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ARTICLE

Cathepsin B and D are Localized at the Surface of Human Breast Cancer Cells*

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Alterations in trafficking of cathepsins B and D have been reported in human and animal tumors. In MCF-10 human breast epithelial cells, altered trafficking of cathepsin B occurs during their progression from a preneoplastic to neoplastic state. We now show that this is also the case for altered trafficking of cathepsin D. Nevertheless, the two cathepsins are not necessarily trafficked to the same vesicles. Perinuclear vesicles of immortal MCF-10A cells label for both cathepsins B and D, yet the peripheral vesicles found in *ras*-transfected MCF-10AneoT cells label for cathepsin B, cathepsin D or both enzymes. Studies at the electron microscopic level confirm these findings and show in addition surface labeling for both enzymes in the transfected cells. By immunofluorescence staining, cathepsin B can be localized on the outer surface of the cells. Similar patterns of peripheral intracellular and surface staining for cathepsin B are seen in the human breast carcinoma lines MCF-7 and BT20. We suggest that the altered trafficking of cathepsins B and D may be of functional significance in malignant progression of human breast epithelial cells. Translocation of vesicles containing cathepsins B and D toward the cell periphery occurs in human breast epithelial cells that are at the point of transition between the pre-neoplastic and neoplastic state and remains part of the malignant phenotype of breast carcinoma cells. (Pathology Oncology Research Vol 1, No1, 43–53, 1995)

Key Words: aspartic proteases, breast cancer, cathepsins, cysteine proteases, oncogenic *ras*

Introduction

Expression, redistribution and/or secretion of the lysosomal proteases cathepsins B, D and L have been reported to parallel malignant progression.[32] Redistribution of cathepsin B has been observed in human colon carcinomas,[5] prostate carcinomas[31] and gliomas;[27] this redistribution parallels increased malignancy and/or decreased patient survival. Recently, the distribution of cathepsin D in phagolysosomes has been suggested to be a prognostic indicator for human breast carcinoma.[6,30] In macrophages and osteoclasts, i.e., cells that like tumor cells participate in degradative or invasive processes, lysosomes undergo translocation from the perinuclear region to the cell periphery. This redistribution is induced by cytoskeletal alterations associated with membrane ruffling. Lysosomes redistribute toward the ruffling membrane of activated osteoclasts and lysosomal enzymes are secreted.[3]

The study of breast cancer progression has been facilitated by the development of the diploid MCF-10 human breast epithelial cell lines. These cells were obtained during reduction mammoplasty from a patient with fibrocystic breast disease and underwent spontaneous immortalization in culture.[35] Transfection of immortal MCF-10A cells with mutated ras^+ results in cells (neoT) that have some of the characteristics of atypical breast epithelial stem cells. *In vitro* they are capable of indefinite proliferation and invade

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Abbreviations: BSA: bovine serum albumin; HEPES: N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid; IgG: immunoglobulin G; IgY: immunoglobulin Y; MPR: mannose 6-phosphate receptor.

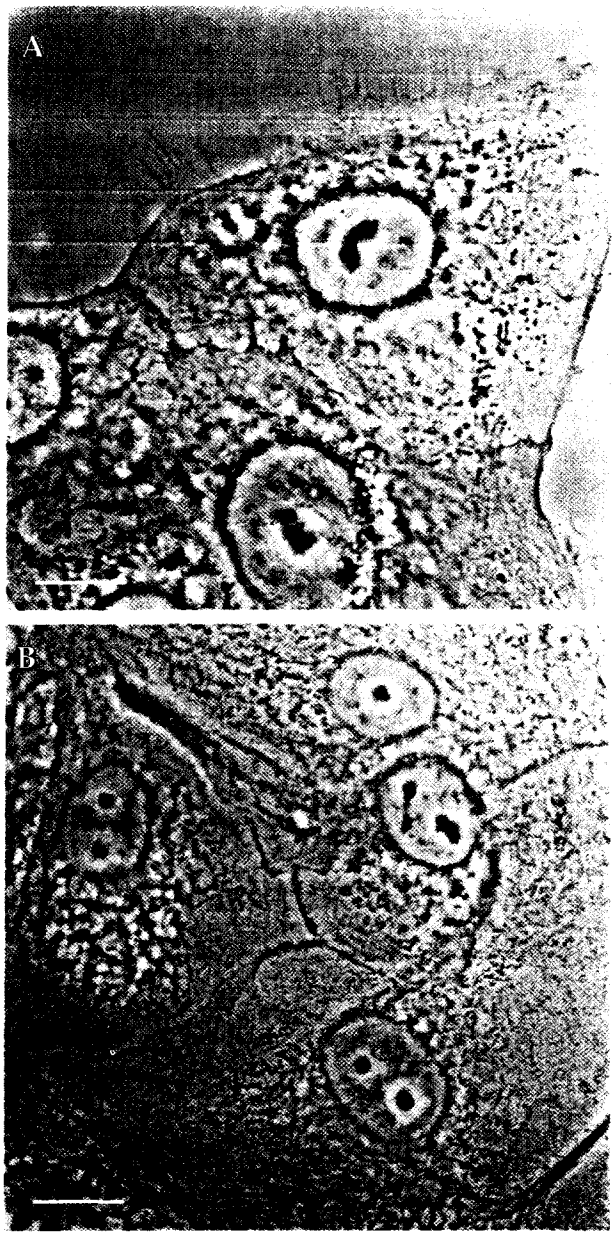


Figure 1. Immunocytochemical localization of intracellular cathepsin D in parental 10A cells (A) and their neoT counterpart transfected with mutated ras (B). The primary antibody was mouse anti-human cathepsin D IgG1. Cathepsin D staining in the 10A cells (A) was concentrated in the perinuclear region, whereas in the ras-transfected cells (B) the distribution of cathepsin D staining was more peripheral. The secondary antibody was Texas red-conjugated donkey anti-mouse IgG. The staining for cathepsin D has been repeated ten times to date with comparable results. Only a weak background fluorescence was observed in controls in which mouse or rabbit pre-immune IgG replaced the primary antibodies (not illustrated). Bars, 10 μm.

through Matrigel[25] and *in vivo* they form persistent palpable nodules that exhibit three pathologicentities: 1) benign ductal aggregates, sometimes with mild hyperplastic changes; 2) atypical hyperplastic lesions; and 3) carcinoma *in situ* and invasive carcinomas.[20] *ras*-transfection of the MCF-10 lines results in altered trafficking of cathepsin B,[33] such that this enzyme is localized in the cell periphery and on the cell surface. As *ras*-transfection of breast cancer cells increases their invasiveness[1] and metastatic ability,[13] the observations on altered trafficking of cathepsin B in *ras*-transfected MCF-10 cells may be of functional significance in the early progression of breast cancer. In the present study, we determined whether transfection of MCF-10 human breast epithelial cells with the c-Ha-*ras* oncogene affects the trafficking of cathepsin D as well as that of cathepsin B, whether cathepsins B and D are trafficked to the same vesicles and whether altered trafficking of these enzymes also is characteristic of fully malignant human breast carcinoma cell lines.

Materials and Methods

Materials

Saponin, Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture, minimal essential medium, HEPES, BSA, insulin, hydrocortisone, antibiotics, fish skin gelatin and methylcellulose were from Sigma (St Louis, MO); equine serum and fetal bovine serum from GIBCO (Grand Island, NY); epidermal growth factor from UBI (Lake Placid, NY); and 4,6-diamidin-2-phenylindol-dihydrochloride from Boehringer-Mannheim (Indianapolis, IN). A monoclonal antibody to human breast cancer cathepsin D was purchased from BioSys (Compiegne, France). Fluorescein-conjugated affinity-purified donkey anti-rabbit IgG, Texas red-conjugated affinity-purified donkey anti-mouse IgG and normal donkey serum were obtained from Jackson ImmunoResearch (West Grove, PA); formaldehyde from Polysciences (Warrington, PA); and SlowFade anti-fade reagent from Molecular Probes (Eugene, OR). The microbiological grade gelatin used for embedding of tissues and glutaraldehyde were purchased from Merck (Darmstadt, Germany); paraformaldehyde from BDH (Poole, United Kingdom); fraction V BSA from Boehringer-Mannheim (Mannheim, Germany); and 10 and 15 nm protein A-gold probes from Drs. Slot and Posthuma, Department of Cell Biology, University of Utrecht, The Netherlands. The rabbit anti-chicken IgY used in immunogold labeling was raised against IgY isolated from eggs of non-immunized chickens using polyethylene glycol precipitation.[26] Rabbit anti-chicken IgY-horseradish peroxidase was prepared as previously described.[8] All other chemicals were of reagent grade and were obtained from commercial sources.

Cell lines and culture

MCF-10 is a diploid human breast epithelial cell line derived from a patient with fibrocystic breast disease. This line underwent spontaneous immortalization in culture and grows attached in the presence of calcium or floating in the absence of calcium.[35] Transfection and cotransfections

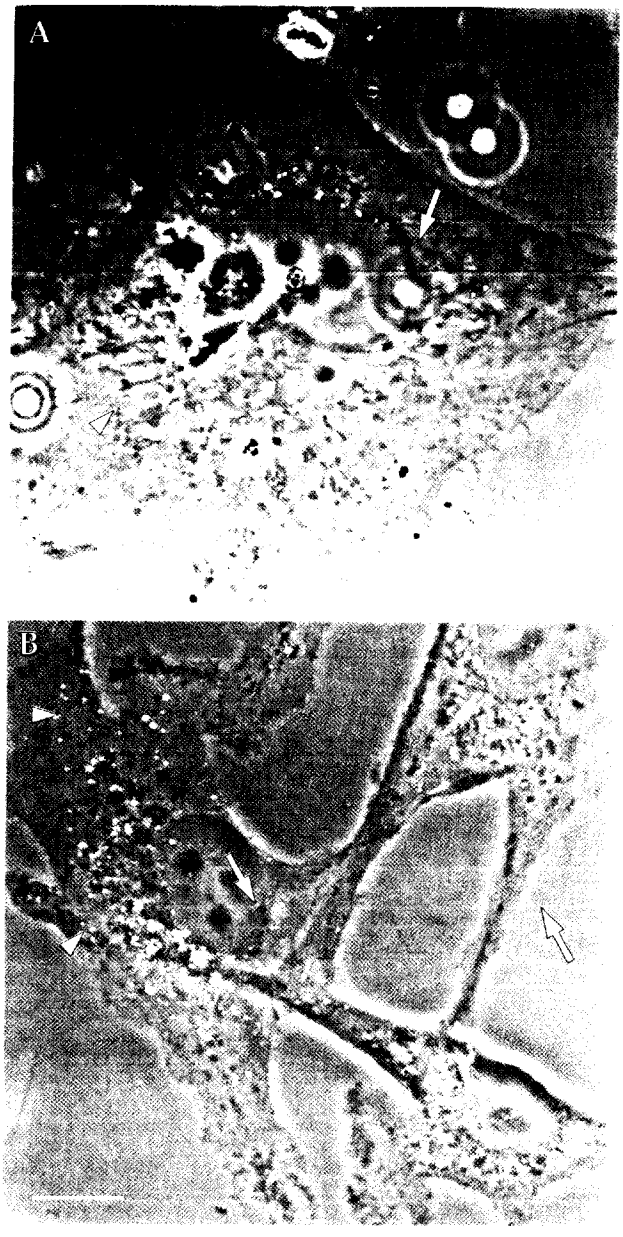


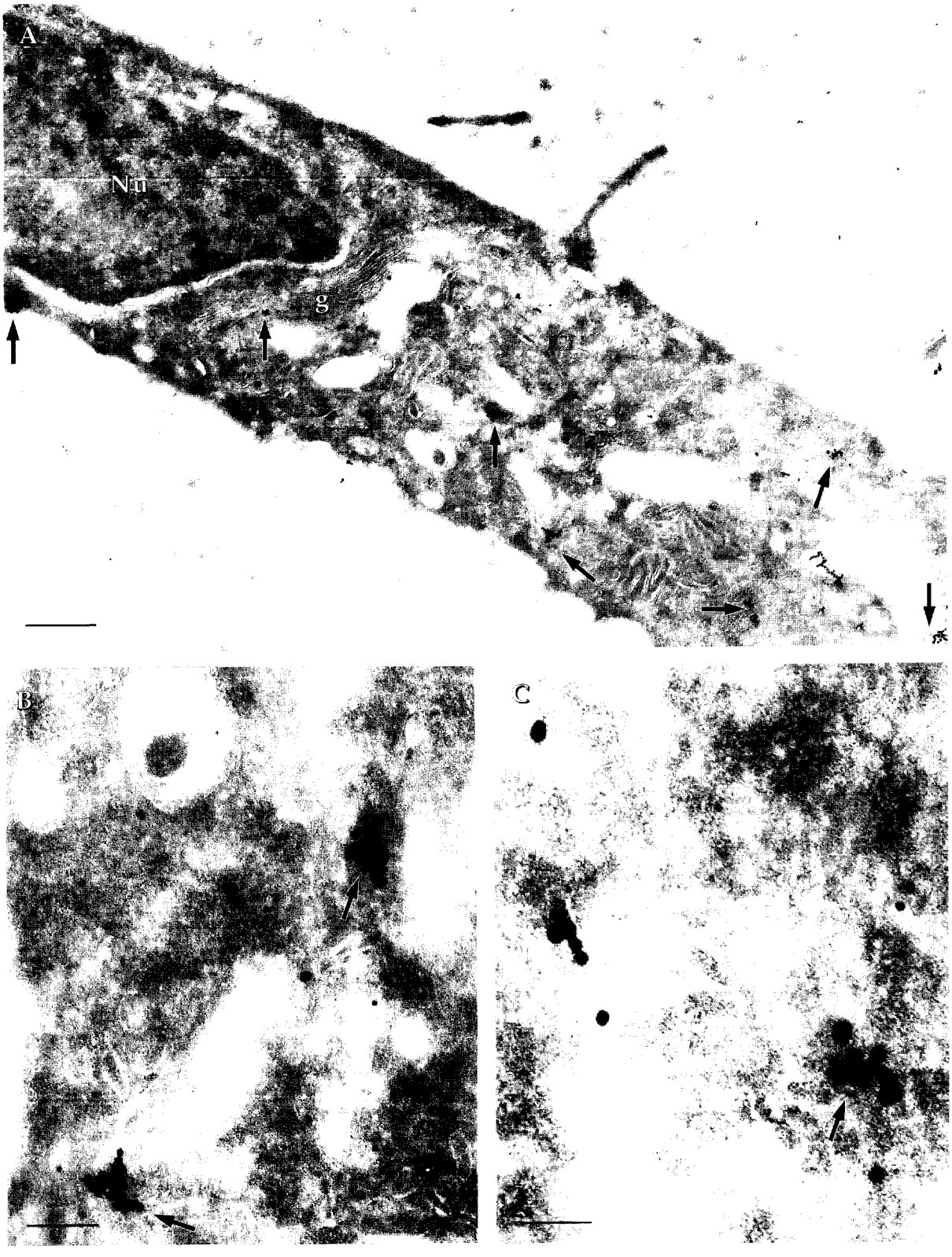
Figure 2. Immunocytochemical colocalization of intracellular cathepsin B and cathepsin D in immortal MCF-10A cells (A) and their counterpart transfected with oncogenic ras (B). Vesicles staining for cathepsin B alone are indicated with arrowheads and those staining for cathepsin D alone are indicated with arrows. Vesicles staining yellow indicate possible colocalization. Primary antibodies were rabbit anti-human cathepsin B IgG and mouse anti-human cathepsin D IgG1. Fluorescein-conjugated affinity-purified donkey anti-rabbit IgG and Texas red-conjugated affinity-purified donkey anti-mouse IgG were used as secondary antibodies. The double labeling for cathepsins B and D has been repeated six times to date with comparable results. Only a weak background fluorescence was observed in controls in which rabbit or mouse pre-immune IgG replaced the primary antibodies (not illustrated). Bars, 10 μm.

were performed using the calcium phosphate method with a plasmid containing the neomycin resistance gene as a transfection vector either alone (MCF-10Aneo) or with constructs containing wild-type (MCF-10AneoN) or mutated (MCF-10AneoT) c-Ha-*ras*.[4] The MCF-10 lines were grown in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture, containing 5% equine serum, supplemented with insulin, hydrocortisone, epidermal growth factor, antibiotics and fungizone,[4,25,35] but without amphotericin and cholera toxin.[33] The MCF-7 and BT20 human breast carcinoma lines were grown in minimal essential medium containing 10% fetal bovine serum as recommended by the ATCC (Rockville, MD). All cell lines were screened on a routine basis with 4',6-diamidin-2-phenylindol-dihydrochloride and shown to be free of *Mycoplasma*.

Immunochemical studies

Preparation of monospecific anti-cathepsin B IgGs and anti-cathepsin D IgY: Cathepsin B antisera were raised in rabbits as described.[21] An IgG fraction was purified and stored at -20°C. The specificity of the IgG used for immunofluorescence labeling of cathepsin B has been confirmed by slotblotting and immunoblotting against crude and purified cathepsin B fractions from human liver and sarcoma,[21] acetone fractions of human colonic mucosa and colon tumors[5] and cell homogenates of human breast epithelial cells.[33] Immunogold labeling for cathepsin B was performed using an affinity purified anti-human liver cathepsin B antibody, kindly supplied by Drs. Lukas Mach and Josef Glössl, Zentrum für Angewandte Genetik, Universität für Bodenkultur, Vienna, Austria. The production and specificity of this antibody was reported previously.[17] Cathepsin D was purified from porcine spleen according to the method of Jacobs et al.[14] Laying hens were immunized with 100 μg of porcine cathepsin D (50 μg into each breast muscle). The antigen was triturated in a 1:1 ratio with Freund's complete adjuvant at 0 wk and in Freund's incomplete adjuvant at 1, 2, 4 and 6 wk and for monthly boosters thereafter. Eggs were collected on a daily basis. IgY was isolated from the yolks by precipitation with polyethylene glycol as described.[26] The specificity of the IgY for cathepsin D was confirmed by immunoblotting against crude and purified cathepsin D fractions (data not shown).

Immunofluorescent staining: Intracellular cathepsins B and D and surface cathepsin B were localized using a modification[33] of the general immunocytochemical methodologies described by Willingham.[36] Cells grown to 60–80% confluence on glass coverslips were fixed with 3.7% formaldehyde in phosphate-buffered saline, pH 7.4. Fixation and subsequent steps were performed at 25 °C for intracellular labeling and at 4 °C for surface labeling. After washing with phosphate-buffered saline, cells were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blocked with phosphate-buffered saline-2 mg/ml BSA. For intracellular labeling, all subsequent antibody and wash solutions contained 0.1% saponin; saponin was not used in the surface labeling studies. Cells were incubated with primary antibody (rabbit anti-human liver cathepsin B, mouse anti-human breast cancer cathepsin D IgG1) for 2h and washed. Surface labeling was performed on cells incubated with primary and secondary antibodies at 4°C prior to fixation for the breast epithelial cells. For the breast carcinoma cells, surface labeling was performed subsequent to fixation at 4°C as these cells detached from the substratum at 40°C. In controls, preimmune serum (rabbit or mouse) was substituted for the primary antibody. After blocking with normal donkey serum (5% in phosphate-buffered saline-0.1% saponin for intracellular staining and without saponin for surface staining), cells were incubated for 60 min with fluorescein-conjugated affinity-purified donkey anti-rabbit IgG or Texas red-conjugated affinity-purified donkey anti-mouse IgG at 20 µg/ml. After washing, the coverslips were mounted upside-down on slides with SlowFade anti-fade reagent and observed with a Zeiss LSM 310 confocal microscope.

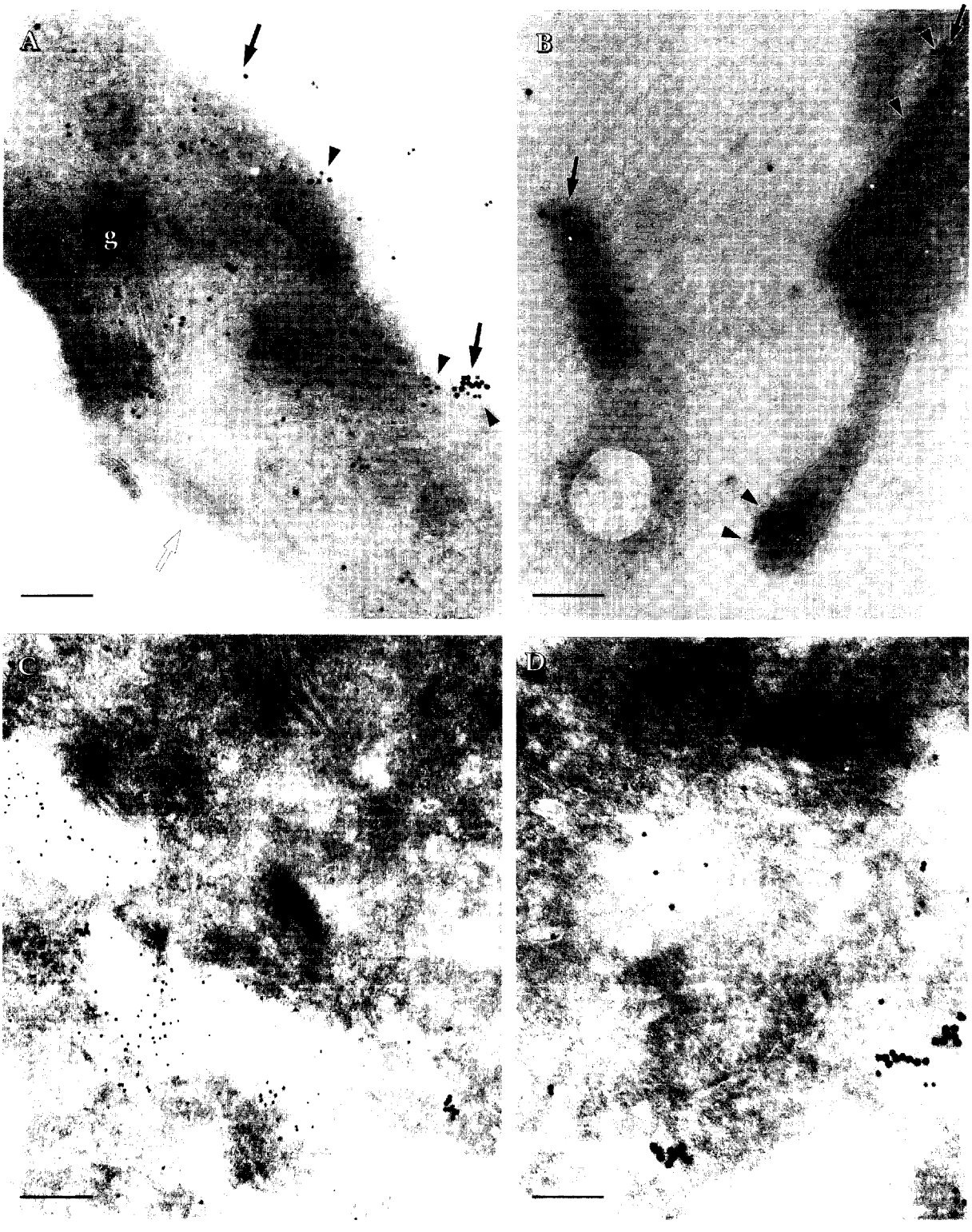
Immunogold staining: Cells grown to 60-80% confluence in T-25 flasks were fixed and processed by a modification of the method of Griffiths et al.[10] Cells were fixed in 2% paraformaldehyde containing 0.02% glutaraldehyde in 200 mM HEPES buffer, pH 7.3, at 4°C for 1h. After washing in phosphate buffer containing 20 mM glycine, the fixed monolayers were infiltrated with gelatin [10% (mass/vol) bacteriological gelatin phosphate buffer, 1h at 37°C] and crosslinked with the primary fixative (10 min at room temperature). The crosslinked, gelatin-infiltrated cell layers were stripped off the plastic and cryoprotected by infiltration with 2.1 M sucrose. Blocks were cut, oriented for vertical sectioning of cells, frozen in liquid nitrogen and ultra-thin frozen sections were cut using an RMC MT6000XL ultramicrotome fitted with a CR2000 cryo-attachment. Sections were collected on 2.3 M sucrose, thawed and mounted on 100 mesh hexagonal copper grids, previously formvar- and carbon-coated and glow-discharged. Thawed, grid-mounted sections were collected on 0.1% fraction V BSA in phosphate buffer prior to labeling. The grids were labeled as described by Slot et al.[32] Non-specific binding sites on the sections were blocked by incubation in 2% fish skin gelatin and 20 mM glycine in phosphate buffer. Incubation on primary antibody (chicken anti-porcine spleen cathepsin D IgY or rabbit anti-human liver cathepsin B IgG, 10 µg/ml) was for 1h at 25°C. Incubation in the anti-porcine spleen cathepsin D IgY required an additional incubation step with a rabbit anti-chicken linker antibody (1:100 dilution for 1h at 25°C). For single labeling, the grids were then incubated for 30 min at 25°C with a 1:40 dilution of protein A-gold probe (mean particle size of 10 nm) before being washed, fixed with 2% glutaraldehyde, counterstained and sealed in a uranyl acetate/methyl cellulose mixture as described by Slot et al.[34] Double labeling was performed by repetition of the blocking and labeling regime described, the detection of antibody-binding to the second antigen being detected using a 1:55 dilution of a second protein A-gold probe (mean particle size of 15 nm). Labeling specificity was verified by the omission of primary and secondary antibodies in various labeling schemes, and the performance of labeling for the two different antigens in different orders, using detection with first the small and then the large gold labels, according to Slot et al.[34] Grids were viewed and photographed in a Jeol 100 CX transmission electron microscope, at 100 kV.

Results

We have previously established that the lysosomal cysteine protease cathepsin B is distributed more peripherally in MCF-10A human breast epithelial cells transfected with oncogenic *ras*.[33] Rochefort and colleagues have shown an association between the presence of cathepsin D in phagolysosomes near the cell surface of human breast carcinomas and prognosis.[30] Therefore, we determined whether the subcellular localization of cathepsin D also was altered. In the parental 10A cells, the staining for cathepsin D was localized primarily to the perinuclear region *(Fig. 1 A)*; a localization consistent with cathepsin D being distributed in

Figure 3. *Immunogold labeling for cathepsin B and cathepsin D in immortal MCF-10A cells. Cell monolayers were fixed with 2% paraformaldehyde containing 0.02% glutaraldehyde, embedded with gelatin, refixed, and the gelatin-infiltrated monolayer, cryoprotected with 2.1 M sucrose, was stripped off the plastic, cut into blocks, mounted for vertical sectioning of cells and frozen for cryoultramicrotomy. Immunolabeling for cathepsin B and D on the sections was performed using an affinity purified rabbit anti human liver cathepsin B antibody and a chicken anti-porcine cathepsin D antibody. For protein A gold labeling using the chicken anti-porcine D antibody, a linker (rabbit anti-chicken antibody) was used. Labeling was performed for cathepsin B and then cathepsin D, and vice versa, labeling being detected using a small (10 nm) followed by a larger (15 nm) protein A gold probe, in each case. Similar colocalization results were observed in either case. Controls for double labeling indicated adequate blocking of sections between double labeling steps. In the micrographs illustrated, localization of cathepsin B was performed first and detected with the 10 nm protein A gold probe and labeling for cathepsin D was performed subsequently and detected with a 15 nm gold probe. A transverse section of an MCF-10A cell is shown (A; basolateral surface indicated with open arrows). Generally, cathepsins B and D were found to colocalize in the more electron-dense, larger vesicles (presumably late endosomal or lysosomal compartments) situated in a perinuclear location (arrowheads). Vesicles selected for enlargement (B and C) indicate colocalization more clearly. Nu = nucleus, g = Golgi apparatus. Bars, 1 µm (A) and 0.2 µm (B and C).*

lysosomes. In the *ras*-transfected neoT cells *(Fig. 1 B)*, both perinuclear and peripheral staining for cathepsin D was observed. Thus, cathepsin D exhibited a more peripheral subcellular distribution in the neoT cells, a pattern similar to that observed previously for cathepsin B.[33] In order to assess whether the two enzymes were distributed in the same vesicles, we performed double labeling studies. In the immortal 10A cells, cathepsins B and D were found to be primarily



colocalized in perinuclear vesicles *(Fig.2 A)*. A different pattern was observed in the neoT cells transfected with mutated *ras (Fig.2 B)*. The distribution of both enzymes was more peripheral and three patterns of vesicular staining were observed: 1) vesicles staining for both enzymes, 2) vesicles staining for only cathepsin B, and 3) vesicles staining for only cathepsin D. Vesicles staining for cathepsins B and D, cathepsin B or cathepsin D appeared to be of similar sizes.

The peripheral vesicles staining for cathepsin D may be endosomes as endosomes containing cathepsin D have been observed in macrophages[29] and hepatocytes[7] or may be the phagolysosomes described by Rochefort and colleagues.[23,30] In order to determine the localization of cathepsins B and D at the ultrastructural level, we employed immunogold double-labeling. In the immortal 10A cells, cathepsins B and D were largely colocalized in perinuclear vesicles *(Figs.3 A, B, and C, arrowheads)*. In contrast, in the neoT cells transfected with mutated *ras*, a more peripheral distribution of the gold labeling for both enzymes was observed, including increased labeling on the cell surface *(Figs.4 A and B)*. The majority of peripheral vesicles exhibited label for only one of the two cathepsins *(Fig.4 A, C and D)*. Gold particles representing cathepsin D protein could be observed apparently in the process of being secreted from surface protrusions of the neoT cells *(Fig.4 B)*. The most numerous cathepsin D- and B-labeled organelles in the immortal 10A cells *(Fig.3)* were of the order of 0.14-0.19 μm in diameter, whereas in the *ras*-transfected neoT cells these vesicles were 0.1-0.13 μm in diameter *(Fig.4)*. In the 10A cells, occasional vesicles (0.5-0.54 μm in diameter) were observed that resembled phagolysosomes and labeled heavily for cathepsin D. Larger phagolysosomes (0.5-1 μm in diameter) were observed in the neoT cells where they labeled more heavily for cathepsin B than for cathepsin D *(Fig.4 C)*.

Cell surface labeling for cathepsin B has been observed by immunofluorescence techniques in human lung carcinoma cells[9] and murine B16 amelanotic melanoma cells.[12] In order to evaluate whether the immunogold labeling for cathepsin B observed in *ras* transfected neoT cells[33] *(Fig.4)* was on the external surface of the cells, we performed immunofluorescence staining in non-permeabilized cells. Staining for cathepsin B was not observed on the surface of the immortal 10A cells *(Fig.5 A)*, but was present on the surface of the *ras*-transfected neoT cells *(Fig.5 B)*. In these latter cells, the staining for cathepsin B was localized to discrete regions on the basal surface. For cathepsin D, some cell surface labeling was observed on immortal 10A cells, yet substantially more cell surface labeling on the *ras*-transfected neoT cells (data not shown). As indicated above, in these confocal studies, the cell surface labeling was localized primarily to the basal surface of the cells with apical labeling only in a few cells. By the immunogold method, apical labeling was observed rather than basal *(Figs.3 and 4)*. However, this latter technique may not be optimal for examining basal membrane expression of cathepsins as the surface-bound cathepsins may be lost when the cells were stripped off the plates (see Materials and Methods). By contrast, in the immunofluorescence method, the cells were examined without removal from the coverslips.

Studies in human colon carcinomas,[5] prostate carcinomas[31] and gliomas[27] suggest that altered trafficking of cathepsin B is part of the malignant phenotype. Rochefort and colleagues.[30] have shown that altered trafficking of cathepsin D may be of prognostic significance in human breast carcinomas. As similar studies have not been performed for cathepsin B, we analyzed the intracellular and surface distribution of cathepsin B in two human breast carcinoma lines, MCF-7 and BT20. In both lines, cathepsin B was found to be distributed throughout the cytoplasm rather than being restricted to the perinuclear region *(Fig.6 A and C)*. The sizes of the vesicles labeling for cathepsin B could not be accurately determined in these immunofluorescent images. In both MCF-7 and BT20 cells, surface labeling for cathepsin B was observed *(Fig.6 B and D)*. As in the *ras*-transfected MCF-10AneoT cells *(Fig.5 B)*, surface staining for cathepsin B was found at discrete regions on the basal surface of the cells.

Discussion

Trafficking by two distinct pathways might be responsible for cathepsins B and D being localized in separate peripheral vesicles in the *ras*-transfected neoT cells *(Figs.2 and 4)*. Studies to date suggest that cathepsin B is trafficked to the lysosomes via a MPR-dependent pathway.[11,17] On the other hand, cathepsin D has been shown to be trafficked by both MPR-dependent[2,16,19] and MPR-independent

Figure 4. *Immunogold labeling for cathepsin B and cathepsin D in MCF-10AneoT cells transfected with oncogenic ras. Cell monolayers were fixed, embedded, and processed for vertical cryoultramicrotomy sectioning and immunolabeling for cathepsins B and D was performed, in both orders, as described in the legend to Fig. 3. In the micrographs illustrated, localization of cathepsin D was performed first and detected with a 10 nm protein A gold probe (A and B) and labeling for cathepsin B was performed subsequently and detected with a 15 nm gold probe. Immunolabeling was also performed in the reverse sequence (C and D), cathepsin B being detected with the small gold probe and cathepsin D with the larger probe. A transverse section of a cell, shows less colocalization of cathepsins B and D in peripheral regions (A) [basolateral surface indicated with an open arrow (A)] and in subperinuclear regions of the cell (C and D), than in the parental MCF-10A cells (cf. Fig. 3). Some peripheral vesicles appear caught in the process of secretion (A). Association of cathepsin B (arrows) and cathepsin D (arrowheads) with the surface of cells is also evident in the ras-transfected neoT cells (A and B). g = Golgi apparatus. Bars, 0.25 μm (A, B and D) and 0.5 μm (C).*

pathways.[28,37] Although the peripheral vesicles labeling for either cathepsin B or cathepsin D might represent two different vesicular compartments, this would appear to be unlikely as the sizes of the vesicles are similar. Another possibility is that one compartment might contain only pro forms of the two cathepsins and the other mature forms. However, as the antibodies used in the present study recognize both pro and mature forms of cathepsins B and D, both compartments should stain for the two enzymes. Thus, at present the identity of the peripheral vesicles staining for only cathepsin B or for only cathepsin D is unknown. Studies to establish the molecular forms of cathepsins B and D associated with the cell surface and these peripheral vesicles and the identity of these peripheral vesicles are in progress.

Rochefort and colleagues have proposed that intracellular cathepsin D plays a functional role in breast carcinoma, specifically in the degradation of extracellular matrix proteins in a peripheral compartment of phagolysosomes.[23] In the present study by immunogold labeling, we localized

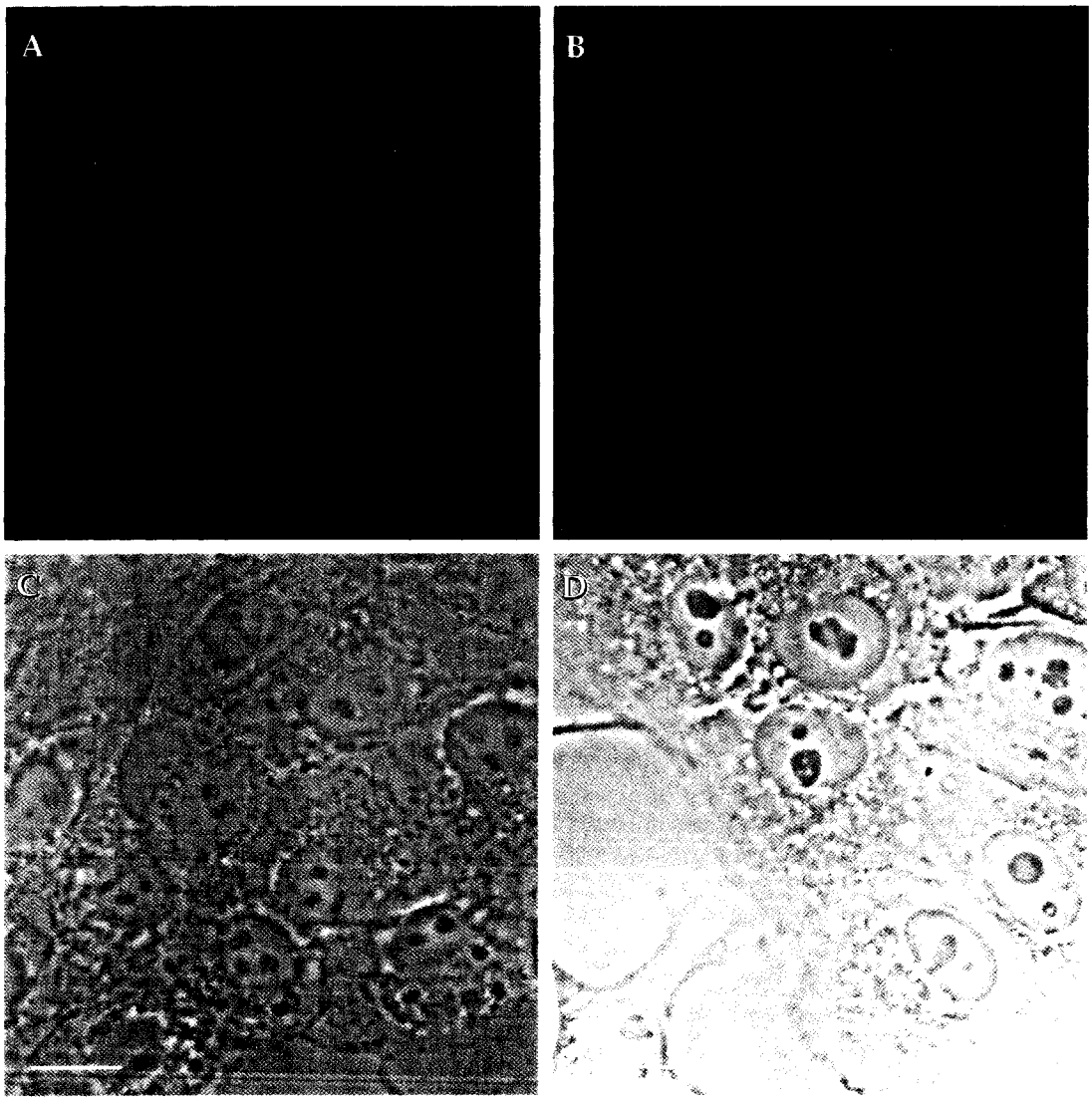


Figure 5. *Immunocytochemical localization of cathepsin B on the surface of non permeabilized MCF-10 human breast epithelial cells. Surface staining for cathepsin B was present in discrete regions on the basal surface of the cells transfected with mutated ras (B). Surface staining could not be visualized on the immortal 10A cells (A). The coverslips were mounted upside-down on slides. Thus, the labeling observed in panel B is underneath the cells. The primary antibody was rabbit anti-human cathepsin B IgG and the secondary antibody Texas red-conjugated donkey anti-rabbit IgG. The staining for cathepsin B has been repeated three times to date with comparable results. Only a weak background fluorescence was observed in controls in which rabbit pre-immune IgG replaced the primary antibody (not illustrated). Panels C and D are the phase contrast images corresponding to the fluorescence images of panels A and B, respectively. Bars, 10 μm.*

cathepsin D to phagolysosomes primarily in immortal 10A cells and cathepsin B to phagolysosomes primarily in *ras*-transfected neoT cells. We also localized both cathepsins B and D to smaller peripheral vesicles and to surface membranes of the *ras*-transfected neoT cells. To our knowledge, the present study is the first to localize cathepsin D to the cell surface by immunogold techniques. Three lysosomal proteases have now been localized to the surface of malignant cells: cathepsin D to the surface of *ras*-transfected neoT cells by immunogold microscopy (present study); cathepsin B to the surface of 1) human lung adenocarcinoma cells by immunofluorescence microscopy,[9] 2) murine B16 amelanotic melanoma cells and *ras*-transfected neoT cells by immunofluoresence microscopy and flow cytometric analysis,[12] 3) *ras*-transfected neoT cells by immunogold microscopy[33] (present study), and 4) *ras*-transfected neoT cells and MCF-7 and BT20 human breast carcinoma cells by immunofluorescence microscopy (present study); and cathepsin L to the surface of human colon adenocarcinoma cells by immunofluores-

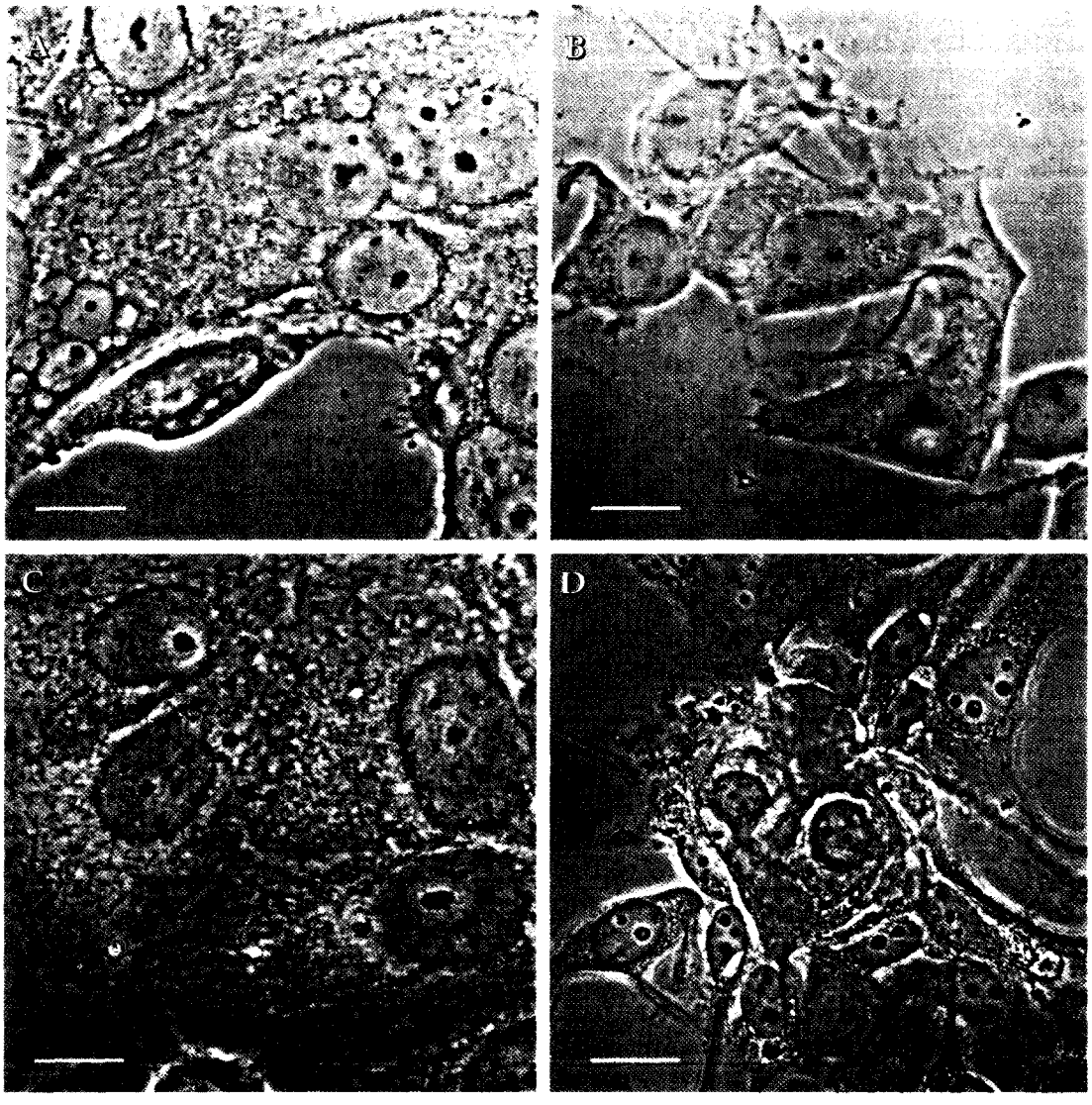


Figure 6. *Immunocytochemical localization in MCF-7 (A, B) and BT20 (C, D) human breast carcinoma cells of intracellular cathepsin B (A and C) and cell surface cathepsin B (B and D). Intracellular cathepsin B staining in the breast carcinoma lines was present throughout the cytoplasm and at the cell periphery. Surface staining for cathepsin B was present on the basal surface of both cell lines (see legend to Fig. 5). The primary antibody was rabbit anti-human cathepsin B IgG and the secondary antibody Texas red-conjugated donkey anti-rabbit IgG. The staining for cathepsin B has been repeated three times to date with comparable results. Only a weak background fluorescence was observed in controls in which rabbit preimmune IgG replaced the primary antibody (not illustrated). Bars, 10 μm.*

cence microscopy.[18] We do not yet know whether surface associated lysosomal proteases play a functional role in tumor progression. This possibility is suggested by the ability to induce concomitantly in malignant cells the surface expression of cathepsin B, the integrin $\alpha_{IIb}\beta_3$, and the autocrine motility factor receptor. These three proteins could mediate the three putative steps in tumor invasion: adhesion, local degradation and migration.[15] Furthermore, the localization of cathepsin B to discrete regions on the basal surface of *ras*-transfected human breast epithelial cells and breast carcinoma cells resembles the localization of proteases to the invadopodia described by Chen and colleagues,[22] a structure shown to be involved in cell adhesion, focal degradation and invasion.[24]

References

1. *Albini A, Graf J, Kitten GT, Kleinman HK, Martin GR, Veillette A and Lippman ME:* 17β-estradiol regulates and v-Ha-*ras* transfection constitutively enhances MCF7 breast cancer cell interactions with basement membrane. Proc Natl Acad Sci USA 83:8182-8186, 1986.
2. *Baranski TJ, Faust PL and Kornfeld S:* Generation of a lysosomal enzyme targeting signal in the secretory protein pepsinogen. Cell 63:281-291, 1990.
3. *Baron R:* Molecular mechanisms of bone resorption by the osteoclast. Anat Rec 224:317-324, 1989.
4. *Basolo F, Elliot J, Tait L, Chen QC, Maloney TM, Russo IH, Pauley R, Momiki S, Caamano J, Klein-Szanto AJP, Koszalka M and Russo J:* Transformation of human breast epithelial cells by c-Ha-*ras* oncogene. Molec Carcinogen 4:25-35, 1991.
5. *Campo E, Munoz J, Miquel R, Palacin A, Cardesa A, Sloane BF and Emmert-Buck MR:* Cathepsin B expression in colorectal carcinomas correlates with tumor progression and shortened patient survival. Am J Pathol 145:301-309, 1994.
6. *Cardiff RD:* Cathepsin D and breast cancer: useful? Hum Pathol 25:847-848, 1994.
7. *Casciola-Rosen L, Renfrew CA and Hubbard AL:* Lumenal labeling of rat hepatocyte endocytic compartments. Distribution of several acid hydrolases and membrane receptors. J Biol Chem 267:11856-11864, 1992.
8. *Coetzer THT, Pike RN and Dennison C:* Localization of a possible immunoinhibitory epitope of the cysteine proteinase, cathepsin L. Immunol Invest 21:495-507, 1992.
9. *Erdel M, Trefz G, Spiess E, Habermaas S, Spring H, Lah T and Ebert W:* Localization of cathepsin B in two human lung cancer cell lines. J Histochem Cytochem 38:1313-1321, 1990.
10. *Griffiths G, McDowall A, Back R and Doubochet J:* On the preparation of cryosections for immunocytochemistry. J Ultrastruc Res 9:65-78, 1984.
11. *Hanewinkel H, Glossl J and Kresse H:* Biosynthesis of cathepsin B in cultured normal and I-cell fibroblasts. J Biol Chem 262:12351-12355, 1987.
12. *Honn KV, Timar J, Rozhin J, Bazaz R, Sameni M, Ziegler G and Sloane BF:* A lipoxygenase metabolite, 12-(S)-HETE, stimulates protein kinase C-mediated release of cathepsin B from malignant cells. Expl Cell Res 214:120-130, 1994.
13. *Ichikawa T, Kyprianou N and Isaacs JT:* Genetic instability and the acquisition of metastatic ability by rat mammary cancer cells following v-H-*ras* oncogene transfection. Cancer Res 50:6349-6357, 1990.
14. *Jacobs GR, Pike RN and Dennison C:* Isolation of cathepsin D using three-phase partitioning in t-butanol/water/ammonium sulfate. Anal Biochem 180:169-171, 1989.
15. *Liotta LA:* Tumor invasion and metastases-role of the extracellular matrix. Cancer Res 46:1-7, 1986.
16. *Ludwig T, Ovitt CE, Bauer U, Hollinshead M, Remmler J, Lobel P, Ruther U and Hoflack B:* Targeted disruption of the mouse cation-dependent mannose 6-phosphate receptor results in partial missorting of multiple lysosomal enzymes. EMBO J 12:5225-5235, 1993.
17. *Mach L, Stuwe K, Hagen A, Ballaun C and Glossl J:* Proteolytic processing and glycosylation of cathepsin B: The role of the primary structure of the latent precursor and of the carbohydrate moiety for cell-type-specific molecular forms of the enzyme. Biochem J 282:577-582, 1992.
18. *Maciewicz RA, Wardale RJ, Etherington DJ and Paraskeva C:* Immunodetection of cathepsins B and L present in and secreted from human pre-malignant and malignant colorectal tumour cell lines. Intl J Cancer 43:478-486, 1989.
19. *Mathieu M, Vignon F, Capony F and Rochefort H:* Estradiol down-regulates the mannose-6-phosphate/insulin-like growth factor-II receptor gene and induces cathepsin-D in breast cancer cells: a receptor saturation mechanism to increase the secretion of lysosomal proenzymes. Mol Endocrinol 5:815-822, 1991.
20. *Miller FR, Soule HD, Tait L, Pauley RJ, Wolman SR, Dawson PJ and Heppner GH:* Xenograft model of human proliferative breast disease. J Natl Cancer Inst 85:1725-1732, 1993.
21. *Moin K, Day NA, Sameni M, Hasnain S, Hirama T and Sloane BF:* Human tumour cathepsin B: comparison with normal human liver cathepsin B. Biochem J 285:427-434, 1992.
22. *Monsky WL, Kelly T, Lin C-Y, Yeh Y, Steiler-Stevenson WG, Mueller SC, Chen W-T:* Binding and localization of M_r 72.000 matrix metalloproteinase at cell surface invadopodia. Cancer Res 53:3159-3164, 1993.
23. *Montcourrier P, Mangeat PH, Salazar G, Morisset M, Sahuquet A and Rochefort H:* Cathepsin D in breast cancer cells can digest extracellular matrix in large acidic vesicles. Cancer Res 50:6045-6054, 1990.
24. *Mueller SC and Chen W-T:* Cellular invasion into matrix beads: localization of β1 integrins and fibronectin to the invadopodia. J Cell Science 99:213-225, 1991.
25. *Ochieng J, Basolo F, Albini A, Melchiori A, Watanabe H, Elliott J, Raz A, Parodi S and Russo J:* Increased invasive, chemotactic and locomotive abilities of c-Ha-*ras*-transformed human breast epithelial cells. Invasion Metastasis 11:38-47, 1991.
26. *Polson A, Coetzer T, Kruger J, von Maltzahn E and van der Merwe KJ:* Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. Immunol Invest 14:323-327, 1985.
27. *Rempel SA, Rosenblum ML, Mikkelsen T, Yan P-S, Ellis KD, Golembieski WA, Nelson KK, Sameni M, Rozhin J, Ziegler G and Sloane BF:* Cathepsin B in glioma progression and invasion. Cancer Res 54:6027-6031, 1994.
28. *Rijnboutt S, Kal AJ, Geuze HJ, Aerts H and Strous GJ:* Mannose 6-phosphate-independent targeting of cathepsin D to lysosomes in HepG2 cells. J Biol Chem 266:23586-23592, 1991.
29. *Rodman JS, Levy MA, Diment S and Stahl PD:* Immunolocalization of endosomal cathepsin D in rabbit alveolar macrophages. J Leukocyte Biol 48:116-122, 1990.
30. *Roger P, Montcourrier P, Maudelonde T, Brouillet J-P, Pages J, Laffargue F and Rochefort H:* Cathepsin D immunostaining in

paraffin-embedded breast cancer cells and macrophages: correlation with cytosolic assay. Hum Pathol 25:863-871, 1994.

31. *Sinha AA, Wilson MJ, Gleason DF, Reddy PK, Sameni M and Sloane BF:* Immunohistochemical localization of cathepsin B in neoplastic human prostate. Prostate 26:171-178, 1995.
32. *Sloane BF, Moin K and Lah TT:* Regulation of lysosomal endopeptidases in malignant neoplasia. In: Aspects of the Biochemistry and Molecular Biology of Tumors. (Eds: Pretlow TG and Pretlow TP), Academic Press, New York, 1994. pp. 411-466.
33. *Sloane BF, Moin K, Sameni M, Tait LR, Rozhin J and Ziegler G:* Membrane association of cathepsin B can be induced by transfection of human breast epithelial cells with c-Ha-*ras* oncogene. J Cell Science 107:373-384, 1994.
34. *Slot JW, Geuze HJ, Gigengack S, Leienhard GE and James DE:* Immuno-localisation of the insulin regulatable glucose transporter in brown adipose tissue of the rat. J Cell Biol 113:123-135, 1991.
35. *Soule H, Maloney TM, Wolman SR, Peterson Jr WD, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF and Brooks SC:* Isolation and characterization of a spontaneously immortalized human breast epithelial cell line MCF-10. Cancer Res 50:6075-6086, 1990.
36. *Willingham MC:* Immunocytochemical methods: useful and informative tools for screening hybridomas and evaluating antigen expression. Focus 12:62-67, 1990.
37. *Zhu Y and Conner GE:* Intermolecular association of lysosomal protein precursors during biosynthesis. J Biol Chem 269:3846-3851, 1994.