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Immunoradiometric Assay of Pro-cathepsin D in Breast Cancer Cytosol: Relative Prognostic Value Versus Total Cathepsin D

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In breast cancer cell lines, the maturation of pro-cathepsin D into enzymatically active cathepsin D is altered, leading to its increased secretion. In order to specifically assay pro-cathepsin D (52 kD form) in breast cancer cytosol, we monitored a solid phase sandwich radioimmunoassay using D9H8 and D7E3 monoclonal antibodies raised against human pro-cathepsin D from MCF7 cells. Pro-cathepsin D was assayed in 108 primary breast cancer cytosols in which total cathepsin D was previously found to be correlated with metastasis. Pro-cathepsin D concentrations were found to be correlated with total cathepsin D and with lymph node invasion, and was slightly higher in premenopausal patients. By contrast, Cox multiparametric analysis showed that pro-cathepsin D status had no prognostic value for survival, or metastasis free survival contrary to total cathepsin D status. This first study shows the technical validity of the pro-cathepsin D assay but indicates that it has less value as a prognostic marker than total cathepsin D. This study also shows that the proportion of pro-cathepsin D recovered *in vivo* (1–6%) is much less than that produced in cell lines and suggests that the secreted pro-enzyme might be activated in the tumour extracellularly or following its reinternalisation.
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INTRODUCTION

CATHEPSIN D (cath D), a lysosomal protease is produced in excess by most breast cancer cell lines [1, 2]. Moreover, in many breast cancer cell lines, maturation of the pro-enzyme and its routing to lysosomes are known to be altered, resulting in its increased secretion. Several monoclonal antibodies have been raised against human pro-cath D secreted by MCF7 cells [3]. Two of them (D7E3 and M1G8) have been used in a solid phase immunoradiometric assay (IMRA) to measure total cath D concentration in breast cancer cytosol, including the pro form (52 kD), the intermediate form (48 kD) and one of the mature chain (34 kD) [4]. Studies using this assay [5] or ELISA [6] have shown that a high cath D level is associated with higher risk of relapse and metastasis even in node-negative breast cancer patients. One frequently proposed mechanism by which proteases might facilitate metastasis is by degradation of the extracellular matrix after secretion [7]. Moreover, pro-cath D has been shown to be mitogenic [8] and to interact with the mannose-6-phosphate/insulin-like growth factor II (Man 6P/IGFII) receptor [9]. Thus the pro-cath D assay might prove to be more

potent for predicting metastasis than the total cath D assay. Alternatively pro-cath D is inactive as a protease and should be activated by removal of its pro-fragment. This is obtained with the pure pro-enzyme, but this autoactivation requires an acidic pH (< 5) and normally takes place in lysosomes and possibly sorting endosomes [10, 11]. One might, therefore, also anticipate that the activated protease level will be more closely correlated with metastasis than the inactive one if proteolytic activity is required. Two monoclonal antibodies were selected for their ability to specifically interact with mature pro-cath D [10]. We, therefore, developed a solid phase immunoradiometric assay using D9H8 and D7E3 antibodies and report the first retrospective clinical study on its prognostic value compared with that of total cath D.

MATERIALS AND METHODS

Patients

108 patients of the Centre Rene Huguenin treated between 1982 and 1984, and corresponding to previous retrospective study on total cath D [5] are included in this study. They were selected according to several criteria: primary and unilateral breast cancer, no other primary cancer, oestrogen receptor (ER), progesterone receptor (PR) and cath D assayed in the cytosol of the primary tumour and a complete follow-up at the Centre René Huguenin. At the time of the diagnosis, all patients were staged according to the UICC TNM classification. Tumours were graded according to the method of Scarff, Bloom and Richardson. ER and PR were assayed by the dextran charcoal method and total cathepsin D was determined using the ELSA Cath D kit (CIS BioInternational, Gif sur Yvette, France) as previously described in [5].

Pro-cathepsin D immunoradiometric assay

Cytosol was prepared in 10 mmol/l Tris–HCl buffer, pH 7.4, containing 1.5 mmol/l EDTA, 0.5 mmol/dithiothreitol, 10% glycerol and kept at −80°C until assay. Cytosols used in this study have been thawed three times, but we verified on a pool of cytosols that two or three cycles of thawing had no effect on pro-cath D determinations. Two monoclonal antibodies raised against pro-cath D were used in a one-step immunoradiometric assay. The first (D7E3), recognising both precursor and mature forms, was coated on the solid phase and the second (D9H8) recognising only pro-cath D [10], was labelled with ^{125}iodine. Fifty microlitres of diluted cytosol, 1/10 and 1/20, were incubated with the two monoclonal antibodies for 3 h under agitation at room temperature. After three washes with distilled water and Tween 20 (0.3%), the ^{125}iodine radioactivity was counted in a gamma counter. MCF7 medium, calibrated against purified pro-cath D with the ELSA Cath D kit, was used as standard, and the standard curve was plotted from 0 to 3000 fmol/ml. The protein concentration in cytosol was measured by the Bradford technique (Bio-Rad Laboratories Gmb, Munich, F.R.G.) with bovine serum albumin as standard.

Statistical analysis

For pro-cath D we used a qualitative cut-off value of 2.5 pmol/mg protein, determined by the Fischer method. The median value was 1.6 pmol/mg protein. The χ^2 test was used for group analysis. Kaplan–Meier metastasis-free survival and disease-free survival were analysed by the logrank test. The most significant prognostic factors were identified by Cox's proportional hazards method.

Menopausal status, UICC stage, histological grade, axillary lymph node status, cytosolic ER and PR, macroscopic tumour size and age, were analysed in addition to pro- and total-cath D.

RESULTS

Validity of the IRMA of pro-cath D

The detection limit was 25 fmol/ml. The coefficient of variation determined with different cytosol at different cath D concentrations, within assays and between 10 assays with several batches of reagents used at different periods of their validity was always lower than 10% (Table 1). Additivity tests were satisfactory when samples from the same origin, cytosol to cytosol, culture medium to culture medium, or purified pro-cath D to either sample were used. When cytosols were added to culture medium, higher values than those expected were obtained suggesting that the D9H8 antibody recognition was dependent on the tertiary structure of the antigen [12]. Both purified pro-cath D and pro-cath D secreted in culture medium were checked to be stable at −80°C for several years.

Distribution and clinical follow-up of patients

During the 7-year mean follow-up period, 20 patients died, 37 had distant metastasis and 6 had isolated local recurrences. Distribution of pro-cath D concentration in these patients is shown in Fig. 1. Pro-cath D concentration varied from 6×10^{-2} to 40.6 pmol/mg protein and from 0.1 to 13.8% of the total cath D concentration.

Pro-cathepsin D correlated with other variables

Using a cut-off of 2.5 pmol/mg protein determined by the Fisher exact test, pro-cath D was correlated with node invasiveness ($P = 0.02$) and with total cath D ($P < 0.001$) (Fig. 2). The correlation was at the limit of significance for menopausal status ($P = 0.051$) (Table 2). Pro-cath D was not an independent factor in this study since it was also correlated with node invasiveness and total cath D, two more potent markers.

Table 1. Reproducibility of the IRMA pro-cathepsin D assay

Samples	n	$\bar{X}$ (fmol/ml)	CV (%)
1			
A	30	70	8.6
B	30	2850	3.4
2			
A	10	72	9.5
B	10	2910	4.4

Two pooleds cytosols were used with low (A) and high (B) levels of pro-cath D assayed in duplicate in each run. 1 = Within-run test; 2 = between-run test; CV: coefficient variation; $\bar{X}$: mean value of duplicates.

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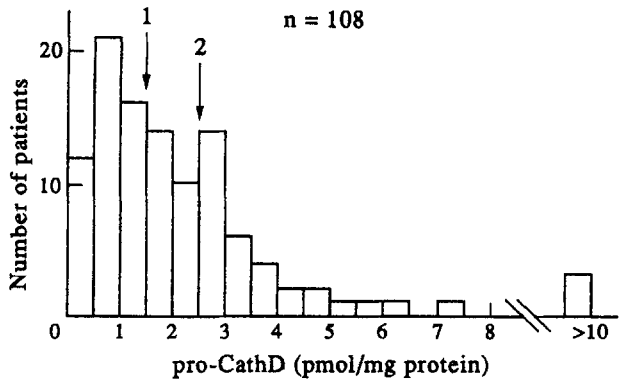

Fig. 1. Distribution of primary breast cancer according to the cytosolic pro-cathepsin D. The class interval is 0.5 pmol/mg protein. The median class (1) is 1.6 pmol/mg protein and the most significant cut-off determined by the Fisher exact test is 2.5 pmol/mg protein (2).

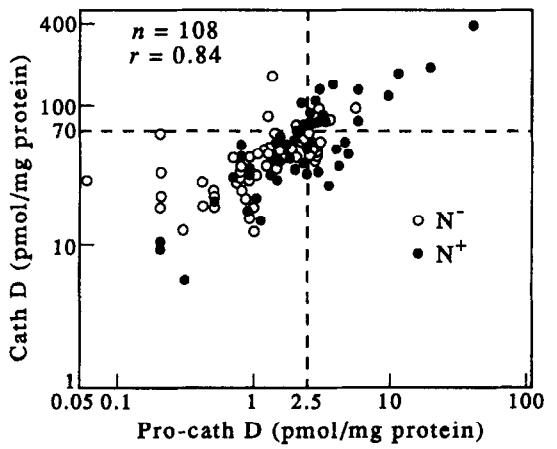

Fig. 2. Correlation between pro-cathepsin D and total cathepsin D concentration (logscale) in 108 breast cancer cytosols.

Univariate prognosis analysis

Using a cut-off value of 2.5 pmol/mg protein, pro-cath D was found to have a significant predictive value with overall survival ($P = 0.044$) (Fig. 3b) and a metastasis-free survival ($P = 0.02$). Using a cut-off of 70 pmol/mg protein, total cath D had a stronger predictive value for overall survival ($P = 0.003$) (Fig. 3a), disease-free survival ($P = 0.002$) and in metastasis-free survival ($P = 0.0002$) (data not shown).

Multivariate prognosis analysis

As shown in Table 3, total cath D, using the cut-off of 70 pmol/mg protein, was the most important prognostic factor both in metastasis-free survival and in disease-free survival. In overall survival, nodal status was the most significant factor, before cath D and ER, respectively. Figure 3 shows that total cath D status remained significant after 7 years follow-up for overall survival but that pro-cath D no longer had a significant prognostic value, as shown by multivariate analysis.

Table 2. Correlation of pro-cathepsin D status with other variables

	Pro-cathepsin D concentration		
	≤ 2.5 pmol/mg protein	> 2.5 pmol/mg protein	*P* value
Menopausal status			
Pre	24	51	0.051 (NS)
Post	17	16	
Lymph node status			
N−	43	11	0.020
N+	32	22	
Cathepsin D			
≤ 70 pmol/mg protein	68	16	< 0.001
> 70 pmol/mg protein	7	17	

Only the significant or near significant correlations with pro-cathepsin D are shown. The other variables studied (age, UICC stage, tumour size, histological grading, ER and PR status) were not significantly correlated.

DISCUSSION

This study shows the validity and limitations of the pro-cath D IRMA. The fact that the pro-cath D level was found to be a less potent prognostic factor than total cath D was unexpected considering that cath D should act following its secretion. However, these results are in agreement with a separate clinical study in which only the mature 34 kD form of cath D had been considered [13] and with a role of cathepsin D in metastasis involving its proteolytic activity. An alternative explanation for the better prognostic values of the total cath D assay is that higher concentrations of total cath D in the cytosol could result in higher reproducibility for the assay.

Pro-cath D concentration is generally proportional to total cath D concentration, however, in some tumours the proportion of pro-cath D varied.

The percentage of pro-cath D found in tumour cytosol was surprisingly low (1–6%) compared to the high proportion of pro-cath D being secreted by breast cancer cells in culture (20–60%). The difference does not seem to be due to an escape of the

Table 3. Significant variables in multivariate Cox analysis

	Overall survival		Metastasis-free survival		Disease-free survival	
Significant variable	*P*	Regression coefficient	*P*	Regression coefficient	*P*	Regression coefficient
Total cathepsin D (< or ≥ 70)	0.02	1.19	3×10^{-5}	1.44	4×10^{-4}	1.6
Nodal status	0.003	1.29	0.04	0.76	0.012	0.79
Oestrogen receptor	0.03	−1.04	NS	NS	NS	NS

Pro-cathepsin D was not significant by multivariate analysis, contrary to total cathepsin D and is therefore not represented. NS = non significant.

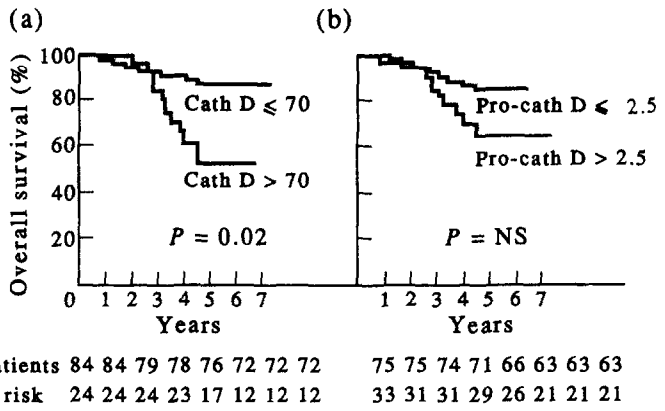

Fig. 3. Overall survival of patients as a function of total cath D (a) or pro-cath D (b) status. The most significant cut-off values were chosen. They correspond to 21.3% of patients with cath D > 70 pmol/mg protein and 28.7% of patients with pro-cath D > 2.5 pmol/mg protein. *P* values were determined by Cox multivariate analysis.

secreted pro-enzyme in the blood, since we found no increase of plasmatic cath D level in metastatic breast cancer patients compared with normal patients [14]. It is more likely that the re-uptake of the pro-enzyme is via mannose-6-phosphate receptors by cancer cells or cells in the stroma, and that activation follows intracellularly. A final hypothesis would be that the pro-enzyme is activated extracellularly at the contact of the basement membrane in an acidic microenvironment.

In practical terms, the pro-cath D assay in breast cancer cytosol appears to be less useful as a prognostic marker than total cath D assay, the reliability and prognostic value of which has been demonstrated in several studies [1]. However, using a similar sandwich immunoassay, the pro-cath D level was found to increase more than that of total cath D following tamoxifen treatment [15]. This is in agreement with the shorter half-life of the precursor compared to the native enzyme [2]. The pro-cath D assay might, therefore, be useful for assessing the efficacy of hormonally active drugs such as anti-oestrogens and generally to evaluate the regulation of pro-cath D according to different hormonal or pharmacological conditions in treated patients.

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