A Two-Site Immunoenzymometric Assay of 52-kDa Pro-Cathepsin D, and Its Use in Human Breast Diseases

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After isolating monoclonal antibodies specific for the 52-kDa precursor of cathepsin D (cath-D), which is secreted in excess in both hormone-dependent and hormone-independent breast cancer, we developed a two-step double-determinant immunoenzymometric assay that is specific for this pro-enzyme. The assay combines the use of a monoclonal antibody specific for the precursor and bound to microtiter plates, and a second antibody directed against a smaller processed form of the mature enzyme, coupled to alkaline phosphatase. The specificity of the assay relies on separate and sequential additions of the antigen and the conjugated second antibody. It allows rapid measurement of the analyte in plasma and cytosols of normal and neoplastic mammary tissues, with a detection limit of 5 fmol and a maximal inter-assay coefficient of variation of 9%. This assay is particularly useful for tissue cytosol samples where the pro-enzyme form co-exists with large quantities of the mature processed forms of the enzyme. Comparative assays of 52-kDa pro-cath-D and total cath-D in cytosols of breast cancers and benign mastopathies indicate that the present assay better discriminates between benign and cancerous mammary tumors.

Additional Keyphrase: distinguishing benign and cancerous tumors

In hormone-responsive breast-cancer cells in vitro, estrogens specifically increase the synthesis and secretion of several proteins (1-4) and later stimulate cell proliferation (4, 5). Among these secreted factors potentially acting as paracrine or autocrine growth stimulants (6), we have focused our studies on a 52-kDa glycoprotein (7). Purification of this protein to homogeneity with monoclonal antibodies (7, 8) and complete sequencing of its corresponding cDNA (9) have allowed us to identify it as the lysosomal aspartyl protease cathepsin D (cath-D, EC 3.4.23.5).

The 52-kDa cath-D secreted by breast-cancer cells displays a mitogenic effect in vitro on estrogen-deprived cells (10) and a proteolytic effect on extracellular matrix after its autoactivation at acidic pH (11). This suggests that it could participate actively in controlling the growth and spread of breast cancer. Clinical studies involving both immunohistochemical and immunoenzymatic assays have shown that the concentration of total cath-D (52-kDa pro-enzyme plus the 48- and 34-kDa/14-kDa "mature" forms) is related to the proliferation of mammary ducts (12) and to shorter relapse-free survival of breast-cancer patients (13, 14, and S. Thorpe, submitted for publication).

The selective increase in the synthesis and secretion of the 52-kDa cath-D precursor in breast-cancer cells compared with normal breast cells in primary culture (F. Capony, in preparation) led us to isolate monoclonal antibodies specific for this pro-enzyme form (15). Their strict specificity for 52-kDa pro-cath-D allowed us to develop an immunoenzymometric assay (IEMA) that is specific for the pro-enzyme and to assess its discriminative clinical relevance compared with evaluation of total cath-D.

Materials and Methods

Chemical Reagents

Bovine serum albumin and diethanolamine were obtained from Sigma Chemical Co., St. Louis, MO. Gelatin and Tween 20 surfactant were from Merck, Darmstadt, F.R.G.; alkaline phosphatase (EC 3.1.3.1) and p-nitrophenyl phosphate from Boehringer Mannheim; and saccharose from Carlo Erba, Milan, Italy. Other chemicals obtained from Prolabo, Paris, France, were analytical grade. The 96-well microtiter plates (NUNC Microwell 96F) were from Prolabo, Strasbourg, France. Dulbecco’s modified Eagle’s medium and fetal-calf serum were obtained from Flow Laboratories (Flow SA, Puteaux, France).

Immunological Reagents

The monoclonal antibodies M2E8*, D9H8*, and M1G8 were purified from ascitic fluids as previously described (15, 16), dialyzed against Dulbecco’s phosphate-buffered saline, and stored at –80 °C. The antibody concentration was assayed in each dialysate by the Bradford technique (17) with use of the Bio-Rad protein assay kit and γ-globulins as the standards (Bio-Rad, Richmond, CA). The nonspecific "scavenger" monoclonal antibody 8A6 was provided by Sanofi Research. The monoclonal antibody M1G8 tracer was conjugated with alkaline phosphatase by use of glutaraldehyde (18). This conjugate is stable in glycerol solution for five months when stored at –20 °C.

Breast-Cancer Cells and Tumors

The MCF7 breast-cancer cell line (19) obtained from the Michigan Cancer Foundation was cultured as previously described (10). Tissues were obtained by mastectomy of non-irradiated primary breast cancers collected from the Cancer Center of Montpellier (Centre Paul Lamarque) and from benign mastopathies from the Department of Gynecology and Obstetrics, Medical Center of Montpellier (Maternité).

Determination of Cytosolic Concentration

Two standard preparations of antigens were used as in ref. 16. We calculated the concentration of 52-kDa pro-cath-D protein in the test cytosol (20) by comparing values obtained from duplicates of the different dilutions of this cytosol with the standard cytosol range present on the same microtiter plate and processed according to a linear function. Reproducibility was better at higher values in the standard range. We therefore chose the two dilutions of test cytosol whose absorbances were the closest to (without exceeding) the absorbance measured in a standard amount.

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of cytosol, 80 fmol per well. The concentration determined by using the dilution factor was then converted into femtomoles per milligram of cytosolic proteins to standardize the values between mammary cancers.

Results

Final Assay Protocol

We took advantage of the fact that two monoclonal antibodies (M2E8* and D9H8*) and the first series of antibodies (7, 15) recognized different cathepsin D domains, and we set up an IEMA specific for the 52-kDa cathe-D pro-enzyme. A sandwich-type assay involving a monoclonal antibody to the pro-enzyme (N-terminal; M2E8* or D9H8*) and a monoclonal antibody (M1G8) to the mature (C-terminal) form ensures highly specific recognition of the entire 52-kDa form.

The first step in developing this assay was to select from the two available antibodies the specific monoclonal antibody for preparing the sensitized solid phase. M2E8* coated on microtiter plates was assayed with a constant concentration of antigen (secreted 52-kDa protein) and conjugate antibody (M1G8-PA). It gave a constant signal over a wide range of concentrations, associated with a low background signal in the absence of antigen. It was therefore more suitable than D9H8* for coating (data not shown) and was used in the following experiments at a concentration of 30 mg/L.

Figure 1 summarizes the final assay protocol. The assay is based on the specific adsorption of pro-cathepsin D via the adsorbed first antibody (M2E8*), followed by protein labeling with the second antibody (M1G8) conjugated to alkaline phosphatase. This assay was designed for clinical determination of 52-kDa pro-cathepsin D in breast cancer and benign disease cytosols in which the pro-enzyme form coexists with the 48-kDa and 34-kDa/14-kDa forms also recognized by the conjugated M1G8 antibody. It was therefore necessary to eliminate the 48-kDa and 34-kDa forms that had not been retained on the coated M2E8* before adding the second antibody. The washes and the incubation

conditions with imidazole buffer (0.1 mol/L), pH 7.4, and Dulbecco’s modified Eagle’s medium with fetal-calf serum (100 mL/L) are particularly crucial for avoiding desorption of the antigen from the first antibody, which would occur with phosphate buffer.

Validation of the Assay

We tested the linearity and sensitivity of the method, using different dilutions of a culture medium in which the 52-kDa cathe-D concentration had previously been estimated by silver staining. The method gave a significant signal at 5 fmol of antigen per well, and the curve was linear up to 80 fmol per well (Figure 2). Moreover, the sensitivity and linearity of the method were equivalent over the same range of concentrations of cytosolic and secreted 52-kDa protein after overloading cytosol dilutions with two constant concentrations of secreted 52-kDa protein (data not shown). These results indicate the immunological identity of the secreted and cytosolic forms of 52-kDa cathe-D, as detected by the two antibodies used in this assay. Assay values for a control cytosol (Table 1) routinely included in each test plate showed that inter- and intra-assay reproducibility was good (16).

Comparative Evaluation of 52-kDa Pro-Cath-D and Total 52-kDa-Cath-D in Breast-Cancer and Benign-Mastopathies Cytosols

We used this assay to measure 52-kDa pro-cath-D in cytosols of both benign mastopathies and breast cancer, and to compare its evaluation in both tissues with estimates of total 52-kDa cathe-D.

In an initial series of 30 different breast-cancer cytosols (13) in which steroid receptors were separately assayed, we measured the concentrations of total 52-kDa cathe-D and 52-kDa pro-cath-D. Figure 3 shows that there was a good correlation (r = 0.82) between the two. However, the percentage of the total cathepsin D that was precursor varied from 0% to 20% according to the sample.

Secondly, we assayed 52-kDa pro-cath-D in the cytosols of a series of 20 benign mastopathies. This series was com-

![Graph](image)

Fig. 2. Sensitivity and linearity of the two-step double-determinant IEMA of the secreted 52-kDa pro-cath-D was performed as in Figure 1 on a medium conditioned by MCF7 breast-cancer cells as a source of antigen. The 52-kDa cathe-D concentration in this secondary standard medium was evaluated by immunoradiometric assay (7) based on a primary standard, as defined in Materials and Methods.

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Table 1. IEMA of 52-kDa Pro-Cath-D: Inter- and Intra-Assay Reproducibility

<table>
<thead>
<tr>
<th>Assay no.</th>
<th>Mean and SD of 5 plates, nmol/L</th>
<th>Intra-assay CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.46 (0.23)</td>
<td>6.5</td>
</tr>
<tr>
<td>2</td>
<td>3.58 (0.60)</td>
<td>16.7</td>
</tr>
<tr>
<td>3</td>
<td>3.57 (0.18)</td>
<td>4.8</td>
</tr>
<tr>
<td>4</td>
<td>3.28 (0.19)</td>
<td>5.8</td>
</tr>
<tr>
<td>5</td>
<td>3.62 (0.16)</td>
<td>4.5</td>
</tr>
<tr>
<td>6</td>
<td>3.48 (0.12)</td>
<td>3.5</td>
</tr>
<tr>
<td>7</td>
<td>3.69 (0.21)</td>
<td>5.8</td>
</tr>
<tr>
<td>Mean</td>
<td>3.54 (0.50)</td>
<td></td>
</tr>
</tbody>
</table>

Inter-assay CV, 8.6%

To estimate the intra- and inter-assay reproducibility of this assay we assayed a control cytosol in duplicate at four successive dilutions in five different plates per assay. Results of seven different such experiments are represented. In each plate, the concentration of 52-kDa pro-cath-D (nmol/L) was calculated from the means of the concentrations as measured with dilutions that gave linear signals.

pared with a series of 25 breast cancers (Figure 4). Although the assay of total 52-kDa cath-D (Figure 4a) did not significantly distinguish between benign and cancer tissues (P = 0.07), the assay of 52-kDa pro-cath-D (Figure 4b) allows a significant (P = 0.02) discrimination between benign and cancerous disease of the breast. An increase in the ratio of 52-kDa pro-cath to total cath-D was significantly associated (P = 0.009) with breast-cancer cytosols, thus suggesting that a higher proportion of pro-enzyme in a cytosol might be an index of cell transformation.

Discussion

The isolation of monoclonal antibodies specific for secreted 52-kDa pro-cath-D (15) allowed us to develop a rapid, sensitive, two-step IEMA of this pro-enzyme form on the basis of a previously described assay of total 52-kDa cath-D (16). By combining antibody to the pro-enzyme form with antibody to the mature enzyme (conjugate), we could assay pro-cath-D in addition to total cath-D in clinical trials. However, mammary tumor cytosols contain both pro-enzyme and mature forms, so we had to eliminate non-reacting mature forms by washing in imidazole buffer before adding the conjugate.

The assay is sensitive (detection limit, 5 fmol) and reproducible (inter-assay CV, 8.6%). It allows measurement of 52-kDa pro-enzyme, not only in plasma or culture medium, where pro-cath-D is the only form, but more importantly in cytosol and cell extract, where the pro-enzyme concentration is only a small proportion of the total cath-D concentration.

The rationale for developing a new assay was based on the observation that cancerous mammary cells show a greater production and secretion of the pro-enzyme form than do normal mammary cells (F. Capony et al., in preparation), which led us to propose that this enzyme, which has both a mitogenic (10) and a proteolytic (11) activity, is involved in or associated with the growth and invasion processes of mammary cancer.

The development of this new IEMA should prove to be clinically worthwhile, because our evaluation of the pro-enzyme in the mastopathies and breast-cancer cytosols of the present study showed that the mean concentration of 52-kDa pro-cath-D and its ratio to total cath-D can be used to distinguish benign and cancer tissue more significantly than is the case for total cath-D assay. However, the limited series of benign and cancer cytosols thus far assayed, although significantly different according to the Mann–Whitney test, do not allow us to select a reliable cutoff value that would permit evaluation of the sensitivity, specificity, and positive predictive values of such assays. The 52-kDa analyte concentration is also more associated with hormone dependency than is total cath-D, because it was found to be specifically increased in primary breast cancers after presurgical treatment with antiestrogen (T. Maudelonde et al., in press). This selective IEMA will allow evaluation of whether such an increase in pro-enzyme form is restricted to breast diseases or is also significantly increased in other cancers such as melanoma and prostate or endometrial adenocarcinoma.

The present IEMA might also be useful for fundamental cellular studies, because it allows one to evaluate the concentration of the pro-enzyme form in all tissues. It should
therefore be possible to estimate accumulation of the pro-
enzyme or a defect in its processing and targeting to
lysosomes within altered cancer cells.

In conclusion: this new immunoenzymometric assay pro-
vides an additional tool for use in fundamental and clinical
studies of mammary and other tumors.

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