Western Blotting and Enzymatic Activity Analysis of Cathepsin D in Breast Tissue and Sera of Patients with Breast Cancer and Benign Breast Disease and of Normal Controls

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ABSTRACT

Increased total antigen amounts of cathepsin D in breast tissue have been reported to be associated with increased disease recurrence, more frequent metastasis, and increased mortality in breast cancer patients. In the present study, Western blotting analysis has been used for the first time to determine the relative amounts of precursor and processed forms of cathepsin D in sera and breast tissue of patients with breast cancer, benign breast disease, and normal controls. Sera gave similar blots for of cathepsin D found in sera and an additional Mr 31,000 form which was found in significantly increased (P < 0.001) relative amounts in breast tissue from 43 breast cancer patients [24 ± 12% (SD)] when compared to 51 benign breast disease patients (13 ± 9.9%) and 23 normal controls (1.8 ± 4.4%). Preliminary analysis of subgroups of benign breast disease patients suggested no significant difference (P = 0.61) in relative amounts of the Mr, 31,000 form of cathepsin D between proliferative-type and non-proliferative-type fibrocystic breast disease.

A cathepsin D assay has been optimized for human breast tissue and used to demonstrate for the first time significantly increased (P < 0.001) amounts of pepstatin-inhibitable, cathepsin D-specific activity in breast tissue from 36 breast cancer patients (2.2 ± 1.4 units/mg of protein) when compared to 47 benign breast disease patients (0.63 ± 0.43) and 23 normal controls (0.24 ± 0.21). Preliminary analysis of subgroups of benign breast disease patients suggested no significant difference (P = 0.21) in pepstatin-inhibitable, cathepsin D-specific activity between benign breast disease patients and normal controls. This enzyme activity in malignant breast tissue suggests that the Mr, 31,000 form is the proteolytically active form of the enzyme which may be involved in the development and/or metastatic spread of breast cancer.

INTRODUCTION

The incidence of breast cancer has been increasing, and breast cancer is now the major cause of cancer deaths for nonsmoking women in Western societies (1). It recently has been estimated that approximately 12% of American women will develop breast cancer during their lifetime and that approximately 3.5% of American women will die of the disease (2). This translates into approximately 45,000 to 50,000 deaths/year. Axillary lymph node involvement occurs in approximately one third of breast cancer patients and is associated with increased disease recurrence and increased mortality when compared to lymph node-negative patients (2, 3). Although lymph node-negative patients have the best prognosis, approximately 20 to 30% of this group will experience recurrent disease within 5 yr (4, 5). Since it is difficult to determine which node-negative patients will relapse, adjuvant treatment is usually recommended for these patients despite the fact that most will not benefit from, and some patients even will be adversely affected by, this therapy. Better prognostic indicators clearly are needed for breast cancer patients (5, 6).

Cathepsin D (EC 3.4.23.5) is a ubiquitous aspartyl endopeptidase which is involved in normal protein degradation within lysosomes (7, 8). This protease appears to be a useful marker for identifying breast cancer patients with increased risk of recurrent disease, more frequent metastatic involvement, and increased mortality (reviewed in Refs. 9–11). It has been hypothesized that breast cancer patients make an abnormally processed cathepsin D which gets secreted rather than transported to the lysosome (10–12). This secreted cathepsin D may be involved in extracellular matrix degradation and the metastatic spread of malignant disease (13, 14).

Most previous studies have determined cathepsin D levels in the cytosols of primary breast cancer tissue by immunoblotting of total antigen (9–11, 15–17). This type of analysis may not provide the most useful information since significant amounts of cathepsin D can exist as precursors which are not active enzymatically (18–20). In the present study, the relative amounts of precursor and processed forms of cathepsin D have been analyzed by Western blotting and densitometric scanning for the first time in breast tissue and sera of patients with breast cancer, benign breast disease, and normal controls. This analysis demonstrated the presence of significantly increased relative amounts of a Mr, 31,000 form of cathepsin D in malignant breast tissue when compared to both benign breast disease and normal breast tissue. In addition, an enzymatic assay for cathepsin D has been optimized for human breast tissue and used to demonstrate for the first time that cathepsin D activity levels are significantly increased in malignant breast tissue when compared to both benign breast disease and normal breast tissues. This increased cathepsin D enzymatic activity correlates positively with the increased relative amounts of the Mr, 31,000 form of this enzyme in malignant breast tissue and, thus, provides evidence for the potential importance of this form of the protease in breast cancer and the metastatic process.

MATERIALS AND METHODS

General. Protein was determined by the method of Lowry et al. (21) using human serum albumin as the standard. Hemoglobin was purchased from Sigma Chemical Co. (St. Louis, MO), and pepstatin was from Boehringer Mannheim (Indianapolis, IN).

Patient Materials. Procedures that involved human subjects and tissues were approved by a Human Subjects Committee. Breast tissue was obtained from surgical biopsies from 53 patients with benign breast disease (primarily fibrocystic breast disease), from 23 normal controls (primarily patients undergoing reductive breast surgery), and from 43 patients with carcinoma of the breast. Blood was drawn preoperatively, allowed to coagulate at room temperature, and centrifuged at 2000 × g for 20 min; the resultant sera were poured into tubes which were sealed. The fresh breast tissues and sera were...
stored frozen at -20°C until used. The patients with breast cancer were included after a definitive diagnosis was made by histopathology by one or more of four hospital staff pathologists. All patients gave informed consent for participation in the study, and confidentiality was protected by assigning numbers to each patient. The characteristics of the groups and subgroups of patients who participated in the study are summarized in Table 1. All of the patients and controls were Caucasian except for five who were Hispanic. Sixty-three percent of the breast cancer patients and 26% of the benign breast disease patients were postmenopausal. The great majority (84%) of breast cancer patients had infiltrating ductal and/or infiltrating lobular carcinoma, and 44% of all the breast cancer patients were node negative. The majority (64%) of the benign breast disease patients had FCBD, of which 14% had nonproliferative-type disease and 20% had proliferative-type disease (defined by the presence of any of the following components: ductal and/or lobular hyperplasia; apocrine metaplasia; papillomatosis; and sclerosing adenosis). The mean (± SD) age of the breast cancer patients (57.5 ± 16.8 yr) was significantly increased compared to benign breast disease patients (44.9 ± 12.5) (P < 0.001) and normal controls (34.8 ± 15.4) (P < 0.001), and the mean age of benign breast disease patients was significantly increased (P < 0.01) compared to normal controls.

Tissue Preparation. Breast tissue from patients with cancer, benign breast disease, and normal controls was extracted after removal of fat using a modification of the procedure of Leto et al. (22). Frozen tissue was cut into small pieces (10-100 mg) with a razor blade with the following extraction buffer [50 mM Tris-HCI buffer (pH 7.4), containing 0.25 mM KCl, 1 mM EDTA, 0.02% (w/v) Na2SO4, and 0.1% (v/v) Triton X-100]. The washed tissue was transferred into a Waring blender with 4 ml of ice-cold extraction buffer per g of tissue and homogenized at low speed for 2-3 min periods with a 3-min interval on ice. The homogenate was centrifuged at 32,000 × g for 30 min at 4°C, and the resulting supernatant fluid (which contained cathepsin D) was stored at -20°C.

SDBS-PAGE. Slab SDS-PAGE was used, according to the method of Laemmli (23), to characterize samples of serum and tissue breast supernatant fluids containing cathepsin D. Antigen samples (3-10 µg), corresponding to approximately 0.05 to 0.2 µl of serum or 1.0 to 5.0 µl of supernatant fluid, were incubated for 5 to 10 min at 100°C in 62.5 mM Tris-HCI buffer (pH 6.8), containing 2.0% (v/v) SDS, 5.0% (v/v) β-mercaptoethanol, 10% (v/v) glycercol, and 0.0012% Bromophenol blue. Samples were electrophoresed on a 3-12% SDS-polyacrylamide slab gel (composed of a 4% stacking gel and a 14% separating gel) at 180 V for 1 h at room temperature in a pH 8.5 running buffer containing 25 mM Tris, 0.2 M glycine, and 0.1% SDS.

Western Blot Analysis. After SDS-PAGE, the gels were allowed to equilibrate for 1 h in 25 mM Tris and 192 mM glycine in 20% (v/v) methanol. The protein was transferred to 0.2-µm pore-sized nitrocellulose with a modified version of extraction buffer [50 mM Tris-HCl (pH 7.4), containing 0.25 mM KCl, 1 mM EDTA, 0.02% (w/v) Na2SO4, and 0.1% (v/v) Triton X-100]. The washed tissue was transferred into a Waring blender with 4 ml of ice-cold extraction buffer per g of tissue and homogenized at low speed for 2-3 min periods with a 3-min interval on ice. The homogenate was centrifuged at 32,000 × g for 30 min at 4°C, and the resulting supernatant fluid (which contained cathepsin D) was stored at -20°C.

RESULTS

Fig. 1 depicts the results of Western blotting analysis of cathepsin D in breast tissue supernatant fluids from patients with breast cancer, benign breast disease, and normal controls. From this blot it can be seen that the major form of cathepsin D for all patients and controls has a molecular weight of 52,000, while another has a molecular weight of 27,000 (as determined by Mr standards; data not shown). A Mr 31,000 form of cathepsin D is prominent in breast cancer tissue (Fig. 1, Lanes 1 and 2), seen in smaller amounts in benign breast disease tissue (Lanes 3 and 4), and not seen in detectable amounts in the two normal control breast tissues (Lanes 3 and 4). This Mr 31,000 form of cathepsin D comigrates with the major form of authentic human liver cathepsin D (Lane 7). Densitometric scanning of Lanes 3-4 revealed a significant increase in the amount of this Mr 31,000 form of cathepsin D in all Western blot analysis.
ANALYSIS OF BREAST CANCER CATHEPSIN D

Table 1

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Benign breast disease

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a C, Caucasian; H, Hispanic.
b Mean ± SD.

Fig. 1. Western blot analysis of cathepsin D in breast tissue supernatant fluids from patients with breast cancer, benign breast disease, and normal controls. Comparable amounts of supernatant protein (10 μg) were run in each lane. See “Materials and Methods” for details. Lane 1, breast cancer patient (C-2); Lane 2, breast cancer patient (C-7); Lane 3, normal control (N-2); Lane 4, normal control (N-4); Lane 5, benign breast disease patient (BC-20); Lane 6, benign breast disease patient (BC-25); and Lane 7, authentic human liver cathepsin D (0.25 μg).

Fig. 2. Densitometric scanning of Western blot of cathepsin D in breast tissue supernatant fluids from a patient with breast cancer and a normal control. A, scan of Fig. 1, Lane 1 (representing breast cancer Patient C-2); B, scan of Fig. 1, Lane 3 (representing normal control N-2).
of a Mr 31,000 form of cathepsin D in breast tissue from patients with breast cancer, benign breast disease, and normal controls.

Patient numbers are necessary before a more definitive analysis can be done. The breast cancer patients have significantly decreased (P < 0.001) relative amounts of the major Mr 52,000 form when compared to both benign breast disease patients and normal controls. The Mr 27,000 form is present in decreased relative amounts in breast cancer patients approximately 12% and 15% when compared to benign breast disease patients and normal controls, respectively.

The optimized assay conditions described above were used to determine cathepsin D activity levels in breast tissue from patients with breast cancer, benign breast disease, and normal controls. Because of the lack of specificity of this type of protease assay on a protein substrate, duplicate assays were run in the presence of pepstatin, a relatively specific inhibitor of cathepsin D (22).

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The lack of linearity beyond 40 min of incubation appears to be due to the thermolability of cathepsin D as indicated by preincubation studies of cathepsin D at 37°C for various times (0 to 60 min) followed by assay of enzymatic activity for 20 min. Cathepsin D retained approximately 90% of its initial activity after 20 min of preincubation, whereas the enzyme retained approximately 73% of its initial activity for a preincubation time of 50 to 60 min. Fig. 5 depicts the pH optimum curve for cathepsin D using 20 μl of breast supernatant fluid and 20-min incubation times. Substantial activity (≥50% of maximal) exists between pH values of 2.8 and 3.8 with maximal activity around pH 3.3. The assay conditions also were optimized for hemoglobin concentration and the concentration of TCA to stop the reaction, resulting in the conditions specified for the cathepsin D assay in "Materials and Methods."

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ANALYSIS OF BREAST CANCER CATHEPSIN D

Fig. 5. pH-optimum curve of cathepsin D from breast tissue supernatant fluid. See "Materials and Methods" for details.

Fig. 6. Frequency plot of pepstatin-inhibitable, cathepsin D-specific activities for breast tissue from patients with breast cancer, benign breast disease, and normal controls.

0.150
0.120
0.090
0.060
0.030
0.000

2.5
3.0
3.5
4.0
4.5
5.0
pH

Cathepsin D Activity (Δ Abs 280 nm)

benign breast disease patients, whereas values of >1.0 unit/mg of protein are found for the great majority (86%) of breast cancer patients. The mean (± SD) values for cathepsin D activity levels, specific activities, percentage of activity which is pepstatin inhibitable, and pepstatin-inhibitable specific activities are summarized in Table 3 for all the patients and controls. The activity and specific activity of cathepsin D in malignant breast tissue are significantly increased (P < 0.001) when compared to both benign breast disease and normal control tissues. The increases of malignant tissue when compared to benign breast disease and normal breast tissues are even larger when pepstatin-inhibitable cathepsin D specific activities are calculated. This is due primarily to the significant increase (P < 0.001) of the percentage of malignant tissue proteolytic activity at pH 3.3 which was pepstatin inhibitable when compared to both benign breast disease and normal control tissues. An analysis of pepstatin-inhibitable cathepsin D specific activities in the two types of FCBD indicated no significant difference (P = 0.21) between 17 patients with proliferative-type disease (0.54 ± 0.36 units/mg of protein) and 11 patients with nonproliferative-type disease (0.76 ± 0.63), but this analysis is very preliminary due to the small number of patients and the large standard deviations in each subgroup. No significant differences were found for mean (± SD) protein concentrations of breast cancer tissue supernatant fluids (6.0 ± 2.3 mg/ml) when compared to supernatant fluids from normal controls (6.7 ± 2.1) (P = 0.243) or benign breast disease patients (5.3 ± 2.3) (P = 0.171). Cathepsin D enzymatic activity, as determined with the breast tissue assay, was not detectable in sera from 15 breast cancer patients and controls which we analyzed.

Race and sex are not related to the increased relative amount of the Mr 31,000 cathepsin D form and increased cathepsin D enzymatic activity in breast cancer patients compared to benign breast disease patients and normal controls, since 96% of the patients were Caucasian and 98% were female. Although the mean age of the breast cancer group is significantly increased compared to both benign breast disease (P < 0.001) and normal control (P < 0.001) groups, age does not appear to be correlated with the two cathepsin D variables which were investigated. This was determined by an analysis of age versus the two cathepsin D variables within each major patient group. For a given age, essentially all values (low, intermediate, and high) for the cathepsin D variables were found, and for a given cathepsin D value, essentially all ages (young, intermediate, and old) were found.

DISCUSSION

Several studies have provided evidence that elevated levels of breast tissue cathepsin D are associated with a higher incidence of disease recurrence, more frequent metastasis, and increased mortality in breast cancer patients (reviewed in Refs. 9–11). Most of these studies have used immunoassays developed using the MCF-7 breast cancer cell line, and total antigen levels of cathepsin D have been determined. Although total antigen levels provide potential independent prognostic information, this type of analysis may not give the most useful information since cathepsin D can exist as precursors which are not active enzymatically (18–20). For cathepsin D to be involved mechanistically in the metastatic spread of breast cancer as hypothesized (13, 14), it must possess enzymatic activity.

Three recent studies have described more specific techniques for assessing cathepsin D levels. In a study by Tandon et al. (15), Western blotting and scanning densitometry were used to retrospectively show that increased amounts of a Mr 34,000 form of cathepsin D were a significant predictor of reduced disease-free survival in node-negative breast cancer patients. This study did not analyze benign breast dis-
were found. In a second study by Krishnan et al., the relative amounts of the Mr 31,000 form of cathepsin D in malignant tissue were positively correlated with increased total cathepsin D antigen levels, and both were associated with poor prognosis in node-negative breast cancer patients. However, the enzymatic cathepsin D assay which was used does not appear to have been optimized since assay incubations were done at 37°C for 5 h, which is well beyond the time for which human breast cancer tissue activity is thermostable. In addition, their assay was not used to assess cathepsin D activities in normal or benign breast disease tissues (27).

Our present study used Western blotting and densitometric scanning to determine for the first time the relative amounts of precursor and processed forms of cathepsin D in breast tissue and sera of patients with breast cancer, benign breast disease, and normal controls. This analysis was done to determine if specific processed (and presumably catalytically active) forms of cathepsin D are increased in breast cancer and/or benign breast disease. The results indicate that the major form of cathepsin D in breast tissue and sera from all patients and controls has a molecular weight of 52,000. Since this form represents the inactive precursor of cathepsin D (18–20), our finding emphasizes the need to measure something other than total cathepsin D antigen levels. Our results indicate that total cathepsin D antigen levels are only modestly increased in breast tissue from breast cancer patients when compared to benign breast disease patients and normal controls. Our results also demonstrate the presence of a Mr 31,000 form of cathepsin D in surgically removed breast tissue from 42 of 43 cancer patients which was detected in only 4 of 23 normal control breast tissues. Although the Mr 31,000 form was found in the majority of breast tissues from benign breast disease patients, it was present in significantly decreased relative amounts (P < 0.001) when compared to malignant breast tissue. The Mr 31,000 form is the major processed form of cathepsin D in breast tissue from breast cancer patients, whereas the Mr 27,000 form is the major processed form in benign breast disease tissue and normal breast tissue. Our results suggest that cathepsin D is processed abnormally in malignant breast tissue. Serum from a number of the breast cancer patients and controls was analyzed for cathepsin D by Western blotting in an attempt to obtain prognostic information using a less invasive procedure. However, the Mr 31,000 form of cathepsin D was not detected in sera from any of the breast cancer patients or controls which were investigated. Our serum results are similar to the results of a recent study which found that plasma cathepsin D concentrations were not a useful marker for breast cancer (28).

Analysis of cathepsin D enzymatic activities in the breast tissues with our assay optimized for human breast provided further evidence for the importance of the Mr 31,000 form in malignant breast disease. The mean cathepsin D specific activity in malignant tissue was increased approximately 3-fold and 2-fold when compared to normal and benign breast disease tissues, respectively. The breast cancer increase was even more pronounced when pepstatin-inhibitable, cathepsin D-specific activities were calculated, with 9-fold and 3.5-fold increases found when compared to normal and benign breast disease tissues, respectively. This suggests that cathepsin D activity is disproportionately increased compared to other proteases in malignant breast tissue. Cathepsin D enzymatic activity was not detected in sera from any of the breast cancer patients or controls which were investigated. Our results demonstrate for the first time that cathepsin D enzymatic activity levels are significantly (P < 0.001) increased in breast tissue from patients with breast cancer when compared to both patients with benign breast disease and normal controls. In addition, the increased activity is correlated positively (r = 0.82) with the increased relative amount of the Mr 31,000 form of cathepsin D in breast tissue of individual breast cancer patients. This correlation suggests that the Mr 31,000 form of cathepsin D is the major proteolytically active form of the enzyme, and that this form may be involved in the development and/or metastatic spread of breast cancer. A preliminary analysis of the two FCBD subgroups suggested no significant difference between proliferative-type and nonproliferative-type patients for the two cathepsin D variables investigated. However, the numbers of patients in these two subgroups were too small for an appropriate statistical analysis.

Our results raise the possibility that the presence and relative amount of the Mr 31,000 form of cathepsin D and/or the amount of pepstatin-inhibitable cathepsin D activity in breast tissue may prove to be better prognostic indicators than total cathepsin D antigen levels for breast cancer patients. Since these cathepsin D values were somewhat increased in our benign breast disease patients, they also may prove to be useful in determining which of these patients will develop malignancies. This is a reasonable expectation since increased cathepsin D levels have been found in cyst fluids from patients with gross cystic breast disease (29, 30) who are known to be at a 2- to 4-fold increased risk of developing breast cancer compared to the normal female population (31–33). Larger numbers of patients are needed to confirm our preliminary results and to allow subdivision of the breast cancer patients to determine whether a number of patient variables (e.g., nodal status, tumor grade and size, DNA ploidy, hormone receptor status) are related to the two cathepsin D variables studied here. In addition, long-term follow-up on patients participating in our ongoing

### Table 3: Cathepsin D enzymatic activities in breast tissue from patients with breast cancer, benign breast disease, and normal controls

<table>
<thead>
<tr>
<th>Group</th>
<th>Activity (units/mg)</th>
<th>Specific activity (units/mg of protein)</th>
<th>% of activity which is pepstatin inhibitable</th>
<th>Pepstatin-inhibitable specific activity (units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer (N = 36)</td>
<td>20 ± 10^a</td>
<td>3.4 ± 1.4</td>
<td>61 ± 21</td>
<td>2.2 ± 1.4</td>
</tr>
<tr>
<td>Benign breast disease</td>
<td>9.3 ± 2.8</td>
<td>2.0 ± 0.84</td>
<td>32 ± 18</td>
<td>0.63 ± 0.43</td>
</tr>
<tr>
<td>Normal control (n = 23)</td>
<td>7.3 ± 1.3</td>
<td>1.2 ± 0.44</td>
<td>21 ± 16</td>
<td>0.24 ± 0.21</td>
</tr>
</tbody>
</table>

^a Mean ± SD.
^b P value for comparing breast cancer to benign breast disease.
^c P value for comparing breast cancer to normal control.
^d P value for comparing benign breast disease to normal control.
study is required before the true value of the $M$, 31,000 form of cathepsin D or pepstatin-inhibitable cathepsin D activities can be evaluated as prognostic indicators for patients with breast cancer and/or as premalignancy markers for benign breast disease.

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REFERENCES

Western Blotting and Enzymatic Activity Analysis of Cathepsin D in Breast Tissue and Sera of Patients with Breast Cancer and Benign Breast Disease and of Normal Controls


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