Immunoenzymatic Assay of M, 52,000 Cathepsin D in 182 Breast Cancer Cytosols: Low Correlation with Other Prognostic Parameters

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ABSTRACT

The M, 52,000 cathepsin-D-like protease induced by estrogens in MCF7 human breast cells was assessed in 182 primary breast cancer cytosols prepared for receptor assays from pre- and post-menopausal patients. Using two solid-phase sandwich immunoenzymatic assays, we quantified the total M, 52,000 cathepsin D (52K-cath-D) (the M, 52,000 precursor protein and its M, 48,000 and 34,000 processed forms) and the M, 52,000 precursor alone. The value of total 52K-cath-D varied between 3 and 165 pmol/mg protein and the proportion of the precursor varied from 0 to 28% of total 52K-cath-D. There was no correlation between the concentrations of 52K-cath-D and estrogen receptor, but the estrogen receptor status (> or <10 fmol/mg protein) was correlated to the 52K-cath-D status (> or <15 pmol/mg protein) according to the χ² test (P < 0.001). The correlation with progesterone receptor concentrations and status was low (0.43) and absent, respectively. There was no correlation with Scarff and Bloom stages, tumor size, or patient’s age. The correlation with progesterone receptor concentrations and status was low (r = 0.43) and absent, respectively. There was no correlation with progesterone receptor status (> or <10 fmol/mg protein) was correlated to the 52K-cath-D (12). The total 52K-cath-D (34K-, 48K-, and 52K-) and the M, 52,000 precursor (pro-cath-D) were quantified separately, using two distinct immunoenzymatic assays.

In the present study, the concentrations of 52K-cath-D were assayed in 182 human breast cancer cytosols and correlated with other clinical and biological parameters.

INTRODUCTION

The choice of breast cancer treatment is aided by several prognostic parameters, including the levels of the cytosolic estrogen and progesterone receptors in tumors collected at surgery (1, 2). Monoclonal antibodies directed against other proteins of putative biological interest in breast cancer can be used to complement the information provided by receptor assays (3). Since 1979, our laboratory has been engaged in the study of a M, 52,000 protein which is synthesized and secreted by breast cancer cells. The protein is stimulated by estrogen and secreted by hormone-dependent breast cancer cell lines (4) and primary culture of pleural effusion (5). It is also produced constitutively in hormone-independent breast cancer cell lines (6).

We have raised monoclonal antibodies against this protein (7), and identified it as the precursor of a cathepsin-D lysosomal protease bearing mannose 6-phosphate signals on its N-glycosylated chains (8, 9). Two in vitro biological activities of the protein have been detected, a mitogenic activity on estrogen-depleted MCF7 cells (10), and an acidic proteolytic activity on various substrates, including proteoglycans and basement membrane (8).

The first clinical studies using semiquantitative immunohistochemistry indicated that the protein was highly concentrated in proliferative ductal or cystic mastopathies (11) and that in breast cancer it was not correlated with cytosolic estrogen and progesterone receptor concentrations (6).

We therefore decided to assay the 52K-cath-D in the same breast cancer cytosol used for receptor assays. While only the M, 52,000 precursor form is secreted, the cytosolic extract of breast cancer cells contains a mixture of the M, 52,000 precursor and the M, 48,000 and M, 34,000 processed mature enzyme (12). The total 52K-cath-D (34K-, 48K-, and 52K-) and the M, 52,000 precursor (pro-cath-D) were quantified separately, using two distinct immunoenzymatic assays.

In the present study, the concentrations of 52K-cath-D were assayed in 182 human breast cancer cytosols and correlated with other clinical and biological parameters.

MATERIALS AND METHODS

Patients. In 219 patients, mammary carcinomas were collected at surgery from February 1985 to June 1986. Pre- and postmenopausal patients had no clinical metastasis and had received no radiotherapy before surgery. They were mainly from the Cancer Research Center (Centre Paul Lamarque) and several hospitals from the south of France. Following exclusion of samples too small for assays of both receptors and 52K-cath-D, and samples that contained no cancer cells after histological examination, 182 primary breast cancer tumors were included in this study.

Pathological Grading. Each tumor was sent to a pathologist and part of it was immediately frozen in liquid nitrogen. Tumor stages were defined according to the International Union against Cancer tumor (T)-node (N)-metastasis (M) classification of breast cancer (13). The number of axillary nodes invaded was determined by histological examination. Carcinomas were classified according to the histological grading of Bloom and Richardson (14).

Preparation of Cytosols. Cytosols were prepared from frozen tumors in 10 mM Tris buffer, pH 7.4, containing 1.5 mM EDTA, 10 mM monothioglycerol, and 10 mM sodium molybdate (15).

Steroid Receptor Assay. The RE concentration was assayed by the dextran-coated charcoal method in the first 128 tumors by using two saturating concentrations (5 and 10 nM) of [3H] Estradiol (40 Ci/mmol) as described (15). In the last 54 tumors, RE were determined by the Abbott enzyme immunoassay, using a monoclonal antibody to the MCF7 human breast cancer estrogen receptor (16). We confirmed a high correlation between the two methods in 20 samples.

RP concentration was assayed by the dextran-coated charcoal technique (15) by using two saturating concentrations (10 and 20 nM) of [3H] ORG-2058 (40 Ci/mmol) from Amersham, Versailles, France, and...
100 nM cortisol after 2 h of incubation at 4°C. Nonspecific binding was assayed in a parallel set of incubations containing 1 μM of nonradioactive ORG 2058 (Amersham). Protein concentration was assayed by the Lowry technique.

Immunoenzymatic Assay of \( M, 52,000 \) Protein (Total and Proenzyme). Using two double determinant solid-phase assays total 52K-cath-D concentration* and 52K-pro-cath-D concentration* were assayed in the same cytosol. Microtiter plates were coated with the monoclonal antibody D7E3, which recognizes the three cellular forms of 52K-cath-D (total 52K-cath-D), or with the monoclonal antibody M2E8, selectively directed against the precursor form (52K-pro-cath-D). After saturation of the remaining free adsorption sites, plates were washed three times with buffer before cytosol addition. For the assay of total 52K-cath-D, cytosol and a second antibody (M1G8 which recognizes the 3 forms) conjugated with alkaline phosphatase were incubated simultaneously for 16 h at 4°C in buffer containing 1% bovine serum albumin and 0.05% Tween 20. For 52K-pro-cath-D, the cytosol was incubated alone in Dulbecco's modification of Eagle's medium containing 10% fetal calf serum for 16 h at 4°C. After a wash with buffer, the second antibody (M1G8) conjugated with alkaline phosphatase was added and left for 2 h at room temperature. The plates were then washed 5 times with 0.9% NaCl solution and 0.05% Tween 20 (pH 8.6) in the case of total 52K-cath-D, and with imidazole 0.1 M buffer for pro-cath-D, and incubated in a 0.1 M diethanolamine (pH 9.8) chromogen solution containing 1 mg/ml p-nitrophenyl phosphate for 1 h (total 52K-cath-D) or 2 h (52K-pro-cath-D) at room temperature. Plates were read at 405 nm by using a multispectral spectrophotometer (Titertek, MC; Flow Laboratories S.A., Puteaux, France) coupled to an Apple II microcomputer.

The cytosolic concentration of 52K-cath-D was obtained from the absorbances of four duplicate dilutions (1/10 to 1/80) by comparison with a linear standard curve obtained in each microtiter plate from five duplicate dilutions of a breast cancer cytosol pool (aliquoted and stored at -70°C). The intraassay and interassay reproducibilities were <10 and 15%, respectively. Beyond these limits, the cytosol assay was repeated or the results were not included.

The concentration of total 52K-cath-D in standard cytosol (90 pmol/ml) has been determined by reference to the concentration of pure 52K-pro-cath-D evaluated by silver staining. This cytosol was used to convert the measured absorbance into 52K-cath-D concentrations expressed as pmolar equivalents of 52K-cath-D, since we verified that the antibody reactivity was similar with the three forms (52K-, 48K-, 34K-) (not shown). The sensitivity of the assay was 5 fmol (52K-pro-cath-D) or 30 fmol (total 52K-cath-D). The standard curves were linear from 0 to 80 fmol/well (52K-pro-cath-D) and to 600 fmol/well (total 52K-cath-D). Total 52K-cath-D was found to be stable in cytosol kept at -70°C for 1 year, or exposed to 3 cycles of freeze-thaw, or kept at 7°C for 4 days prior to analysis. Details and validation of the two assays are described elsewhere.45

Statistical Methods. Statistical differences within the population were determined by the Kruskall-Wallis or Mann-Whitney nonparametric tests. The \( \chi^2 \) test was used for qualitative parameters. Linear regression was calculated by Pearson's least squares method. Pearson's correlation coefficients were analyzed by Student's \( t \) test.

RESULTS

Characteristics of the Population. In 182 patients, both total 52K-cath-D and the receptors for estrogen (RE) and progesterone (RP) were assayed. The 52K-pro-cath-D level was measured in only 136 patients (78%). Clinical and histological parameters could be obtained in 73% of the population: 55.5% of the breast cancer tumors were at stage T2 of the T-N-M classification and less than 7% were at T0 or T4; 43% of the tumoral tissues were at stage 3 of the Scarff and Bloom classification and 39% at stage 2; in 52% of the patients, axillary lymph nodes were histologically invaded (N+). The age of the patients was 56.1 ± 13.5 years (SD). The mean concentration of total 52K-cath-D and pro-cath-D in tumor cytosol was, respectively, 24.4 ± 22.2 and 3.6 ± 5.9 pmol/mg protein. The distribution was in favor of tumors with low concentrations (Fig. 1). The concentration of 52K-pro-cath-D was correlated with that of total 52K-cath-D (\( r = 0.79 \)), but the proportion of the \( M, 52,000 \) precursor compared to the total fluctuated from 0 to 28%, depending on the tumor.

\( M, 52,000 \) Protein and Lymph Node Invasion. The concentrations of total 52K-cath-D were plotted in four groups according to the presence or absence of histologically invaded axillary lymph nodes and of RE (Fig. 2). The tumors from lymph node-positive patients (N+) contained the highest total 52K-cath-D concentrations and a higher mean concentration than those from lymph node-negative patients (N-), but the difference was not significant (\( P = 0.88 \)) (Fig. 2A). In the N+ group, however, the total 52K-cath-D concentration was significantly higher (\( P = 0.05 \)) in the RE-positive subgroup than in the RE-negative subgroup (Fig. 2B). The 52K-pro-cath-D concentration was not correlated to lymph node invasion, although its mean value was higher in the N+ group (\( n = 36 \)) than in the N- group (\( n = 182 \)).

![Fig. 1. Distribution of primary breast cancer tumors as a function of the 52K-cath-D concentration. Total 52K-cath-D (a) and 52K,000 precursor (b) concentrations were measured in breast cancer cytosol by double-determinant immunoenzymatic assay. a, total 52K-cath-D concentration: the class interval is 3 pmol/mg protein. The distribution of patients corresponds to a composite curve close to a Poisson distribution, with most of the tumors containing less than 42 pmol/mg protein. b, 52K-pro-cath-D concentration: the class interval is 0.2 pmol/mg protein. n, number of patients assayed.](https://example.com/fig1.png)
52K-Cath-D and Steroid Receptor Concentrations. The 182 cytosols showed no significant correlation between the concentrations of total 52K-cath-D and RE (r = 0.20) (not shown). There was a low correlation between total 52K-cath-D and RP (r = 0.43, P < 0.01) similar to that found between RE and RP (r = 0.42, P < 0.01). The correlation of receptors with 52K-pro-cath-D was also low. The mean level of total 52K-cath-D is not significantly higher in RE-positive tumors than in RE-negative tumors (29.4 ± 25.2 versus 16.5 ± 12.2 pmol/mg proteins). We therefore conclude that the concentrations of 52K-cath-D in breast cancer cytosol cannot help to predict hormone responsiveness. Table 1 shows a qualitative correlation between receptors and 52K-cath-D status. Three groups (low, moderate, and high 52K level) were defined using the cutoff level of 15 pmol/mg protein corresponding to a lower limit of positive immunostaining of breast cancer tissue found in previous studies (6) and a cutoff level of 42 pmol/mg protein that discriminates the patients running a higher risk of lymph node invasion. The only correlation was found between RE and 52K-cath-D concentration when taking 10 fmol and 15 pmol/mg protein.

Table 1 Distribution of patients according to total 52K-cath-D and other parameters

<table>
<thead>
<tr>
<th>Node invasiveness</th>
<th>Low 52K (&lt;15 pmol)</th>
<th>Moderate 52K (15-42 pmol)</th>
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* n, number of tumors studied; NS, not significant.
* Using the cutoff level of 42 pmol/mg protein.
* Using a cutoff level of 42 pmol/mg protein.
mg protein as a limit for RE and 52K positivity.

Relationship with Other Prognostic Parameters. There was no relationship between the concentration of total 52K-cath-D or 52K-pro-cath-D and the Bloom and Richardson stages (Fig. 4). By contrast, the RE and RP concentrations decreased progressively from grades 1 to 3. This is in agreement with the finding (2) that RE are more concentrated in well-differentiated mammary cancers, but suggests that the concentration of 52K-cath-D is not related to tumor differentiation.

The 52K-cath-D concentration (total and pro-) are not related to tumor size. There was a slight but insignificant decrease in concentration from T1 (19.2 ± 16.3) to T2 (15.8 ± 13.3) and T3 (14.8 ± 11) tumors. The mean concentrations of total 52K-cath-D and 52K-pro-cath-D did not differ between patients older or younger than 50. The RE concentration, however, was 2-fold higher in postmenopausal patients than in premenopausal patients as described (2).

DISCUSSION

This is the first report on the assay of M, 52,000 cathepsin D in the cytosol of primary breast cancers. The total 52K-cath-D assay gives an overall estimate in pmol equivalents of M, 52,000 precursor and its M, 48,000 and 34,000 processed products. As far as the 52K-pro-cath-D is concerned, tumor cytosol probably contains a mixture of the cellular precursor and the M, 52,000 protein secreted into the interstitial fluid. At present, it is not possible to discriminate between these two compartments.

Even though biosynthesis of 52K-pro-cath-D is regulated by estrogens in estrogen-responsive breast cancer cell lines (4, 12) no overall correlation was found between the concentration of total 52K-cath-D, or its precursor, and that of RE sites in whole tumors. These results indicate that hormone-independent tumors constitutively produce the M, 52,000 protein, which is consistent with other studies. First, immunoperoxidase staining of 52K-cath-D in breast cancer (6) indicated the same lack of correlation with RE and RP concentrations. Second, quantification of 52K-cath-D in RE-negative breast cancer cell lines (MDA-MB231 and BT20) showed a constitutive production of both cellular and secreted cathepsin D, which was even higher than in MCF7 cells not treated by estrogens (6). Nevertheless, we found a correlation with RE status (but not with RP status) when the M, 52,000 protein status (high or low) was considered, rather than its concentration. The absence of correlation with Bloom and Richardson grading suggested that 52K-cath-D does not characterize well-differentiated breast cancers, in contrast with the RE.

When the total population was considered, the mean concentration of total 52K-cath-D in the cytosol of primary tumors was higher in tumors from N+ patients than in those from N− patients, but the difference was not significant. However, the tumors with the highest 52K-cath-D level (only 12% of the population) were correlated with lymph node invasiveness. This is in agreement with the mitogenic (10) and proteolytic (9) activities of this protease, which can degrade extracellular matrix. Though proteases probably play a role in metastatic processes (for review see Ref. 17), previous clinical studies have generally failed to show any relationship between protease assay and the prognosis of breast cancer (18, 19).

Immunoassay of the M, 52,000 protein is more sensitive than detection by immunoperoxidase staining, since all tumors and normal mammary glands contain assayable levels of total M, 52,000 protein even though they are negative by immunostaining (11). This is in agreement with the ubiquitous distribution of cathepsin D (20). The 40% of breast cancers that were negative according to immunohistochemistry (6) corresponds in this study to concentrations below 15 pmol/mg protein, which can therefore also be considered to be the cutoff level between positive and negative staining of frozen sections in the earlier study (7). The cytosol assay does have several limitations compared to immunohistochemistry since 52K-cath-D distribution is often heterogeneous in breast cancer. However, it can be applied to the same extract used for RE and RP determinations and the content of a great number of cells can be assayed. While the cytosolic 52K-cath-D assayed here with monoclonal antibodies does not seem to be an additional marker of estrogen responsiveness, it might be used to detect invasive tumors. However, its prognostic value needs to be ascertained by clinical follow-up of the patients and by retrospective studies initiated with other clinical centers.

ACKNOWLEDGMENTS

We are grateful to Professor Laffargues and Drs. Domergue, Lavie, and Camo for their participation in this study, and to Michèle Egéa for secretarial assistance.

* D. Derocq, unpublished experiments.
* P. Briozzo et al., manuscript submitted for publication.
* F. Capony, unpublished data.
REFERENCES


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