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 cathepsin D in sera and breast tissue of patients with breast cancer, benign breast disease, and normal controls. Sera gave similar blots for cathepsin D 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%) and 23 normal controls (1.8 ± 4.4%). Preliminary analysis of subgroups of benign breast disease patients suggested no significant difference (P = 0.41) 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 and proliferative-type and nonproliferative-type fibrocystic breast disease. The positive correlation (r = 0.82) of increased amounts of the Mr 31,000 form of cathepsin D and increased pepstatin-inhibitable, cathepsin D enzymatic 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 immunoassay 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 clot 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,3 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 (2- to 3-mm cubes) with a razor blade and washed with 10 volumes of 0.1% SDS-polyacrylamide slab gel (composed of a 4% stacking gel and a 14% resolving gel) containing 2.0% (w/v) SDS, 5.0% (v/v) β-mercaptoethanol, 10% (v/v) glyc erol, and 0.0012% Bromophenol blue. Samples were electrophoresed on a 0.1% 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 and was transferred to 0.2-μm pore-sized nitrocellulose (Schleicher and Schuell, Keene, NH), either at 150 mA or 100 V for 1 h by using a Bio-Rad Mini Trans-Blot electrophoresis cell according to the method described in the manual accompanying the unit. A portion of the nitrocellulose which contained molecular weight standards (bovine serum albumin, Mr 67,000; immunoglobulin G, Mr 25,000 and 55,000; ovalbumin, Mr 45,000 from Schwarz/Mann Biotech, Cleveland OH; and carbonic anhydrase, Mr 29,000; lysozyme, Mr 14,400 from Sigma Chemical Co.) was stained for 5 min with 0.1% Amido Black 10B (Bio-Rad Laboratories, Richmond, CA) in propan-2-ol-acetic acid:water (3:16:1, by volume) followed by destaining with propan-2-ol-acetic acid:water (3:16:1, by volume). The remainder of the nitrocellulose was incubated in 20 mM Tris-HCl buffer (pH 7.4), containing 2% (w/v) Tween 20 with cold water fish skin collagenase (Sigma, St. Louis, MO), 50 mM NaCl, 10 mM MgCl2, 10 mM CaCl2, and 10 mM MnCl2 for 12 h. Before incubation with antibody against cathepsin D, the nitrocellulose was incubated in 50 mM Tris-HCl buffer (pH 7.5), containing 150 mM NaCl for 30 min. The same procedure was used for blotting serum samples except that the pH 7.5, 50 mM Tris-HCl buffer was pH 7.4 and 10 mM and contained 0.1% (w/v) Tween 20. The nitrocellulose was incubated in a 10-fold dilution of polyclonal rabbit anti-human liver cathepsin D (American Research and Technology, Inc., Athens, GA) for 1 h, followed by three consecutive washes in the 50 mM Tris-HCl buffer (pH 7.5), containing 150 mM NaCl (10 min/wash). The nitrocellulose was incubated in a 10-fold dilution of sheep anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 1 h. After three more 10-min washes in 50 mM Tris-HCl buffer (pH 7.5), containing 150 mM NaCl, the nitrocellulose samples were placed into developing buffer (2.5 mg of NitroBlue tetrazolium and 1.9 mg of 5-bromo-4-chloroindol-3-yl phosphate in 10 ml of 0.1 Tris-HCl (pH 9.5), containing 0.1 mM NaCl and 5 mM MgCl2) for 5 to 10 min. The color development reactions were stopped by immersing the nitrocellulose in water.

Densitometric Scanning. Color-developed nitrocellulose was optically scanned using an LKB Ultrascan XL enhanced laser densitometer. Relative amounts (percentages) of the peak areas were assessed by cutting the peaks and weighing them on an analytical balance.

Cathepsin D Enzymatic Activity Assay. Cathepsin D enzymatic activity in breast tissue supernatant fluids was assayed using hemoglobin as a substrate after acid denaturation of the hemoglobin using the method of Anson (with 1.35 mM acetic acid and 0.02 mM ammonium sulfate) (24). The conditions of the assay were optimized for human breast tissue cathepsin D with regard to hemoglobin concentration, buffer type, concentration and pH, length of incubation at 37°C, amount of supernatant fluid, and concentration of TCA to stop the reaction. The final assay conditions which follow were approximately linear with regard to enzyme concentration and time of incubation at 37°C: breast tissue supernatant fluid (20 to 40 μl) was added to 680 or 660 μl of 0.1 mM sodium formate buffer (pH 3.3), which had been preincubated for 3 min at 37°C with 100 μl of 4.0% (w/v) acid-denatured hemoglobin. Incubations were done in duplicate for various times (usually 20 to 40 min) at 37°C, and reactions were stopped by addition of 0.5 ml of 10% (w/v) TCA. The reaction mixtures were vortexed and centrifuged at 1800 × g for 10 min at 2°C, and the absorbances of the supernatant fluids (which contained the hemoglobin peptides) were determined at 280 nm. These absorbances were corrected by subtracting 280-nm absorbances of tissue/substrate blanks which were done as described above except that the hemoglobin was added after stopping the reaction with TCA. Parallel assays were also run in the presence of 1.6 μg of pepstatin per ml [added in 0.1 mM sodium formate buffer (pH 3.3)] to determine what percentage of the activity was pepstatin inhibitable (22). A unit of cathepsin D activity is defined as the amount of enzyme necessary to cause an absorbance change of 1.0 at 280 nm for 60-min incubation using acid-denatured hemoglobin as substrate and under the above-described optimized conditions.

Thermastability and pH Optimum Analysis. Breast tissue supernatant fluid (20 μl) was preincubated at 37°C for various times (0 to 60 min) and then assayed at 37°C for 20 min as described above for cathepsin D activity. The pH optimum curve of cathepsin D was determined from pH 2.3 to 4.6 using a constant ionic strength buffer system (25) which consisted of 0.5 mM chloroacet ic acid:0.5 mM KOH (pH 2.3 to 3.4), 0.5 mM formic acid:0.5 mM KOH (pH 3.4 to 3.7), and 0.5 HCl:0.5 NaOH (pH 3.7 to 4.6). Incubations were done in duplicate on 20 μl of breast tissue supernatant fluid for 20 min at 37°C, and actual pH values were determined on a set of identical tubes which were not incubated.

Statistical Analysis. Means, standard deviations, and correlation coefficients (from regression analysis) were calculated using a Casio Super-FX scientific calculator. Analysis of the difference of means was performed using the t test on SigmaStat (Jandel Scientific, San Rafael, CA). Frequency plots were done using KaleidaGraph, Version 2.0 (Synergy Software, Reading, PA).

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 27,000, 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 6), 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

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3 The abbreviations used are: FCBD, fibrocystic breast disease; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.
Table 1: Summary of characteristics of patient groups and subgroups

<table>
<thead>
<tr>
<th>Patient group</th>
<th>n</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Race*</th>
<th>Nodal status</th>
<th>Menopausal status</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>F/M</td>
<td>C</td>
<td>H</td>
<td>N(+)</td>
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<tr>
<td>Breast cancer</td>
<td>43</td>
<td>57.5 ± 16.8b</td>
<td>43</td>
<td>0</td>
<td>42</td>
<td>21</td>
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<tr>
<td>Infiltrating ductal</td>
<td>28</td>
<td>27 ± 8.4</td>
<td>28</td>
<td>0</td>
<td>27</td>
<td>16</td>
</tr>
<tr>
<td>Infiltrating lobular</td>
<td>6</td>
<td>6 ± 2.3</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Infiltrating ductal/lobular</td>
<td>2</td>
<td>2 ± 1.0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Intraductal</td>
<td>4</td>
<td>4 ± 2.0</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
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<tr>
<td>Inflammatory</td>
<td>1</td>
<td>1 ± 0.0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Infiltrating mucinous</td>
<td>2</td>
<td>2 ± 1.0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
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<tr>
<td>Benign breast disease</td>
<td>53</td>
<td>44.9 ± 12.5</td>
<td>51</td>
<td>2</td>
<td>50</td>
<td>3</td>
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<tr>
<td>Fibrocystic</td>
<td>34</td>
<td>34 ± 12.5</td>
<td>34</td>
<td>0</td>
<td>33</td>
<td>1</td>
</tr>
<tr>
<td>Proliferative</td>
<td>20</td>
<td>20 ± 10.0</td>
<td>20</td>
<td>0</td>
<td>19</td>
<td>1</td>
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<tr>
<td>Nonproliferative</td>
<td>14</td>
<td>14 ± 7.4</td>
<td>14</td>
<td>0</td>
<td>14</td>
<td>0</td>
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<td>Fibroadenoma</td>
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<td>16 ± 11.5</td>
<td>16</td>
<td>0</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Gynecomastia</td>
<td>2</td>
<td>0 ± 0.0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
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<td>Atypical hyperplasia</td>
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<td>1 ± 0.0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Normal controls</td>
<td>23</td>
<td>34.8 ± 15.4</td>
<td>23</td>
<td>0</td>
<td>22</td>
<td>1</td>
</tr>
<tr>
<td>Reductive surgeries</td>
<td>20</td>
<td>20 ± 10.0</td>
<td>20</td>
<td>0</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>Nonpathological biopsies</td>
<td>3</td>
<td>3 ± 0.0</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

* 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).

1 and 3 of Fig. 1 led to the profiles depicted in A and B, respectively, of Fig. 2. The three major forms of cathepsin D (i.e., Mr 52,000, 31,000, and 27,000) in cancerous breast tissue can be seen in A, while the Mr 31,000 form is not detectable in normal breast tissue (B). The relative amounts of the three major cathepsin D forms were determined by densitometric scanning of breast tissue blots from all the patients and controls. A frequency plot of the data, depicted in Fig. 3, indicates that the Mr 31,000 form of cathepsin D was not detected in the great majority (83%) of normal controls, was present in relative amounts <15% in most (75%) of the benign breast disease patients, and in relative amounts >15% in most (77%) of the breast cancer patients. Table 2 summarizes the mean (± SD) relative amounts of the three major cathepsin D forms for the three groups of patients. Statistical analysis of the data indicates that the relative percentage of the Mr 31,000 form of cathepsin D is significantly increased (P < 0.001) in the 43 breast cancer patients when compared to both the 51 benign breast disease patients and the 23 normal controls. An analysis of the relative percentage of the Mr 31,000 form in the two types of FCBD (see Table 1) indicated no significant difference (P = 0.41) between them even though a trend of increased relative amounts of the Mr 31,000 form was found for 20 patients with proliferative-type disease (14.2 ± 9.1%) compared to 13 patients with nonproliferative-type disease (11.5 ± 9.2%). These findings are very preliminary, and larger
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.

Fig. 4 depicts the results of Western blotting analysis of sera from patients with breast cancer (Lanes 1 and 2), benign breast disease (Lanes 4 to 6), and a normal control (Lane 3). All the sera gave comparable blots and contained two major forms of cathepsin D (Mr 52,000 and 27,000), with a possible minor form at a slightly higher molecular weight than the Mr 52,000 form. The Mr 31,000 form, which was found in breast tissue supernatant fluids in Fig. 1 (and which is represented in Fig. 4 by authentic cathepsin D in Lane 7), was not detected in any of the sera. The results in Fig. 4 are representative of Western blots done on sera from 8 patients with breast cancer, 5 patients with benign breast disease, and 3 normal controls.

One aim of the present study was to develop a cathepsin D enzymatic activity assay for human breast tissue supernatant fluids. The optimized conditions resulted in an assay which was approximately linear for 20 to 40 μl of supernatant fluid from 20 to 40 min of incubation with minor deviation from linearity seen for 40 μl incubated for 40 min. Activity was not linear for supernatant fluid amounts below 20 μl or above 40 μl, or for incubation times below 20 min or above 40 min. 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."

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). Fig. 6, which is a frequency plot of pepstatin-inhibitable cathepsin D specific activities for all patients and controls, indicates specific activities ≤1.0 unit/mg of protein for all the normal controls and the great majority (83%) of above 31 kDa cathepsin D.

**Table 2** Relative amounts of precursor and processed forms of cathepsin D in breast tissue of patients with breast cancer, benign breast disease, and normal controls as determined by densitometric scanning of western blots.

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative % of three major forms of cathepsin D</th>
<th>Mr 52,000</th>
<th>Mr 31,000</th>
<th>Mr 27,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer (n = 43)</td>
<td>58 ± 11a</td>
<td>24 ± 12</td>
<td>18 ± 7.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.001b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.001c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign breast disease (n = 51)</td>
<td>67 ± 13</td>
<td>13 ± 8.9</td>
<td>20 ± 9.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.001b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.001c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control (n = 23)</td>
<td>70 ± 8.6</td>
<td>1.8 ± 4.4</td>
<td>28 ± 7.7</td>
<td></td>
</tr>
</tbody>
</table>

*a* Mean ± SD.

*b* P value for comparing breast cancer to normal control.

*c* P value for comparing breast cancer to benign breast disease.

*d* P value for comparing benign breast disease to normal control.

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). Fig. 6, which is a frequency plot of pepstatin-inhibitable cathepsin D specific activities for all patients and controls, indicates specific activities ≤1.0 unit/mg of protein for all the normal controls and the great majority (83%) of above 31 kDa cathepsin D.
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.

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 breast cancer indicated no significant difference (P = 0.21) between 17 patients with proliferative-type disease (0.54 ± 0.56 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-
ease tissues and only analyzed a few normal breast tissues in which low or undetectable amounts of the Mr 34,000 form of cathepsin D were found. In a second study by Krishnan et al. (26), SDS-PAGE and scanning densitometry were used to quantify amounts of the Mr 52,000 form of cathepsin D secreted from MCF-7 breast cancer cells grown under various conditions. However, these investigators indicated that their technique was not applicable to breast cancer tissue cytosols because of the presence of too many proteins. A third study by Kute et al. (27) developed an enzymatic assay for cathepsin D and demonstrated that increased cathepsin D activity levels 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 cathepsin D activity is thermostable. In addition, their assay was not used to assess cathepsin D activities in normal or benign breast control tissues. Cathepsin D enzymatic activity was not detected in sera from any of the breast cancer patients or controls which were investigated. Our results also demonstrate the presence of a Mr 31,000 form in breast tissue of individual breast cancer patients. This correlation suggests that the Mr 31,000 form 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 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 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 34,000 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/ml)</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></td>
<td>&lt;0.001^b</td>
<td>&lt;0.001^b</td>
<td>&lt;0.001^b</td>
<td>&lt;0.001^b</td>
</tr>
<tr>
<td>Benign breast disease (n = 47)</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></td>
<td>&lt;0.001^c</td>
<td>&lt;0.001^c</td>
<td>&lt;0.001^c</td>
<td>&lt;0.001^c</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 normal control.
^c P value for comparing breast cancer to benign breast disease.
^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
