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Quantification and Molecular Analysis of Cathepsin D in Breast Cyst Fluids

Luis M. Sánchez, Francisco Vizoso, M. Teresa Allende, Alvaro Ruibal and Carlos López-Otín

Cyst fluids from 55 premenopausal women with gross cystic breast disease were classified by K^+/Na^+ ratio: 19 with ratio over 1 (type I) and 36 with ratio less than 1 (type II). Immunoradiometric assay of cathepsin D in both types of cyst fluids revealed the presence of large amounts of this proteinase. The average concentration of cathepsin D in type I cyst fluids was 63.3 nmol/l, which was significantly higher than that corresponding to type II cyst fluids (35.1 nmol/l). Immunoprecipitation analysis of intracystic cathepsin D demonstrated that this protein was present as the 52 kD non-processed precursor form of the molecule. Since procathepsin D is a useful prognostic marker in breast carcinoma, we suggest that cyst fluid quantification of cathepsin D could aid to detect patients affecting of gross cystic disease with higher risk for developing breast cancer.
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INTRODUCTION

GROSS CYSTIC breast disease is the most common mammary pathology in premenopausal women, affecting about 7% of women in Western populations [1]. Although the relationship of gross cystic disease to breast carcinoma is controversial [2], there is statistical evidence indicating that patients with gross cystic lesions are at a 2–4-fold greater risk of developing breast cancer than the normal female population [3, 4].

The biochemical composition of cyst fluid aspirated from patients with gross cystic breast disease has been studied in an attempt to understand the mechanisms involved in cyst formation and to define their possible role in carcinogenesis. These analyses have demonstrated that cyst fluids contain a wide variety of substances at concentrations more than 100 times higher than the levels found in plasma. These substances include conjugated steroids [5, 6], tumour markers [7, 8], epidermal growth factor [9, 10] and several unusual proteins that seem to be specific secretory products of epithelial cells surrounding the cysts [11–13].

In addition, analyses of the composition of fluid filling the cysts have allowed cysts to be typed into two principal categories according to the concentrations of different intracystic sub-

stances, being the ratio K^+/Na^+ the most convenient marker to distinguish both types [14–19]. Epidemiological studies have suggested that incidence of subsequent breast cancer is more frequent in women with type I cysts than in those with type II cysts [20], which has led to the proposal that those substances found primarily in type I cysts might be useful markers for increased breast cancer risk [21].

In this work we present evidence that cyst fluids and mainly those of type I accumulate large amounts of cathepsin D, which has been recently introduced as a marker for predicting early recurrence in node-negative breast cancer patients [22]. The possible implications of cathepsin D in the development of breast gross cystic disease are also discussed.

MATERIALS AND METHODS

Samples

Cyst fluids were obtained, with informed consent, by needle aspiration from 55 women with gross cystic breast disease. The patients were aged 32–48 (mean 43.8). Cancer was excluded by clinical, echographic, mammographic and cytological studies. All women had regular menstrual cycles and none was taking any hormonal medication at the time of the study or during the preceding 6 months. Cyst fluids were drawn during the luteal phase of the menstrual cycle, which was confirmed by measuring luteinising hormone (LH), follicle stimulating hormone (FSH) and progesterone in blood samples. These hormones were quantified by immunoradiometric assay using kits from Ire-Medgenix (Fleurus, Belgium) for LH and FSH and from CIS Bio-International (Gif-sur-Yvette, France) for progesterone. Cyst fluid samples ranged from 1.5 to 48 ml and after collection, they were centrifuged at 35000 *g* for 1 h at 4°C and the supernatants stored at −20°C.

Biochemical determinations

Sodium and potassium concentrations were determined with a flame photometer "Model 450" (Corning Medical) with an internal standard of lithium. The cystic fluid values of oestriol, oestradiol, dehydroepiandrosterone sulphate (DHAS) and CA 15.3 were determined by radioimmunoassay using commercially available kits from DPC (Los Angeles) for oestriol and oestradiol; DSL (Texas) for DHAS and CIS Bio-International for CA 15.3.

Cathepsin D immunoassay

Cyst fluids were assayed by a solid phase immunoradiometric assay with the ELSA–cathepsin D kit obtained from CIS Bio-International. The assay involves two monoclonal antibodies, one (D7E3) coated on the ELSA solid phase and the other (M1G8) radiolabelled with ^{125}I. In each ELSA tube 300 µl of ^{125}I monoclonal anticathepsin D and 50 µl of each standard or sample dilution were incubated for 3 h at room temperature under agitation. The tubes were then washed and radioactivity measured in a gamma scintillation counter LKB (Uppsala), model 1271.

Protein labelling and immunoprecipitation

Cyst fluid (500 µl) were incubated with 1.48 TBq of ^{35}S-labelling reagent (Amersham International) in 120 µl of 0.5 mol/l borate buffer pH 8.5. After 30 min at 0°C, the reaction was quenched with 50 µl of 2 mol/l glycine in borate buffer. The labelled sample was diluted in 1 ml of immunoprecipitation buffer (Tris–HCl 10 mmol/l pH 7.5, NaCl 150 mmol/l, bovine serum albumin (BSA) 0.1%, sodium dodecyl sulphate (SDS) 0.1%, NP 40 1%, sodium deoxycholate 1%, NaN_3 0.02%) and incubated for 1 h at room temperature with 20 µl of rabbit antibodies against mouse IgGs (Sigma). Protein A sepharose (Sigma) was then added, continuously mixed for 30 min at room temperature, and sedimented. The precleared supernatant was collected and incubated with 50 µl of mouse monoclonal antibodies anticathepsin D D7E3 and M1G8. After 1 h at room temperature, rabbit antibodies against mouse IgGs, and protein A sepharose were added and allowed to react as above. The resin was pelleted, washed with immunoprecipitation buffer and sedimented through 0.5 ml of sucrose buffer (Tris–HCl 10 mmol/l pH 7.5, NaCl 150 mmol/l, sucrose 30%, SDS 0.1%, NP 40 1%, sodium deoxycholate 1%, NaN_3 0.02%). Immunoprecipitated proteins were dissociated from the resin by heating for 5 min at 90 °C in SDS reducing sample buffer, electrophoresed through 12% SDS–PAGE (polyacrylamide gel electrophoresis) and autoradiographed.

Statistical methods

For analysis of data, the patients were subdivided into two groups on the basis of their K^+/Na^+ ratio [23]. Student's *t* test for unpaired data was performed to assess differences between the two groups of cysts. Correlation coefficients were calculated by the least-squares method. Significance was established at the 0.05 level.

RESULTS

Quantification of cathepsin D concentrations in breast cyst fluids

Cyst fluids aspirated from 55 women with gross cystic breast disease were divided into two groups according to their K^+/Na^+ ratio. 19 cysts (35%) were of type I, defined by a K^+/Na^+ ratio higher than 1. The remaining 36 cysts (65%) were of type II with a K^+/Na^+ ratio lower than 1.

Cathepsin D was measured in cyst fluid of both group of patients and the results obtained are shown in Table 1. Cathepsin D levels differ substantially when the two groups of patients above mentioned were considered. Thus, the average concentration of cathepsin D found in type I cyst fluids was 63.3 ± 9.4 nmol/l, whereas the value corresponding to the type II was significantly ($P < 0.05$) lower [mean (S.E.M.) 35.1 (5.8) nmol/l].

Relationship of cathepsin D levels with other parameters

Intracystic cathepsin D concentrations were first compared with the corresponding values of K^+/Na^+ ratio. As Table 2 shows, there is a positive association between both parameters; however, separate analysis of the different cyst types revealed that such association is not homogeneous, since it was present only in type I cysts and could not be detected in those of type II.

On the other hand, since cathepsin D is an oestrogen-inducible protein in breast cancer cells [24], we tried to establish the possible relationship between intracystic oestrogens and cathepsin D. To do that, cyst fluid contents of oestriol and oestradiol were measured and the results are shown in Table 1. Type I cysts had very high levels of oestriol [mean (S.E.M.) 61.6(6.9) µg/l) and oestradiol [3.2(0.4) µg/l] when compared with those found in type II cysts (average 38.5(4.3) µg/l of oestriol and

Correspondence to C. López-Otín.
C. López-Otín and L.M. Sánchez are at the Departamento de Biología Funcional, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain; F. Vizoso is at the Servicio de Cirugía, Hospital de Jove, Gijón, Spain; and M.T. Allende and A. Ruibal are at the Servicio de Medicina Nuclear, Hospital Central de Asturias, Oviedo, Spain.
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Table 1. Intracystic concentrations of cathepsin D, oestriol, oestradiol, DHAS and CA 15.3 in type I and type II breast cyst fluids

	Type I cysts ($K^+/Na^+ > 1$)	Type II cysts ($K^+/Na^+ < 1$)	P^*
Cathepsin D (nmol/l)			
Mean	63.3	35.1	<0.05
S.E.M.	9.4	5.8	
Range	8.1–125.1	1.0–147.6	
Oestriol (μg/l)			
Mean	61.6	38.5	<0.001
S.E.M.	6.9	4.3	
Range	2.5–109.0	2.5–100.1	
Oestradiol (μg/l)			
Mean	3.2	2.1	<0.01
S.E.M.	0.4	0.2	
Range	1.1–7.2	0.4–4.8	
DHAS (mg/dl)			
Mean	10.1	1.8	<0.001
S.E.M.	1.9	0.5	
Range	1.8–26.8	0.1–14.4	
CA 15.3 (U/ml)			
Mean	59.3	31.8	<0.01
S.E.M.	5.2	2.0	
Range	33.1–121.0	12.1–52.3	

*Student's unpaired *t* test.

2.1(0.2) μg/l of oestradiol]. Analysis of these data along with those of cathepsin D revealed a significative correlation between the oestrogens and the protease in type I cysts, but we did not find any correlation in type II cysts. It is remarkable that the intracystic concentrations of oestriol and oestradiol are higher than those previously reported by other groups [5, 25]. One possibility to explain these discrepancies is that all our measurements were performed during the mid luteal phase of the menstrual cycle while the patient population analysed by the other authors was not clearly defined on this point. However, additional explanations such as cross-reactivity with other steroids which are highly elevated in cyst fluid, cannot be ruled out.

We also studied the possible association between cathepsin D and DHAS. The concentrations of DHAS were much higher in type I cysts [10.1 (1.9) mg/dl] than in type II ones [1.8 (0.5) mg/dl], however, they were not apparently correlated with cathepsin D levels neither in type I nor in type II cyst fluids.

Finally, since CA 15.3 is also an efficient marker to check the course of breast cancer [26], the cathepsin D concentration in breast cyst fluid was compared to the intracystic values for CA 15.3. As Table 2 shows, no correlation was seen between both markers. Type I breast fluids presented an average concentration of CA 15.3 of 59.3 (5.2) U/ml while Type II fluids showed lower levels of CA 15.3 [31.8 (2.0) U/ml].

Table 2. Correlations of K^+/Na^+, oestriol, oestradiol, DHAS and CA 15.3 with cathepsin D in breast cyst fluids

	All cysts			Type I cysts			Type II cysts		
	n	*r*	*P*	*n*	*r*	*P*	*n*	*r*	*P*
K^+/Na^+	55	0.46	<0.001	19	0.59	<0.01	36	0.09	n.s.*
Oestriol	55	0.35	<0.01	19	0.51	<0.05	36	0.01	n.s.
Oestradiol	55	0.38	<0.01	19	0.47	<0.05	36	0.12	n.s.
DHAS	55	0.35	<0.01	19	0.13	n.s.	36	0.31	n.s.
CA 15.3	55	0.22	n.s.	19	0.20	n.s.	36	0.02	n.s.

n: number of samples; *r*: correlation coefficient; *P*: significance level; *n.s. = non-significant.

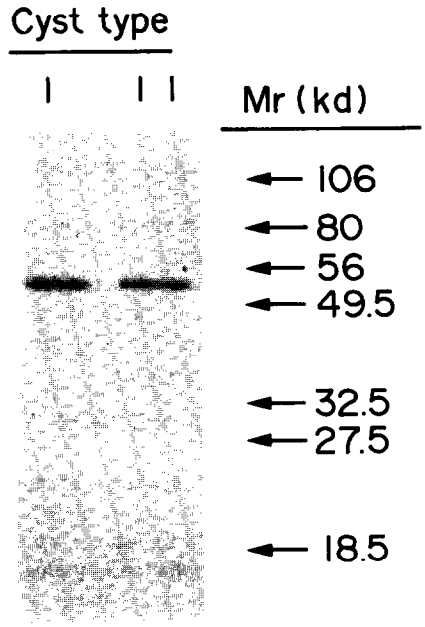

Fig. 1. Immunoprecipitation of cathepsin D from type I and type II cyst fluids. Cyst fluid proteins were radiolabelled, immunoprecipitated with monoclonal antibodies anticathepsin D and analysed by SDS–PAGE and autoradiography.

Analysis of molecular forms of cathepsin D in breast cyst fluids

It has been established that cathepsin D is actively secreted by breast cancer cells as a precursor form of 52 kD. However, in normal cells this precursor is rapidly processed into active products of 48 and 34 kD. In order to characterise the molecular forms of the enzyme present in cyst fluids, intracystic cathepsin D was immunoprecipitated with monoclonal antibodies D7E3 and M1G8, which recognise all the possible forms of the enzyme. The immunoprecipitated proteins were then analysed by SDS–PAGE, and the results obtained in a representative experiment with both types of cyst fluids are shown in Fig. 1. A single band of about 52 kD could be detected in all cases, indicating that the non processed precursor of cathepsin D is the only molecular form present in significative amounts in cyst fluids.

DISCUSSION

In this work we have shown that cyst fluids from women with gross cystic breast disease accumulate large amounts of cathepsin D. Type I cysts, classified according to K^+/Na^+ ratio, have cathepsin D concentrations of about 63.3 (9.4) nmol/l, whereas those designated type II show lower levels of this protein [35.1 (5.8) nmol/l]. These results represent the first classification of gross cystic breast disease patients into different subgroups by considering the concentration of cathepsin D. In addition, the finding that this protein is a marker of different cyst types provides an explanation to the variable levels of cathepsin D found in a previous study of its distribution in benign breast diseases [27].

In type I cysts there was a positive correlation between cathepsin D concentration and K^+/Na^+ ratio as well as between

cathepsin D and the levels of the oestrogens oestriol and oestradiol. However, these correlations were not significative in type II cysts. Moreover, no correlations could be found between the content of cathepsin D and those of DHAS or CA 15.3.

The observation that type I cyst fluids present higher levels of cathepsin D may be of interest in relation to the natural history of breast gross cystic disease. Several studies have shown that evolution of the disease depends on the cyst type, being the risk of developing breast cancer greater in women with type I cysts than in those with type II [20]. According to our results, quantification of cathepsin D in the fluid filling the cysts could be useful to identify a subset of women with gross cystic disease who are at higher-risk of breast carcinoma.

In addition to its possible value as a marker for monitoring breast gross cystic disease, cathepsin D may play a role in the induction of the pathological process characteristic of the disease. At present, the mechanism of cyst formation is unknown, however, the finding of active proteinases in the fluid filling the cysts has led to the proposal that these enzymes may account for gross cyst formation and enlargement [28]. According to Kesner *et al.*, intracystic proteinases would produce a series of peptides which would increase the oncotic pressure inside the cyst capsule. The subsequent accumulation of water to reestablish osmotic equilibrium would cause the enlargement of the cavity resulting finally in gross cyst formation. However, the nature and specificity of the proteinase or proteinases involved in this process remain unclear. It has been suggested that one of these enzymes could correspond with the major cyst fluid protein (designated GCDFP-24), but our recent finding that this protein is apolipoprotein D does not support this hypothesis [13]. The intracystic accumulation of large amounts of cathepsin D presented herein points to the possibility that this proteinase could be one of the active enzymes above mentioned. However, such an effect would require an acidic pH since the optimal pH of cathepsin D is 3.5 using methemoglobin and about 5.0 using proteoglycans from human cartilage [29]. In relation to this it is worthwhile mentioning that type I cysts present a pH which is more acidic than type II cysts [18], but in any case, it is not acidic enough to give an account of the proteolytic activity of cathepsin D.

To provide further insight on this question, studies were undertaken to perform molecular characterisation on the cathepsin D present in cyst fluid. This proteinase is secreted by breast cancer cells as an inactive precursor of 52 kD, which can subsequently be processed into 48 and 34 kD products. The total 52 kD-cathepsin D assay used for quantification in cyst fluids gives an overall value of the 52 kD precursor protein and its processed products. Therefore, cyst fluid could contain a mixture of these three molecular forms. However, immunoprecipitation analysis clearly indicates that the only molecular form detected in the cyst fluid is that corresponding with the 52 kD precursor. Taken together, these results suggest that breast epithelium surrounding the cysts is able to secrete large amounts of inactive cathepsin D. Activation of this proteolytic enzyme by transient creation of an acidic microenvironment, in a similar way to that proposed for cathepsin D activation in breast cancer [30], could initiate events leading to proliferative breast diseases. Studies are in progress to evaluate this possibility as well as the possible clinical value of cathepsin D quantification in cyst fluid as indicative of the increased risk of breast cancer.

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Radiotherapy of Aggressive Fibromatosis

G. Schmitt, E.E.D. Mills, V. Levin, B.J. Smit, H. Boecker and H. Pape

The long term results of 24 patients treated with postoperative irradiation for aggressive fibromatosis are presented. Tumour sites were the pelvis (8), chest wall (5), shoulder (5), extremities (4) and head and neck (2). Macroscopic complete resection (R1) was performed in 3 cases. 17 patients presented postoperatively with gross disease (R2), 8 of which were recurrent tumours. 4 patients with inoperable disease had biopsies only. Radiation doses ranged from 28 to 64 Gy at a fractionation of 5 × 2 or 4 × 2,5 Gy/week. 4 patients had external irradiation in combination with ^{192}Ir implants, 2 were irradiated with implants alone. In the combined treatment group, external doses ranged from 28 to 52 Gy and additional interstitial doses from 35 to 50 Gy. ^{192}Ir treatment alone was given with 45 and 57 Gy to the contour of the target volume. The 10 year recurrence free survival rate is 75%. A dose response relationship has been established in the dose range of 30–60 Gy revealing an expected 80% persistent tumour control rate at 60 Gy. A dose volume relationship however, could not be derived from our data. Moderate fibrosis without functional impairment developed in 5 patients (21%). These data support a policy of postoperative radiotherapy with 60 Gy in patients with incompletely excised or gross residual tumour following surgery.
Eur J Cancer, Vol. 28A, No. 4/5, pp. 832–835, 1992.

INTRODUCTION

AGGRESSIVE FIBROMATOSIS or desmoid tumour is a non-metastasing, locally infiltrative lesion of heterogeneous fibroblastic origin derived primarily from fascial sheaths and musculo-aponeurotic structures. The histological picture comprises uniformly elongated, slender cells with few mitoses, interspersed in abundant collagen. The incidence is 0.03% of all neoplasms with a predilection for young females [1]. The tumour characteristically infiltrates surrounding vital tissues causing severe morbidity and if untreated, may ultimately be the cause of death. Local resection results in a 25–58% failure rate. Radiotherapy, first suggested by Ewing in 1929 [2], has been shown to substantially reduce the recurrence rate in incompletely resected tumours. This report of 24 cases supports these data and updated our long term results.

PATIENTS AND METHODS

Between 1972 and 1983, 24 patients with histologically proven aggressive fibromatosis were treated at the departments of radiotherapy, Groote Schuur Hospital and University of Düsseldorf. There were 13 females and 11 males including 6 black and 18 white patients. The age distribution was 10–62 years (mean: 25 years). Tumour sites were the pelvis (8), chest wall (5), shoulder (5), extremities (4) and head and neck (2).

Macroscopic complete resection was performed in 3 cases. However, margins were histologically doubtful in these cases (R1). 17 patients presented postoperatively with gross disease (R2), 8 of which were recurrent tumours. 4 patients with inoperable disease had biopsies only. The time interval from surgery to radiotherapy ranged from 0.5 to 108 months.

External beam irradiation was administered with ^{60}Co-gamma rays, 5.7 and 12 MV photons as well as 15 MeV electrons as a boost. 4 patients received external irradiation in combination with ^{192}Ir implants, 2 were irradiated with implants alone. The treatment volume included the tumour or tumour bed with a safety margin of 2–3 cm. Parallel opposed isocentric fields were used for the extremity and head and neck lesions whereas

Correspondence to G. Schmitt.
G. Schmitt, H. Boecker and H. Pape are at the Klinik für Strahlentherapie und Radiologische Onkologie, Universität Düsseldorf, Moorenstr. 5, 4000 Düsseldorf 1, Germany; E.E.D. Mills is at the Department of Radiotherapy, Tygerberg Hospital, University of Stellenbosch; V. Levin is at the Department of Radiotherapy, University of Witwatersrand, and B.J. Smit is at the Department of Radiotherapy, Tygerberg Hospital, University of Stellenbosch, South Africa.
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