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In Vivo Stimulation by Tamoxifen of Cathepsin D RNA Level in Breast Cancer

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We have previously shown that 3 weeks of treatment with tamoxifen, of patients with primary breast carcinomas, increased cytosolic cathepsin D protein in oestrogen receptor (ER) positive tumours [Maudelonde *et al.*, *Cancer* 1989, 63, 1265–1270]. In order to investigate the mechanism of this increase and to eliminate a transient flare-up effect, we semi-quantified cathepsin D RNA levels by *in situ* hybridisation in 32 breast carcinomas from patients treated with tamoxifen for 3 weeks prior to surgery and in 35 breast cancer patients receiving no tamoxifen. We found that tamoxifen increased cathepsin D RNA level regardless of the ER status of the tumours. In ER positive tumours, tamoxifen increased the cathepsin D RNA level to the same extent as cytosolic cathepsin D protein but not in ER negative tumours. The induction of cathepsin D RNA by tamoxifen in ER positive tumours was probably due to its agonist activity, also observed *in vitro* in breast cancer cell lines. These results suggest that the cathepsin D gene is inducible by oestrogens in ER positive breast cancer as it is in breast cancer cell lines.

Key words: breast cancer, cathepsin D, tamoxifen, antioestrogen, *in situ* hybridisation
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INTRODUCTION

A HIGH cytosolic level of cathepsin D (cath D) is a marker of poor prognosis in breast cancer [1]. In oestrogen receptor positive (ER+) breast cancer cell lines, the cath D gene is regulated by oestrogen at the transcriptional level and its secretion is mostly inhibited by antioestrogens such as tamoxifen. However, *in vivo*, following a presurgical treatment for 3 weeks, tamoxifen increases the cytosolic cath D in ER+ breast cancers [2]. This is in contrast to the fact that these ER+ tumours respond favourably to tamoxifen treatment. One reason for this apparent paradox could be that the cath D increase is linked to the transitory flare-up preceding tumour regression. Since proteins are generally more stable than RNA, the cath D RNA could thus be already decreased in most of the ER+ tamoxifen treated breast cancers. In an attempt to specify the mechanism of this increase at the protein level, we have assayed cath D RNA in breast cancers using semiquantitative *in situ* hybridisation to evaluate the effect of tamoxifen on cath D RNA and correlate the results to the corresponding cytosolic cath D levels.

MATERIALS AND METHODS

Patient population

Sixty seven samples of primary breast cancer were included in our study between June 1989 and December 1990. They were collected from patients who underwent surgery without preoperative radio- or chemotherapy at the Cancer Centre (Centre Val d'Aurelle) of Montpellier. 32 patients were treated with tamoxifen (20 mg/day) before surgery, of which 27 took tamoxifen for 3 weeks before surgery, and 5 developed a new breast cancer after 2 years of adjuvant therapy with tamoxifen. The 35 remaining patients were untreated and used as a control group. The project has obtained the agreement of the Ethical Committee of Montpellier and is in agreement with Helsinki recommendations (1967), amended in 1975 (Tokyo), 1983 (Venice) and 1989 (Hong Kong).

Tumour stages were evaluated according to the International Union Against Cancer (UICC) classification of breast cancer [3]. The histopathological grading of Bloom and Richardson [4] was used to classify the tumours. The number of invaded axillary lymph nodes was determined by histological examination.

Immediately after surgical resection, each breast cancer was cut into three parts. The main part was sent to the pathologist for histological examination. Two samples were stored in liquid nitrogen. One was used to perform the cytosol assays of steroid receptors and cath D protein. 23 tamoxifen-treated cancers and 33 cancers of the control group had enough cytosol to perform the cath D precursor (52K) assay. The other sample was used for *in situ* RNA hybridisation experiments on frozen sections.

Preparation of tissue extracts

Each breast cancer tissue was homogenised in 10 mmol/l-Tris, pH 7.4, containing 1.5 mmol/l ethylenediamine tetraacetic acid (EDTA), 10 mmol/l monothioglycerol, and 10 mmol/l sodium molybdate (TET molybdate buffer) and ultracentrifuged at 105 000 *g* for 60 min. Aliquots of the high speed supernatant

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(cytosol) were used for measuring the concentrations of total cath D, its precursor, ER and progesterone receptor (PR).

Cytosolic assays

ER and PR were assayed using the Abbot enzyme immunoassay (ER-EIA and PR-EIA, Abbot Diagnostic, Rungis, France) as described [5]. A maximum of 10 fmoles/mg protein were considered to be the limit of positivity for ER and PR. Protein concentration was assayed using a Bio-Rad kit (Lab GmBh, Munich, Germany) using bovine serum albumin (BSA) as a standard. Total cath D was measured in a one-step double determinant solid-phase immunoradiometric assay (IRMA) as previously described [6]. Procathepsin D was also assayed with a similar one-step double determinant solid-phase method, using the D7E3 monoclonal antibody recognising all the forms of cath D on the solid phase and the D9H8 labelled with 125 iodine recognising only the precursor [7].

Quantitative in situ *hybridisation*

In situ RNA hybridisation was performed on 5 μm frozen sections as previously described [8]. Briefly, slides were hybridised with 1.5×10^{-6} dpm[35S] alpha UTP-labelled RNA probes for 15 h at 42°C in mineral oil. After hybridisation, oil was removed from slides in three chloroform washes, and coverslips were allowed to slide off in 2 × SSC (150 mM NaCl, 15 mM sodium citrate pH = 7) at room temperature. Excess radioactivity was washed off by incubating the slides once for 30 min at 37°C with 20 μg/ml RNAse-A (Sigma) and 400 U/ml RNAse-T1 (Boehringer, Mannheim, Germany) in 10 mM Tris-HCL (pH8)–0.5 M NaCl, and rinsed once in the same buffer without RNAses for another 30 min at 37°C. Slides were then washed twice in 0.1 × SSC, first at 65°C and then at room temperature. Hybridised sections were exposed for autoradiography to Ilford K5 emulsion developed in Kodak Dektol D19 (Eastman Kodak, Rochester, New York, U.S.A.) for 2 min 30 s at 22°C, and then stained with haematoxylin–eosin.

Cellular RNA integrity was verified by hybridisation with 28S ribosomal RNA. Any samples with null or weak ribosomal expression levels were eliminated from the study. A negative control involved *in situ* hybridisation of sections with RNA synthesised from linearised pS64 vector without insert. Each biopsy was analysed in three independent experiments, and hybridisation signals were quantified in at least 300 cells located in the tumour area for each experiment. Sections hybridised with cath D, 28S RNA or pSP64 were exposed for 3 days for autoradiography, and cath D RNA levels were quantified by semiautomatic counting of silver grains ("Starwise grains" Programme, IMSTAR image analyser, France), as previously described [9]. Background values for each slide were measured in cell-free areas surrounding the tissue sections and then subtracted from cell area values. The number of silver grains in one area was divided by the mean epithelial cell surface, determined by surrounding an average of 10 individual cells on eosin–haematoxylin-stained sections. Results are given in grains/cells (G/C).

Probes

Cathepsin D:953 bp kpnI-EcoRI cDNA probe [10] subcloned in psp64 vector. 28S ribosomal RNA:1.3kb BamHI/EcoRI fragment [11] subcloned in psp64.

Statistics

Statistical analyses were performed using the STAT-ITCF package (Institut Technique des Cereales et des Fourrages, Paris, France). Significance of the quantitative parameters were tested with non-parametric tests for small populations or with Student's *t*-test, when the patient number was higher than 30. Qualitative parameters were tested with the chi square test.

RESULTS

Characteristics of the population

The control and tamoxifen treated populations were comparable according to tumour stage, histopathological grade and lymph node invasiveness, but the control population was significantly younger with approximately a third being premenopausal patients (Table 1). ER status and cytosolic ER concentration were also similar in both populations, contrasting with the statistically non-significant increase of PR in tamoxifen treated tumours already described [12]. Using univariate analysis, no correlation between cath D RNA level and any of the classical prognostic factors of Table 1 was found.

Correlation between cath D RNA and protein in control breast carcinomas according to their ER status

Cathepsin D RNA levels detected by *in situ* hybridisation were quantified in epithelial tumoural cells (Figure 1). Expression in the stroma was generally weak and probably corresponded to macrophages [9]. As described previously [12], cath D RNA expression was mainly observed in epithelial tumour cells, and not in the stroma. Cathepsin D RNA expression was not homogenous among the epithelial cells of a single structure. Indeed, cells with low, medium or high expression could be found in the vicinity of each other. Cathepsin D RNA level

Table 1. Distribution of the clinico-biological parameters in control and tamoxifen patient populations according to their status and/or their values

Parameters	Control (n = 35)	TAM (n = 32)	P
Age (years)			
(Mean ± S.D.)	55 ± 15.4	68.7 ± 9.7	0.0001
Menopausal status			
Pre	13	2	
Post	22	30	0.003
Tumour stage			
T1	9	6	
T2	19	18	
T3–T4	7	8	0.75
Histopathological grade*			
I	1	2	
II	14	13	
III	10	9	0.82
Lymph node invasion			
–	17	17	
+	18	15	0.71
ER status			
–	12	10	
+	23	22	0.79
Mean ± S.D. (fmol/mg P)	58.5 ± 71.7	47.6 ± 51.8	0.48
PR status			
–	4	9	
+	31	23	0.08
Mean ± S.D. (fmol/mg P)	114.3 ± 181	189.2 ± 404	0.23

S.D., standard deviation; P, P value using the chi square test or the t-test; n, the number of patients in each group.
*Data missing for some patients.

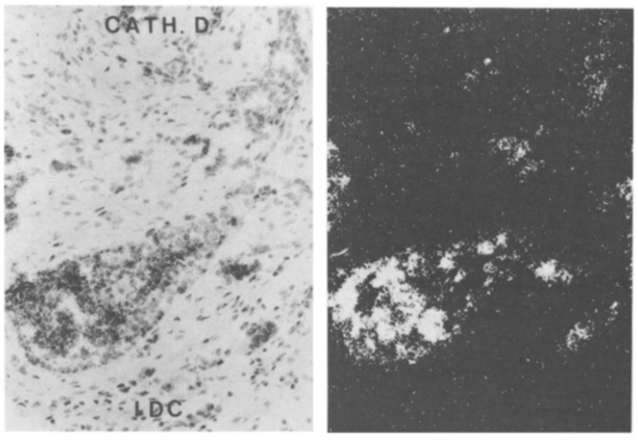

Figure 1. Photographs of bright and dark fields of cath D expression detected by RNA *in situ* hybridisation of a breast carcinoma from a patient treated with tamoxifen for 3 weeks prior to surgery. As described in Materials and Methods, the 35S alpha UTP labelled cath D probe was used to hybridise 5 μm thick frozen sections. RNA levels were revealed by autoradiography after 3 days of exposure of the hybridised section to the emulsion.

ranged from 1.1–11.5 grains/cell (G/C) (mean = 3.1 G/C, Figure 2; Table 2) and there was no significant difference between the means of RNA levels according to the ER status of the tumours (Figure 3b).

The levels of cytosolic total cath D protein ranged from 8 to 313 pmol/mg P (mean = 59 pmol/mg P) (Figure 2; Table 2) and

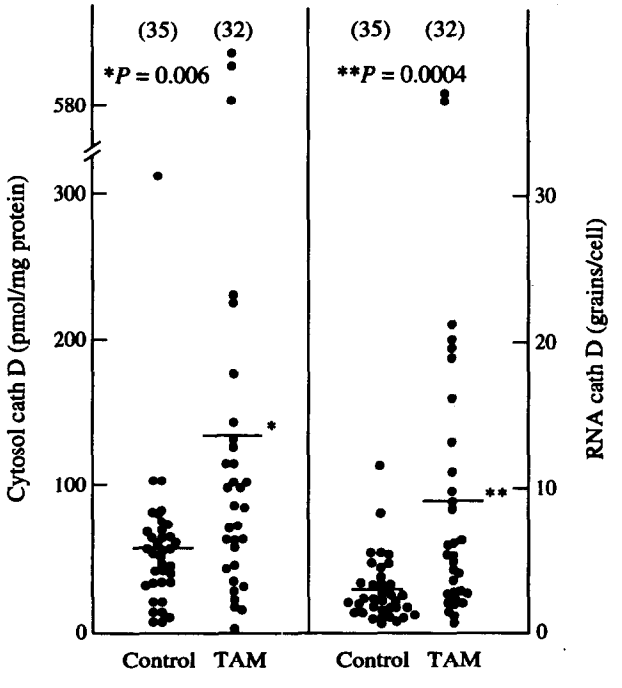

Figure 2. Cytosol cath D and RNA cath D values in the tamoxifen treated (TAM) and in the control groups. Cytosol cath D concentrations were obtained with the one-step double determinant solid-phase immunoenzymatic assay and *in situ* hybridisation of the RNA cath D was realised in frozen sections as described in the Materials and Methods. *P* values were obtained using the Wilcoxon-Mann Whitney non-parametric test. Numbers in brackets are the numbers of patients in each group.

Table 2. Range and mean value of RNA cath D, pro- and total cath D according to the treatment (control or tamoxifen treated groups)

	RNA (grains/cell)	Pro-cath D (pmol/mg P)	Total cath D (pmol/mg P)
Control			
Range	1.1–11.5	0.18–33.1	8–313
Mean	3.1	4.45	59
n	35	33	35
Tamoxifen			
Range	0.9–37	0.4–202	3–618
Mean	9.1	17.6	134
n	32	21	32
P (control/TAM)	0.0009	0.047	0.0144

P, *P* value using Student's *t*-test; *n*, the number of patients in each group.

those of its precursor (52K protein) ranged from 0.18 to 33.1 pmol/mg P (n = 33; mean = 4.45 pmol/mg P, Table 2). These results on total cath D and its precursor measurements were comparable with those obtained in our previous study [12]. There was no statistical association between the ER status and total cath D protein (Figure 3a) or its precursor.

As shown in Figure 4, there was a good correlation (r = 0.74) between cath D RNA and protein in the ER+ population, even when the high cath D sample was removed. However, we found no significant correlation between protein and RNA cath D levels in the ER− population (r = 0.16).

Effect of tamoxifen on cath D RNA and protein

Mean cath D RNA levels measured in tamoxifen treated tumours were higher than those of the control group (9.1 G/C versus 3.1 G/C; P = 0.0004, Figure 2; Table 2). As in the control population, there was, however, no statistical difference in mean RNA levels according to the ER status (Figure 3b). Tamoxifen increased cath D RNA expression in three tamoxifen treated ER+-tumours. In tamoxifen treated patients, the range of cath D concentration was wide (3–618 pmol/mg P), and the mean levels were statistically higher than those of the control group (mean: 134 versus 59 pmol/mg P; P = 0.006) confirming results of a previous study ([2], Figure 2). The increase in cytosolic cath D by tamoxifen was limited to the RE+ population (P = 0.005; Figure 3a). The 52K precursor concentration was statistically higher (P = 0.05) in the treated (n = 21; mean: 17.6 pmol/mg P; range: 0.4–202) than in the control group (mean: 4.45; range: 0.18–33.1; Table 2). Overall, these results indicate that in ER+ breast cancer, tamoxifen has an agonistic effect on cath D expression at both RNA and protein levels. Figure 4 shows the correlation between cytosolic cath D protein and cath D RNA in the ER+ treated population with, however, a lower correlation coefficient than in the control group (r = 0.47 versus r = 0.74). The ratio of the mean levels of each of the various cath D measurements in the tamoxifen group to the control group showed that cath D RNA levels in the tamoxifen population were twice those of the control group, regardless of the ER status (Table 3). However, the number of ER− patients was too low to draw definite conclusions concerning this category. The cytosolic cath D and pro-cath D were increased by tamoxifen in the total and the ER+ populations but not in the ER− group.

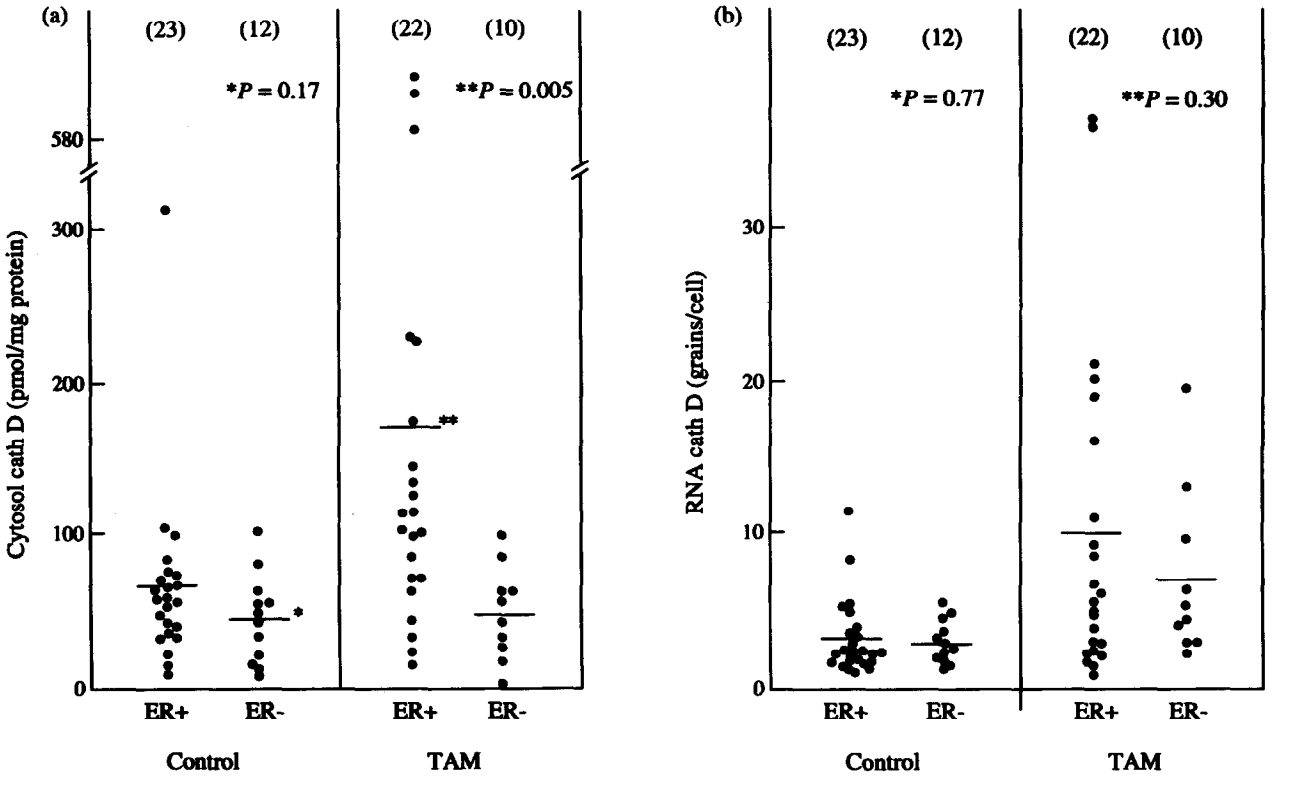

Figure 3. Cytosol (a) and RNA (b) cath D in control and tamoxifen treated (TAM) groups according to the ER status. Numbers in brackets are the numbers of patients in each group.

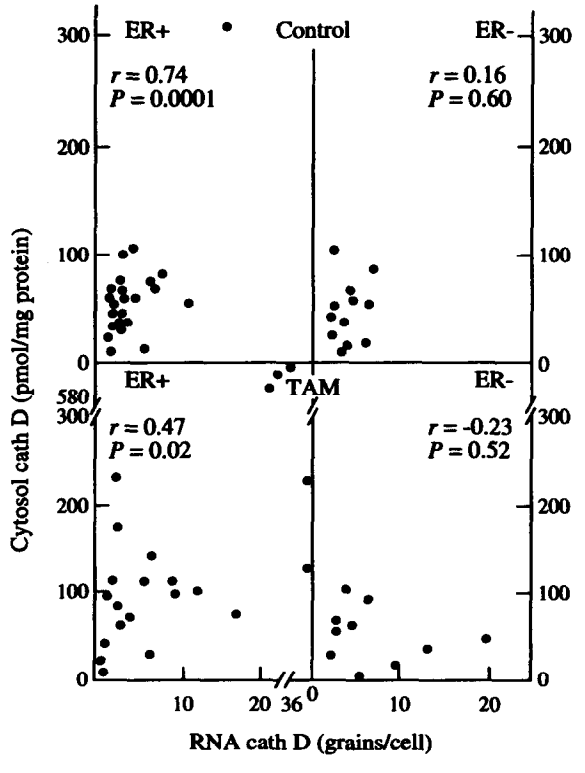

Figure 4. Correlation of cytosol cath D with the RNA cath D according to the ER status in the control and treated groups. *r* is the coefficient of correlation. The *P* value was determined by variance analysis.

DISCUSSION

Using semi-quantitative *in situ* hybridisation of frozen tissue sections, we meaured cath D RNA in a series of breast carcinomas from patients treated with tamoxifen for 3 weeks prior to surgery. Since a previous study showed an increase of cytosolic cath D in ER+ breast cancers after three weeks of pretreatment by tamoxifen, one hypothesis was that the protein level could still be high as a consequence of the partial agonist effect of the tamoxifen, but that the RNA might already be decreased in a larger part of the ER+ population. The present analysis showed that the RNA level measured in tumoural cells was correlated to the cytosolic protein levels of ER+ breast cancers in control and tamoxifen treated groups, although it should be mentioned that different parts of the tumour were examined. This suggests that

Table 3. Ratio of the mean value of each cath D parameter in the tamoxifen treated to that in the control group in total, ER+ and ER− populations

	Ratio TAM/control		
	Tissue	Cytosol	
Population	RNA	Total cath D	Precursor
Total	2.37 (32/35)*	2.59 (32/35)	3.97 (23/33)
ER+	2.46 (22/23)	2.93 (22/23)	4.43 (16/21)
ER−	2.12 (10/12)	1.21 (10/12)	1.41 (7/12)

*Number of patients in tamoxifen treated group over the number of patients in the control group.

the increase of cytosolic cath D with tamoxifen was not due to a higher stability of the protein compared with the RNA, but rather to a true agonist effect of tamoxifen on the cath D gene expression, as for the PR gene [13], and not the result of a flare-up, which is transitory and appears in only 5% of the ER+ breast cancers treated by hormonal therapy. This is in agreement with the partial oestrogenic activity of the 4-hydroxytamoxifen, *in vitro*, on the regulation of cath D RNA level in MCF7 cells [14, 15]. Therefore, the prognostic significance of the high cath D level in breast cancer would be different depending on whether patients have been pretreated by tamoxifen or not. While the high cath D level is a factor of bad prognostic significance in non-treated patients, following tamoxifen therapy, it would be a marker of responsiveness to antioestrogen therapy. Increased cath D cellular levels have previously been described during apoptosis induction in rat prostate of castrated rats [16]. Similarly, in breast cancer, tamoxifen might contribute to the apoptotic process by inducing proteases such as cath D. Another possible explanation for increased cath D accumulation is that tamoxifen could enhance the recruitment of macrophages in the tumour which also expresses cath D [17]. However, preliminary observations of paraffin embedded sections of control and treated breast cancers showed no difference in the amount of macrophages between both groups. Histological analysis of the stroma reaction (fibrosis, lymphocytes, macrophages) by a pathologist did not show any obvious difference between control and tamoxifen pretreated groups. Moreover, macrophages are mostly located in the stroma surrounding the tumour, while the increased cath D level was observed mainly within the tumour area. This strongly suggests that the increase of cytosolic cath D level by tamoxifen is mainly the consequence of enhanced cath D by gene expression in tumoural cells.

In conclusion, we have confirmed by *in situ* RNA hybridisation that tamoxifen acts *in vivo* as an oestrogen agonist for cath D induction in ER+ breast cancer tissue. Therefore, the increase in cath D cytosolic protein observed after 3 weeks of treatment was unlikely to be due to a biological flare-up mechanism, since it is also observed at the RNA level. The significance of the increased RNA level in some ER− breast cancers is not clear since it was not observed in the cytosolic study. In our previous study, some of the breast cancer tissue contained a very high proportion of pro-cath D after tamoxifen treatment. Whether or not pro-cath D maturation and intracellular routing is altered *in vivo* by tamoxifen should be investigated in future studies.

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