Cathepsin D in Breast Cancer Cells Can Digest Extracellular Matrix in Large Acidic Vesicles

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ABSTRACT

In breast cancer cell lines, pro-cathepsin D is synthesized in excess and abnormally processed, resulting in its slower maturation and increased secretion into the culture medium. Since this lysosomal protease is only active at acidic pH, we have searched for acidic compartments other than lysosomes where cathepsin D might be active when MCF7 cells are plated on corneal extracellular matrix. We found large acidic intracellular vesicles (1.5 to 20 μm in diameter) by acridine orange and 3-(2,4-dinitroanilino)-3'−amino-N-methyl dipropylamine staining, two fluorescein probes which reveal acidic compartments. These vesicles were actively acidified. They were 2- to 20-fold more abundant in MCF7 breast cancer cells and primary cultures of human breast cancers cells than in primary cultures of normal mammary epithelial cells. In living MCF7 cells, high resolution video-enhanced microscopy showed that these vesicles were mobile and intracellular. Double immunolocalization indicated that they contained mature cathepsin D (but no detectable pro-cathepsin D) and endocytosed extracellular material. This material (dextran, transferrin, and extracellular matrix) and the association with other lysosomal enzymes varied according to the vesicles, suggesting their heterogeneity (large endosomes or phagosomes).

We conclude that, in breast cancer cells, cathepsin D may digest intracellularly phagocytosed and/or endocytosed extracellular matrix in large acidic vesicles. We propose that the higher expression of cathepsin D associated with the increased number of large acidic vesicles in breast cancer cells may facilitate digestion of basement membrane and consequently metastasis.

INTRODUCTION

Neutral proteases have long been thought to be involved in the degradation of ECM and tumor invasion (for review see Ref. 1), mainly plasminogen activator (for review see Ref. 2) and collagenases (3). Among the cathepsins, whose normal function is to digest proteins in lysosomes at acidic pH, cathepsin B-like enzymes (4) and cathepsin L (5), both of which are secreted as proenzymes (6), have also been suspected to play a role in metastasis (7, 8).

In breast cancer cells, however, the aspartyl protease pro-cathepsin D appears to be more important (Ref. 9 and references therein). While in normal cells, including human mammary epithelial cells (10), more than 90% of the lysosomal enzymes bearing mannose-6-phosphate residues are sorted in the Golgi apparatus and targeted to lysosomes (for review see Refs. 11 and 12), up to 50% of the pro-cathepsin D is misrouted and secreted by breast cancer cells. Compared to normal cells, breast cancer cells contain a greater but variable amount of cytosolic cathepsin D and secrete 30-fold more pro-cathepsin D. The processing of this proenzyme into mature cathepsin D is much slower (10). At low pH, the secreted M, 52,000 pro-cathepsin D can be autoactivated into a M, 51,000 cathepsin D, which in vitro can digest ECM prepared from bovine corneal endothelium (13). Cultured breast cancer cells can also digest ECM (14). Finally, retrospective clinical studies indicate that a high concentration of cathepsin D in breast cancer cytosol is a marker of aggressive breast cancer (15, 16). Together, these data suggest that cathepsin D may behave in vivo as a protease, digesting the basement membrane underlying the noninvasive transformed cells and consequently facilitating tumor invasiveness and metastasis. However, such an effect would require sufficiently acidic pH, which has not been demonstrated outside the cells.

An alternative possibility is that misrouted cathepsin D may digest endocytosed or phagocytosed extracellular material within cancer cells. Hence, we plated normal and tumoral mammary cancer cells onto bovine corneal extracellular matrix, in order to search for acidic microenvironments in which cathepsin D may digest extracellular material.

MATERIALS AND METHODS

Cell Culture. Human breast carcinoma cell line MCF7 (17) (originally supplied by the Michigan Cancer Foundation, Detroit, MI) and MDA-MB231 (18) from V. Piczac (Masonic Research Institute, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum supplemented with 0.6 μg/ml bovine insulin (Collaborative Research, Lexington, MA), 25 units/ml penicillin/streptomycin, phenol red, and 2 mM L-glutamine (Flow Laboratories, Ltd, Ayrshire, UK). Normal human mammary cells were obtained immediately after mammoplasty surgery performed for cosmetic purposes, and were cultured as described (19). Following enzymatic digestion, glandular epithelial structures 40- to 300-μm in size were plated on glass coverslips coated with ECM and were allowed to grow for 10 to 20 days with F12/Dulbecco's modified Eagle's medium (1/1) containing the same additives as for the cell lines. Human breast cancer cells were obtained either from a primary tumor after enzymatic digestion or directly from pleural effusions of metastatic breast cancers, following collection by thoracentesis from patients who had not received chemotherapy. Cells from primary tumor were cultured for 10-20 days like the normal cells. Cells from pleural effusion were cultured as described (20) and generally studied 4-5 h following plating. The nontumorigenic human breast epithelial cell line HMT 3522 was a gift from P. Briand (The Fübig Institute, Copenhagen, Denmark) and was maintained in defined medium, as described (21). For evaluating the estradiol effect, MCF7 cells were first grown for 6 days on ECM in medium without phenol red, containing serum depleted in steroids by treatment with dextran-coated charcoal, and then treated or not with 10 nM estradiol for 2 days.

Unless specified, all mammary cells were plated on ECM substrate, which was synthetized by corneal endothelial cells of freshly enucleated bovine eyes collected from a local slaughterhouse and prepared according to the method of Gospodarowicz (22). Fluorescence of Acidic Vesicles. Acidic vesicles were detected using two fluorescent probes. In living cells, we used acridine orange (23), a fluorescent tertiary amine which becomes concentrated in its protonated...
counted in double-blind manner in the indicated number of cells (corresponding to approximately 20 fields for each experimental condition). Two typical experiments are represented in a and b. Other experiments performed under the same conditions gave similar results. The range represents the variability of LAV content/100 cells/field counted. The P values were calculated in each experiment using two nonparametric signed rank tests: the Kruskal-Wallis test for three groups of data and the Wilcoxon-Mann-Whitney test for paired data. a. Absence of effect of estradiol (E2) on cells cultured in medium with fetal calf serum (FCS) or with fetal calf serum deprived of estrogens by a dextran-coated charcoal (DCC) treatment in medium without phenol red, with or without 10 nM estradiol. b. Absence of effect of cell detachment by trypsin digestion on the number of LAVs in MCF7 cells.

**Table 1** Quantification of large acidic vesicles in estrogen-treated and detached MCF7 cells

<table>
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<tr>
<th>Total no. of vesicles</th>
<th>Total no. of cells</th>
<th>No. of vesicles/100 cells</th>
<th>Mean</th>
<th>Range</th>
<th>P value</th>
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<td>a. Effect of estradiol</td>
<td></td>
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<td>MCF7 FCS</td>
<td>104</td>
<td>1132</td>
<td>9.4</td>
<td>7.01;14.3</td>
<td>0.01;17.1</td>
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<tr>
<td>MCF7 FCS + E2</td>
<td>183</td>
<td>1903</td>
<td>9.3</td>
<td>5.3;14.9</td>
<td>NS*</td>
</tr>
<tr>
<td>b. Effect of cell detachment</td>
<td>MCF7 on ECM</td>
<td>141</td>
<td>1499</td>
<td>9.2</td>
<td>6.3;12.3</td>
</tr>
<tr>
<td>Detached MCF7</td>
<td>203</td>
<td>2102</td>
<td>9.7</td>
<td>4.2;12.7</td>
<td>NS*</td>
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* NS, not significant.

In fixed cells, we stained acidic vesicles using DAPM (a gift from R. G. W. Anderson, University of Texas, Dallas, TX), a tertiary amine with the same properties as acridine orange but which can be detected by antidiitrophenol antibodies after aldehyde fixation (24). Cells incubated for 30 min in 30 nM DAPM at 37°C were fixed in 4% paraformaldehyde in phosphate-buffered saline. They were then used for immunocytochemical fluorescence detection of the antigen to be colocalized with DAPM (see below) and treated with 0.1% Triton X-100 (3 min, -10°C). The DAPM was visualized with rabbit antidiitrophenol antibodies (ICN ImmunobiologicaLs, Lisle, IL).

**High-Resolution Video-enhanced Microscopy.** Live or fixed cells were viewed at 37°C under differential interference contrast microscopy or epifluorescence at low UV illumination levels, using a Reichert Polyvar microscope with a x100 NA. 1.32 planapochromat lens. Video frames were attained using a Chalnicon (for DICC microscopy) or a silicon-intensiﬁed target camera (for fluorescence microscopy) (LHESA Electronique, Saint-Ouen l’Aumône, France). The appropriate grey level setting was kept constant throughout the experiments. Video frames were digitized on a 256-grey level scale with an A/D converter in a Crystal image processor (Quantel Micro Consultants, Montigny le Bretonneux, France) connected to a microcomputer. Fluorescence video images were obtained by averaging from 4–16 (real-time images) to 256 (fixed images) frames, which were recorded in real time on 1⁄4-inch video cassettes (Sony) or in time lapse on a hard disk. For fixed images, the camera background light and auto-fluorescence were recorded and subtracted from the registered image. Photographs were taken directly from the video monitor screen.

**Immunoctytochemistry and Enzyme Detection.** For the localization of cathepsin D, MCF7 cells were fixed in a mixture of 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M cacodylate buffer and were permeabilized with 0.05% saponin. Cells were incubated with M2E8 (25) or M1G8 (26) monoclonal antibodies, which interact specifically with the M, 52,000 pro-cathepsin D or total cathepsin D, respectively, labeled with RITC- or FITC-conjugated rabbit anti-mouse antibodies (Bio-Veda, Rehovot, Israel), and observed by epifluorescence. Double labelings were performed by association of FITC and RITC labelings. Breast cancer cells in primary culture were tested for staining with monoclonal antipankeratin (Biosoft, Paris, France), 115D8 anti-milk-fat globules (27), and MY-4 antimacrophages (Coulter

![Fig. 2. Quantification of large acidic vesicles in breast cancer cells and normal mammary cells. MCF7 cells and primary cultures of a primary breast cancer, pleural effusions, and normal mammary cells. MCF7 cells and primary cultures of a primary breast cancer, pleural effusions, and normal mammary cells.](image-url)

![Fig. 1. Evidence for LAVs in breast cancer cells. The MCF7 cell lines and primary cultures of mammary epithelial cells were plated on ECM from bovine corneal endothelium (ECM) and allowed to grow until confluence in medium with fetal calf serum (FCS). Cells were then detached with trypsin, washed, and incubated in acridine orange, as described in Fig. 1.](image-url)
Fig. 3. Intracellular localization of LAVs. MCF7 cells on ECM were incubated with acridine orange, as described in Fig. 1. The cells were observed with conventional light microscopy (a and b) and with video-enhanced microscopy (c to e), under phase contrast (a), differential interference contrast (c and e), and epifluorescence (b and d). Two different fields are represented in a plus b and c plus d. Bars = 10 μm (a and b) or 5 μm (c to e). a and b. Following staining with acridine orange, MCF7 cells were detached from their substrate by trypsin digestion and observed after cytocentrifugation. One LAV is indicated by an arrow. LAV numbers were similar to those found in plated cells (see also Table 1), indicating that they were intracellular. c and d. Three rough large vesicles are shown (arrows), having heterogeneous contents (c). Two of them are intensely stained with acridine orange and included within the cell, as observed by varying the focusing. Smaller vesicles, probably representing lysosomes, are clustered around the nucleus (N). e. A LAV (* in e) moves laterally and rotates within the cell, strongly suggesting its intracellular location. These movements are evidenced on time-lapse images and video-enhanced microscopy, taking the notch in the nucleus (N in e) as a fixed reference position. Video frame time lapses are indicated in s in the lower left.
Fig. 4. Colocalization of cathepsin D with DAMP and endocytosed extracellular markers. The same MCF7 cells were compared by double fluorescence staining in an attempt to show the colocalization of cathepsin D (b, f, and h) or pro-cathepsin D (d) with the DAMP probe for acidic vesicles (a and c) or with endocytosed RITC-dextran (e) or RITC-transferrin (g). Bar = 10 μm. a and b, DAMP (30 μM, 30 min, 37°C), which concentrates in acidic cell compartments and is recognized by antinitrophenol antibodies, was used together with the M1G8 anticathepsin D antibody (26). In addition to lysosomes, two LAVs (arrows) are stained with both FITC-labeled DAMP (a) and RITC-labeled cathepsin D (b). c and d, DAMP, as above, was used together with the M2E8 antipro-cathepsin D antibody (25). Very small vesicles are stained by anti-DAMP (c) or antipro-cathepsin D (d) but the two LAVs (arrows) are stained by anti-DAMP and not by the antipro-cathepsin D. e and f, MCF7 cells incubated with RITC-dextran, M, 70,000 (500 μg/ml, 3 min), as a marker for fluid phase endocytosis, were fixed and processed for staining with FITC-coupled M1G8 anticathepsin D antibody. One LAV (arrow) contains both internalized M1G8 anticathepsin D (f) and engulfed RITC-dextran (e). Another LAV (arrowhead) contains RITC-dextran but no cathepsin D, g and h, MCF7 cells incubated for 10 min with RITC-transferrin (500 μg/ml) were fixed and stained by the M1G8 anticathepsin D antibody. A LAV (arrow) contains both internalized transferrin (g) and cathepsin D (h).
Immunology. Hialeah, FL) with a secondary antibody coupled to horse-radish peroxidase.

Negative controls were obtained by incubation with irrelevant primary mouse antibodies (MOPC 21; Litton Bionetics, Kensington, MA) before peroxidase or fluorescein labeling or with secondary antibodies alone. Acid phosphatase activity was revealed by histochemistry according to the method of Barka and Anderson (28), using 1.25% sodium β-glycerophosphate as substrate with 0.2% lead nitrate in Tris maleate buffer, pH 5.2, at 37°C for 45 min. β-Glucuronidase activity was detected according to the method of Hayashi et al. (29), with a 90-min incubation time.

Internalization of Fluorescent Extracellular Material. Cells were grown to near confluence on glass coverslips, incubated in 500 μg/ml RITC-dextran 70s, M, 70,000 (Sigma Chemicals, St. Louis, MO), in complete medium for 3 to 15 min at 37°C, and then extensively washed. The coverslips were mounted so as to form sterile chambers (see above) for observing alive or fixed immunostained cells. RITC was coupled to ferrotransferrin (Sigma) in 0.1 M borate buffer, pH 9.0, 0°C, and dialyzed in phosphate-buffered saline. One mg/ml of RITC-transferrin was allowed to bind to receptor for 1 h at 4°C in medium containing 0.1% bovine serum albumin (w/v). After removal of unbound transferrin by washings, internalization was performed at 37°C for 10 min, before cell fixation and immunochemical staining.

Fluorescent ECM was obtained on coverslips by covalent coupling with 0.01 mg (0.05 μg/ml) of DTAF (Sigma) for 10 min in 0.2 M bicarbonate buffer, pH 9.0, at room temperature. The reaction was stopped by adding 50 mM Tris buffer. MCF7 cells were then plated onto the DTAF-coupled ECM, incubated under normal culture conditions for 2 days, and mounted to form a sterile chamber. Control MCF7 cells were plated on plastic and incubated for 2 days in complete medium containing 0.05 μg/ml DTAF.

The fate of RITC-dextran or DTAF-coupled ECM was monitored in the cells at 37°C, using reflected fluorescence (545 nm excitation, 590 nm emission and 490 nm excitation, 515 nm emission, respectively) with low light-level illumination and high resolution video-enhanced microscopy, in parallel with DIC microscopy. At these low radiation levels, cells remained alive for several hours.

RESULTS

Cultured Breast Cancer Cells Contain Large Acidic Vesicles Stained by Acridine Orange. MCF7 cells cultured on bovine ECM to confluence (Fig. 1a) were stained with acridine orange, which gives a red-orange fluorescence when concentrated in acidic vesicles (23) (Fig. 1b). Most cells contained many small acidic vesicles (∼0.5 μm) forming small bright spots and corresponding to lysosomes and possibly endosomes. They were variably clustered in the cytoplasm or concentrated in the Golgi zone close to the nucleus. In addition, LAVs (1.5 to 20 μm in diameter) were seen in both confluent and isolated cells; their numbers varied from 1 to 10/cell (Fig. 1b).

Addition of 20 mM ammonium chloride 15 min before incubation with acridine orange, raising the internal pH of acid cell compartments, totally removed the fluorescence of lysosomes and LAVs (Fig. 1c). Without alkalinization, acridine orange staining of cellular vesicles was constant for more than 8 h. Fluorescence reappeared following incubation in the absence of ammonium chloride (Fig. 1d), indicating that LAVs were able to actively acidify their contents. To accurately quantify LAVs (Table 1, Fig. 2), we counted only the large LAVs of ≥5 μm diameter, allowing us to use a low magnification for counts of...
FIG. 6. Uptake of extracellular rhodamine-labeled dextran in LAVs. MCF7 cells on ECM were incubated for 3 min (a and b) and 15 min (c to f) with rhodamine-coupled dextran, M, 70,000, and observed by high resolution video-enhanced microscopy, using differential interference contrast (a, c, and e) and epifluorescence (b, d, and f). Bars = 5 μm. a and b. A rough and heterogeneous LAV (arrow), close to the nucleus (N), is stained 3 min after incubation with fluorescent dextran. c and d. Within the same cell, several smooth and homogeneous LAVs do not contain dextran, whereas several small vesicles do. N, nucleus. e and f. The degree of dextran uptake into LAVs is highly variable according to the vesicles. In this figure, one smooth vesicle shows very strong fluorescence (thin arrow), whereas others do not (arrowheads), one of the more heterogeneous vesicles shows only faint fluorescence (curved arrow). In other fields, rough vesicles incorporate dextran more actively than smooth vesicles (not shown). N, nucleus.

at least 20 fields or 1000 cells in each experimental condition. As shown in Table 1a, the presence of estradiol and cell detachment by trypsin had no significant effect on the number of these LAVs in MCF7 cells. Fig. 2 shows that LAVs (≥5 μm) were observed in 2–23% of MCF7 cells, depending on the experiments, with a mean of 9.2%. Their numbers were not modified by culturing MCF7 cells on plastic.

LAVs were also present in estrogen-independent mammary breast cancer cells (MDA-MB231) (Fig. 2). The mean number of vesicles/100 cells was consistently and significantly (P < 0.01) higher in breast cancer cell lines (mean, 9.4%) than in normal mammary cells (mean, 0.8%; Fig. 2), suggesting that LAVs are associated with cell transformation. LAVs were not restricted to permanent cell lines but were also observed in breast cancer cells plated in primary culture. Cells from a primary breast cancer contained 7.5% LAVs. The metastatic breast cancer cells from pleural effusion contained a similar amount of LAVs (mean, 8.8%), significantly higher (P < 0.01) than in normal mammary cells (Fig. 2). LAV numbers in cancer cells of pleural effusion did not significantly vary with culture time (from plating up to 17 days) or with subculturing, suggesting that they are not a culture artifact (data not shown). The epithelial and mammary origins of normal and cancer cells analyzed in primary culture were evidenced by staining with...
cunae between the basal side of the cells and the ECM. Secondly, we visualized the LAVs in living cells using video-enhanced microscopy. By combined fluorescence and DIC microscopic observation, the large acidic fluorescent compartments were clearly located between the basal and apical plasma membrane focal planes, generally close to the nucleus (Fig. 3, c and d). Most were round-shaped but some were distorted (not shown), and the contents, as seen by DIC and fluorescence, were either smooth and homogeneous or rough and heterogeneous (Fig. 3c; see also Figs. 6 and 7). Thirdly, real-time video images showed that these vesicles were mobile. The small acidic vesicles (perhaps primary lysosomes) moved along tracks at rates of up to 5 μm/s over distances of 15 μm, whereas LAVs mostly rotated, as shown by time-lapse images and comparison with more static structures such as nuclei (Fig. 3e). We conclude that most LAVs seen by video-microscopy are intracellular.

LAVs Contain Cathepsin D But No Detectable Pro-cathepsin D. By immunofluorescence, cathepsin D was found to be located in the lysosomes, which also are stained with acridine orange. Most LAVs were also labeled by the fluorescent anticathepsin D antibody. A short incubation of living cells with ammonium chloride, which abolishes acridine orange staining, did not alter fluorescence detection of cathepsin D in lysosomes (not shown).

To demonstrate that the same large vesicles were acidic and contained cathepsin D, we used double fluorescence labeling of cathepsin D and of DAMP, which concentrates in the acidic compartments of cells. In all cases, LAVs containing cathepsin D also reacted with antibodies to DAMP, indicating that cathepsin D was in an acidic milieu allowing its activity (Fig. 4, a and b). Some smaller vesicles were sometimes revealed by cathepsin D antibody but not by DAMP antibody. However, when using monoclonal antibodies raised against pro-cathepsin D, only small vesicular compartments of the cells (possibly Golgi and prelysosomal compartments) were stained, suggesting that in the LAVs most of the pro-cathepsin D had already been processed into the mature enzyme (Fig. 4, c and d). Some of the LAVs in cancer cells also contained high concentrations of acid phosphatase and β-glucuronidase, suggesting that they may correspond to large secondary lysosomes (phagolysosomes) (Fig. 5).

Endocytosed Markers Are Recovered in Large Acidic Vesicles. We used different markers to specify the mechanism of internalization of extracellular material into LAVs of MCF7 cells. Figs. 4e and 6 demonstrate the ability of MCF7 cells to take up RITC-dextran (M, 70,000) as a marker of fluid-phase endocytosis. As early as 3 min (Fig. 6, a and b) and 15 min (Fig. 6, c-f) after the addition of dextran, some of the large vesicles were labeled, which suggests that these LAVs may be formed by fusion of endocytic vesicles. Some of the vesicles containing dextran also contained cathepsin D, as shown by double immunostaining, whereas other vesicles contained dextran but no cathepsin D (Fig. 4, e and f) and vice versa (not shown). Normal mammary epithelial cells, which contained fewer LAVs than breast cancer cells (Fig. 2), were, however, also able to internalize and concentrate RITC-dextran (not shown). RITC-labeled transferrin, a marker of receptor-mediated endocytosis, was also present together with cathepsin D in most of the LAVs (Fig. 4, g and h). However, some LAVs were not labeled by transferrin (not shown). When MCF7 cells were cultured onto fluorescent extracellular matrix for 3 days, under conditions used to study ECM degradation by cancer cells (14), they incorporated the ECM intracellularly in some LAVs (Fig. 7). The intensity of vesicle fluorescence varied irrespective of size and rough or smooth aspects. Control MCF7 cells plated on
plastic with free DTAF in the medium (0.05 μg/ml) also took up some DTAF, but in much lower quantities than when cells were plated onto DTAF-coupled ECM (not shown). This indicated that the fluorescent dye was endocytosed in greater amounts when coupled with ECM and suggested that the ECM and not only the fluorescent probe was endocytosed and accumulated in these vesicles.

DISCUSSION

We report that human breast cancer cell lines contain a high proportion of large (1.5- to 20-μm) intracellular acidic vesicles when cultured on extracellular matrix. LAVs were also found in primary culture on ECM of breast cancer cells from primary tumors and metastatic pleural effusions, even when analyzed directly after collection. In primary culture, the number of LAVs did not increase with time (up to 17 days) or with the number of passages. These results clearly indicated that LAVs do not result from an artifact of continuous cell lines. They strongly suggest that LAVs are also present in vivo, even though they were not directly demonstrated in vivo since the addition of a fluorescent dye required primary culture. Most of the LAVs contain mature cathepsin D, which probably acts as a protease since it is present in an acidic medium. They actively acidify their contents and concentrate endocytosed and/or phagocytosed material such as extracellular matrix. It was particularly interesting to find that LAVs were 2 to 20 times more abundant in breast cancer cells than in normal mammary epithelial cells, suggesting that LAVs may be associated with cell transformation and possibly contribute to the invasiveness of the cells containing them. Since ECM can be digested by breast cancer cells (14) and by cathepsin D (13), these acidic compartments could be involved in digestion of the ECM.

These results are in agreement with the first retrospective clinical studies that showed that high cathepsin D concentrations (precursor and mature enzyme) are correlated with frequency of metastasis (15, 16). Cancer cells from solid tumors can penetrate basement membranes and connective tissues following their digestion (1–3). Several proteases and enzymes probably act together to promote invasion of ECM by cancer cells. The mechanism by which cathepsin D might degrade ECM in vivo is unknown. Secreted pro-cathepsin D could act directly and extracellularly. Acidic extracellular compartments have previously been found between resorbing osteoclasts and bone matrix (30) and, although they have not been demonstrated in cultured breast cancer cells, it is possible that LAVs may release their acidic content, thus transiently creating an acidic microenvironment in which ECM could be digested by cathepsin D. In addition, this study clearly shows that cathepsin D is able to degrade ECM intracellularly following its ingestion by breast cancer cells in large acidic vesicles. Cooperation with other proteases is likely. For instance, pro-cathepsin B, which is also secreted by cancer cells, can be activated by cathepsin D (31), which itself is autoactivatable. In turn, activated cathepsin B or L can activate pro-cathepsin D. Whether, this activation occurs in LAVs or before reaching this compartment is unknown. The LAVs contained no pro-cathepsin D detectable by the antibodies, but a rapid activation in LAVs is not excluded.

The significance of the LAVs is not clear and may be variable. Some could correspond to phagosomes or phagolysosomes, since their contents appear to be heterogeneous and include other lysosomal enzymes such as β-glucuronidase and acid phosphatase. Phagocytosis is generally considered to be a specialized function of macrophages and leukocytes. It has, however, been proposed to occur also in cancer cells (32, 33), and our results would strongly support these findings.

Other LAVs seem to correspond to fused endosomes or large prelysosomal compartments, since they were able to endocytose RITC-dextran within the first 3 min and to contain fluorescent transferrin, a receptor-bound protein present in endosomes and absent in lysosomes. Hence, in breast cancer cells, as in macrophages, cathepsin D may be highly concentrated in endosomes (34) and, therefore, able to digest proteins like ECM and other biologically important proteins such as growth factor receptors. Even though estrogens in hormone-dependent breast cancer cells do not increase the number of LAVs, they may increase their functionality by increasing their cathepsin D content. The proteolytic activity of cathepsin D in these large intracellular compartments, different from the normal lysosomes, may, therefore, facilitate steps involved in vivo in the growth and/or invasiveness of mammary cancer cells.

ACKNOWLEDGMENTS

We are grateful to Dr. R. G. W. Anderson and P. Briand for the generous gift of the DAMP probe and the HMT-3522 cell lines, respectively, and to Dr. D. Louvard for constructive suggestions. Drs. Reynaud, Romieu, and Pr Laffargue are greatly acknowledged for providing normal and tumoral breast tissues. We thank E. Barrié and M. Egéa for typing the manuscript.

Note Added in Proof

Recent immunofluorescent studies using monoclonal antibodies (Dakopatts, Glostrup, Denmark) have confirmed the presence, in some LAVs, of human transferrin receptor.

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