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Malignant Transformation Alters Intracellular Trafficking of Lysosomal Cathepsin D in Human Breast Epithelial Cells

Yukio NISHIMURA,[1] Mansoureh SAMENI,[2] Bonnie F SLOANE[2]

[1]Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-0054, Japan;
[2]Department of Pharmacology, Wayne State University, Detroit, Michigan, 48201, USA

Increased expression and alteration of intracellular trafficking of lysosomal cathepsins have been reported in malignant tumors, or in cells transformed by the transfection with the *ras* oncogene. In the present study, immortal MCF-10A human breast epithelial cells were transformed with the mutated *ras* oncogene. Both cell lines were investigated for changes in the intracellular localization of lysosomal cathepsin D and lamp-1 (lysosome-associated membrane protein) employing specific antibodies and confocal immunofluorescence microscopy. The results revealed that staining for cathepsin D along with for lamp-1 was mostly localized in the perinuclear region of MCF-10A cells. In contrast, the staining for these proteins was found to be widely distributed throughout the cytoplasm and at the cell periphery in MCF-10AneoT cells. The organization of microtubules, but not actin, appeared to differ between MCF-10A cells and their oncogenic *ras* transfectants. When the microtubules were depolymerized by treatment of MCF-10A cells with nocodazole, vesicles containing the lysosomal cathepsin D were dispersed in the cytoplasm and translocation of these vesicles to the cell periphery was observed. The intracellular localization of cathepsin D in the nocodazole-treated MCF-10A cells seemed to be similar to that observed in the oncogenic *ras* transfectants of these cells. When taxol, which inhibits microtubule depolymerization, was added to the culture medium of neoT cells, a polymerized microtubule network was observed, and the reclustering of cathepsin D and lamp-1 occurred in a unidirectional manner towards the perinuclear region. These findings support a model in which cytoskeletal microtubule organization is closely related to the trafficking of lysosomes/endodomes, and in which oncogenic *ras* interferes with such organization in human breast epithelial cells. (Pathology Oncology Research Vol 4, No 4, 283–296, 1998)

Key words: cathepsin D, aspartic proteinase, lamp, microtubule, trafficking, breast cancer, *Ras*

Introduction

An increased expression of lysosomal cathepsins has been reported for a variety of human and animal tumors. Altered expression and localization of lysosomal cathepsins are considered to play important roles in the proliferative, invasive, and metastasizing process of malignant cells, particularly because of their destructive effects on the extracellular matrix (reviewed by Sloane et al.[43])

Lysosomal cysteine proteinases, cathepsins B and L, have been implicated in tumorigenesis and reported to be associated with plasma membranes of a wide variety of animal and human tumors and transformed cells.[41,42,44] Similarly, cathepsin L was shown to be overexpressed and secreted from c-Ha-*ras* oncogene transformed mouse fibroblast cell lines.[15] The secretion from and/or plasma membrane association of cathepsin B,H,L, and D in tumor cells has been implicated in tumor invasion and metastasis.[14] In human breast carcinoma, cathepsin D, a major lysosomal aspartic proteinase, was shown to be overexpressed at the mRNA and protein levels; *in vitro* studies indicated that considerable amounts of cathepsin D were secreted into extracellular media of the cultured cell lines which might lead to degradation of basement membrane and metastasis.[31,32]

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Correspondence: Yukio NISHIMURA, PhD, Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi , Higashi-ku, Fukuoka 812-0054, Japan; Tel: +81-92-642-6617; Fax: +81-92-622-7402; e-mail: nishimur@bioc.phar.kyushu-u.ac.jp

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Several clinical studies have correlated a high level of cathepsin D expression with increased risk of metastasis in breast cancer patients.[4,13,33] Thus increased expression and secretion of lysosomal cathepsins in malignant tumors possibly reflect a series of alterations in intracellular trafficking resulting in the delivery of cathepsins to lysosomes/endosomes in the cell periphery or secretion. Recently it was demonstrated that c-Ha-*ras* oncogene was capable of transforming the spontaneously immortalized human breast epithelial cell line MCF-10A.[3,27,45] The diploid MCF-10A human breast epithelial cell lines originated from spontaneous immortalization of breast epithelial cells obtained from a patient with fibrocystic disease, and cell line MCF-10AneoT was created with mutated c-Ha-*ras*. MCF-10AneoT cells were reported to have a transformed phenotype, be capable of indefinite proliferation, invade through Matrigel *in vitro*,[27] and be tumorigenic in nude mice.[21] In contrast, MCF-10A cells are non-tumorigenic.[3,21] In breast cancer cells, it has been shown that the transfection of mutated *ras* oncogenes increases their invasiveness[1] and metastatic ability,[12] partly due to increase in the expression of lysosomal cathepsins. In MCF-10AneoT cells, it was published previously[22,37,44] that a more peripheral distribution of cathepsins B, and D was observed, while in the parental MCF-10A cells, cathepsins B and D were primarily colocalized in the perinuclear vesicles. These results suggest that lysosomal cathepsins B and D are redistributed towards the cell periphery in *ras*-transfected neoT cells where they may participate in the degradative or invasive processes.

Previous studies demonstrated that intracellular translocation and clustering of endosomes and lysosomes depend on microtubule organization, because the microtubule-disrupting agent nocodazole inhibits the movement of endosomes and lysosomes toward the microtubule organizing center.[20] Also an acidic pericellular pH has been shown to induce a redistribution of vesicles staining for cathepsin B toward the cell periphery, and enhanced secretion of active cathepsin B.[36] Both the redistribution of vesicles staining for cathepsin B toward the cell surface induced by acidic pH and induced secretion of active cathepsin B could be inhibited by microtubule poisons, therefore, it was suggested that translocation and secretion of cathepsin B are dependent on a functional microtubular system.[36]

To date, there is no paper demonstrating the mechanism by which intracellular movement and trafficking of lysosomal cathepsins is altered in *ras*-transformed cells. In the present study we have examined by confocal immunofluorescence microscopy whether c-Ha-*ras*-transfection results in depolymerization of microtubules thereby altering the intracellular trafficking of lysosomal cathepsin D. The results presented in this paper suggest that the translocation of lysosomes/endosomes toward the cell periphery may be closely correlated with microtubule reorganization by mutated *ras* in the MCF-10AneoT cells.

Materials and Methods

Materials

Protein A Sepharose CL-4B, and the molecular weight mass markers for immunoblotting were from Pharmacia Biotech (Piscataway, NJ, USA). The enhanced chemiluminescence reagent for staining the immunoreactive bands after immunoblot experiments was purchased from DuPont NEN (Mass, USA). A monoclonal anti-human lysosome-associated membrane protein-1 (lamp-1) H4A3 was purchased from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD, USA), and the Department of Biological Sciences, University of Iowa (Iowa City, IA, USA), under contract NO1-HD-2-3144 from the National Institute of Child Health and Human Development. A monoclonal antibody to α-tubulin was purchased from Amersham (Arlington Heights, IL, USA). Mouse H1 monoclonal IgG to kinesin heavy chain and mouse monoclonal IgG2b to dynein were obtained from Chemicon International INC (Temecula, CA, USA). Fluorescein isothiocyanate (FITC) and Texas red-labeled affinity-purified goat anti-rabbit IgGs, and FITC and Texas red-labeled affinity-purified goat anti-mouse IgGs and normal goat serum were purchased from Jackson ImmunoResearch (West Grove, PA, USA). FITC-

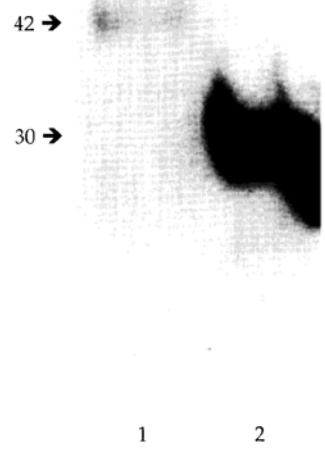


Figure 1. *Immunoblot analysis of human cathepsin D. The human cathepsin D was applied to SDS-PAGE followed by immunoblot analysis. The immunoblot analysis was done with specific IgG against rat liver lysosomal cathepsin D after SDS-PAGE. Lane 1, rat liver lysosomal fraction; lane 2, human cathepsin D. The cathepsin D polypeptides on the left are indicated by their apparent molecular weights (in kilodaltons).*

conjugated phalloidin, Texas red-conjugated phalloidin, and SlowFade anti-fade reagent were purchased from Molecular Probes (Eugene, OR, USA). Nocodazole, taxol, and cytochalasin D were purchased from Calbiochem-Behring (La Jolla, CA, USA). Other chemicals were of reagent grade and were obtained from commercial sources.

Cell Culture and Drug Treatment of Cells

MCF-10A is a diploid human breast epithelial cell line derived from subcutaneous mastectomy in 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.[45] Transfections and cotransfection were performed using the calcium phosphate method with a plasmid containing the neomycin resistance gene as a transfection vector with the mutated (MCF-10AneoT) c-Ha-*ras*.[3] MCF-10A cells and MCF-10AneoT stably transfected with the mutated c-Ha-*ras* were grown in Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture, containing 5% equine serum, supplemented with antibiotics and epidermal growth factor,[3,27,45] but without amphotericin and cholera toxin. The depolymerization of microtubules or microfilaments was achieved by incubation of cells in culture medium containing 2.5 μM nocodazole for 20 min or 1 μM cytochalasin D for 20 min at 37°C.

To determine whether stabilization of the microtubules will result in reclustering of cathepsin D and lamp-1 in MCF-10AneoT cells, 2 nM taxol, which inhibit the microtubule depolymerization, was added to the culture medium and incubated for 4 hr.

Preparation of Monospecific Anti-Cathepsin D IgG

Cathepsin D was purified to homogeneity as described previously.[25,26] Antisera was raised in rabbits (New Zealand white male) against the native mature form of rat liver lysosomal cathepsin D as described previously.[25,26] An IgG fraction was affinity-purified by protein A Sepharose CL-4B, followed by immunoaffinity chromatography using antigen-Sepharose 4B. This antibody has been used here for staining of cathepsin D in human cells. The purified monospecific anti-cathepsin D IgG recognizes single-chain and two-chain forms of the mature enzyme in immunoblots.[14] This antibody has been used previously for staining of cathepsin D in human normal and tumor tissues.[14]

Immunoblot Analysis of Cathepsin D

To examine whether anti-rat cathepsin D antibody was immunoreactive with human cathepsin D, purified human cathepsin D was obtained from commercial sources (Athens Research and Technology, Ga, USA) and this enzyme

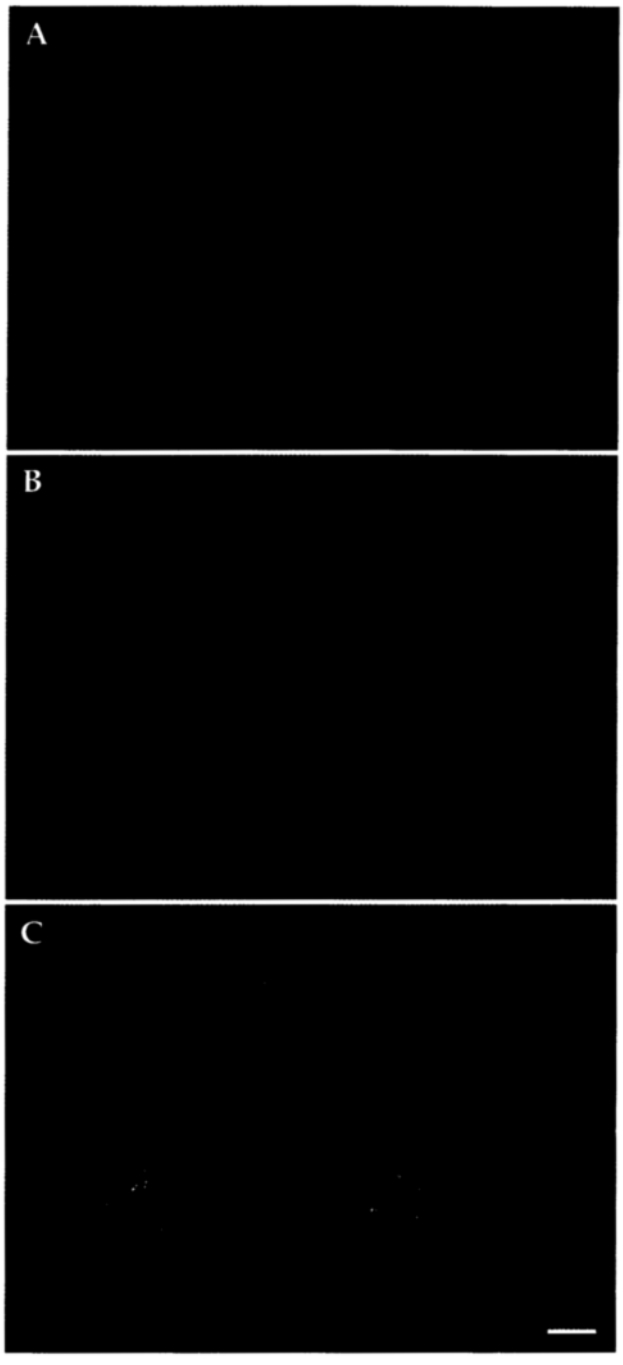


Figure 2. *Intracellular localization of cathepsin D and lamp-1 in MCF-10A cells. Cells were double-labeled to localize cathepsin D and lamp-1 by immunofluorescence labeling with specific antibodies (for see details in Materials and Methods). Cells were fixed, and stained with antibodies against cathepsin D and lamp-1, respectively. The secondary antibodies were FITC-conjugated anti-rabbit IgG and Texas red-conjugated anti-mouse IgG. Cathepsin D staining (green fluorescence in A) is concentrated in punctate structures around the perinuclear region as is lamp-1 staining (red fluorescence in B). The colocalization of cathepsin D and lamp-1 in perinuclear lysosomes is evident by the presence of yellow spots in the superimposed image (C). Bar, 10 μm.*

(approximately 200 ng) was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (10–20% SDS-PAG Gel Plate, Daiichi Pure Chemical, Tokyo, Japan) and then immunoblot analysis was performed as described previously.[25,26]

The crude lysosomal fraction was prepared from rat liver as described previously[25] and a sample of lysosomal fraction (approximately 100 μg) was taken for the immunoblot analysis. For immunostaining, the nitrocellulose strip was first soaked in phosphate-buffered saline containing 10% dry milk and 0.05 % Tween for 60 min followed by incubation with 50 μg/ml of a rabbit monospecific IgG against rat cathepsin D for 60 min. The strip was then soaked in horseradish peroxidase-labeled goat anti-rabbit Fab (Cappel, UK) for 60 min and washed with PBS containing 0.05% Tween three times, and then stained with chemiluminescence reagent (DuPont NEN, Mass, USA).

Immunofluorescence Microscopy

Cells were grown for 3 days in 6-well plates on glass coverslips in DMEM-5% equine serum. Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, permeabilized in PBS containing 0.1% saponin. After washing with PBS, cells were blocked with PBS-2 mg/ml bovine serum albumin. All subsequent antibody and wash solutions contained 0.1% saponin. Cells were incubated with specific primary antibodies (rabbit anti-cathepsin D IgG, mouse anti-lamp-1 IgG, mouse anti-α-tubulin IgG, mouse anti-dynein IgG, and mouse anti-kinesin IgG) for 2 hours and washed. Then cells were incubated for 1 hour with either FITC-conjugated or Texas red-conjugated affinity-purified goat anti-rabbit IgG and FITC-conjugated or Texas red-conjugated affinity-purified goat anti-mouse IgG at 20 μg/ml, followed by three washes in PBS containing 0.1 % saponin. For actin staining, cells were incubated with FITC-phalloidin or Texas red-phalloidin (diluted to 1 U/ml) for 30 min at room temperature followed by three washes PBS containing 0.1 % saponin. In controls, either preimmune serum (rabbit or mouse) was substituted for the primary antibody or no primary antibody was used. To depolymerize microtubules, nocodazole dissolved in DMSO was added to the culture medium to a final concentration of 2.5 μM and incubated for 20 min before

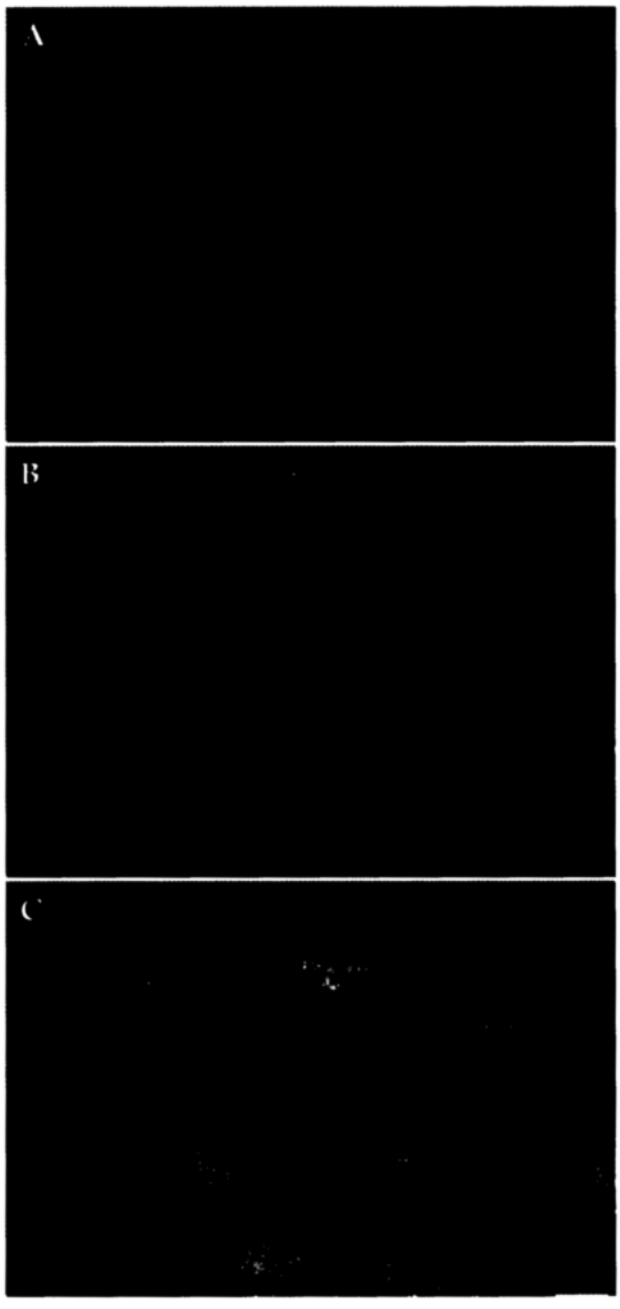


Figure 3. *Immunocytochemical colocalization of intracellular cathepsin D and lamp-1 in MCF-10AneoT cells. Cells were double-labeled to localize cathepsin D and lamp-1 by immunofluorescence labeling with specific antibodies as in the legend to Figure 2. Cells were fixed, and stained with antibodies against cathepsin D and lamp-1, respectively. The secondary antibodies were FITC-conjugated anti-rabbit IgG and Texas red-conjugated anti-mouse IgG. Superimposed images of cathepsin D and lamp-1 in neoT counterpart transfected with oncogenic ras cell lines (C) is shown. Intracellular expression of cathepsin D appears to be increased in neoT cells (A) and cathepsin D staining is present in small punctate structures in the cell periphery and perinuclear region. Lamp-1 staining (B) is also seen in small punctate structures throughout the cytoplasm. Merging of the images results in colocalization in the perinuclear region of neoT cells (C). However, part of the staining in the cell periphery is distinct with structures that stain for cathepsin D, but not lamp-1. Threre are also some lamp-1 positive structures that do not stain for cathepsin D. Note that the colocalization of cathepsin D and lamp-1 in perinuclear lysosomes of neoT cells (C) is evident by the presence of yellow spots in the superimposed image. Bars, 10 μm.*

cell fixation. In some experiments, MCF-10AneoT cells were incubated for 4 hr in the presence of 2 nM taxol at 37°C. To depolymerize microfilaments, cytochalasin D was added to the cell culture medium to a final concentration of 1 μM and incubated for 20 min. Finally, cells were mounted with SlowFade anti-fade reagent and observed on a Zeiss LSM310 confocal laser scanning microscope. Images were converted to TIFF format, and contrast levels of the images were adjusted using Adobe Photoshop software (Adobe Co.) on a Power Macintosh (7600/120; Apple Computers).

Results

Altered intracellular localization of cathepsin D in human breast epithelial cells transfected with mutated ras

Alterations in subcellular distribution of lysosomal cathepsins B and D were observed in human breast epithelial cells transfected with oncogenic *ras*, but not with wild-type *ras*.[22,37,44] An increased association of cathepsin B with plasma membrane/endosomal fractions could be demonstrated by immunocytochemical staining, suggesting that an alteration in the trafficking of cathepsin B is closely linked to malignant progression in epithelial cells.[44] Here we tried to determine by confocal immunofluorescence microscopy whether there was an alteration in the intracellular localization of the lysosomal aspartic proteinase cathepsin D between the parental MCF-10A cells and the MCF-10AneoT cell line created with mutated c-Ha-*ras*.

First, we sought to demonstrate that the purified anti-rat cathepsin D antibody was indeed immunologically cross-reactive with human cathepsin D. When purified human cathepsin D was applied to SDS-PAGE and reacted with anti-cathepsin D antibody in the immunoblot analysis, as shown in *Figure 1*, human cathepsin D was found to be immunoreactive with anti-rat cathepsin D antibody. The immunoreactive molecular forms of the human cathepsin D were slightly different from that of rat cathepsin D. The results indicated that most of the immunoreactive human cathepsin D corresponded to the processed form of 30-kDa; in rat, the immunoreactive band of cathepsin D was the single-chain 42-kDa form.

The activated mutant of *ras* induces a change in cell morphology, producing cell processes and protrusions, as well as affecting the intracellular trafficking of lysosomal cathepsins.[44] These results suggest that there is a link between lysosomal protein traffic and cytoskeletal organization in the breast epithelial cells. Indeed, the cell shapes were apparently different between subconfluent wild-type MCF-10A and subconfluent mutated *ras* tarnsfectants (MCF-10AneoT). A dramatic change in cell shape occurred in the *ras* transfectants, including the formation of cell processes (data not shown).

We analyzed the intracellular localization of cathepsin D and lamp-1, lysosome-associated membrane protein, both in the MCF-10A and its *ras* transfected counterpart by confocal immunofluorescence microscopy. To determine the intracellular localization of cathepsin D and lamp-1 in wild-type MCF-10A, we double- stained for cathepsin D and lamp-1 using secondary antibodies conjugated to either FITC *(Figure 2 A and C)* or Texas red *(Figure 2 B and C)*, respectively. The staining for cathepsin D was present in small vesicles in the perinuclear region *(Figure 2 A)*. Lysosomes as revealed by lamp-1 staining were primarily in the perinuclear region with fewer in the peripheral cytoplasm *(Figure 2 B)*. Merging of these images showed that cathepsin D and lamp-1 staining colocalized in some vesicles in the perinuclear region *(Figure 2 C)*. The colocalization of cathepsin D and lamp-1 in the perinuclear region corresponded to the typical pattern of lysosomal vacuoles.

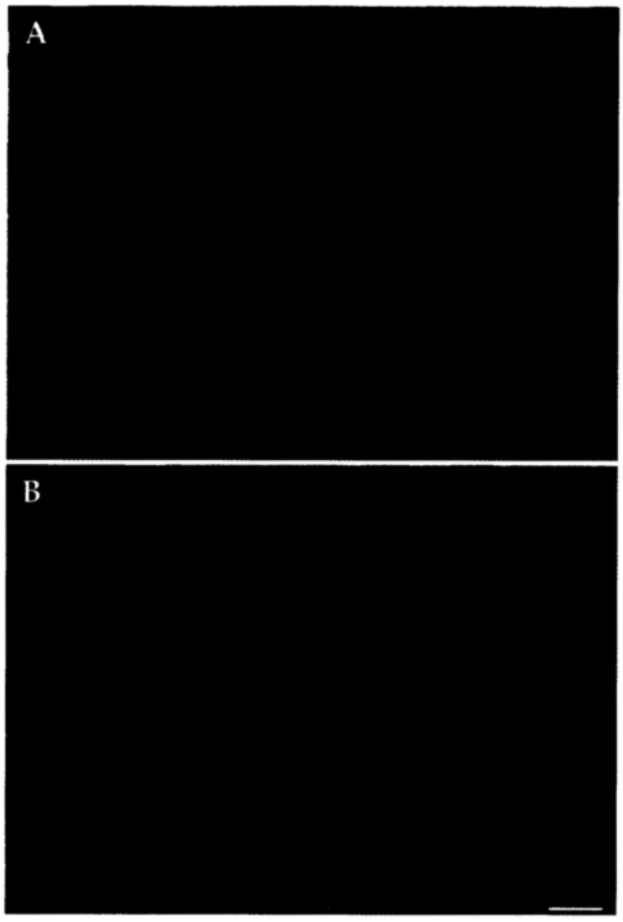


Figure 4. *Localization of microtubules in MCF-10A cells and MCF-10AneoT cells. Cells were fixed and stained with antibody against α-tubulin as described in Materials and Methods. Note that in MCF-10A cells (A) the microtubules are organized into bundles of uniform polarity, but MCF-10AneoT cells (B) lack such polarized bundles of microtubules. Bar, 10 μm.*

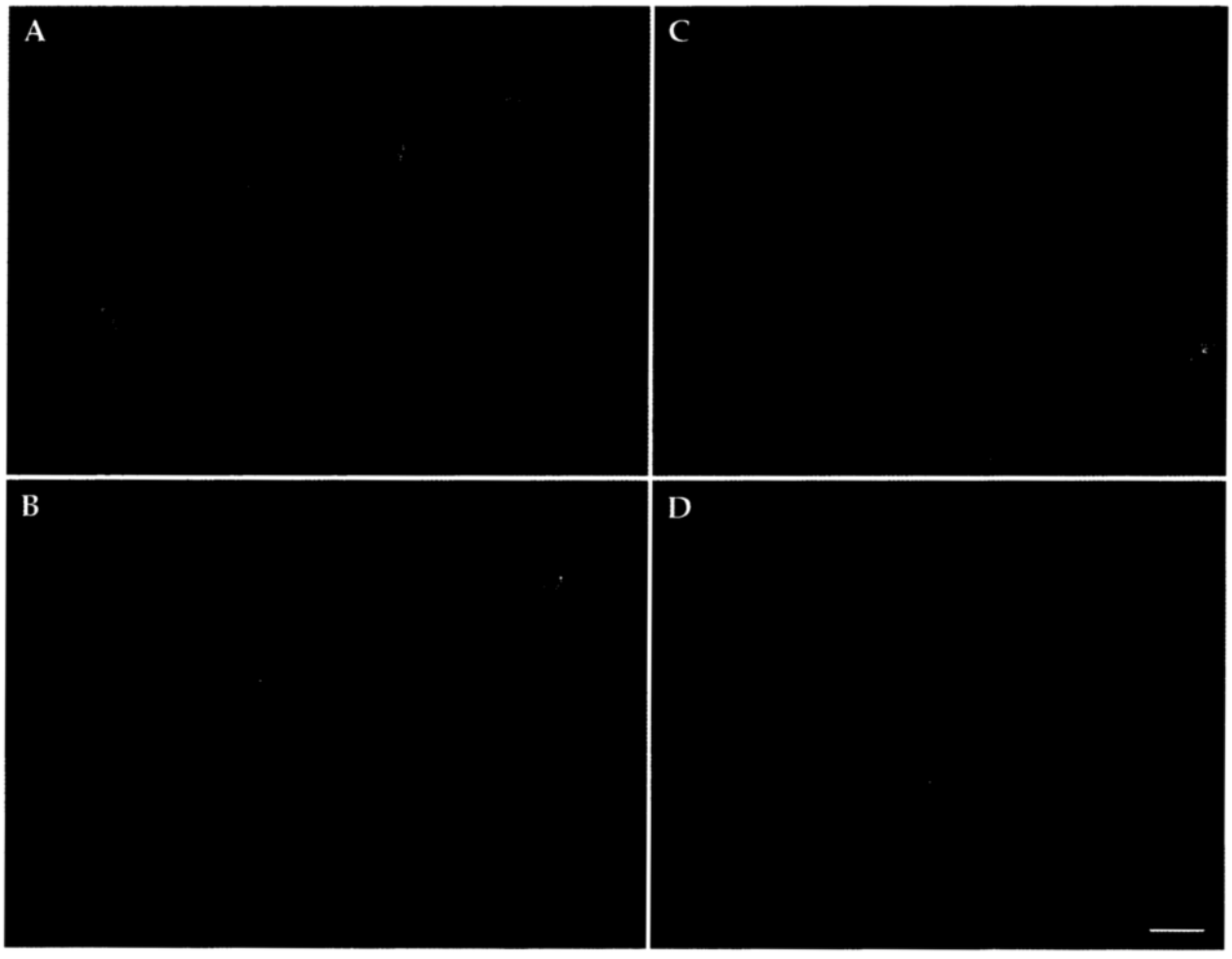
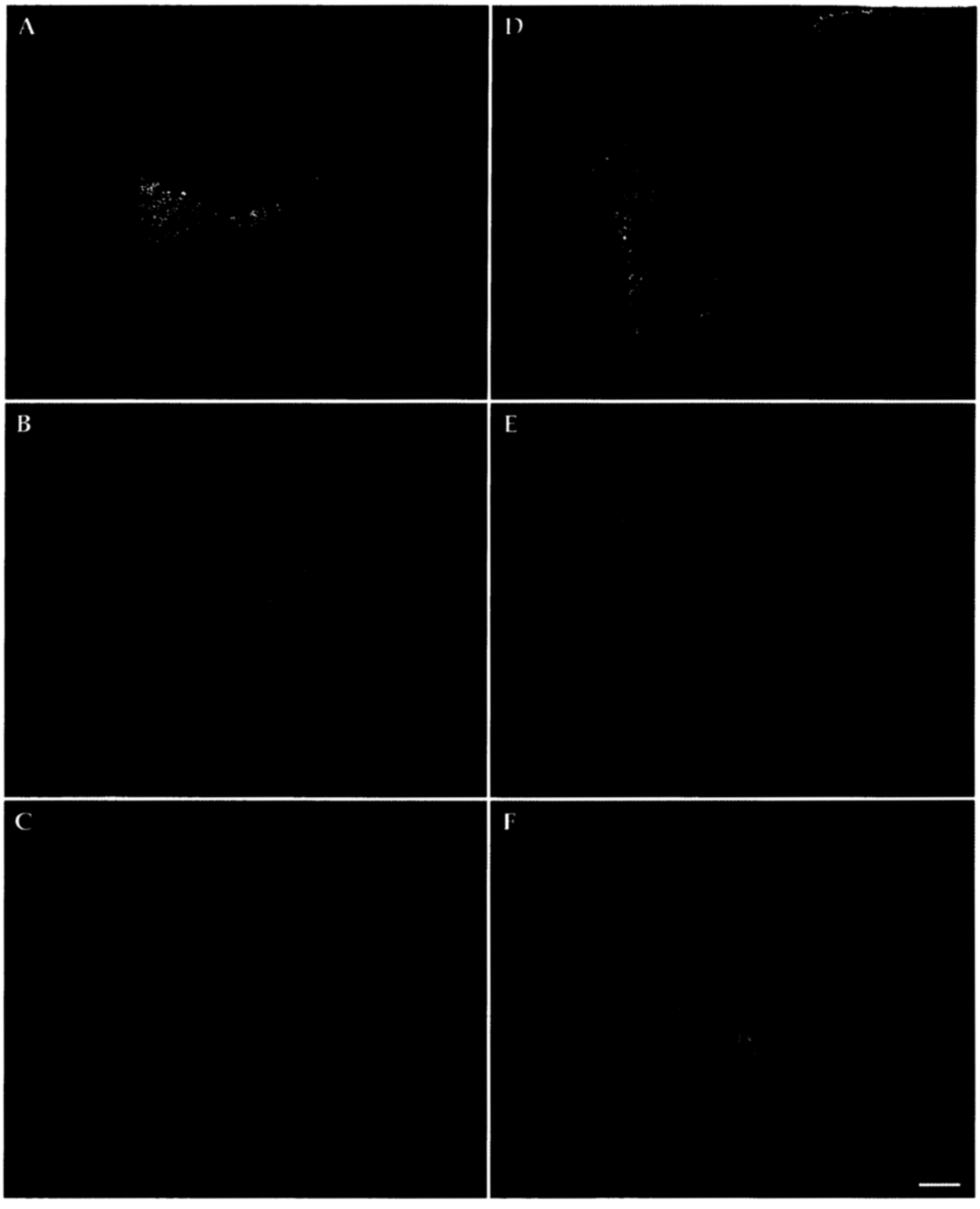


Figure 5. *The effect of nocodazole upon microtubule organization and the intracellular localization of cathepsin D and α-tubulin in MCF-10A cells. Cells were incubated with nocodazole for 20 min (B, D) or without nocodazole (A, C), then cells were fixed, and stained with antibodies against cathepsin D and α-tubulin, respectively, as described in Materials and Methods. Superimposed images of cathepsin D or lamp-1 and α-tubulin are shown. In A and B, cells were double-labeled to localize cathepsin D (green fluorescence) and α-tubulin (red fluorescence) and in C and D, lamp-1 (red fluorescence) and α-tubulin (green fluorescence). Treatment of MCF-10A cells with nocodazole induces a concomitant loss of lysosome clustering and microtubule organization toward the area of the microtubule organizing center, and cathepsin D and lamp-1 staining appears to be scattered in the cytoplasm (B, D). Bar, 10 μm.*

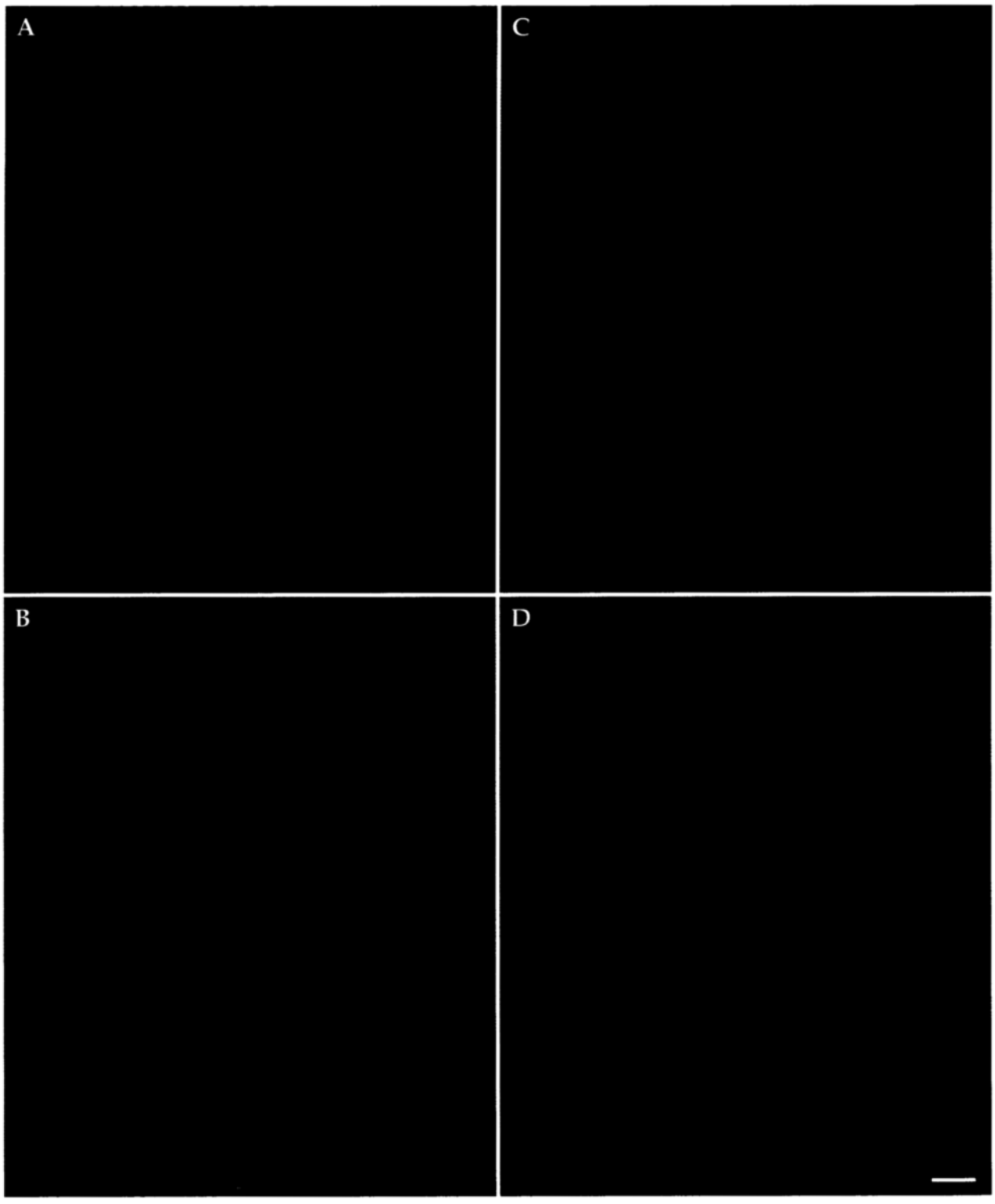
The MCF-10AneoT cells were more spread than the 10A cells and exhibited cell protrusions *(Figure 3)*. Cathepsin D staining in punctate structures was strong *(Figure 3 A)*, suggesting that cathepsin D may be somewhat up-regulated in the transformed cells although a quantitative analysis was not performed. Strong staining was found in small punctate structures throughout the cytoplasm of the neoT cells *(Figure 3 A)*.

Figure 6. *The effect of nocodazole or taxol upon microtubule organization and the intracellular localization of cathepsin D and α-tubulin in MCF-10AneoT cells. MCF-10AneoT cell lines were incubated with nocodazole for 20 min (B, E) or with taxol (C, F) for 4 h after three washes of culture medium. Then cells were fixed, and stained with antibodies against cathepsin D and a-tubulin, respectively, as described in Materials and Methods. Cells were double-labeled to localize cathepsin D or lamp-1 and α-tubulin, and superimposed images of cathepsin D and α-tubulin are shown. In A–C, cells were double-labeled to localize cathepsin D (green fluorescence) and α-tubulin (red fluorescence) and in D–F, lamp-1 (red fluorescence) and α-tubulin (green fluorescence). Treatment of MCF-10AneoT cells with nocodazole does not change the intracellular localization of cathepsin D (B) or lamp-1 (E) which are scattered in the cytoplasm. In the presence of taxol which promotes tubulin polymerization and inhibits its depolymerization, cathepsin D (C) and most of lamp-1 (F) staining in punctate structures becomes localized at the perinuclear region. Tubulin gathers in bundles mainly located at the perinuclear region after 4 h of taxol treatment. These results suggest that the alteration of intracellular trafficking of cathepsin D and lamp-1 is correlated with microtubule reorganization in MCF-10AneoT cells. Bar, 10 μm.*

Lamp-1 staining was also seen in the small punctate structures throughout the cytoplasm and in vesicles at the plasma membrane *(Figure 3 B)*. Cathepsin D and lamp-1 staining were observed in cell processes, suggesting that lysosomes are moving into the cell processes and that cathepsin D may be in the exocytotic pathway. Merging of the images resulted in some colocalization in the perinuclear region of neoT cells *(Figure 3 C)*. However, the



staining pattern in the cell periphery appeared to be distinct. There were some spots that contained cathepsin D staining that did not contain lamp-1 staining, and some lamp-1 positive spots that did not stain for cathepsin D *(Figure 3 C)*. These results indicate that the increased small vacuolar structures may be endosomes rather than lysosomes. Our observations suggest that lysosomal cathepsin D may be targeted to specialized transporting vesicles which function in the process of tumor development in malignant cells. Also the identification of lamp-1



positive-lysosomes that do not stain for cathepsin D at the plasma membrane raises the posssibility that different classes of lysosomes/endosomes, not as yet identified, exist within cells.

Alteration of microtubule organization in ras transfectants

In living cells, the intracellular movement of organelles such as lysosomes and endosomes occurs along microtubules,[20] yet an active role for microfilaments and intermediate filaments has also been proposed.[9,28] Therefore, we examined by confocal immunofluorescence staining whether microtubule organization was altered in the *ras* – transfectants. To probe microtubules, an anti-α-tubulin monoclonal antibody was used. The immunofluorescence study revealed that the microtubule organization was markedly different in the two cell lines. In the parental MCF-10A cells, microtubules formed uniformly and developed predominantly in an unidirectional manner from the microtubule organizing center *(Figure 4 A)*. In contrast, neoT cells had microtubule bundles in the cell processes; the microtubule organizing center was difficult to distinguish and microtubules developed in random directions *(Figure 4 B)*.

To delineate the intracellular localization of cathepsin D or lamp-1 staining in the MCF-10A and neoT cells, we double-stained for cathepsin D and α-tubulin or lamp-1 and α-tubulin using secondary antibodies conjugated to either Texas red or FITC. To study the possible role of microtubules in translocating lysosomal cathepsin D, MCF-10A cells were treated for 20 min with 2.5 μM nocodazole which disrupts microtubules[20] and immunofluorescence labeling of cells with anti-cathepsin D and α-tubulin or with anti-lamp-1 and α-tubulin was carried out *(Figure 5)*. Superimposed images of cathepsin D and α-tubulin *(Figure 5 A)* or lamp-1 and α-tubulin *(Figure 5 C)* showed that cathepsin D and lamp-1 is present in small punctate structures in the perinuclear region. When the microtubules were depolymerized by treatment with nocodazole, cathepsin D and lamp-1 labeling became scattered in the cytoplasm, respectively, *(Figure 5 B, D)*. In contrast, no significant effect on the intracellular localization of cathepsin D or lamp-1 was observed after the addition of nocodazole to neoT cells *(Figure 6 B, E)*. The organization of microtubules and localization of cathepsin D and lamp-1 in nocodazole-treated MCF-10A cells *(Figure 5 B, D)* was found to be similar to those in untreated neoT cells *(Figure 6 A, D)*.

To determine whether stabilization of the microtubules will result in reclustering of cathepsin D and lamp-1 in neoT cells, 2 nM taxol, which inhibits microtubule depolymerization, was added to the culture medium. After incubation for 4 hr, immunofluorescence labeling of cathepsin D and α-tubulin or lamp-1 and α-tubulin was performed. The microtubules were stabilized, and a polymerized microtubule network was observed *(Figure 6 C, F)*. Furthermore, the reclustering of cathepsin D occurred in an unidirectional manner towards the area of the microtubule organizing center *(Figure 6 C)*. Similar observations were made in regard to lamp-1 staining in neoT cells in the presence of taxol *(Figure 6 F)*.

These results suggest that enhanced microtubule stability has an important role in maintenance of lysosomal cathepsin D and lamp-1 positive vesicles in the juxtanuclear region.

Microtubules are involved in the alteration of lysosomal cathepsin trafficking in ras transfected cells

The loss of the clustering of cathepsin D and lamp-1 towards the microtubule organizing center after microtubule disruption with nocodazole suggested that microtubule motor proteins are involved in their inward movement. It is generally believed that two microtubule motor proteins are responsible for intracellular vesicular and organelle transport: the microtubule plus-end-directed motor protein kinesin is thought to be involved in membrane transport to the plus-end of microtubules, and on the other hand, cytoplasmic dyneins are involved in minus-end-directed organelle transport.[10,18,24]

To determine the role of microtubule motor proteins in regulating the intracellular trafficking of cathepsin D in neoT cells, immunofluorescence labeling of cytoplasmic dynein and kinesin using monoclonal antibodies was performed. Double labeling studies with anti-dynein *(Figure 7 B)* and anti-cathepsin D *(Figure 7 A)* or anti-kinesin *(Figure 7 D)* and anti-cathepsin D *(Figure 7 C)* in permeabilized cells of MCF-10A showed that cathepsin D staining was observed in small punctate structures in perinuclear region *(Figure 7 A, C)*, and that staining for dyneins and kinesins was also intense in the juxtanuclear region *(Figure 7 B, D)*.

In contrast, dynein and kinesin staining was spread throughout the cytoplasm of the *ras* transfectants and intense staining at the perinuclear region was not observed (data not shown).

Figure 7. *Colocalization of cathepsin D and microtubule motor proteins in MCF-10A cells. Cells were labeled to localize cathepsin D (A, C), dynein (B), and kinesin (D) by immunofluorescence staining with respective antibodies as described in Materials and Methods. The secondary antibodies were FITC-conjugated anti-rabbit IgG (A, C) and Texas red-conjugated anti-mouse IgG (B, D). Cathepsin D staining (green fluorescence in A, C) is concentrated in punctate structures around the perinuclear region and dynein (B) or kinesin (D) staining (red fluorescence) is also seen in the perinuclear region. Bar, 10 μm.*

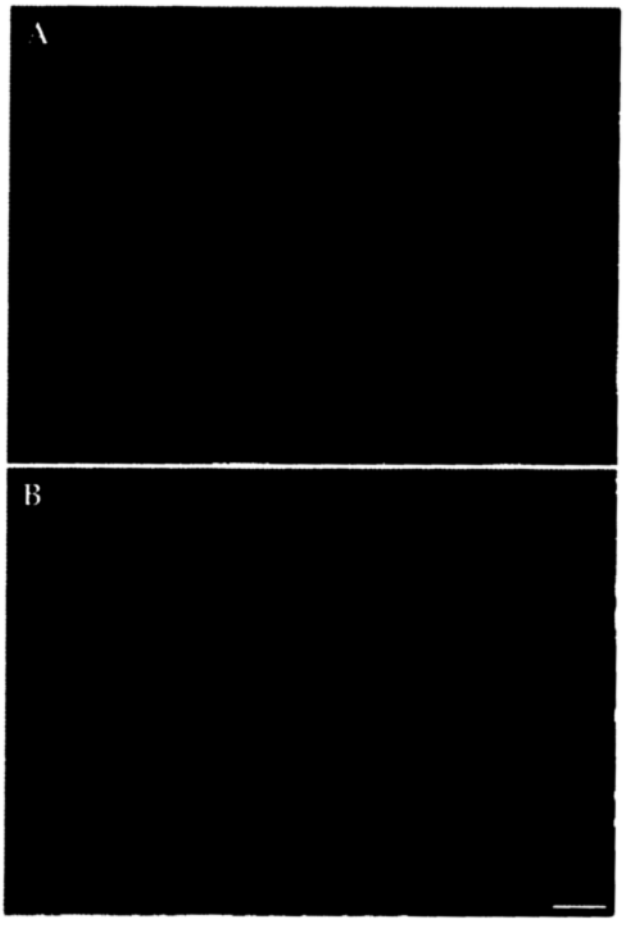


Figure 8. *Localization of actin filaments in MCF-10A cells and MCF-10AneoT cells. MCF-10A cells (A) or MCF-10AneoT cells (B) were fixed, and then filamentous actin was stained with FITC-phalloidin. Bar, 10 μm.*

The actin cytoskeleton is not involved in altered trafficking of lysosomal cathepsin D in MCF-10A cells

Because changes in the actin cytoskeleton are often correlated with microtubule rearrangements,[7,8,17] we analyzed whether there were any changes in the actin network in neoT cells. Actin cables were stained with the FITC-phalloidin. As shown in *Figure 8*, the actin filament organization was found to be similar between MCF-10A cells (A) and MCF-10AneoT cells (B).

The microfilament cytoskeleton was completely depolymerized by treating MCF-10A cells *(Figure 9 B, C)* or MCF-10AneoT cells *(Figure 10 B, D)* with 1 μM cytochalasin D for 20 min. Actin cables were stained with Texas red-phalloidin or FITC-phalloidin and cathepsin D and lamp-1 were stained with secondary antibody conjugated to FITC and Texas red, respectively. The transfection of *ras* into the parental MCF-10A cells did not cause a dramatic change in the actin stress fibers *(Figure 10 A, C)*. Small punctate structures staining for cathepsin D or lamp-1 were found to be spread throughout the cytoplasm of neoT cells despite the presence of cytochalasin D which inhibits actin polymerization *(Figure 10 B, D)*. When the parental MCF-10A cells were treated with cytochalasin D, an alteration in the intracellular localization of cathepsin D and lamp-1 was not observed *(Figure 9 B, C)*. These results suggest that neither microfilaments nor intermediate filaments are involved in regulating retention of lysosomes in the juxtanuclear region of MCF-10A cells. Thus, our findings support the notion that the alteration of intracellular trafficking of cathepsin D and lamp-1 may be closely associated with the reorganization of microtubules in MCF-10A cells transfected by active *ras*.

Discussion

The important finding of this study is that the transfection of activated *ras* in human breast epithelial cells leads to a dramatic change in the intracellular localization of lysosomal cathepsin D and lamp-1 as demonstrated by confocal immunofluorescence microscopy. Cathepsin D and lamp-1 staining was clustered and colocalized around the perinuclear region in parental MCF-10A cells *(Figure 2)*. In contrast, in neoT cells expressing the mutant *ras* gene, cathepsin D and lamp-1 staining was randomly spread throughout the cytoplasm and at the cell periphery *(Figure 3)*. Cathepsin D labeling in small punctate structures colocalized with lamp-1 staining in the vicinity of the nucleus in 10A cells. In neoT cells, the cell morphology was changed and cells had protrusions and processes. Small vesicles containing cathepsin D seemed to be targeted towards these cell protrusions. Lamp-1 staining was also seen in the small punctate structures throughout the cytoplasm and appeared to move towards the plasma membrane. Merging of the images resulted in considerable colocalization in the perinuclear region in neoT cells *(Figure 3 C)*, however, costaining in the cell periphery was less pronounced. There were some spots that contained cathepsin D staining, but not lamp-1. Some lamp-1-positive spots that did not stain for cathepsin D were observed. Thus increased small vacuolar structures in neoT cells may be related to early endosomes rather than lysosomes.[34]

Sloane et al[44] previously reported the altered trafficking and plasma membrane-association of enzymatically active cathepsin B in MCF-10Aneo T cells as revealed by multiple procedures such as cell fractionation techniques, immunofluorescent procedures, and immunoelectron microscopy. They suggested that transport of lysosomes to the cell periphery may be a mechanism common to cells that are involved in local degradative and invasive processes. MCF-10A neoT cells transfected with mutated *ras* have been shown to invade through Matrigel *in vitro*[27] and produce preneoplastic lesions in nude beige mice that progress to neoplasias in 30% of the mice.[21] Since trans-

fection of *ras* oncogene did not result in increased expression of cathepsin B in MCF-10A human breast epithelial cells, they suggested that the induced secretion and/or membrane-association of active cathepsin B in the *ras* transfectants might be related to the invasive process in neoplastic regions.[36,44] Secretion and plasma membrane-association of cathepsin B have been documented in tumor derived cell lines.[5,16,23,29,30,38,39,40] Therefore, it has been suggested that lysosomal cysteine proteinases could participate in the local dissolution of the extracellular matrix during the invasive stages of the tumor metastasis. Our data for the altered localization of lysosomal aspartic proteinase cathepsin D in *ras* transfected human breast epithelial cells suggest this enzyme might also function in tumor invasion, however, the low pH optimum of cathepsin D raises a question about an extracellular role for this enzyme.[25]

The perinuclear location and the multiple functions of lysosomes are strongly dependent on the microtubule network and this organized intracellular distribution is disrupted by drugs such as nocodazole which alter microtubule structure, and cause dispersion of lysosomes throughout the cytoplasm.[20] The dispersed clusters of lysosomes are reclustered to the perinuclear region upon removal of drug, indicating the involvement of microtubules. In the present study, we have demonstrated that exposing MCF-10A cells to nocodazole induced a disruption of microtubule organization and a spreading of cathepsin D and lamp-1 into the cytoplasm and movement of these proteins toward the cell periphery *(Figure 5)*. These findings were in good agreement with the notion that cytoskeletal structures like microtubules are required for the intracellular trafficking of lysosomes and endosomes. It was also surprising to find that the organization of microtubules was markedly different in MCF-10A cells from that in MCF-10AneoT cells in which microtubules appeared to be disorganized and microtubule bundles were found in the cell processes *(Figure 4)*. Thus altered intracellular trafficking of lysosomal cathepsin D correlated well with the disorganization of microtubules. It remains to be seen how oncogenic *ras* alters the organization of microtubules and intracellular trafficking of lysosomes.

To further investigate the involvement of microtubules in the trafficking of lysosomes, we addressed the effects of taxol which inhibits microtubule depolymerization.[6,35] Our results revealed that in the taxol-treated neoT cells microtubules were stabilized, a polymerized microtubule network to the microtubule organizing center was seen, and cathepsin D staining moved in a unidirectional man-

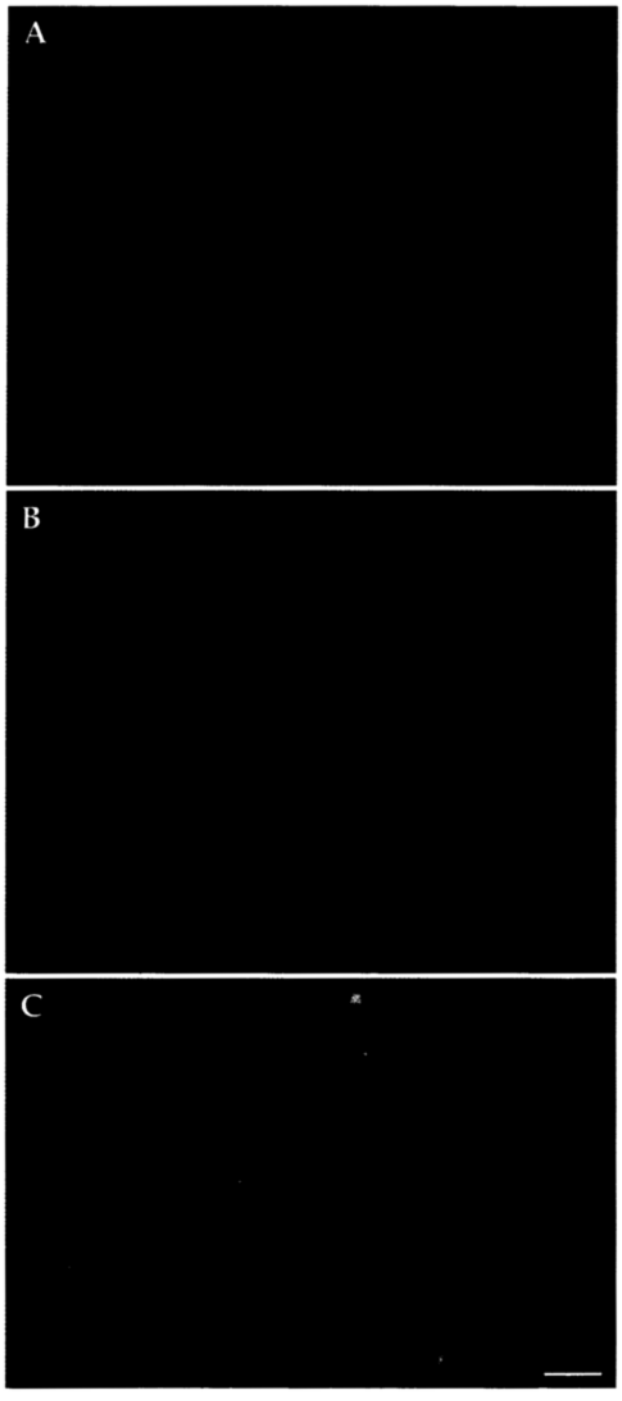


Figure 9. *Cathepsin D and actin localization in MCF-10A cells. Cells were incubated with cytochalasin D (B, C) for 20 min or without cytochalasin D (A), then cells were fixed, and double-staining was performed. Cells were stained with antibodies against cathepsin D (green fluorescence in A, B). Filamentous actin was stained with Texas red-phalloidin (red fluorescence in A, B). In C, lamp-1 (red fluorescence) and filamentous actin (green fluorescence) were stained. Superimposed images of cathepsin D or lamp-1 and filamentous actin are shown. In MCF-10A cells, the actin cytoskeleton contains stress fibers (A). In contrast, cytochalasin D treatment causes a redistribution of the actin cytoskeleton (B, C). The effect of cytochalasin D on the intracellular localization of cathepsin D and lamp-1 is less pronounced; these lysosomal proteins are still localized in the perinuclear region (B, C). Bar, 10 μm.*

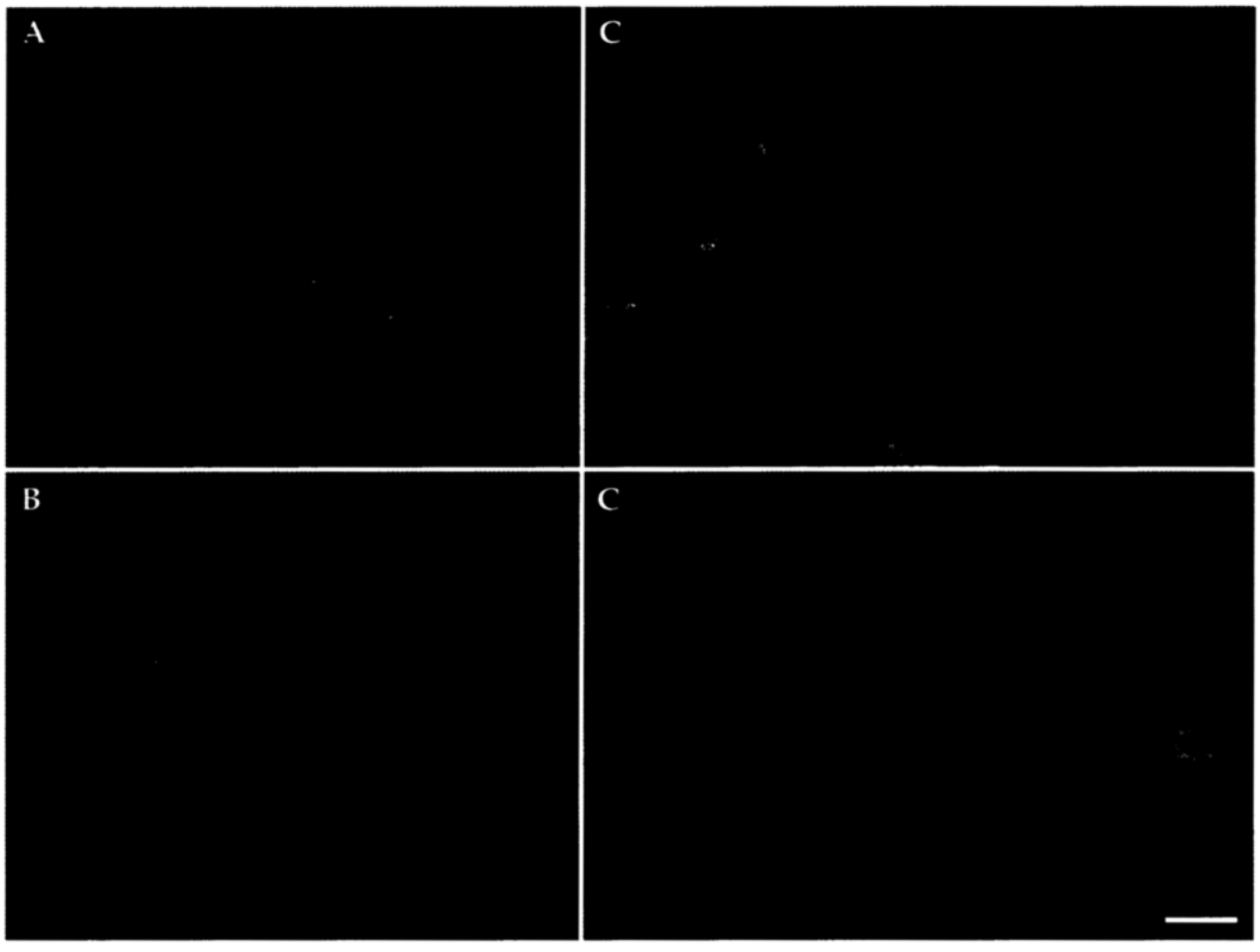


Figure 10. *Cathepsin D and actin localization in MCF-10AneoT cells. Cells were incubated with cytochalasin D (B, D) for 20 min or without cytochalasin D (A, C), then cells were fixed, and double-staining was performed. Cells were stained with antibodies against cathepsin D (A, B) or lamp-1 (C, D), and filamentous actin was stained with Texas red- phalloidin (A, B) or FITC-phalloidin (C, D) as described in the legend to Figure 9. Superimposed images of cathepsin D or lamp-1 and filamentous actin are shown. In MCF-10AneoT cells, the actin cytoskeleton appears to contain stress fibers (A, C). Cytochalasin D treatment causes a dramatic redistribution of the actin cytoskeleton to the cell periphery (B, D). The effect on the localization of cathepsin D and lamp-1 is less pronounced, such that the staining of cathepsin D and lamp-1 remains scattered throughout the cytoplasm. Bar, 10 μm.*

ner towards the perinuclear region *(Figure 6)*. A similar observation was found for lamp-1 staining. These results indicated that microtubule stability has an important role in retention of lysosomes in the juxtanuclear region. The reclustering of lysosomal cathepsin D and the restoration of microtubules in the presence of taxol in neoT cells suggested an involvement of microtubule motor proteins such as the plus-end directed motor protein kinesin or the minus-end directed motor protein dynein. Kinesin is involved in the transport of lysosomes[10] and the plus-end directed Golgi-to-ER transport,[19] and dynein has been implicated in the *in vitro* fusion of endosomes.[2] Confocal immunofluorescence studies with anti-dynein and anti-cathepsin D or anti-kinesin and anti-cathepsin D using permeabilized cells of MCF-10A showed that dyneins and kinesins were intensely stained in the juxtanuclear region *(Figure 7)*. In contrast, the dynein and kinesin staining pattern was somewhat diffuse and spread throughout the cytoplasm of *ras* transfectants; punctate or filamentous staining was not observed (data not shown). Therefore, the immunofluorescence data showing the diffuse cytoplasmic staining of these motor proteins suggests that these cytoplasmic motor proteins might be released from microtubules possibly by hydrolyzing ATP in neoT cells.[24]

Regarding the intracellular translocation of cathepsin B in malignant cells, it was demonstrated previously that protein kinase C-stimulation with 12-lipoxygenase metabolite of arachidonic acid, 12-(S)-hydroxyeicosatetraenoic acid [12-(S)-HETE], in concentrations which are optimal for integrin expression, induces cytoplamic translocation toward the cell periphery of vesicles stain-

ing for cathepsin B and cathepsin B secretion in the more malignant cell lines.[11] Further studies demonstrated that in metastatic B16a murine melanoma cells, the translocation of $\alpha_{IIb}\beta_3$-integrin molecule from a intracellular pool, a cytoplasmic tubulovesicular structure (TVS) most probably corresonding to endosomes, to the plasma membrane is modulated by 12-(S)-HETE.[46,47] The functional significance of this was further supported by the observation that disruption of microtubules or intermediate filaments prevents both the vesicular to tubular transition of TVS as well as the increased surface expression of $\alpha_{IIb}\beta_3$-integrin induced by 12-(S)-HETE.[47] Therefore, translocation of lysosomes and secretion of lysosomal cathepsin B may be mediated through cytoskeleton rearrangements by protein kinase C-regulated process. Further studies will be needed to demonstrate the involvement of microtubule motor proteins in the intracellular trafficking of lysosomal cathepsins in neoT cells. Microinjection of specific antibody to inhibit the motor activity may provide a useful tool.

In the present study, we have investigated the effect of perturbation of the actin cytoskeleton (using cytochalasin D) on the trafficking of lysosomal cathepsin D. This drug did not affect the localization of cathepsin D or lamp-1 in MCF-10A or 10AneoT cells. It is conceivable that the actin cytoskeleton might not be involved in lysosome dynamics in *ras* transfectants. In the case of cell migration where membrane dynamics are critical, actin is thought to be involved in the cellular movements of lysosomes. There are other indications that actin-based processes are involved in regulating intracellular membrane transport.[8] Although cytochalasin D, known to disrupt the supramolecular oraganization of the actin network, did not change the trafficking of lysosomal cathepsin D in MCF-10A cells, it remains to be seen whether all actin filaments are affected equally.

Taken together, our results support a model in which cytoskeletal microtubule organization is closely related to the trafficking of lysosomes/endosomes, with oncogenic *ras* interfering with such organization in human breast epithelial cells.

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