Increased Secretion, Altered Processing, and Glycosylation of Pro-Cathepsin D in Human Mammary Cancer Cells

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ABSTRACT

In human mammary cancer cells, pro-cathepsin D (pro-Cath-D) is induced by estrogens and 50% of it is secreted. To determine whether its secretion is characteristic of mammary cells or transformed cells, we compared its production, processing, and glycosylation in primary cultures of normal mammary epithelial cells to those found in breast cancer cell lines. The cytosolic concentration of total cathepsin D (precursor and mature enzyme) measured by enzyme-linked immunosorbent assay was 8 times higher in cancer cells. Its mRNA level estimated by Northern blot analysis was 8 to 50 times higher and its secretion was 30 times higher in cancer cells. Using pulse-chase labeling, the cellular processing of pro-Cath-D was altered in hormone-dependent and -independent breast cancer cells in comparison to normal cells. This alteration resulted in a lower accumulation of mature enzyme, while the secretion and cytoplasmic accumulation of pro-Cath-D were greater in breast cancer cells than in normal cells. NH4Cl increased secretion of the proenzyme in normal cells but not in cancer cells. The secreted proenzyme was markedly heterogeneous and had a much acidic pi in MCF7 cells than in normal mammary cells. These acidic forms disappeared following endo-β-N-acetylglucosaminidase H treatment indicating that the structural difference between pro-Cath-D of normal and of cancer mammary cells was located on high mannose or hybrid N-linked oligosaccharides. This difference may be responsible for the altered routing of the pro-Cath-D in breast cancer cells.

INTRODUCTION

Secreted proteases are thought to have important functions in carcinogenesis, facilitating tumor invasion and growth (1, 2), and some of them are reported to be secreted more abundantly by cancer cells than by normal cells (3–5). In breast cancer cell lines, a M, 52,000 protein, identified as a pro-cathepsin D (6), is induced by estrogens in estrogen receptor-positive cell lines (MCF7, ZR75-1) and is also produced constitutively at high levels in hormone-independent cell lines (MDA-MB231, BT20). This protease can also be detected in primary breast cancer cells and assayed in cytosol using specific monoclonal antibodies (7). In vitro, the secreted M, 52,000 pro-Cath-D4 displays an autocrine mitogenic activity on MCF7 cells (8) and interacts with the mannose 6-phosphate/IGFII receptor via mannose 6-phosphate signals (6, 9). At acidic pH in vitro, this protease can also degrade the extracellular matrix (10), suggesting that it may be involved in mammary carcinogenesis (11), as proposed for cathepsin L in other cancers (4).

Oncogene products are thought to be carcinogenic following alterations in the structure, regulation, or degree of expression of normal protooncogenes (Ref. 12 and references therein). We have considered the possibility that pro-Cath-D also could favor some steps of mammary carcinogenesis by acquiring different properties during or following transformation. The amino acid sequence of the pro-Cath-D of MCF7 cells, deduced from sequencing its cloned cDNA (13), was found to be almost identical (except for one Ala to Val change in the profragment) to that of the pro-Cath-D of normal human kidney (14). However, the production and secretion of this protease were abundant in the breast cancer cell lines studied, and in vivo the cellular Cath-D concentration as estimated by immunohistochemistry appeared to be elevated in 70% of primary breast cancers and in benign mastopathies compared to normal resting mammary glands (15). However, this difference in concentration might have been due to a higher proliferative rate of cancer cells compared to normal mammary cells, which are mostly quiescent in nonpregnant women (doubling time, 100 days). Moreover, the proportion of pro-Cath-D secreted varies considerably according to the cell type (16). Although it is low (5–10%) in fibroblasts (17) it can reach 50% in kidney cells (18), hepatocytes (19), and endothelial cells (20). There are no data in the literature on the processing and secretion of pro-Cath-D in normal mammary epithelial cells. It was therefore crucial to compare the concentration and processing of pro-Cath-D in normal mammary cells and mammary cancer cells growing in culture at similar rates before concluding that differences in concentration and/or secretion are linked to cell transformation.

We show in the present study that in cancer cells pro-Cath-D is produced in larger amounts and is processed differently than in normal mammary cells. We also report on the first indication of structural differences related to posttranslational modifications producing more acidic N-glycosylated chains.

MATERIALS AND METHODS

Cell Culture. MCF7 cells obtained from the Michigan Cancer Foundation (Detroit, MI; MCF7 R) and Marc E. Lippman (Bethesda, MD; MCF7 L) and MDA/MB231 cells (21) were cultured in DMEM with fetal calf serum (10%) as described previously (22). "Normal" mammary cells were prepared as described previously (23) with minor modifications from mammary tissues collected at surgery from patients undergoing reduction mammoplasties. Histological diagnosis was performed in paraffin sections. Briefly, glandular epithelial structures (organoids) were isolated after digestion for 1–3 days with collagenase IA (250 IU/ml; Sigma) and hyaluronidase IS (200 IU/ml; Sigma). Dissociated organoids, 40 to 300 μm, were then cultured in Ham's F-12/DMEM (1/1), supplemented with 10% fetal calf serum, penicillin-streptomycin (25 IU/ml), and insulin (0.5 μg/ml) in 25-cm² culture flasks or 2-cm² 24-well dishes (Primaria; Falcon). After 15–20 days of culturing and before complete confluence, cells were analyzed for Cath-D content and secretion. Twelve of 33 samples were discordant for high contamination with fibroblasts. In the 21 remaining cultures, the proportion of epithelial cells was 80 ± 17% (mean ± SD) as checked by immunohistochemistry (9).

Received 2/1/89; revised 4/7/89; accepted 4/17/89.

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1 This work was supported by the Institut National de la Santé et de la Recherche Médicale, the Faculty of Medicine of Montpellier, the Association pour la Recherche sur le Cancer, the Groupement des Entreprises Françaises dans la Lutte contre le Cancer, and the Ligue Nationale contre le Cancer.

2 Recipient of the Ministère de la Recherche et de l'Enseignement Supérieur Fellowship Grant.

3 To whom requests for reprints should be addressed.

4 The abbreviations used are: pro-Cath-D, cathepsin D precursor (M, 52,000); Cath-D, cathepsin D (EC 3.4.23.5); DMEM, Dulbecco's modified Eagle's medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mann-6-P, mannose 6-phosphate; cDNA, complementary DNA; IGF, insulin-like growth factor; Endo H, endo-β-N-acetylglucosaminidase H; Endo F, endo-β-N-acetylglucosaminidase F.
Preparation of Conditioned Media and Cytoassay of Cath-D. Subconfluent cultures of normal mammary cells and cancer cells in T25 flasks were washed three times for 30 min with Ham's F-12 and DMEM, respectively, and conditioned in 2 ml of the same media for 6 or 18 h. The conditioned media were collected and centrifuged at 1800 × g for 5 min. Cells were harvested with phosphate-buffered saline (Gibco-BRL) containing 1 mm EDTA and subsequently centrifuged at 1800 × g for 5 min.

Cytosols were prepared at 0–4°C in Tris, EDTA, monothioglycerol and sodium borohydride buffer from frozen tissues or from pelleted cells obtained before or after culturing (24). Tissue pieces were homogenized using an Ultraturrax device. Cell pellets and cultured cells were disrupted by sonication at 100 W 3 times for 10 s followed by 60 s of cooling. Homogenates were then centrifuged at 15,000 × g for 30 min to obtain the cytosols. The pellets were used for DNA assay. Quantitation of total Cath-D (including the precursor and the mature enzyme) in media and cytosols was performed by enzyme-linked immunosorbent assay (24, 25).

Radioimmunoassay of Cath-D. Subconfluent cells were washed twice for 30 min in methionine-free DMEM (MCF7, MDA/MB) or Ham's F-12 (normal mammary cells) and then labeled for 6 or 18 h with 200 µCi/ml of [35S]methionine in methionine-free medium. Pulse-chase experiments were performed as described previously (26) except that the cells were preincubated for 30 min in methionine-free medium prior to addition of [35S]methionine. Labeled and Nonidet P-40 cell extracts were obtained as described previously (6). The Cath-D was immunoprecipitated using protein A-Sepharose, as described (27) with 6 µg/ml purified D8F5 monoclonal antibody to Cath-D and 1% Nonidet P-40 (w/v).

RNA Preparation and Northern Blot Analyses. Total RNA was extracted by the method of Auffray and Rougeon (28), electrophoresed on a 1% agarose-formaldehyde denaturing gel, and then transferred to nitrocellulose. As a cDNA probe, we used the 52K-9 cDNA (13), which contains most of the coding sequence of pro-Cath-D; it was 32P-labeled, using random primers (29), to a specific activity of 1 to 3 × 10^7 dpm/µg. Filters were hybridized and autoradiographed as described previously (13). The amount of RNA was determined by densitometric scanning of different exposures of the autoradiographs.

Treatment with Endoglucosidases. The secreted 35S-labeled pro-Cath-D was purified by immunoprecipitation from MCF7 cells or normal mammary cells treated with NHECl and then treated for 24 h at 37°C with the following enzymes: Endo H as described previously (6); Endo F (from Flavobacterium meninosepticum; Boehringer, Mannheim, France) at 3.6 units/ml in 100 mM sodium acetate (pH 7.0), 10 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 2 µM pepstatin A (Sigma). Control samples were incubated under the same conditions.

Isoelectric Focusing. Analytical isoelectric focusing in polyacrylamide slab gels was performed on an FBE 3000 apparatus from Pharmacia. Proteins in the gel were fixed and stained as recommended in "Materials and Methods." Pro-Cath-D was purified by immunoprecipitation from MCF7 cells or normal mammary cells treated with NH4Cl and then treated for 24 h at 37°C (from Flavobacterium meningosepticum; Boehringer, Mannheim, France) at 3.6 units/ml in 100 mM sodium acetate (pH 7.0), 10 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 2 µM pepstatin A (Sigma). Control samples were incubated under the same conditions.

RESULTS

Immunooxassay of Cath-D in Normal Mammary Cells Compared to Breast Cancer Cells. In previous experiments using immunohistochemistry, 70% of the breast cancer tissues were positively stained for Cath-D (7) whereas normal resting mammary glands collected from plastic surgery were negative (15). Since this difference could be due to the much slower growth rate in normal mammary glands, we cultured these glands prepared from reduction mammoplasties under conditions allowing their proliferation and assay Cath-D in the corresponding cytosols. Table 1 indicates that there was no significant increase in Cath-D concentration following mammary gland isolation or primary culturing. In primary culture, these cells grew with a doubling time of 3.0 ± 1.4 days (mean of 3 experiments ± SD) confirming the results of Yang et al. (34) and Stampfer et al. (35) and contrasting with their doubling time in vivo (100 days). Cath-D concentration appeared to be similar in normal mammary epithelial cells and connective tissue since there was no enrichment following isolation of epithelial organoid structures. The cytosol concentration of Cath-D in normal mammary epithelial cells was in fact 8 to 16 times lower than in breast cancer cells (Table 2). A similar difference was found between the mean values for normal mammary tissue and cancer tissues directly collected from patients (24) and that of organoids directly analyzed before culture.

Northern Blot Analysis of Pro-Cath-D mRNA. In an attempt to explain the higher cytosol concentration of Cath-D in cancer cells, we estimated the level of its mRNA in both normal and cancer mammary cells. Fig. 1 shows that, in MCF7 cells, the steady state level of the 2.2-kilobase Cath-D mRNA which is induced 5-fold by estradiol treatment (Fig. 1, Lanes a–b) was 8 to 50 times higher than in primary cultures of normal mammary epithelial cells prepared from three different patients (Fig. 1, Lanes c–e). This indicated that the expression of cytosolic Cath-D in human breast cancer cells is associated with an increase of its mRNA concentration.

Lower Secretion of Pro-Cath-D in Normal Mammary Cells Compared to Breast Cancer Cells. The amount of pro-Cath-D secreted by normal mammary cells in primary culture was found to be very low and in some cases undetectable. In 19 different
patients, a mean of 12–14 fmol of pro-Cath-D per µg of DNA per 6 h was found to be secreted by normal cells whereas breast cancer cells secreted 30 times more pro-Cath-D. A similar 30-fold difference was found after [35S]methionine labeling of proteins followed by immunoprecipitation of pro-Cath-D (Table 2). The antibodies to Cath-D react with similar affinity with the pro-Cath-D of MCF7 cells and that of normal cells indicating that the lower secretion by normal cells was real and not due to an altered interaction of antibodies with different antigens. When analyzed by SDS-PAGE, the pattern of proteins labeled by [35S]methionine and secreted by normal epithelial cells in culture differed from that of breast cancer cells (Fig. 2, Lanes a and b) and from that of cellular proteins, indicating protein secretion rather than cell lysis (not shown). The pro-Cath-D was generally not visible in medium conditioned by normal mammary cells in contrast with that produced by MCF7 cells.

Altered Cellular Processing of Cath-D in Mammary Cancer Cells Compared to Normal Cells. The steady-state level of the three forms of Cath-D can be visualized by silver staining of SDS-PAGE following purification (Fig. 2, Lanes c and d). In MCF7 cells, all three forms were stained, with a high proportion of the nonprocessed precursor (M, 52,000) and of the intermediate (M, 48,000) form (Fig. 2, Lane c), whereas in normal mammary cells, only the mature M, 34,000 form was stained (Fig. 2, Lane d) which is consistent with a normal processing of Cath-D into lysosomes.

The altered processing of cellular pro-Cath-D was confirmed by pulse-chase experiments. About 45–59% of the precursor was secreted by estradiol-treated MCF7 cells (Tables 2 and 3), whereas only 3–5% is secreted in the absence of estrogens (26). Two estrogen receptor-negative cell lines (MDA-MB231 and BT20) constitutively secreted high levels of precursor, indicating that this high secretion is not related to the estrogen responsiveness of cancer cells. In normal mammary cells, the percentage of the secreted precursor varied between 0 and 12% (Tables 2 and 3). It reached 20% in one benign ductal hyperplasia (not shown). The increased secretion in cancer cells was accompanied by an altered intracellular processing of pro-Cath-D.

**Table 3** Processing of [35S]pro-Cath-D in cancer and normal mammary cells

<table>
<thead>
<tr>
<th>Secreted pro-Cath-D' at 10 h (%)</th>
<th>Cellular pro-Cath-D half-life (h)</th>
<th>M, 34,000' at 10 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>59</td>
<td>6</td>
</tr>
<tr>
<td>L</td>
<td>56</td>
<td>3</td>
</tr>
<tr>
<td>MDA/MB231</td>
<td>66</td>
<td>4</td>
</tr>
<tr>
<td>BT20</td>
<td>40</td>
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<td>NMC&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>2</td>
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<tr>
<td>NMC&lt;sub&gt;c&lt;/sub&gt;</td>
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<sup>a</sup> The [35S]-labeled pro-Cath-D (M, 52,000) secreted following 10 h of chase was immunoprecipitated with the D85 monoclonal antibody.

<sup>b</sup> The cellular pro-Cath-D half-life was evaluated from Fig. 3.

<sup>c</sup> M, 34,000 is the large chain of the mature Cath-D.

<sup>d</sup> The 100% value is the number of densitometric units of cellular pro-Cath-D at 0 h of chase.

<sup>e</sup> NMC<sub>c</sub>, and NMC<sub>c</sub>, normal mammary epithelial cells from two different patients.

D. In MCF7, BT20, and MDA-MB231 cells, cellular pro-Cath-D decreased slowly with a half-life of 3–6 h, whereas the labeled M, 48,000 and M, 34,000 mature forms increased slowly with time (Fig. 3, Lanes a–d). By contrast, the cellular processing of pro-Cath-D was faster (half-life, 2 h) in normal mammary cells (Fig. 3, e–f). The proportion of the M, 34,000 mature form at 10 h of chase was much higher in normal cells than in breast cancer cells. This was also observed by immunoprecipitation of the Cath-D following [35S]methionine labeling (Table 3; Fig. 3).
Fig. 3. Pulse-chase experiments with normal mammary cells and cancer cells. Normal mammary cells (NMC) and breast cancer cells (MCF7 L and R, BT20, and MDA/MB231) were labeled for 1 h with [35S]methionine and chased for the indicated times with unlabeled methionine. The same amounts of Nonidet P-40 cell extracts, based on trichloroacetic acid-precipitable counts, were immunoprecipitated with the Cath-D monoclonal antibody. The immunoprecipitates were analyzed on a 15% acrylamide gel and the bands were quantified by scanning the fluorogram as described by Morisset et al. (26). A, molecular weight in thousands.

Since the cellular processing of lysosomal enzymes and their routing to lysosomes is mostly mediated by their interaction with Man-6-P receptors, a defect in the interaction of pro-Cath-D with this receptor was suspected. A pH increase in acidic organelles following NH4Cl treatment of cells decreases the concentration of available Man-6-P receptors leading to an increased secretion of lysosomal enzymes (36, 37). As expected, NH4Cl markedly increased the secretion of pro-Cath-D by normal mammary cells (Fig. 4A, Lanes a and b; Fig. 4B, Lanes a and c). By contrast, NH4Cl had no effect on pro-Cath-D secretion in MCF7 and MDA-MB231 cells (Fig. 4A, Lanes c and d; Fig. 4B, Lanes b and d) which was already maximal before alkalinization. The NH4Cl resistance of MCF7 cells suggests that the Cath-D interaction with Man-6-P receptors is already modified in transformed cells. In MCF7 cells, the resistance to NH4Cl appeared to require a functional and activated estrogen receptor, since in estrogen-deprived cells, NH4Cl was again effective in increasing the secretion of pro-Cath-D (Fig. 4, Lanes b and d).

Differences in the N-Glycosylation of Pro-Cath-D. Modifications in the intracellular routing to lysosomes could be due to structural alterations of pro-Cath-D. Nucleotide sequencing of the complete cDNA cloned from MCF7 Cath-D (13) showed a single amino acid (Ala to Val) modification compared to normal kidney Cath-D (14). This was not observed in another human breast cancer cell line, ZR75-1 (38) suggesting that it may correspond to polymorphism. Several sequences of cloned cDNA corresponded to a single sequence of the protein, but isoelectric focusing analysis of the secreted pro-Cath-D indicated a high heterogeneity of pIs (Fig. 5) suggesting different

Fig. 4. Effect of NH4Cl on the secretion of the pro-Cath-D in normal mammary, MCF7, and MDA-MB 231 cells. A, normal mammary cells (NMC) and MCF7 cells grown in DMEM plus 10% fetal calf serum, were labeled for 18 h with [35S]methionine in the presence (+) or absence (−) of 10 mM NH4Cl. Immunoprecipitates from the collected media were run on SDS-PAGE and revealed by fluorography as in "Materials and Methods." B, same experiments as above except for cultures 7 and 10 where cells were grown in DMEM plus 10% fetal calf serum treated with dextran-coated charcoal to remove steroid hormones. The cells were either unlabeled and Cath-D was assayed by enzyme-linked immunosorbent assay (Lanes a and b) or labeled as in A (Lanes c and d) and Cath-D was immunoprecipitated as described by Morisset et al. (26). Numbers above columns, 4 cultures of NMC from different patients (Columns 1-4) and 6 different passages of MCF7 cells (Columns 5-10). MDA-MB 231 (MDA) were tested in parallel. Note the different scale of ordinates for MCF7 and MDA cells and normal mammary cells.
may be involved in both cell proliferation and tumor invasion. In the present study, we compared for the first time Cath-D produced by normal and cancerous mammary cells growing at similar rates. We show two differences. (a) The steady state cellular concentrations of Cath-D and its 2.2-kilobase mRNA are markedly increased in cancer cells, suggesting that the increase is more associated with the transformation of mammary cells than with their proliferation. (b) There is an even larger increase in the secretion (30-fold) of pro-Cath-D by cancer cells. This increased secretion is associated with, and may be the consequence of, altered processing of pro-Cath-D, which is totally processed into the mature form (M, 34,000) in normal mammary cells, whereas at least 45% of it is secreted as the proenzyme by breast cancer cells. The altered processing of Cath-D was observed in both the hormone-dependent and -independent breast cancer cell lines studied. This is also in agreement with the immunoblot assay of pro-Cath-D in the cytosol of mammary cells which represented an average of 10% of total Cath-D in 136 breast cancers (24), whereas it was undetectable in normal mammary epithelial cells. The molecular mechanism for this altered processing may be related to the structure of pro-Cath-D, since its secretion appears to be increased more than those of other lysosomal enzymes.7-9 The amino acid sequence of the pro-Cath-D of breast cancer cells (13) and the amino acid analysis of the purified secreted pro-Cath-D of MCF7 cells10 are very similar to those of normal pro-Cath-D. By contrast, pro-Cath-D of MCF7 cells contains more acidic groups located on N-glycosylated chains sensitive to Endo H digestion. Further work is required to identify the groups (sialic acids, phosphates, sulfates, etc.) responsible for the lower pl of isoenzymes in cancer cells.

DISCUSSION

Two types of proteins induced by estrogens and secreted by breast cancer cells are thought to mediate the stimulatory effect of estrogens on the growth and invasiveness of breast cancer cells. Growth factors such as transforming growth factor α and IGFI appear to be mostly associated with the cell growth rate since their concentration is similar in normal proliferating epithelial mammary cells and in breast cancer cells (39). Proteases such as plasminogen activator (40) and pro-Cath-D (11) have been found to be increased in NIH 3T3 fibroblasts after transfection of cathepsin L (4) and transin (5) were shown to be secreted by cancer cells (43). Our results with Cath-D suggest that the precursor of cathepsin B is abnormally produced by normal mammary cells growing at similar rates. We show two differences, (a) The steady state concentration of Cath-D in NIH 3T3 fibroblasts after transformation by oncogenes or after growth factor treatment. It has also been shown that the precursor of cathepsin B is abnormally secreted by cancer cells (43). Our results with Cath-D suggest a general characteristic of cancer cells that have acquired the ability both to secrete more lysosomal proteases and to be more invasive. They also indicate that the kind of protease altered varies according to the type of tissue which is being transformed.

Increased production of proteases has previously been implicated in the transformation process (1, 43). Recently, the production and secretion of cathepsin L (4) and transin (5) were found to be increased in NIH 3T3 fibroblasts after transformation by oncogenes or after growth factor treatment. It has also been shown that the precursor of cathepsin B is abnormally secreted by cancer cells (43). Our results with Cath-D suggest a general characteristic of cancer cells that have acquired the ability both to secrete more lysosomal proteases and to be more invasive. They also indicate that the kind of protease altered varies according to the type of tissue which is being transformed.

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1 F. Capony, unpublished results.
2 T. Braulke and K. Von Figura, personal communication.
In mammary cancer, pro-Cath-D is the acidic protease most abundantly secreted in conditioned media (10); whereas in other transformed cells, the amount of cathepsin L or cathepsin B is more important. Transfection with the full-length cDNA of pro-Cath-D in normal and cancer cells will indicate whether or not increased production and secretion of pro-Cath-D can facilitate tumor invasion and may also reveal the mechanism of the altered processing of pro-Cath-D.

ACKNOWLEDGMENTS

We would like to thank Drs. J. P. Reynaud and A. de Ricard for kindly providing human mammary tissue, and Dr. Per Briand (Fibiger Institute, Copenhagen, Denmark) and Drs. F. Vignon and M. Garcia for critical reading of the manuscript. We are grateful to J. Duponté, S. Khalaf, and D. Francès for the Cath-D enzyme-linked immunosorbent assay and to M. Egela and E. Barrie for typing the manuscript.

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