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Serum Proteinase-like Peptidase Activities and Proteinase Inhibitors in Women with Breast Disease*

ANIL VASISHTA, PETER R. BAKER,† PAUL E. PREECE, ROBERT A. B. WOOD and ALFRED CUSCHIERI

University Department of Surgery, Ninewells Hospital and Medical School, Dundee DD1 9SY, U.K.

Abstract—*The pre-treatment serum activities of several proteinase-like peptidases and the proteinase inhibitors, α_1-antitrypsin (α_1AT) and α_2-macroglobulin (α_2M), have been determined in 102 women with breast cancer and compared with those in 20 women with benign disease and in 30 healthy women of cancer bearing age. There were no significant differences in serum proteinase-like peptidase activities associated specifically with breast cancer. However, trypsin-like and plasmin-like activities were significantly lower than normal in women with breast disease. Serum α_1AT and α_2M levels were higher in patients with breast cancer than in healthy women or women with benign breast disease. These results indicate that, at presentation, breast cancer is not associated with abnormal serum levels of the proteinase-like peptidases studied, possibly as a result of an increase in the concentration of proteinase inhibitors.*

INTRODUCTION

THE MECHANISMS of tumour invasion are poorly understood. It has been proposed that malignant cells secrete substances which destroy the surrounding tissue and so aid the spread of tumour to distant sites [1]. Sylvén suggested that proteolytic enzymes might be involved in the destruction of extracellular matrix and may also facilitate cellular detachment from tumour masses [2]. Subsequently several endopeptidases or proteinases, including plasminogen activators [3], collagenase [4] and the lysosomal cysteine proteinase cathepsin B [5], have been implicated in the destructive action of tumours. Recently it has been shown that breast cancer tissue in culture released more cathepsin B-like activity than normal breast tissue [6] and cultures of rat mammary carcinoma cells released active collagenase [7]. If such a process occurs *in vivo* the proteinases may enter the general circulation. There has been no systematic study undertaken of serum proteinase activities in breast cancer, although increased cathepsin B-like activities in women with various cancers, including five with breast cancer, have been reported [8]. In serum, proteinases are complexed to naturally occurring inhibitors, particularly α_1AT (or α_1-antiproteinase) and α_2M [9]. Determination of the activity of proteinases in serum therefore requires reversal of this inhibition or the use of low molecular weight substrates which can gain access to the catalytic site of the enzyme complexed with α_2M.

In the present study specific low molecular weight 4-methyl-7-coumaryl-amide (MCA) peptides were used as substrates to assay several proteinase-like peptidase activities in the sera of women of cancer bearing age who had normal breasts or benign breast disease, and in women with breast cancer. In addition, two of the major serum proteinase inhibitors have also been studied since circulating levels of these inhibitors may influence, or be influenced by, serum proteinase activities.

MATERIALS AND METHODS

Subjects and patients

Blood samples were obtained at presentation from women with breast symptoms attending the Breast Clinic at Ninewells Hospital, Dundee, and from healthy women attending the Well-Women

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†To whom correspondence and requests for reprints should be addressed.

Clinic at Dundee Royal Infirmary. Of the 152 women included in the study (Table 1), 30 were without breast symptoms and had normal breasts on clinical examination (healthy group); 20 had benign breast lesions, diagnosed clinically, by aspiration cytology and on mammography (benign group); and 102 had breast cancer which was confirmed in 77 by histology. In 25 women with obvious malignancy for whom non-surgical treatment was indicated, diagnosis was made by cytology and radiology. The women selected for the healthy and benign groups were of cancer bearing age. The patients with breast cancer were sub-divided on the basis of the extent of their tumour burden. The size of the tumour mass was measured in both horizontal and vertical planes with special callipers at initial examination. Patients in group Ca1 had tumours $\leqslant$20 mm, patients in group Ca2 had tumours >20 mm and group Ca3 included patients with metastases, as shown by radiological and scintographic survey, or whose tumours were fixed to the skin or chest wall. The largest recorded tumour in group Ca2 was 80 mm. Of the patients with advanced breast cancer (group Ca3) 14 had died of the disease at the time of data analysis (survival time 1–29 months, mean 13.0) and 15 were still alive (7–38 months, mean 15.9).

Histological examination of axillary nodes following axillary node clearance was made in 69 of the 73 patients with primary tumours (all in groups Ca1 and Ca2) and nodal involvement was present in 28.

Blood was withdrawn by venepuncture, allowed to clot at room temperature for 45–60 min and serum obtained following centrifugation at 1600 *g* for 10 min at 4°C. Small aliquots of serum were stored at below -70°C until required for assay. 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.

Chemicals

All the MCA substrates listed in Table 2 and the elastase inhibitor elastatinal were purchased from the Peptide Research Foundation (Osaka, Japan). Leupeptin, phenyl-methyl-sulphonyl-fluoride (PMSF), iodoacetamide and *N*α-benzoyl-DL-arginine-*p*-nitroanilide were all obtained from Sigma (Poole, England). Aprotinin (Trasylol) was from Bayer (Sussex, England). All other

Table 1. Control and patient groups

Group	Number	Age (yr) (mean and 95% CL)	Range	Tumour burden
Healthy	30	52 (49–55)*	40–68	-
Benign	20	53 (46–60)	28–81	-
Breast cancer				
Ca1	26	57 (52–63)	36–80	$\leqslant$20 mm
Ca2	47	60 (57–63)	32–85	>20 mm
Ca3	29	69 (63–75)†	42–89	Fixation and/or metastases

*Significantly different from groups Ca2 and Ca3 ($P < 0.001$).
†Significantly different from other groups ($P < 0.001$).

Table 2. Serum proteinase-like peptidase assay conditions

Peptidase activity	Substrate* (final conc.)	Reference	Buffer pH	Serum dilution	Coefficient of variation (%)
CL-	Suc-Gly-Pro-Leu-Gly-Pro-MCA (1.8 mM)‡	[14]	8.0	None	3.16
Cat BL-	Z-Phe-Arg-MCA† (0.4 mM)	[15]	6.5	1:100	4.20
Cat HL-	Arg-MCA (1.0 mM)	[15]	6.8	1:100	1.73
EL-	Suc-Ala-Pro-Ala-MCA (0.4 mM)		7.4	1:10	2.56
TL-	Bz-Arg-MCA (0.08 mM)	[16]	8.0	1:10	2.67
PL-	Boc-Val-Leu-Lys-MCA (0.15 mM)	[17]	7.4	1:10	3.39

*Z = Carbobenzoxy, Bz = benzoyl, Boc = *t*-butyloxycarbonyl.
†PMSF present in assay to inhibit serum kallikrein-like activity.
‡This substrate was reconstituted in assay buffer, all the other MCA substrates were reconstituted in dimethylsulphoxide.

reagents were of Analar grade and obtained from BDH (Poole, England).

Proteinase-like peptidase activities

Collagenase-like (CL-), cathepsin B-like (Cat BL-), cathepsin H-like (Cat HL-), trypsin-like (TL-) and plasmin-like (PL-) peptidase activities in serum were determined using MCA substrates by reported methods modified for use with human serum (Table 2). Elastase-like (EL-) peptidase activity was also assayed with an MCA substrate using the buffer system of Castillo *et al.* [10]. The highly fluorescent product 7-amino-4-methyl-coumarin (AMC) released by enzyme activity on MCA substrates was detected in a ratio-recording spectrofluorimeter (Model SFR 100, Baird Atomic, Braintree, England) with excitation at 380 nm and emission at 460 nm, and concentrations were determined by comparison with AMC standards. All incubations were carried out in duplicate at 37°C and the reactions terminated by the addition of 100 μmol of sodium acetate, pH 4.3. Activities are expressed as the amount of product formed per minute per ml of serum at 37°C and 400 μl was sufficient to assay all the enzymes. The affinity of the serum enzymes for the MCA substrates was assessed by determination of their apparent K_m values from Lineweaver–Burk plots, and the relative specificity of the substrates evaluated by the use of appropriate inhibitors (Table 3).

Proteinase inhibitor assays

Alpha$_1$-antitrypsin and α_2-macroglobulin activities were determined as the trypsin inhibitory capacity [11] and the trypsin binding capacity [12], respectively, using the synthetic chromogenic substrate *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). The chromogenic product *p*-nitroaniline was detected at 400 nm in an SP8-100 spectrophotometer (Pye Unicam, Cambridge, England).

Data analysis

Overall, 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 the 95% confidence limits (CL). All other values are presented as the arithmetic means with 95% CL (i.e. mean ± 2 S.E.M.). All the data was analysed by Student's *t*-test for independent groups, with Cochrane's modification where required, and by Wilcoxon's rank sum test [13]. The precision of each assay was determined from 40 serum duplicates and expressed as the coefficient of variation.

RESULTS

Although women in the control group were of cancer bearing age, on average the healthy women were significantly younger than the patients in groups Ca2 and Ca3 (Table 1). This indicates the relative low attendance at Well-Women Clinics of older women. The patients with advanced breast cancer were, not surprisingly, older on average, than women in the other groups.

The coefficients of variation of the proteinase-like peptidases were under 5% (Table 2) and values of 1.5 and 1.9% were obtained for α_1AT and α_2M, respectively. In general, K_m values obtained for serum using the MCA substrates were similar to reported values for purified enzymes and activities were usually 60–100% inhibited by appropriate inhibitors (Table 3).

There were no significant differences between the different groups for CL-, Cat BL-, Cat HL- and EL-activities (Table 4). However, serum TL- and

Table 3. Substrate and inhibition characteristics for serum proteinase-like peptidase activities

Peptidase activity	Observed K_m* (mM)		Reported K_m* for proteinase (ref) (mM)	Inhibitor (final conc. mM)	% activity inhibited
CL-	0.71	0.40	chick embryo collagenase [14]	EDTA (30)	90
				L-cysteine (100)	60
Cat BL-	0.29	0.29	human liver cathepsin B [15]	IAA† (60)	90
				Leupeptin (0.05)	100
Cat HL-	0.25	0.15	human liver cathepsin H [18]	IAA (80)	100
				Na chloroacetate (10)	100
EL-	0.40	0.29	human leukocyte elastase [10]‡	PMSF (2)	100
				Elastatinal (0.04)	100
TL-	0.08	0.11	bovine trypsin [16]	PMSF (1.6)	85
				Trasylol (100 KIU)	85
PL-	0.11	0.77	human plasmin [17]	PMSF (8)	75

*Substrates as listed in Table 2.

†IAA = Iodoacetamide.

‡K_m value with the substrate Methoxy-Suc-Ala-Ala-Pro-Val-MCA.

Table 4. Serum proteinase-like peptidase activities in patients with breast disease and in healthy controls

Group	Arithmetic or geometric means and 95% CL†					
	CL-	Cat BL- (nmol/min/ml)	Cat HL-	EL-	TL- (pmol/min/ml)	PL-
Healthy	2.3 (2.0–2.6)	43 (33–35)	3.3 (2.9–3.7)	51 (43–59)	158 (137–183)*	581 (487–675)*
Benign	2.5 (2.2–2.8)	54 (36–82)	3.9 (3.5–4.3)	50 (44–56)	89 (72–111)	395 (309–481)
Ca1	2.4 (2.2–2.6)	48 (36–64)	3.5 (2.9–4.1)	53 (45–61)	87 (67–113)	448 (382–514)
Ca2	2.3 (2.1–2.5)	56 (45–71)	3.3 (3.0–3.6)	53 (45–61)	79 (67– 93)	407 (355–459)
Ca3	2.5 (2.2–2.8)	50 (38–64)	3.8 (3.3–4.3)	54 (44–64)	98 (81–119)	407 (349–465)
Alive	2.5 (2.1–2.9)	49 (34–71)	3.7 (3.0–4.4)	58 (41–75)	96 (73–127)	441 (347–535)
Dead	2.6 (21.–3.1)	50 (35–72)	3.9 (3.2–4.6)	51 (41–61)	100 (75–134)	368 (302–434)

*Significantly different from all other groups ($P < 0.05$–0.001).
†Cat BL- and TL-peptidase values are given as geometric means, other activities as arithmetic means (see text).

PL-activities were significantly lower in women with benign or malignant breast disease compared with healthy women, although all the patient groups had similar mean values of these serine-like proteinases. There did appear to be some differences in the distribution of both TL- and PL-peptidase activities in healthy women and the patient groups, although larger group sizes would be required to confirm this. Similar enzyme activities were found in patients who had subsequently died of advanced disease, compared with those still alive at the time of data analysis (Table 4).

Serum proteinase-like activities in patients with involved nodes were not significantly different from those without nodal involvement, although on average activities were higher in the former.

Serum trypsin inhibitory capacity (α_1AT) in patients with advanced breast cancer (group Ca3) was significantly higher than in all the other groups studied, and women with early breast cancer (groups Ca1 and Ca2) had significantly higher α_1AT activities than women with benign disease (Table 5). Mean serum trypsin binding capacity, or α_2M, levels were higher in patients with breast cancer, although this only reached significance when patients in group Ca2 were compared with healthy women (Table 5).

Table 5. Serum proteinase inhibitor activities

Group	Arithmetic means and 95% CL	
	α_1-Antitrypsin (μmol/min/ml)	α_2-Macroglobulin (nmol/min/ml)
Healthy	4.4 (4.2–4.6)	269 (244–294)
Benign	4.1 (3.7–4.5)	279 (247–311)
Ca1	4.5 (4.3–4.7)*	316 (276–356)
Ca2	4.4 (4.2–4.6)*	311 (287–335)‡
Ca3	5.2 (4.8–5.6)†	295 (266–324)
Alive	5.1 (4.5–5.7)	282 (246–318)
Dead	5.3 (4.8–5.8)	318 (268–368)

*Significantly different from benign group ($P < 0.05$).
†Significantly different from all other groups ($P < 0.001$).
‡Significantly different from healthy women ($P < 0.05$).

DISCUSSION

We found the synthetic MCA peptide substrates to be extremely suitable and convenient for studying serum proteinase-like peptidase activities, and the assays were highly reproducible and sensitive. Furthermore, the good agreement between the K_m values obtained for serum and those reported for the purified enzymes and the degrees of inhibition observed indicate the relative specificity of the MCA substrates, and suggests that the enzyme activities measured are probably due to the presence of the endopeptidases. For example, the pattern of inhibition of serum CL-peptidase activity by EDTA and L-cysteine reported here is similar to that for purified specific mammalian collagenase [4]. Similarly, the inhibition of Cat BL- and Cat HL-peptidase activities by iodoacetamide, and the inhibition of EL-, TL- and PL-peptidases by PMSF suggests that the activities detected are probably due to the presence of cysteine and serine proteinases, respectively [15]. As might be expected with unfractionated serum, the inhibitor concentrations required to give maximum inhibition were generally higher than those reported for purified or partially purified proteinases [4, 19]. The degree of specificity of the MCA substrates for the proteinases and/or peptidases present in serum is unknown, although the assay conditions used and the inhibitor responses observed presumably narrows the range of possibilities. However, Z-Phe-Arg-MCA can be hydrolysed by cathepsin L and plasma and tissue kallikreins [20], Arg-MCA by certain aminopeptidases [20] and Boc-Val-Leu-Lys-MCA by urinary and tissue kallikreins [17].

Apart from TL- and PL-peptidase, the serum activities of the other enzymes studied were similar in all the groups of women. In a previous study we observed similar levels of cathepsin D-

like peptidase activities in the sera of women with benign and malignant breast disease [21].

Although mean serum Cat BL-peptidase activities in patients with breast disease were higher than those in healthy women and were more variable in patients with breast cancer, we have not observed the large differences reported by Pietras *et al.* [8] in women with gynaecological cancers and 5 patients with breast cancer, compared with a control group of younger women. These workers used the synthetic substrate Z-Ala-Arg-Arg-4-methoxy-β-naphthylamide while we have used Z-Phe-Arg-MCA and our different findings suggest that the enzymes assayed may not be the same. We chose the MCA peptides as opposed to the naphthylamides because of their greater solubility, higher fluorescence efficiencies and non-carcinogenic properties [15]. Although, at the time of our study, Z-Phe-Arg-MCA was reported to be highly specific for cathepsin B, it has recently been reported that this substrate is also hydrolysed by cathepsin L [20]. It is therefore possible that the activity detected in serum with Z-Phe-Arg-MCA may be due to either cathepsin B or cathepsin L, a combination of both or to peptidases with similar substrate specificities. To our knowledge levels of cathepsin L activity in serum have not been reported. Recently Kirschke *et al.* [22] have introduced the substrate Z-Arg-Arg-MCA, which appears to be ideal for assaying cathepsin B.

Several workers have reported that breast tumours contain higher levels of plasminogen activator than normal tissue [23, 24], so the apparent depression of TL- and PL-peptidases in women with benign and malignant breast disease found in our study was unexpected and of unknown cause. A more detailed investigation of the fibrinolytic system in breast disease is required to resolve this apparent discrepancy.

The observed increase in pre-treatment serum α_1AT with disease progression has been reported previously in patients with lung cancer [25] and breast cancer [26]. However, these changes in serum α_1AT are probably non-specific since elevated α_1AT levels have been found in a wide variety of non-neoplastic conditions [27]. Lamoureux *et al.* [26], using a radial immunodiffusion method, found that mean serum α_2M levels in 134 patients with breast cancer were higher, although not significantly so, compared with 70 healthy controls which included men and women. Our findings, using a different assay method, are consistent with this, and in fact a significantly higher mean α_2M was found in women with tumours >20 mm when compared with healthy women. It is tempting to speculate that the increase in α_2M may be in response to release of proteinases into the circulation, and the relatively small differences may reflect rapid removal of the α_2M–proteinase complex from the circulation. Such a process may explain the absence of increased proteinase-like peptidase activities in women with breast cancer.

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