Interaction of Human Breast Fibroblasts with Collagen I Increases Secretion of Procathepsin B*

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Interactions of stromal and tumor cells with the extracellular matrix may regulate expression of proteases including the lysosomal proteases cathepsins B and D. In the present study, we determined whether the expression of these two proteases in human breast fibroblasts was modulated by interactions with the extracellular matrix component, collagen I. Breast fibroblasts were isolated from non-malignant breast tissue as well as from tissue surrounding malignant human breast tumors. Growth of these fibroblasts on collagen I gels affected cell morphology, but not the intracellular localization of vesicles staining for cathepsin B or D. Cathepsins B and D levels (mRNA or intracellular protein) were not affected in fibroblasts growing on collagen I gels or plastic, nor was cathepsin D secreted from these cells. In contrast, protein expression and secretion of cathepsin B, primarily procathepsin B, was induced by growth on collagen I gels. The induced secretion appeared to be mediated by integrins binding to collagen I, as inhibitory antibodies against α1, α2b, and β1 integrin subunits prevented procathepsin B secretion from fibroblasts grown on collagen. In addition, procathepsin B secretion was induced when cells were plated on β1 integrin antibodies. To our knowledge, this is the first examination of cathepsin B and D expression and localization in human breast fibroblasts and their regulation by a matrix protein. Secretion of the cysteine protease procathepsin B from breast fibroblasts may have physiological and pathological consequences, as proteases are required for normal development and for lactation of the mammary gland, yet can also initiate and accelerate the progression of breast cancer.

The interaction of cells with extracellular matrix proteins can modulate cell proliferation, polarity, survival, differentiation, adhesion, migration, and tumorigenicity (1, 2). The tight regulation of the spatial and temporal organization of extracellular matrices is integral to physiological processes such as embryonic development, whereas disruptions in this organization can lead to pathologies such as tumor invasion, metastasis, and fibrosis (2, 3). Degradation of the extracellular matrix accompanies physiological and pathological processes; the regulation of this degradation is altered in pathological processes. The proteases responsible for this degradation are of the serine, cysteine, aspartic, and metalloprotease classes, the various proteases interacting to activate one another in a proteolytic cascade (4, 5).

Extracellular matrix proteins (6, 7) and intact basement membrane (8, 9) can be digested by the cysteine protease cathepsin B and the aspartic protease cathepsin D in vitro. The in vitro biochemical data are supported by immunohistochemical studies of several human carcinomas in which the intensity of staining for cathepsin B and type IV collagen or laminin were inversely correlated. This is true for bladder (10), colon (11), gastric (12), and lung (13) carcinomas. In breast carcinoma an inverse correlation between staining for cathepsin D and basement membrane has been observed (14). We have recently shown that living human breast cancer cells employ multiple proteases, including the cysteine protease cathepsin B, serine proteases of the plasminogen cascade, and matrix metalloproteases (MMPs), to digest type IV collagen (5). Both cathepsin B and D may play a role in many different pathological processes, e.g. Alzheimer’s disease (15), arthritis (16, 17), and cancer (4), perhaps reflecting alterations in regulation of these enzymes.

Extracellular matrix is not only a substrate for proteases, but also plays a role in regulating their expression. For example, growth of epithelial cells, tumor cells, fibroblasts, and macrophages on vitronectin, fibronectin, laminin, or collagen I increased expression and/or activation of the serine protease urokinase plasminogen activator and of MMPs (18–21). This increase in expression may be mediated through integrins, as ligation of integrins with collagen I increases the expression of collagenase-1 (MMP-1) and -3 (MMP-13) (18, 22). This induction is broad-based as MMP-1, gelatinase A (MMP-2), stromelysin-1 (MMP-3), MMP-13, and membrane type 1 MMP are all induced by growing fibroblasts on collagen I (18, 22). Interestingly, fibroblasts surrounding malignant breast tumors exhibit an increased expression of MMP-13 and stromelysin-3 (MMP-12).
11) (23, 24). We have shown that cathepsin B expression is increased in breast tumor-associated stromal cells as well as in the tumor cells themselves (25). Joensuu et al. (26) made a similar observation for cathepsin D in breast carcinomas. The ability of extracellular matrix components to mediate expression of cysteine and aspartic proteases has not yet been examined.

We hypothesize that interactions of breast cancer and stromal cells with extracellular matrices can modulate expression of cathepsins B and D and thereby modulate the contribution of these enzymes to degradative activities of human breast cancer. Expression of both enzymes has been assessed in breast tumor cells and lymphoyctic cells (27) of and 5mM HEPES. The isolation and characterization of these fibroblasts from F-12 medium enriched with 10% heat-inactivated fetal bovine serum (28) were maintained in DMEM/H9252 (Cambridge, MA) and Chemicon (Temecula, CA), respectively. The differential induction of the two proteases may indicate that tridiopeptidase A, EC number 3.4.2.4.3) from Invitrogen; DIG-RNA and DIG-alkaline phosphatase conjugate Fab fragment from Roche Molecular Biochemicals; ECL-Plus and Hyperfilm ECL from Amersham Biosciences; and Vitrogen-100 collagen (type I) from Coheion (Palto Alto, CA). Horseradish peroxidase-labeled goat anti-rabbit and anti-mouse IgGs were obtained from Pierce. Monoclonal mouse anti-human liver cathepsin D monoclonal antibody (OS-13A) and anti-mouse IgGs were obtained from Sigma; fetal bovine serum, trypsin-EDTA, and collagenase (closed mixture (DMEM/F-12), Hanks/H9252/H9252/H9252) from Invitrogen; and Z-Arg-Arg-NHMec and NH2Mec from Bachem (King of Prussia, PA). CA-O74 was a kind gift from Dr. Mark A. Karmanos Cancer Institute, Detroit, MI) were maintained in DMEM/H9252 (Cambridge, MA) and Chemicon (Temecula, CA), respectively.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F-12), Hanks' balanced salts, saponin, guanidinium thiocyanate, and Hoechst 33258/bisbenzimide were obtained from Invitrogen; DIG-RNA and DNA labeling kits, non-radioactive nucleic acid detection kits, CDPPTM, Star, and anti-DIG-alkaline phosphatase conjugate Fab fragment from Roche Molecular Biochemicals; ECL-Plus and Hyperfilm ECL from Amersham Biosciences; and Vitrogen-100 collagen (type I) from Coheion (Palto Alto, CA). Horseradish peroxidase-labeled goat anti-rabbit and anti-mouse IgGs were obtained from Pierce. Monoclonal mouse anti-human liver cathepsin D monoclonal antibody (OS-13A) and anti-mouse IgGs were a generous gift from Dr. Kenneth M. Yamada (National Institutes of Health, Bethesda, MD). Sheep anti-human cathepsin D antibody was a kind gift from Dr. Paul Matthews (Dana S. Kline Institute, Orangeburg, NY), and mouse anti-human cathepsin D antibody (D101) was obtained from RRKA (Ljubljana, Slovenia). Fluorescein-conjugated, Texas Red-conjugated, and unjugated affinity-purified donkey anti-mouse, anti-rabbit, and anti-goat IgGs, as well as normal donkey serum, were obtained from Jackson Immunoresearch (West Grove, PA); Slow Fade from Molecular Probes (Eugene, OR); and Z-Arg-Ang-NHMec and NH2-Mec from Bachem (King of Prussia, PA). CA-O74 was a kind gift from Dr. Nobuhiko Katunuma (Tokushima Bunri University, Tokushima, Japan).

Cell Culture—Human breast fibroblasts (a kind gift from Drs. Rafael Fridman and Robert Pauley, Wayne State University and Barbara Ann Karmanos Cancer Institute, Detroit, MI) were maintained in DMEM/F-12 medium enriched with 10% heat-inactivated fetal bovine serum and 5 mM HEPES. The isolation and characterization of these fibroblasts has been described elsewhere (29). Briefly, fibroblasts were obtained from three groups of patients: 1) fibroblasts surrounding malignant human breast tumors (9T, 10T, 11T, and 12T); 2) those from benign tissues at a grossly non-malignant site as distant as possible from the tumor (11B and 12B); and 3) those from reduction mammoplasties from patients without cancer (14RM, 31RM, and 33RM). The human breast fibroblasts were infected at passages 5–7 with the LXS1N16EB27 recombinant retrovirus encoding the human papilloma virus serotype 16 E6 and E7 transforming proteins (30), which extended their life but did not result in immortalization (29). All cell lines were shown to be free of Mycoplasma by routine screening with 4,6-diamidino-2-phenylindol dihydrochloride.

Collagen Gels—The human breast fibroblasts were grown on either uncoated or Vitrogen-100 bovine collagen (type I)-coated tissue culture plastic. The latter were prepared by mixing, on ice, eight volumes of collagen I (2.9 mg/ml), pH 2.5, with 1 volume of 0.2 M NaHPO4, 1.3 M NaCl, pH 7.4, followed by one volume of 0.1 M NaOH. Neutralized collagen was added at 1 ml/well to a six-well plate resulting in a collagen concentration of 0.25 mg/cm2. The formation of gels was initiated by incubating at 37 °C for 1 h. The human breast fibroblasts were confluent at a density of 0.5 × 105 or 1.5 × 105 cells/well and grown for either 1 or 3 days.

Preparation of Cell Lysates and Conditioned Media—All human breast fibroblast cell lines, including fibroblast lines that were not infected with the papilloma virus, were grown for 12 or 60 h on uncoated or collagen I-coated plates and then serum-starved for 12 h. After the 12-h serum starvation, the cells (~80% confluent) were harvested using 0.1% collagenase at 37 °C for 30 min. The resulting cell suspension was centrifuged at 100 × g for 10 min and the cell pellet resuspended in cold (4 °C) 250 mM sucrose, 25 mM MES, 1 mM EDTA, pH 6.5, and 0.1% Triton X-100 (SME buffer). The cells were lysed by sonication (two times, 30 s each with 2-s pulses). An aliquot was used for determination of DNA as described by Downs and Wilfinger (31). The conditioned medium was centrifuged at 150,000 g, passed through Millipore UltraFree 100 K (Burlington, MA) concentrators to remove large collagen fragments, and then concentrated by centrifugation through Millipore UltraFree 10 K concentrators. Samples were assayed as described below for cathepsin B protein by SDS-PAGE and immunoblot analysis and for cathepsin B activity.

SDS-PAGE and Immunoblot Analysis—Samples (cell lysate or conditioned media) were normalized based on DNA determinations were subjected to SDS-PAGE using 12% (w/v) gels. The protein in these gels was transferred to nitrocellulose and then immunoblotted using 3 µg/ml rabbit anti-human liver cathepsin B polyclonal antibody (32) or 10 µg/ml mouse anti-human liver cathepsin D monoclonal antibody (OS-13A) in 5% nonfat milk-PBS with 0.05% Tween 20 (T-PBS). Membranes were probed with a 1:16,000 dilution of horseradish peroxidase-labeled secondary antibodies (goat anti-rabbit or anti-mouse IgG) in 5% nonfat milk-T-PBS and reactive proteins detected using ECL-PlusTM. To reprobe, the blots were stripped for 35 min at 65 °C with 2% (w/v) SDS, 62.5 mM Tris, pH 6.8, and 100 mM β-mercaptoethanol and then washed three times with T-PBS. Immunoblots were quantified using NIH Image 1.62 and expressed as relative density units. Standards of cathepsin B and cathepsin D were used to establish linear ranges for quantification.

Continuous Assay for Activity of Secreted Procathepsin B—Lactate cathepsin B was assayed as previously described (33) with the following modifications. To assess the amount of procathepsin B present, 100 µl of conditioned medium or PBS (control) were incubated for 30 min at 37 °C with 25 µl of 0.5 mM sodium formate, 20 mM EDTA, pH 3.2, and 0.2 mg/ml pepsin. Additionally, two controls in the absence of pepsin were performed: 1) incubated at 37 °C for 2 h or 3) incubated at 37 °C for 0.5 h with buffers. The amount of active cathepsin B in the conditioned media. The second assessed the amount of active cathepsin B generated autocatalytically during the incubation at acid pH. Cathepsin B activity was then assayed by adding 125 µl of 200 mM sodium phosphate buffer, pH 6.7, containing 4 mM EDTA, 10 mM diithiothreitol, 0.1% Triton X-100, and 200 µM Z-Arg-Ang-NHMec (final concentration, 100 µM). The fluorescence of the assay was 6.0. Duplicate samples and controls contained 10 µl CA-O74. Samples and controls were transferred into 96-well plates and the fluorescence intensities read. Results were expressed as picomoles (NH2-Mec formed)/min/mg of DNA. To determine statistical differences among the samples, one-way analysis of variance was performed. Dunnett's multiple comparison test was used as the post-test to compare pairs of group means with the control.

RNA Isolation and Northern Blot Hybridization—Total cellular RNA from fibroblasts grown on collagen I gels and plastic was collected according to the procedure of Chomczynski and Sacchi (34), electrophoresed (4 µg/tube) on a 0.8% agarose formaldehyde denaturing gel according to the procedure of Maniatis et al. (35), and transferred overnight to a positively charged nylon membrane using a TurboBlotter (Schleicher & Schuell). The membranes were UV-cross-linked and analyzed by hybridization (68 °C, overnight) to 50 ng/ml DIG-labeled antisense riboprobe containing exons 7–11 (500 bp) of human cathepsin B in 0.02% SDS, 5% SSC, 50% deionized formamide, 0.1% sodium lauryl sarcosine, and 2% blocking reagent. Following post-hybridization washes (two at 22 °C in 2× SSC, 0.1% SDS, and two at 85 °C in 0.5× SSC, 0.1% SDS, for 15 min
each), the hybridized probes were detected using anti-DIG-alkaline phosphatase conjugate Fab fragment and CDP-Star™ chemiluminescent substrate. Equal loading was determined using a DIG-labeled glyceraldehyde-3-phosphate dehydrogenase probe (Ambion, Austin, TX).

**Immunofluorescence Staining**—We localized cathepsins B and D using a modification (36) of the general immunocytochemical method described by Willingham (37). Human breast fibroblasts were grown to ~80% confluence for 1 or 3 days on glass coverslips either uncoated or coated with a thin layer of type I collagen. For surface staining of cathepsins B and D and for staining of actin, cells were fixed for 10 min in 3% formaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 1 mM CaCl2, and 0.5 mM MgCl2, pH 7.4). For intracellular staining of cathepsins B and D, cells were fixed for 5 min in cold methanol. Fixation and subsequent steps were performed at 25 °C for intracellular staining and at 4 °C for surface labeling. After washing, the nonspecific binding was blocked with 0.2% bovine serum albumin in PBS for 45 min. For intracellular labeling, 0.1% saponin was added to all subsequent antibody and wash solutions.

The cells were incubated for 2 h with primary antibodies (for intracellular double labeling, 230 μg/ml rabbit anti-human liver cathepsin B IgG and 20 μg/ml mouse anti-human liver cathepsin D IgG (D101); for surface labeling, 230 μg/ml rabbit anti-human liver cathepsin B IgG or 100 μg/ml sheep anti-human cathepsin D (D-23)) or FITC-conjugated phallolidin (1:1000). In controls, pre-immune serum (rabbit or mouse) or 100 μg/ml purified anti-mouse IgG and 20 μg/ml sheep anti-human liver cathepsin D IgG (D101) was substituted for the primary antibody. After washing, the cells were incubated for 1 h with fluorescein-conjugated affinity-purified anti-mouse IgG and/or Texas Red-conjugated affinity-purified donkey anti-rabbit or anti-goat IgG (20 μg/ml) containing 5% normal donkey serum. The cells were then washed, fixed, and mounted upside-down on slides with SlowFade reagent and observed with a Zeiss LSM 310 confocal microscope.

**Identification of Integrin Expression Using Flow Cytometry**—Human breast fibroblasts grown on collagen or plastic for 1 day were collected by first treating the cells for 30 min with 0.1% collagenase and then trypsin-EDTA for 3 min. Integrin expression was determined following the protocol of Henriet et al. (38) using 10 μg/ml anti-integrin antibodies (α5 (FB12), αv (PI6E), αv (PI5B), αv (PS58), β5 (6S6), and β3 (25E11)) or control antibodies (pre-immune mouse IgG antibodies and FITC-conjugated donkey anti-mouse IgG). Ten thousand cells were sorted and their fluorescence quantified using a FACS Calibur and analyzed by Cell Quest version 3.1 (Becton Dickinson Immunocytochemistry System, San Jose, CA).

**Influence of Inhibitory Anti-integrin Antibodies on Cell Shape and Cathepsin B Secretion from Fibroblasts Grown on Collagen I**—Collagen I gels were prepared as described above but with the addition of 5 μg/ml inhibitory anti-integrin antibodies (α1 (FB12), α2 (PI6E), and β1 (Ab13)) or negative control (pre-immune mouse IgG) antibodies. Fibroblasts were then treated with 5 μg/ml amounts of these antibodies for 10 min at 37 °C before seeding on collagen I gels containing the same anti-integrin or control antibodies. Untreated fibroblasts seeded on collagen I gels were used as controls. Twelve hours after seeding, the cells were changed to serum-free media for an additional 12 h. The fibroblasts were observed with a Zeiss LSM 310 confocal microscope, and then the cells and media were harvested and assayed for cathepsin B activity as described previously.

**Incubation of Fibroblasts Grown on Plastic with Anti-integrin Antibodies**—Experiments were performed using immobilized anti-integrin antibodies (inhibitory α1 (FB12), α2 (PI6E), and β1 (Ab13)); non-inhibitory β3 (K20); and activating β1 (12G10) and αvβ3 (JBS2)) coated onto bacterial culture plates (Falcon, Franklin Lakes, NJ). The plates (60 mm) were coated with 5, 10, or 20 μg/ml anti-integrin antibodies for 16 h in 50 ml Tris, pH 8.0, at 4 °C and then washed three times in PBS. The fibroblasts were seeded on these plates (4.5 × 105 cells/60-mm plate) and incubated for 12 h in DMEM/F-12 medium containing 10% fetal bovine serum. Cells were serum-starved for an additional 12 h before collecting the media and cells to measure cathepsin B activity. Fibroblasts seeded on plastic tissue culture dishes, mouse pre-immune IgG (20 μg/ml), or collagen I-coated tissue culture dishes were used as controls. The fibroblasts would only bind to the bacterial culture dishes that were coated with anti-integrin antibodies; therefore, tissue culture dishes had to be used for the controls.

**RESULTS**

**Expression of Cathepsins B and D in Human Breast Fibroblasts Grown on Plastic or Collagen I**—Interactions with collagen I can increase expression of MMPs in fibroblasts (18, 22).

In the present study, we determined whether interaction with collagen I affects expression in fibroblasts of proteases from two other classes: the cysteine protease cathepsin B and the aspartic protease cathepsin D. These two proteases have been proposed as prognostic markers for breast cancer (39, 40). We evaluated by immunoblotting the levels of these enzymes in nine different clones of human breast fibroblasts (9T, 10T, 11B, 11T, 12B, 12T, 14RM, 31RM, 33RM) comparing cells grown on either plastic or collagen I-coated plastic for 1 or 3 days. Similar results were obtained for all cell lines, including fibroblasts that were not infected with the papilloma virus (data not shown). Only the mature (active) single-chain (SC) and double-chain (DC) forms of cathepsin B (Fig. 1A, top panels) and cathepsin D (Fig. 1A, bottom panels) were present in cell lysates. Only the heavy chain of the double-chain form (i.e. H-DC) is observed in the 12% gels used here. Conversion from the single-chain forms of these enzymes to the double-chain forms occurs in the lysosome (41). Intracellular levels of cathepsins B and D were comparable in human breast fibroblasts whether grown on plastic or collagen I for 1 day (data not shown) or 3 days (Fig. 1A).

Furthermore, levels were comparable in fibroblasts isolated from benign tissue (Fig. 1A) and malignant human breast tumors (Fig. 1B). Cathepsin D was not secreted from the fibroblasts in response to growth on collagen I (Fig. 1B), yet cathepsin D was not (Fig. 1C). Neither enzyme was secreted from fibroblasts grown on plastic (Fig. 1, B and C). One might have anticipated that both enzymes would be secreted from fibroblasts, as both are secreted from breast cancer cells (42–44). The form of cathepsin B secreted in re-
response to collagen I was predominantly the inactive precursor form, i.e. procathepsin B (Fig. 1B). The lower band of ~10 kDa may represent a degradation product of cathepsin B or an artifact. When we quantitated cathepsin B protein in the cell lysate and conditioned media (Fig. 1D), it was apparent that interaction of breast fibroblasts with collagen I preferentially increased the total amount (lysate + media) of cathepsin B protein. This was not the case for cathepsin D protein (data not shown). The percentage of the total expressed cathepsin B protein that was secreted ranged from 39 to 84%, indicating a substantial induction of secretion by cellular interaction with collagen I.

To determine whether the increase in cathepsin B protein induced by collagen I reflected an increase in cathepsin B transcripts, we isolated total cellular RNA from fibroblasts grown on plastic or collagen I and evaluated it by Northern blot analysis. Both major transcripts, 4 and 2.2 kilobases, of cathepsin B were observed (45); however, the levels of the transcripts were not increased in fibroblasts grown on collagen I (Fig. 2). Fig. 2 illustrates transcript levels in 12T fibroblasts grown on plastic or collagen I; comparable results were obtained in 12B fibroblasts. Increases in cathepsin B protein in the absence of increases in cathepsin B transcripts have previously been observed in human breast cells, i.e. MCF-10A epithelial cell lines (43).

**Cathepsin B Activity in Fibroblasts Grown on Plastic or Collagen I—**Collagen I-stimulated changes in cathepsin B protein expression should be predictive of changes in cathepsin B activity. This was true in cell lysates where cathepsin B activity was not altered (data not shown), a finding consistent with there being comparable levels of cathepsin B protein in cell lysates (Fig. 1A). Both the cell lysates and the conditioned media contain many proteolytic enzymes. Therefore, we verified that our assay procedure would assess only cathepsin B activity by establishing that CA-O74, a highly selective cathepsin B inhibitor (46), totally blocked activity in both cell lysates and conditioned media (data not shown).

We observed small increases in cathepsin B activity in the conditioned media of cells grown on collagen I as compared with those grown on plastic (Fig. 3, bars labeled C–). Once again this was consistent with the small amounts of mature cathepsin B secreted into the media in response to growth on collagen I (Fig. 1B). Procathepsin B, which was the predominant form of cathepsin B secreted from the fibroblasts (Fig. 1B), requires proteolytic activation. Therefore, we incubated the conditioned media with pepsin and determined the amount of “activatable procathepsin B” present in the conditioned media (Fig. 3, bars labeled C+). A small amount of activatable procathepsin B was present in conditioned media of fibroblasts cultured on plastic (Fig. 3, bars labeled P+). Growing the fibroblasts on collagen I whether they were from reduction mammoplasties (31RM and 33RM) or from areas surrounding a tumor (12T (Fig. 3B), 12B, 11B, and 11T (data not shown)) increased the amount of activatable procathepsin B secreted into the conditioned media (Fig. 3, bars labeled C+). The amount of procathepsin B secreted varied among the cell lines, but was increased by growth on collagen I. Comparisons in the same assay of cathepsin B activity in conditioned media of cells grown on collagen I and those grown on plastic revealed that as much as ~250-fold more activatable procathepsin B was secreted from those breast fibroblasts grown on collagen I (Figs. 3 and 7, bars labeled P and C).

**Localization of Cathepsins B and D in Fibroblasts—**Increases in cathepsin B secretion and alterations in the localization of cathepsin B are dependent on a functional cytoskeleton (42, 47). Some investigators suggest that increased secretion and cell surface localization are solely caused by changes in cell shape, but we have not found this to be the case either in vitro (43) or in vivo (48). We, therefore, compared the morphologies of living breast fibroblasts grown on glass to fibroblasts grown on collagen I. On glass, the fibroblasts were flat and spread (Fig. 4A); on collagen I, they were spindle-shaped (Fig. 4B), resembling fibroblasts in vivo (49). The morphological differences that we observed appeared to reflect changes in the actin cytoskeleton, as actin-rich processes/ruffles were present in the fibroblasts grown on collagen I (Fig. 4D), but not in those grown on glass (Fig. 4C). Results are shown for 12T, and similar results were observed for 12B, 14RM, 31RM, and 33RM. The presence of ruffles suggests that the fibroblasts grown on collagen I were actively engaged in endo/exocytic processes (50), an observation consistent with the increased secretion of procathepsin B from all nine clones of fibroblasts when grown on collagen I.

We had previously established that increases in cathepsin B secretion are associated with an altered subcellular localization of the enzyme, most often a localization at or adjacent to the cell membrane (42, 49). Therefore, we compared the subcellular localizations of both cathepsins B and D in the fibroblasts grown on collagen I and those grown on glass coverslips. Antibodies against cathepsins B and D used in this study detect both the pro forms and mature single-chain and heavy double-chain forms of these enzymes (see Fig. 1). Staining for both cathepsins B and D was vesicular and was localized primarily to the perinuclear region of the fibroblasts whether grown on glass (Fig. 4E) or on collagen I (Fig. 4F). There was heterogeneity in staining for cathepsins B and D with some fibroblasts staining more intensely for cathepsin B than cathepsin D. In addition, three patterns of vesicular staining were observed: 1) vesicles...
Fibroblasts were cultured on uncoated (left panels, glass) or collagen I-coated (right panels, collagen I) coverslips for 3 days. Phase contrast images of live fibroblasts (A and B). Actin cytoskeleton stained with phalloidin-FITC (C and D). Intracellular staining for cathepsins B (red) and D (green) superimposed on a phase contrast image of the fibroblasts (E and F). Colocalization of the two enzymes (yellow) can be seen in some vesicles. Staining for cathepsin B (G and H) or cathepsin D (I and J) on the surface of non-permeabilized fibroblasts. All images were taken with a Zeiss LSM 310 microscope in the confocal mode; bars, 10 μm. This figure illustrates results from 12T fibroblasts; comparable results were obtained with other fibroblast lines.

Thus, the increased secretion of procathepsin B from fibroblasts grown on collagen I was not accompanied by increased surface localization of this enzyme.

**Integrin Expression**—Integrins link the cytoskeleton to the extracellular matrix, and the ligation of integrins has been shown to transduce expression of proteases, e.g., expression of MMP-1 is increased in fibroblasts when α2β1 binds to collagen I (52). As integrin ligation might modulate cathepsin B expression in breast fibroblasts, we analyzed by immuno-flow cytometry the integrins expressed on the surface of the human breast fibroblasts studied herein. Integrins α1, α2, α3, αv, and β1 were present on the surface of the breast fibroblasts whether grown on plastic or on collagen I; β3 was not present (Fig. 5). Because α1β1, α2β1, and α3β1 (53) are the main collagen I binding integrins, our results suggest β1 integrins might mediate cell shape of the breast fibroblasts when grown on collagen I. When cultured on collagen I in the presence of an inhibitory antibody to human β1 integrin (mAb 13), the fibroblasts were small and did not exhibit the extensive cell processes indicative of adhesion to the underlying collagen I matrix (Fig. 6F). The presence of blocking antibodies to human α1 (FB12), α2 (PIE6), or α1 and αv in combination (Fig. 6, C, D, and E, respectively) did not have as profound an effect on cell shape as the β1 integrin antibody. These cells had less extensive processes and appeared thinner but were still well spread as compared with the control cells grown on collagen I only or in the presence of pre-immune IgG (Fig. 6, A and B). Our results thus indicate that β1 integrin mediates the interaction of the breast fibroblasts with collagen I; the cell spreading on collagen I may involve modulation of the actin cytoskeleton via outside-in signaling through the β1 integrin.

**Stimulation of Cathepsin B Secretion by Integrins**—To investigate the role that integrins have on secretion of procathepsin B, we performed two experiments: 1) fibroblasts were grown on collagen I in the presence of inhibitory anti-integrin antibodies (α1 [FB12], α2 [PIE6], and β1 [mAb13]) to see whether the procathepsin B secretion induced by interaction of the cells with collagen I could be reduced, and 2) fibroblasts were cultured on anti-integrin antibodies immobilized on the culture substrate (inhibitory α1 [FB12], α2 [PIE6], and β1 [mAb13]; non-inhibitory β1 [R20]; and activating β1 [12G10] and αvβ3 [JBS2]) to see whether procathepsin B secretion could be induced by integrin redistribution and/or activation. Because the breast fibroblasts examined here expressed mainly α1β1 and
$\alpha_2\beta_1$ integrins, we chose to look at these receptors. The inhibitory antibodies against $\alpha_1$, $\alpha_2$, and $\beta_1$ significantly reduced (~50%) procathepsin B secretion from fibroblasts grown on collagen I (Fig. 6G), suggesting that all three of these integrin subunits mediate secretion of procathepsin B when fibroblasts are grown on collagen I. Fibroblasts cultured on antibodies against $\beta_1$ and $\alpha_2\beta_1$ integrins showed an enhanced secretion of procathepsin B (Fig. 7), but no change in intracellular cathepsin B was seen under any of the experimental conditions (data not shown). No increase in procathepsin B secretion was seen with the inhibitory $\alpha_1$ and $\alpha_2$ antibodies. A dose-dependent increase in procathepsin B secretion was found only with the antibody that recognizes active $\beta_1$ integrins. Our data are consistent with increased expression of cathepsin B in fibroblasts grown on collagen I being mediated at least in part by $\alpha_2\beta_1$ integrins.

**DISCUSSION**

Culturing fibroblasts in collagen I gels is a well established model for studying the three-dimensional matrix surrounding fibroblasts in vivo (for review, see Ref. 54). Increased expression of several MMPs, e.g. MMP-1, -2, -3, and -13 and membrane type 1 MMP, is seen when skin fibroblasts are grown on collagen I (18, 22). Here we have demonstrated that the interaction of human breast fibroblasts with collagen I increases total cathepsin B protein levels, including increased secretion of procathepsin B. As integrins binding to collagen I mediate the increased expression of MMP-1 and -13 in fibroblasts (18, 22), we investigated whether interaction of integrins with collagen I was also responsible for the increased expression of the lysosomal cysteine protease cathepsin B and for increased secretion of procathepsin B.

Integrins function as transmembrane linkers between the extracellular matrix and the actin cytoskeleton and thus influence cell morphology (55). When human breast fibroblasts were grown on collagen I, we observed changes in cell morphology and the actin cytoskeleton, i.e. more actin-rich processes/ruffles. The formation of these processes could be blocked using an inhibitory $\beta_1$ integrin antibody, indicating that $\beta_1$ integrins are important in regulating the cell shape of human breast fibroblasts when they are grown on collagen I. The $\alpha_1$ and $\alpha_2$ inhibitory antibodies did not affect cell shape as profoundly as the $\beta_1$ inhibitory antibody, suggesting that other $\beta_1$ integrins may also be involved. All three of these inhibitory antibodies were able to significantly reduce procathepsin B secretion from the fibroblasts grown on collagen I, suggesting that $\alpha_2\beta_1$ and $\alpha_2\beta_2$ integrins are important in the induction of procathepsin B secretion.
Previous studies have found that cell shape changes can regulate protease activity (56). To confirm that our findings were the result of inhibition of integrin binding to collagen I and not simply the result of changes in cell shape, we plated fibroblasts on anti-integrin antibodies immobilized on plastic. Anti-integrin antibodies immobilized on plastic, regardless of whether they are inhibitory, activating, or non-inhibitory, can mimic ligand and induce redistribution and/or aggregation of the integrin. These anti-integrin antibodies allowed the cells to spread and form processes similar to those seen when the cells were grown on collagen I. All immobilized anti-β1 integrin antibodies were able to induce secretion of procathepsin B. The same was true for an activating antibody directed against a heterodimer of β1, α3β1. Inhibitory antibodies to the individual α1 and α2 integrin subunits were not able to induce secretion. Our results indicate that stimulation of procathepsin B secretion from breast fibroblasts can be induced through redistribution of β1 integrin subunits. In addition, activation of β1 integrins seems to be important because only the antibody that recognizes active β1 integrin showed a dose response effect.

The increased expression of cathepsin B that resulted from the interaction of breast fibroblasts with collagen I may be regulated at the post-transcriptional level, because we found no change in mRNA message level. Interaction of the fibroblasts with collagen I could increase translation or stabilize procathepsin B protein. Translational regulation of cathepsin B has been reported by Yano et al. (57), who found that cathepsin B mRNA levels in Sarcophaga peregrina (flesh fly) larval and pupal hemocytes are not different, yet increased cathepsin B protein is present in the pupal hemocytes. The translational repression of cathepsin B mRNA in the larval hemocytes is dependent on a 3′-untranslated region-specific binding protein. Whether a similar mechanism can regulate cathepsin B translation in human cells is not known.

Both cathepsins B and D are synthesized as preproenzymes. The propeptide serves as a signal sequence to direct these enzymes to the endoplasmic reticulum. In the Golgi network, these enzymes acquire phosphomannosyl residues that target them to the lysosomes primarily via mannose phosphate receptor pathways. In the acidic environment of the late endosome, the proenzyme disassociates from the mannose phosphate receptors. The propeptide is also cleaved at this point, activating the proteases (for a recent discussion on the trafficking and processing of cathepsins, see Ref. 58). The collagen I-stimulated secretion of cathepsin B observed here was predominantly of the inactive precursor form, i.e. procathepsin B, suggesting that the enzyme had not yet reached the late endosomes or lysosomes. The minor amount of mature single-chain cathepsin B that was secreted in response to collagen I could reflect processing of procathepsin B extracellularly. It could also reflect exocytosis of cathepsin B from late endosomes or lysosomes, as a stimulated exocytosis of active lysosomal enzymes has been shown to occur in a variety of cells (59).

We speculate that integrin signaling interferes with the targeting of procathepsin B to the lysosomal pathway. The fact that cathepsin D was not secreted may reflect differential sorting of the two enzymes in breast fibroblasts. Putative sorting receptors that bind procathepsin D, but not procathepsin B, have been identified (60). Clearly the mechanisms for secretion of cathepsins are complex as both active and pro forms of cathepsin B are secreted, whereas cathepsin D and cathepsin L, another lysosomal cysteine protease, are secreted primarily as proenzymes (9, 44, 61). The ras oncogene is able to induce secretion of cathepsins B (43) and L (58, 61). This may be linked to integrin signaling as both α5β1 and α2β1 signal through the Ras/mitogen-activated protein kinase pathway (22). In ras-transfected murine fibroblasts, procathepsin L is localized in exosomes (58). Exosomes arise from fusions of late multivesicular endosomes with the plasma membrane (62, 63) and contain annexin II (63), the binding partner for procathepsin B on the tumor cell surface (51). We speculate that association of procathepsin B with annexin II may be responsible for its secretion. We have shown that this association leads to activation of procathepsin B (51); thus, procathepsin B secreted from the breast fibroblasts could be activated at the surface of breast fibroblasts and participate in local degradation of extracellular matrices. The multiple pathways for secretion of cathepsin B in both its active and latent pro form suggest that the secretion of cathepsin B may have functional consequences for extracellular degradation of matrix proteins. In this regard, a role for secreted cathepsin B in digestion of type IV collagen by living human breast cancer cells has recently been demonstrated (5).

In the present study, we obtained similar results with presumably normal fibroblasts isolated from reduction mammoplasties and those isolated from areas surrounding malignant tumors. This included localization of both cathepsins B and D on the cell surface. What might be the physiological role for this association of cathepsins B and D with the surface of human breast fibroblasts? Mammary gland development is a complex process that involves numerous changes in the extracellular matrix, including remodeling during puberty, pregnancy, lactation, and menopause. Perhaps cathepsins B and D are involved in this reorganization. Cathepsin B can degrade extracellular matrix proteins directly at neutral pH (5, 7) and indirectly through its ability to activate pro-urokinase and thus the plasminogen cascade (17, 64). Alternatively, cathepsin B may be involved in matrix remodeling through its ability to directly activate transforming growth factor-β (65), a growth factor that regulates the expression of collagen I in the postnatal mammary gland (66). Recently, mature cathepsin B secreted from human breast epithelial cells has been shown to initiate the plasminogen cascade leading to activation of transforming growth factor-β (67). An involvement of cathepsin D in degrading the extracellular matrix at the fibroblast cell surface is unlikely because cathepsin D is active at very acidic pH (9). On the other hand, cathepsin D may be acting as a mitogen rather than a protease, as procathepsin D on the surface of breast and prostate cancer cells increases cell proliferation and tumor growth (68, 69). Increased stromal growth accounts for most of the increase in breast volume in the post-pubertal years and also occurs in breast tumors. Cathepsin D at the surface of breast fibroblasts might well be a mitogen that induces stromal proliferation in both instances.

We have shown that growth of human breast fibroblasts in collagen I gels in vitro can differentially affect the expression of two lysosomal proteases, cathepsins B and D. We found that collagen I, through its interaction with α1β1 and α2β1 integrins, stimulated increased secretion of procathepsin B. In addition, we found that the redistribution of β1 integrins increased secretion of procathepsin B. This is the first examination of cathepsin B and D expression and localization in human breast fibroblasts and their regulation by a matrix protein. Whether cathepsins B and D are localized on the surface of fibroblasts from other tissues and integrins can mediate regulation of cathepsin B in those fibroblasts is not known. In breast tissues, the mechanism(s) responsible for differential induction of cathepsins B and D by collagen I is of potential importance as both enzymes have been linked to progression of breast cancer.

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