tissue specimens were cut into small pieces, frozen in liquid nitrogen and stored at below -70°C prior to homogenisation.

Proteinase-like peptidase assays

Collagenase-like (CL-), cathepsin B-like (Cat BL-), cathepsin H-like (Cat HL-), elastase-like (EL-), trypsin-like (TL-) and plasmin-like (PL-) peptidase activities were determined using synthetic low molecular weight fluorogenic 4-methyl-7-coumarylamide (MCA) peptide substrates as reported previously [7]. The serum samples were stored at below -70°C prior to assay.

When required for assay, small pieces of tissue were cooled in liquid nitrogen and crushed to a powder in a stainless steel mortar, also cooled in liquid nitrogen. The powder was then homogenised on ice with a Potter–Elvejham glass-Teflon homogeniser, in 10 mM Tris–HCl buffer, pH 7.5, containing 0.01% Triton X-100. The homogenate was centrifuged at 100,000 *g* in a Beckman L2-65 B Ultracentrifuge at 4°C. The supernatant was assayed for proteinase-like peptidase activity and protein content [8] on the same day as the preparation using the methods reported for serum with only minor modifications. Approximately 0.5–0.8 mg of supernatant protein was sufficient for all the assays.

Proteinase inhibitor assays

Serum and tissue activities of α_1-antitrypsin (α_1AT) and α_2-macroglobulin (α_2M) were determined as the trypsin inhibitory capacity [9] and the trypsin binding capacity [10], respectively, using the synthetic low molecular weight chromogenic substrate *N*α-benzoyl-L-arginine-*p*-nitroanilide.

Units of activity and data analysis

Enzyme activities are expressed as the amount of product formed per min per ml of serum or per mg of protein at 37°C. Serum Cat BL- and TL-peptidase activities appeared to have a log-normal type of distribution and values are therefore presented as geometric means with 95% confidence limits (CL). All other values are presented as arithmetic means with 95% CL (i.e. mean ±2 S.E.M.). Statistical analysis was performed using Student's *t*-test for independent groups (with Cochrane's modification as required), paired *t*-test, Wilcoxon's rank sum test or the signed rank test as appropriate [11]. For the latter two tests the normal deviate was calculated when $n > 16$ [11].

RESULTS

Seven to sixteen months after total mastectomy there was a significant decrease in serum CL-, Cat BL- and Cat HL-peptidase activities when compared with pre-operative levels (Table 1). Furthermore, these lower enzyme activities were accompanied by a significant increase in serum α_2M (Table 2). There were no significant changes in the mean activities of the other enzymes.

Serum proteinase-like peptidase activities in patients with involved nodes were not significantly different from those in the other patients, although on average Cat BL-peptidase activities were higher in the former, both before and after total mastectomy. Patients without nodal in-

Table 1. Serum proteinase-like peptidase activities before and after total mastectomy (n = 40)

	Arithmetic or geometric means and 95% CL*					
	CL-	Cat BL- (nmol/min/ml)	Cat HL-	EL-	TL- (pmol/min/ml)	PL-
Pre-treatment	2.4 (2.2–2.6)	65 (52–83)	3.3 (3.0–3.6)	52 (44–60)	73 (60–89)	387 (331–443)
Post-treatment	2.0 (1.8–2.2)	32 (26–39)	2.8 (2.6–3.0)	57 (50–64)	79 (67–94)	365 (305–425)
Paired *t*-test	$P < 0.01$	$P < 0.001$	$P < 0.02$	NS	NS	NS
Signed rank test	$P < 0.03$	$P < 0.001$	$P < 0.04$	NS	NS	NS

*Cat BL- and TL-peptidase values given as geometric means, all other activities as arithmetic means.
NS = not significant.

Table 2. Serum proteinase inhibitor activities before and after total mastectomy (n = 40)

	Arithmetic means and 95% CL	
	α_1-Antitrypsin (μmol/min/ml)	α_2-Macroglobulin (nmol/min/ml)
Pre-treatment	4.3 (4.1–4.5)	300 (276–324)
Post-treatment	4.5 (4.3–4.7)	322 (296–348)
Paired *t*-test	NS	$P < 0.05$
Signed rank test	NS	$P < 0.05$

volvement had significantly lower post-operative serum activities of CL-, Cat BL- and Cat HL-peptidase activities when compared with pre-operative levels, whereas in patients with involved nodes only Cat BL-peptidase activities were significantly lowered post-operatively (Table 3).

Activities of CL- and Cat BL-peptidases were significantly higher in malignant tissue than in either non-malignant breast tissue or benign breast lesions (Figs 1 and 2). Furthermore, Cat HL- and EL-peptidase activities were significantly higher in malignant tissue than in benign lesions (Figs 3 and 4). Cat HL-peptidase activity was higher in non-malignant tissue compared with benign lesions (Fig. 3). All these differences were similar or even greater when activities in malignant and non-malignant tissue obtained from the same patients were compared. There were no significant differences in the activities of TL- and PL-peptidases between non-malignant ($n = 11$) and malignant tissue homogenates [TL-: non-malignant = 12 (5–17), malignant = 18 (9–27); PL: non-malignant = 112 (36–188), malignant = 102 (46–158) pmol/min/mg]. In homogenates of non-malignant ($n = 11$) and malignant ($n = 10$) breast tissue there were detectable, although similar, activities of α_1AT [non-malignant = 0.24 (0.06–0.42), malignant = 0.23 (0.14–0.32) μmol/min/mg] and α_2M [non-malignant = 4.0 (2.2–5.8), malignant = 3.0 (1.6–4.4) nmol/min/mg].

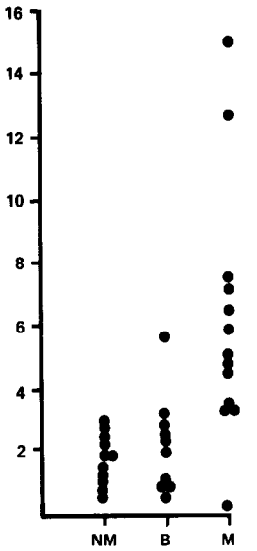

Fig. 1. CL-peptidase activities (nmol/min/mg) *in non-malignant (NM), benign lesion (B) and malignant (M) breast tissue homogenates. M V NM and M V B,* $P < 0.01$ *(rank sum test).*

Serum and tissue homogenate showed several similarities in the properties of CL- and Cat BL-peptidase activities, although different values for K_i were obtained for leupeptin inhibition of the latter (Table 4). The plot of leupeptin concentration against the ratio of substrate concentration and fluorescence gave parallel straight lines, indicating purely competitive inhibition for both serum and tissue Cat BL-peptidase activity.

DISCUSSION

Significantly increased serum cathepsin B-like activity has been reported in women with gynaecological cancer, with a decrease following

Table 3. Axillary node involvement and serum proteinase-like peptidase activities

	Arithmetic or geometric means and 95% CL					
	CL-	Cat BL- (nmol/min/ml)	Cat HL-	EL-	TL- (pmol/min/ml)	PL-
Pre-treatment						
Not involved (27)	2.4 (2.1–2.7)	63 (48–82)	3.3 (2.9–3.7)	51 (42–60)	80 (62–103)	406 (338–474)
Involved (13)	2.4 (2.1–2.7)	71 (44–113)	3.2 (2.7–3.7)	54 (36–72)	62 (48–80)	349 (251–447)
Post-treatment						
Not involved (27)	2.0 (1.7–2.3)	28 (22–35)	2.7 (2.4–3.0)	58 (51–65)	80 (65–100)	390 (306–474)
Involved (13)	2.1 (1.7–2.5)	42 (29–62)	3.0 (2.5–3.5)	53 (37–69)	78 (59–103)	313 (263–363)
*Not involved						
Significance level	$P < 0.05$†	$P < 0.001$	$P < 0.02$	NS	NS	NS
*Involved						
Significance level	NS	$P < 0.03$	NS	NS	NS	NS

*Comparison of pre-treatment and post-treatment values by paired *t*-test and signed rank test.
†NS with signed rank test.

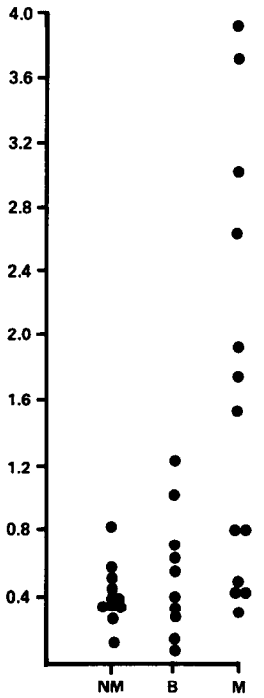

Fig. 2. Cat BL-peptidase activities (nmol/min/mg) *in non-malignant (NM), benign lesion (B) and malignant (M) breast tissue homogenates. M V NM,* $P < 0.01$ *and M V B,* $P < 0.02$ *(rank sum test).*

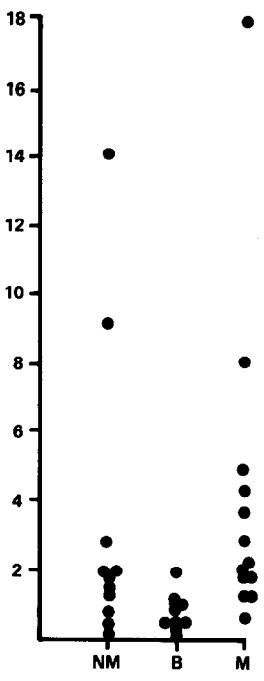

Fig. 3. Cat HL-peptidase activities (nmol/min/mg) *in non-malignant (NM), benign lesion (B) and malignant (M) breast tissue homogenates. NM V B,* $P < 0.05$ *and M V B,* $P < 0.01$ *(rank sum test).*

surgical excision or chemotherapy [6]. However, we were unable to confirm this for either serum Cat BL- or other proteinase-like peptidases in a comparative study of women with breast cancer,

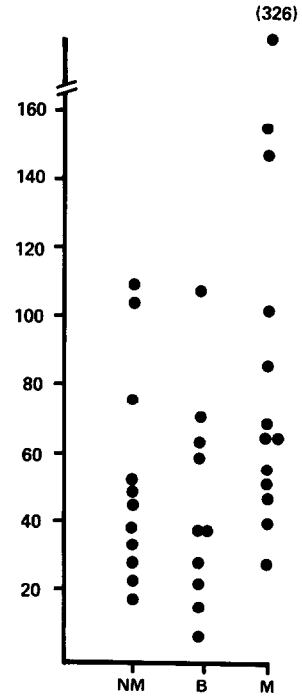

Fig. 4. EL-peptidase activities (pmol/min/mg) *in non-malignant (NM), benign lesion (B) and malignant (M) breast tissue homogenates. M V B,* $P < 0.05$ *(rank sum test).*

benign breast disease and healthy women of cancer bearing age [7]. In the present report patients who had undergone total mastectomy exhibited significantly decreased activities of CL-, Cat BL- and Cat HL-peptidase activities after operation, with the most striking difference being for Cat BL-peptidase activity. Whether these findings can be related to the removal of tumour tissue containing high levels of proteinases must await further study, but it is interesting to speculate that the higher Cat BL-peptidase activities in patients with involved nodes may be due to the presence of as yet clinically undetectable distant micro-metastases. This hypothesis is, however, supported by the results of the tissue studies which show that malignant breast tissue contains significantly higher CL, Cat BL-, Cat HL- and EL-peptidase activities. If the breast is normally a major contributor of serum Cat BL-peptidase activity, then removal of the breast in patients with cancer may explain the apparent decrease in Cat BL-peptidase activity to levels below that observed in control subjects [7].

Human breast cancer tissue in culture has been shown to secrete higher levels of cathepsin B-like thiol proteinase, but not collagenase, than normal breast tissue [5]. While the former observation is consistent with our findings for Cat BL-peptidase activity in tissue homogenates, there are several possible reasons for the apparent differences for collagenase/CL-peptidase activity.

*Table 4. Kinetic constants for CL- and Cat BL- peptidases from serum and tissue homogenates**

Proteinase-like peptidase activity	Source	Optimal pH	K_m (mM)	K_i (mM)	Type of inhibition
CL-	Serum	8.0	0.71	3.8	Non-competitive
	Homogenate	8.0	0.67	5.7	Non-competitive
Cat BL-	Serum	6.5	0.29	1×10^3	Competitive
	Homogenate	6.5	0.33	0.03×10^3	Competitive

*Serum was obtained from a healthy volunteer and the homogenate was from non-malignant tissue. CL- was assayed with the substrate Suc-Gly-Pro-Leu-Gly-Pro-MCA and Cat BL- with Z-Phe-Arg-MCA. The apparent K_m were determined from Lineweaver–Burk plots and the apparent K_i and type of inhibition by the graphical method of Cornish-Bowden [12]. Inhibition studies were with EDTA (CL-) and leupeptin (Cat BL-).

Firstly, the rate of secretion of collagenase may not reflect the amount of proteinase present in cells of breast tissue. There is, however, also evidence that collagenase may be secreted in different forms by malignant and non-malignant tissue [13]. O'Grady *et al.* [13] have reported that cultures of malignant rat mammary carcinoma cells secreted collagenase in an active form while cultures of lung fibroblasts secreted the enzyme in a latent form. Since Recklies *et al.* [5] routinely included a collagenase activator (*p*-aminophenylmercuric acetate) in their assay medium differences of latency would not have been detected. Finally, the different assay methods used by Recklies *et al.* and ourselves may not detect the same enzymes. Whereas the former used the fibril collagen assay, we have used a synthetic low molecular weight substrate which detects collagenase-like peptidase activity [7]. Although this activity was optimal at pH 8 and was inhibited by EDTA and L-cysteine [7], confirmational studies on purified human collagenase from breast cancer tissue and serum of women with breast cancer are required.

On the other hand, some workers have reported that human epithelial tumours in culture release increased collagenase activity compared with non-malignant tissue [14], and Hornbeck *et al.* [15] found that infiltrating human breast carcinoma tissue contained significantly higher levels of elastin and elastase than fibroadenoma tissue. Our findings in breast cancer are consistent with these reports.

Activities in non-malignant breast tissue and benign lesions were similar apart from the significantly higher levels of Cat HL-peptidase in the former, although the values were variable. The reason for this is unknown but may reflect a response of the normal tissue to the presence of malignant cells, since all the non-malignant tissue was obtained from mastectomy specimens in women with breast cancer.

Since lysosomal enzymes are associated with phagocytosis, differences in proteinase activities in heterogenous tissue samples could reflect differences in the macrophage population, especially as the latter can actively infiltrate malignant tumours [3]. However, it has been shown that the increased cathepsin B activity released from mouse B_{16} melanoma cells was due to the tumour and not to the invading macrophages [16].

If excess cathepsins and collagenase are released into the circulation they are complexed and inhibited by α_2M and subsequently removed by the reticuloendothelial system [17]. The subsequent increase of α_2M after mastectomy may reflect, therefore, a reduction in the proportion of complexed inhibitor as a result of a decrease in proteinase or proteinase-like peptidase activity since the assay employed measures the active, or 'slow' form, of α_2M [17]. Alternatively, the effects of surgery may elicit a non-specific response.

Detectable levels of trypsin inhibitory capacity (α_1AT) and trypsin binding capacity (α_2M) were found in both malignant and non-malignant tissue, an observation also reported by Twining and Brecher [18]. However, specific cellular inhibitors are more likely to play an important role in influencing any invasive properties of tumours mediated by proteinases active on connective tissue [19]. Studies of these inhibitors in breast tissues are currently being undertaken in our laboratories.

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