The Role of Cathepsin D in the Invasiveness of Human Breast Cancer Cells

Michael D. Johnson, Jeffrey A. Torri, Marc E. Lippman, and Robert B. Dickson
Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, DC 20007

ABSTRACT

The aspartyl protease cathepsin D has been shown to be a marker of poor prognosis when found at high levels in primary breast tumors. It has been suggested that this is because the production of cathepsin D increases the invasive potential of the tumor cells, thus increasing the probability of metastasis. We have therefore conducted experiments to determine if secreted cathepsin D makes a significant contribution to the invasive phenotype of breast cancer cells in the Boyden chamber assay of invasion, which measures the ability of a cell to invade through an artificial basement membrane. Cathepsin D secretion and Boyden chamber invasiveness were measured in nine clones of the breast cancer cell line MCF-7, and no correlation was found between cathepsin secretion and invasive behavior. Invasion assays were also conducted in the presence of the aspartyl protease inhibitor pepstatin A, and no inhibition of the invasive behavior of cells was seen. Since low-pH environments are required for both the activation of pro-cathepsin D and the activity of the mature enzyme, assays were also conducted in the presence of chloroquine to neutralize the pH in the acidic compartments of the cells. This treatment did not inhibit invasiveness. Cathepsin D secretion by the breast cancer cell lines MDA-MB-231, MDA-MB-435, MDA-MB-435s, MDA-MB-468, SK-Br-3, and MCF-7-ADR was also measured. Again, there was no correlation with invasion. In fact, cathepsin D levels were inversely correlated with aggressive behavior in vivo and in vitro in previously reported studies. These data suggest that cathepsin D secretion by tumor cells is not an important determinant of the invasive potential of the tumor cells per se. These data also reinforce the view that the poor prognosis in clinical breast cancer linked to high tumor levels of cathepsin D is probably due to high levels of cathepsin D in the stromal components of the tumor such as infiltrating inflammatory cells.

INTRODUCTION

Patients who die of breast cancer are almost always killed by recurrence of their disease at a site removed from the location of their primary tumor. Thus, an understanding of the processes that are involved in metastasis and its regulation is crucial to the development of new strategies for the treatment and prevention of this disease. In order for a tumor cell to metastasize to another organ, it must pass through at least one basement membrane to gain entry to a blood or lymphatic vessel, and then having been deposited in the capillary bed of another organ, it must again traverse one or more basement membranes to invade and colonize this new site. Passage through basement membranes is thought to depend on the ability of the cell to degrade the proteolytic enzymes that are secreted by cancer cells.

Breast cancer is often characterized by a sensitivity to the hormone estrogen, with 30% to 50% of patients showing some response to antiestrogenic therapies (1). Consequently, the discovery that secretion of the aspartyl protease cathepsin D by estrogen-responsive breast cancer cells is greatly stimulated by treatment with this hormone has resulted in the suggestion that this enzyme is important in the invasive process (2, 3). Studies to determine if the level of cathepsin D found in primary tumor cytosols correlates with prognosis have shown that patients whose tumors contained high levels of cathepsin D have both shorter disease-free and overall survival times (4). These data would seem to support the attractive hypothesis that cathepsin D secreted by the tumor cells helps to degrade the basement membrane, thereby facilitating invasion and metastasis (4).

However, immunohistochemical studies of the localization of cathepsin D expression in breast tumors have shown that the presence of high levels of the enzyme, specifically in the tumor cells, rather than in the stromal components of the tumor, is a marker of good prognosis (5). Also, cathepsin D is a lysosomal protease with a pH optimum of 2.5–3.5, depending on the substrate: a pH much lower than would usually be found in the extracellular fluid (6). These points do not seem to be consistent with the above hypothesis, and consequently this study set out to determine whether cathepsin D secretion by tumor cells is important to the invasive process as measured in a model system: the Boyden chamber assay of chemoinvasion (7).

MATERIALS AND METHODS

Cell Culture. The cell lines used in this study were obtained from the following sources: MCF-7, Dr. Marvin Rich (Michigan Cancer Foundation, Detroit, MI); MCF-7-ADR, Dr. Kenneth Cowan (Clinical Pharmacology Branch, NIH, Bethesda, MD); MDA-MB-435, Dr. Janet Price (M. D. Anderson Cancer Center, Houston, TX); MDA-MB-231, MDA-MB-435s, MDA-MB-468, and SK-Br-3, American Type Culture Collection (Rockville, MD). All cell lines were maintained in serum-reduced modified Eagle's medium (Gibco, Rockville, MD) supplemented with 10% fetal calf serum (Biofluids, Rockville, MD). The MCF-7 clones were generated by limiting dilution in a 96-well plate. Each well contained a single cell that was identified using an inverted microscope. These nine clones were then expanded and frozen down. All experiments were conducted using cells cultured for no more than 10 passages after cloning.

The clones were characterized for their responses to estrogens and antiestrogens in both anchorage-independent and -dependent growth assays and for growth in the nude mouse, using methods described before (8–10).

For the measurement of cathepsin D secretion, 100,000 cells of each clone in 100 μl of medium were plated into quadruplicate wells on a 96-well plate and allowed to attach overnight. The following day the medium was replaced with 50 μl of methionine-free modified Eagle's medium (Gibco, New York, NY) supplemented with [35S]methionine (200 μCi/ml) (Amersham, Arlington Heights, IL). After 6 h of incubation at 37°C the conditioned medium was removed from the 4 wells, centrifuged at 1500 X g for 5 min to remove any contaminating cells, pooled, and frozen at –20°C until required.

Immunoprecipitation. [35S]-labeled conditioned medium from each clone (68 μl) was immunoprecipitated with a monoclonal antibody (1 μg) raised against cathepsin D (kindly provided by Dr. Henri Rochefort, INSERM, Montpellier, France), using a rabbit anti-mouse antibody (Organon Teknika, West Chester, PA) and protein A Sepharose (Pharmacia, Piscataway, NJ) to recover the antibody-cathepsin complexes. Precipitates were analyzed by fractionation on 10% tricine-sodium dodecyl sulfate-polyacrylamide gels (11), which were processed for fluorography and exposed to X-ray film (Fujifilm, Japan) at –70°C (12). 14C-labeled molecular weight standards were purchased from Gibco BRL (Gaithersburg, MD). The intensity of the bands on the autoradiogram was measured by densitometric scanning. Incorporation of [35S]methionine into secreted proteins by the 9 clones was determined by measuring TCA-precipitable radioactivity in the conditioned medium by liquid scintillation counting.

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2 To whom requests for reprints should be addressed, at Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, DC 20007.
3 G. R. Pasternack, personal communication.
4 The abbreviation used is: TCA, trichloroacetic acid.
**Boydne Chamber Assay.** The Boydne chamber chemoinvasion and migration assays were performed essentially as described previously (7, 13, 14). Matrigel and collagen IV were prepared from Engelbreth-Holm-Swarm tumors, kindly provided by Dr. Hynda Kleinman (Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research, NIH), as previously described (15). Polycarbonate filters (12-μm pore; polyvinylpyrrolidone free; Poretics, Livermore, CA) were coated with 25 μg of matrigel/filter, which was then reconstituted at 37°C. Cells were harvested with trypsin/EDTA (Gibco), washed twice with Iscove's minimal essential medium containing 0.1% bovine serum albumin (Miles Biochemicals, Kankakee, IL) and 10% fetal calf serum, and added to the top chamber (70,000 cells/chamber). Treatment media were added to the cells immediately after addition to the chamber, such that the final concentration in the chamber would be that specified. Mammary fibroblast-conditioned medium obtained by incubating confluent monolayers of primary human mammary fibroblasts for 24 h with Iscove's minimal essential medium containing 0.1% bovine serum albumin and 0.05 mg/ml ascorbic acid was used in the lower compartment of 0.2-ml blind well-modified Boydne chambers. Chambers were incubated in a humidified incubator at 37°C in 5% CO₂/95% air for 18 h, after which the cells that had traversed the matrigel and spread on the lower surface of the filter were stained with Diff-Quik (American Scientific Products, McGaw Park, IL) and quantitated electronically with the Zeiss IBIS 2000 image analysis system using a Kontron Aiaig processor interfaced with a Zeiss Axiophot microscope equipped with an automated stage.

Migration assays were performed as described for the chemoinvasion studies with the single exception that the filter surfaces were coated with 5 μg of collagen IV instead of the 25 μg of matrigel. Coatings ranging between 5 and 60 μg of collagen IV promote even attachment to and migration across the filter, without presenting a significant barrier to invasion (13). Migration assays were performed in parallel to the chemoinvasion assays, using the same cells and conditioned media, and were quantified similarly.

In some experiments, pepstatin A (Boehringer Mannheim, Indianapolis, IN) or chloroquine (Sigma, St. Louis, MO) was added to the media. The diffusion of pepstatin A across matrigel-coated filters was measured by placing phosphate-buffered saline containing bovine serum albumin (0.1%) and pepstatin A (10 μg/ml) in the upper chamber of a Boydne chamber and then assaying for the presence of pepstatin in the bottom chamber after various times using the pepstatin assay described below.

**Pepstatin Assay.** Pepstatin was assayed by a slight modification of a standard cathepsin D assay (6). Briefly, a solution containing sodium citrate (5.7 mM, pH 3) (Fisher, Pittsburgh, PA), hemoglobin (1.4%) (Sigma), and the relevant amount of pepstatin A (Boehringer Mannheim), was prepared in an Eppendorf tube and incubated at 37°C for 5 min. Cathepsin D (1.4 units; Sigma) was then added, and the solution was incubated at 37°C for 10 min, after which TCA (Fisher) was added to a final concentration of 3%. Precipitated protein was removed by centrifugation at 12,000 × g, and the absorbance of the supernatant was measured at 280 nm. Increasing levels of pepstatin progressively inhibited the liberation of TCA-soluble material. Pepstatin concentrations were calculated by reference to a standard curve.

All of the assays performed in this study were conducted at least twice and found to be reproducible. Representative data are presented throughout.

**RESULTS**

**Relationship between Cathepsin D Secretion and Invasiveness.** Nine clones of the breast cancer cell line MCF-7 were assayed for the secretion of cathepsin D by immunoprecipitation and were found to secrete markedly different levels (Fig. 1). Clone 3 secreted the least, approximately one-sixth the level produced by clone 2, which secreted the most. These differences do not reflect differences in the incorporation of [35S]methionine into the proteins secreted by the clones, since TCA-precipitable radioactivity in the conditioned medium from the same number of cells did not vary by more than 10% between the clones (data not shown). The anti-cathepsin D antibody used recognizes both the M, 52,000 pro-form of the enzyme and the M, 34,000 chain of the mature enzyme, but as previously reported the cells secreted almost exclusively the M, 52,000 form of the enzyme (4). The apparent molecular weight of pro-cathepsin D (46,000 or 52,000) depends on the weight assigned to the ovalbumin marker (43,000 or 46,000).

The invasiveness of the 9 MCF-7 clones was measured in a Boydne chamber assay, and Fig. 2 shows these results expressed as the number of cells having passed through the filter. The chemotactic migration of the clones was determined at the same time in parallel assays and was found not to vary significantly from clone to clone (not shown).
Fig. 2. Invasiveness of the MCF-7 clones. Invasion assays were conducted on the nine clones as described in "Materials and Methods." The cells that traversed the matrigel and spread on the lower surface of the filter were stained and quantitated electronically with the Zeiss IBIS 2001 image analysis system using a Kontron Aiag processor interfaced with a Zeiss Axiophot microscope equipped with an automated stage. The results are expressed as the mean number of cells (and the SE) per 10 high-power fields.

**DISCUSSION**

Since 1988 numerous studies examined the value of cathepsin D level as a prognostic indicator in breast cancer. The consensus of these studies is that high levels of cathepsin D in tumor cytosols are an indicator of poor prognosis, both in terms of shorter disease-free survival and overall survival (reviewed in Ref. 4). It remains to be seen whether this relationship is causal, and if so, what the underlying mechanism might be. Cathepsin D is a protease, and it is not surprising that a role for the enzyme in the degradation of extracellular matrix and hence in invasion and metastasis has been suggested (18). Studies have been conducted to try and demonstrate that cathepsin D can indeed degrade extracellular matrix, despite the fact that the...
The two sets of data (Pearson's r = 0.14), suggesting that cathepsin D (Figs. 1 and 2). However, there was apparently no correlation between pro-cathepsin D secreted varied significantly among the 9 clones. Conversely, epidermal growth factor receptor, and erb-B2 levels are all determined in whole tumor extracts primarily signify inflammatory infiltrates. It may be, therefore, that high cathepsin D levels associated with poor prognosis. There is evidence in the literature that these infiltrates. It may be, therefore, that high cathepsin D levels determined in whole tumor extracts primarily signify inflammatory cell involvement in the tumor and that such immune infiltration is associated with poor prognosis. There is evidence in the literature that lymphocytic infiltrates are associated with poor prognosis (19).

It was in light of this conflicting evidence that this study set out to determine the importance of tumor cell-derived cathepsin D to the invasive potential of human breast cancer cells. The Boyden chamber assay of chemo-invasion (7) was chosen to measure invasive potential in vitro because it is versatile and the accompanying migration assay allows one to discriminate between effects on the motility and viability of cells versus effects on their invasiveness. It was decided to use a series of single cell clones derived from the same cell line in initial experiments so that the cells would have generally similar properties, and indeed, the growth rate, hormonal responsiveness, estrogen receptor, epidermal growth factor receptor, and erb-B2 levels are all very similar among the clones. Both the invasiveness and the level of pro-cathepsin D secreted varied significantly among the 9 clones (Figs. 1 and 2). However, there was apparently no correlation between the two sets of data (Pearson’s r = 0.14), suggesting that cathepsin D is not an important determinant of invasiveness. Alternatively, the data could be consistent with the hypothesis that all of the clones secrete enough of the enzyme for it not to be rate limiting.

To assess more directly whether secreted pro-cathepsin D is important in invasion, assays were conducted in the presence of high concentrations of the specific aspartyl protease inhibitor pepstatin A. Cathepsin D is an aspartyl protease that is normally found in the lysosomes. It is secreted by breast cancer cells in its inactive pro-form, and low pHs (pH 3–5) are required to allow both autoactivation and subsequent activity of the mature enzyme (6, 16, 20, 21). Pepstatin A is a cleavage site analogue peptide and has little affinity for the enzyme at neutral pHs. However, under acidic conditions when the enzyme is only active at pHs far lower than would be expected at the well-perfused invading edge of a tumor (16).

It is notable that all the studies that have found cathepsin D to be a marker of poor prognosis have measured the protein levels in tumor cytosols. This means that the stromal component of the tumor contributes to the overall level of cathepsin D measured. Two immunohistochemical studies that examined cathepsin D levels in breast tumors showed that if the tumor cells alone were scored for cathepsin staining then high levels of the enzyme were found to be a marker of good prognosis, with increased overall and disease-free survival (5). This is in fact what one might expect from a protein the expression of which is stimulated by estrogen, as is found with the progesterone receptor. One of the studies (5) also noted that there were significant numbers of infiltrating inflammatory cells that stained strongly for cathepsin D, and it seems likely that some of the protein being detected in the studies that assayed tumor cytosols was extracted from these infiltrates. It may be, therefore, that high cathepsin D levels determined in whole tumor extracts primarily signify inflammatory cell involvement in the tumor and that such immune infiltration is associated with poor prognosis. There is evidence in the literature that lymphocytic infiltrates are associated with poor prognosis (19).

Table 1: The invasiveness, tumorigenicity, and cathepsin D secretion of 7 breast cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Invasion*</th>
<th>Nude Mouse*</th>
<th>Cathepsin D secretion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7-2</td>
<td>++</td>
<td>P</td>
<td>100</td>
</tr>
<tr>
<td>MCF-7-ADR</td>
<td>+++</td>
<td>P</td>
<td>0</td>
</tr>
<tr>
<td>MDA-MD-231</td>
<td>++++</td>
<td>LI</td>
<td>2</td>
</tr>
<tr>
<td>MDA-MB-435</td>
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<td>++++</td>
<td>P</td>
<td>25</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>+</td>
<td>N</td>
<td>48</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>+</td>
<td>N</td>
<td>98</td>
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</table>

* Data from Ref. 14, except where noted. P, primary tumor formation only, no local invasion or metastasis; LI, local invasion through peritoneum, colonization of visceral organs; M, metastasis to lungs and other organs; N, nontumorigenic. Activity in Boyden chamber assay graded as percentage MDA-MB-231: +, 0-20%; ++, 21-40%; ++++, 41-60%; ++++, 61-80%; +++++, >80%.
* Generated by densitometric scanning of the film from Fig. 6 and presented as a percentage of the MCF-7 clone 2 level.
* Data from Ref. 24.
* Unpublished observation.
enzyme is active, the peptide is an extremely potent inhibitor (20). The results from these invasion assays, shown in Fig. 3, demonstrate clearly that even when pepstatin was present in very high concentrations (100 μg/ml) it did not inhibit invasion by MCF-7 cells and indeed seemed to be slightly stimulatory. This stimulation is difficult to explain but was quite reproducible and might indicate that the pepstatin was inhibiting the degradation of other proteases that are important in invasion. The ease with which pepstatin is able to diffuse across matrigel-coated filters (Fig. 4) suggests that it is unlikely that the pepstatin was excluded from some extracellular "microenvironment" in which the cathepsin was active.

It has been reported that MCF-7 cells when plated on extracellular matrix produce large, actively acidified vesicles which contain mature cathepsin D and endocytosed extracellular material (16). It was proposed that in these vesicles cathepsin D might digest endocytosed extracellular matrix and thereby facilitate invasion and metastasis. Pepstatin A is a small molecule (MW 685), and it seems likely that if large molecules such as dextran can be endocytosed and conveyed to these large acidic vesicles (16), then the pepstatin would be carried along with any extracellular matrix being endocytosed for degradation, particularly since the data from the pepstatin diffusion experiments show that a layer of matrigel presents no barrier to the rapid diffusion of pepstatin. However, despite the huge concentrations used, it is possible that insufficient pepstatin was taken up to inhibit the lysosomal aspartyl proteases, particularly in view of a recent study demonstrating the poor uptake of leupeptin, a similar molecule (22).

To further assess the role of acidic compartments in the invasive- ness of breast cancer cells we conducted a series of invasion assays in the presence of concentrations of chloroquine shown to completely neutralize all the acidic compartments visible within the cell by light microscopy. This neutralization was verified by acridine orange staining. The neutralization of the acidic compartments did not produce any significant inhibition of the invasiveness of the cells, and indeed at some concentrations invasion seemed to be potentiated (Fig. 5). These data would again seem to argue strongly that cathepsin activity in these "large acidic vesicles" (16) or, for that matter, in the lysosomes is not important to the invasiveness of these cells in this model. The potentiation of invasion seen at some chloroquine concentrations may again be evidence that there are other proteases important in invasion, the degradation of which is an acid-dependent process.

To extend this study to other breast cancer cell lines with different properties in terms of Boyden chamber invasiveness and behavior in the nude mouse, pro-cathepsin D secretion by six other cell lines was measured (Fig. 6). Table 1 lists the level of cathepsin secretion for each cell line, along with data about their invasiveness in the Boyden chamber and behavior in the nude mouse. The mouse data are important because it is not clear how relevant the in vitro Boyden chamber assay data are to invasion in vivo. As is clear from this table, far from being associated with a more aggressive behavior, high levels of pro-cathepsin D secretion seem to be associated with a less tumorigenic and less invasive phenotype, at least for this series of cells. Of particular interest is the pair of cell lines MCF-7 and MCF-7ADR. The MCF-7ADR cells are an Adriamycin-resistant variant of MCF-7 (23) that have become hormone independent, lost expression of the estrogen receptor, and become more invasive and tumorigenic, and yet have virtually completely stopped secretion of cathepsin D. These data again suggest that cathepsin D secretion by breast cancer cells is not an important causal determinant of tumor cell aggressiveness.

The data presented in this paper provide evidence that cathepsin D is not important to the invasive phenotype of breast cancer cells, at least as measured by the Boyden chamber assay, and that high levels of intracellular and secreted cathepsin D are an indicator of a less aggressive phenotype in the small panel of cells examined. Taken together, these data argue against a causal involvement of tumor cell-derived cathepsin D as a marker of poor prognosis. This observation may be important in determining the appropriate protease to target for experimental antimitastatic therapies for breast cancer. The possibility remains that cathepsin D may be involved directly in the invasive process, but it is the enzyme derived from the stromal components of the tumor that is important. Alternatively, it has been suggested that high levels of cathepsin D are a marker of significant inflammatory cell involvement in the tumor and that this is associated with a poor prognosis. Further studies will be required to determine what, in fact, is the case.

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