Expression of Matrix Metalloprotease-2-Cleaved Laminin-5 in Breast Remodeling Stimulated by Sex Steroids

Gianluigi Giannelli,*† Ambra Pozzi,*
William G. Stetler-Stevenson,‡
Humphrey A. Gardner,* and Vito Quaranta*

From the Department of Cell Biology,* The Scripps Research Institute, La Jolla, California, Institute of Clinica Medica II,† Università degli Studi di Bari, Bari, Italy, and Laboratory of Pathology,‡ National Cancer Institute, National Institutes of Health, Bethesda, Maryland

The extracellular matrix plays an important role in breast remodeling. We have shown that matrix metalloprotease-2 (MMP2) cleaves laminin-5 (Ln-5), a basement membrane component, generating a fragment called $\gamma 2x$. Human breast epithelial cells, while constitutively immobile on intact Ln-5, acquire a motile phenotype on MMP2-cleaved Ln-5. We hypothesize that this mechanism may underlie cell mobilization across the basement membrane during branching morphogenesis in breast development regulated by sex steroids. We report that the expression of MMP2 and cleavage of Ln-5 correlate well with tissue remodeling and epithelial rearrangement of the breast both *in vivo* and *in vitro*. Thus, the Ln-5 $\gamma 2x$ fragment was detected by immunoblotting in sexually mature, pregnant, and postweaning, but not in prepubertal or lactating mammary glands. Furthermore, cleaved Ln-5, as well as MMP2, became detectable in remodeling glands from sexually immature rats treated with sex steroids. In rat mammary gland explants, epithelial reorganization and luminal cell morphological changes were induced by the addition of exogenous MMP2, in parallel to the appearance of cleaved Ln-5. Similar effects were observed in epithelial monolayers plated on human Ln-5 and exposed to MMP2. These results suggest that cleavage of Ln-5 by MMP2 might be regulated by sex steroids and that it may contribute to breast remodeling under physiological and possibly pathological conditions. (*Am J Pathol* 1999, 154:1193–1201)

Tissue organization and specialization is a challenging issue in medical science. In many physiological and pathological conditions such as tissue repair and tumor invasion, tissue architecture and cell-stroma boundaries are rearranged, resulting in the reformation of pre-exist-

ing structures or in the generation of new structures. This process, commonly known as tissue remodeling, requires the interaction between epithelium and mesenchyma mediated, at least in part, by extracellular matrix (ECM) proteins. ECM components, including laminin-1 and laminin-5 (Ln-5), collagen type IV, and fibronectin, are assembled in a complex network known as the basement membrane (BM) which separates the epithelium from the stroma.¹ Ln-5, a heterotrimer glycoprotein formed by three disulfide-bonded subunits, $\alpha 3$, $\beta 3$, and $\gamma 2$,¹ regulates different cell functions such as adhesion, hemidesmosome formation, and migration.^{2,3,4,5} It has been reported that Ln-5 is a major component of the BM, and its absence is responsible for lethal diseases.^{6,7} Epithelial cells interact with Ln-5 via a family of transmembrane receptors, the integrins, which mediate cell adhesion as well as signal transduction.⁸

The BM regulates many cellular functions such as adhesion, migration, differentiation, and survival,⁹ and its integrity is crucial to preserving epithelial architecture and organization.¹⁰ The mammary gland represents an excellent model for studying interactions between the BM and epithelial cells.¹¹ At birth the mammary gland is histologically organized as a simple tree-like structure in which the ducts present only a few lateral branches.¹² As the duct tips cells are assembled in bulbous structures, the end buds specifically penetrate the surrounding stroma.¹² The epithelial cells located at the end tips are known as cap cells and are responsible for the elongation and the ramification of the ductal tree.¹² Mammary gland development and branching morphogenesis is controlled by sex steroids, as well as growth hormone, prolactin, and epidermal growth factor, each of which plays a mammary role by stimulating epithelial cell proliferation and differentiation.^{13,14} Also, other local factors, such as proteolytic remodeling of the ECM, are believed to be involved in mammary gland development and branching morphogenesis.^{15,16} It has been reported that members of the matrix metalloprotease family, including stromely-

Supported by Grant DAMD 17-97-1-7218 from the Department of the Army (to G.G.) and by National Institutes of Health Grants CA47858 and GM46902 (to V.Q.).

Accepted for publication December 22, 1998.

Address reprint requests to Gianluigi Giannelli, Department of Cell Biology, The Scripps Research Institute, 10550 N. Torrey Pines, SBR-12, La Jolla, CA 92037. E-mail: gianlu@scripps.edu.

sin-1 and matrix metalloprotease-2 (MMP2), are up-regulated in several conditions in which tissue remodeling occurs.¹⁷ These enzymes are ubiquitously located in the body, are capable of degrading several proteins, including BM components,¹⁰ and are considered to have a key role in tissue morphogenesis and in cancer metastasis as well.^{10,18} In a transgenic mouse model, stromelysin-1 has been shown to induce branching morphogenesis^{16,19} and also to trigger a malignant phenotype in epithelial mammary cells after extended contact.²⁰ While sex steroids regulate branching morphogenesis of the mammary gland, as is proteolytic ECM remodeling, no mechanistic link between these two processes has yet been established.

We have recently reported that human breast epithelial cells are able to migrate *in vitro* on MMP2-cleaved but not on intact Ln-5.⁴ MMP2 cleaves the γ chain of Ln-5, generating an 80-kd fragment referred to as γ 2x. This fragment is present in tumors and in some tissues undergoing remodeling but absent in quiescent tissues.⁴ Together, these findings support a model whereby local secretion and/or activation of MMP2 in the proximity of the epithelial BM results in the cleavage of Ln-5 to promote epithelial cell migration. This model may be particularly suitable in mammary gland studies, since this organ undergoes dramatic remodeling after puberty.

The goal of this study was to investigate the cleavage of Ln-5 by MMP2 in breast tissue remodeling. Initially, using the γ 2x fragment as a marker for MMP2 cleavage, we could show that its presence occurs exclusively during mammary gland tissue remodeling. We further showed that treatment with sex steroids can induce the cleavage of Ln-5 in the mammary gland of sexually immature rats and that epithelial reorganization and tissue remodeling occur in cultured explants of rat mammary gland where Ln-5 is cleaved by the addition of MMP2. Similar epithelial rearrangements and morphological changes were induced also in cells in contact with human Ln-5 after exposure to MMP2.

Materials and Methods

Induction of Mammary Gland Development *in Vivo*

Sexually immature (12 days old, less than 30 g of body weight) female Wistar rats were treated with a combination of sex steroids (estrogen and progesterone) (Sigma, St. Louis, MO) as previously described.²¹ Briefly, hormones were dissolved in sesame oil and injected subcutaneously in animals at a dosage of 500 μ g of estrogen once a day and 2 mg of progesterone twice a day. Control animals received vehicle alone. Animals were treated for 3 days and euthanized 24 hours after the last injection by CO₂ inhalation. Mammary glands were explanted and either immediately snap frozen in liquid nitrogen or imbedded in OCT.

All of the experiments were approved by and conformed to the guidelines of the Institutional Animal Care Committee.

Tissue Harvesting

Mammary gland tissue was collected from rats or mice less than 2 weeks of age for the sexually immature stage, at 8 weeks for the sexually mature stage, at 13.5 days of pregnancy, at 12 days after the onset of lactation, or at 8 days after weaning.

Western Blot Analyses

Tissues were pulverized, washed, and resuspended in sample buffer as previously described.⁴ The total amount of protein was measured using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL) and normalized amount (300 μ g of protein loaded into each lane) was separated by SDS-PAGE on a 6% polyacrylamide gel under reducing conditions and transferred to polyvinylidene difluoride membranes (BioRad, Hercules, CA) for western blot analyses, performed as described.⁴

Antibodies

Rabbit antiserum 1963, which recognizes the γ 2 subunit of rat Ln-5, was prepared as described.⁴ Rabbit polyclonal antiserum Ab45 to MMP2 was also described.²² Antiserum 2794 was prepared by immunizing rabbits with a glutathione-S-transferase fusion protein prepared by cloning in the vector pGEX the coding region of the Ln-5 γ 2 chain corresponding to the last 126 residues of the COOH terminus (residues 1046–1171).

Protease Activation

Human recombinant pro-MMP2²³ was activated with 1 mmol/L paraminophenylmercuric acetate in a buffer containing 50 mmol/L NaCl, 5 mmol/L CaCl₂, and BRIJ 35% 0.01%, pH 7.2, for 30 minutes at 37°C²⁴. Human recombinant pro-MMP9²⁵ was incubated with the activating buffer for 3 hours at 45°C. Enzyme activation was verified by zymography.

Mammary Gland *ex Vivo* Explants

Mammary glands were explanted under sterile conditions, washed with phosphate-buffered saline (PBS), sliced into 1-mm cubes, cultured in DFCI medium without serum or in serum-free DMEM supplemented with L-glutamine and antibiotics, submerged on 24-transwell filters (Corning Costar, Cambridge, MA). Explants were incubated for 24 hours at 37°C in a CO₂ incubator in the presence of recombinant active MMP2 at concentrations of 30, 10, or 3 nmol/L, MMP9, or plasmin (Enzyme Research Laboratory, South Bend, IN). In some cases, the MMP inhibitor, BB94²⁶ (kindly provided by British Biotechnology, Ltd), at concentrations of 500 nmol/L, was added.

Electron Scanning Microscopy, Histology, and Immunohistochemistry

Mammary gland explants were either paraffin-embedded, sectioned, and analyzed by scanning electron microscopy (stereoscan 360 scanning electron microscope at an accelerating voltage of 10 kV) or snap-frozen in liquid nitrogen, embedded in OCT compound (Miles Laboratories Inc., Naperville, IL), cut into 5- μ m sections with a microtome (model HM 505E, Carl Zeiss, Oberkochen, Germany), and stained with antibodies.²⁷

Whole-mount staining of mammary glands was performed as described.¹² Briefly, glands were flattened on a tissue capsule, fixed in Telly's fixative, defatted in three changes of acetone, hydrated in 95% ethanol, and stained with hematoxylin (0.65 g of FeCl₃, 67.5 ml of H₂O; 8.7 ml of 10% hematoxylin in 95% ethanol, pH 1.25). Glands were rinsed in water, destained in acid ethanol, dehydrated in increasing ethanol concentrations, indefinitely stored in methyl salicylate, and photographed using a Zeiss microscope.

Cell Cultures

MCF-10 cells, a spontaneously immortalized human breast epithelial cell line,²⁸ was maintained in culture in DFCI medium composed of a 1:1 mixture of modified Eagle's medium and Ham's F12 media (Gibco, Grand Island, NY), and enriched with 1% fetal bovine serum and growth factors.²⁹

804G cells, derived from a rat urinary bladder carcinoma, were cultured in DMEM medium, supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, penicillin (20 U/ml), and streptomycin (20 mg/ml).

Deposited ECM Preparation

Deposited Ln-5 was prepared from the human cell lines MCF-10³⁰ or the rat 804G cell line.^{31,32} Briefly, cells were cultured for 3 days to confluency and then removed according to described procedures³³ that leave behind functional ECM. The ECM from the above cells is known to be highly enriched in Ln-5.^{30,34}

Cell Morphology Assays

Glass coverslips were coated with Ln-5-enriched ECM by cell deposition, as described above. In some cases, glass coverslips were coated with ECM secreted by the cell line 804G by incubating them in 804G conditioned medium overnight. For Ln-5 depletion, the 804G medium was passed through an anti-Ln-5 TR1 antibody affinity column before incubation with coverslips. Coated coverslips were treated with MMP (130 nmol/L in DMEM) or control medium as described above. MCF-10 cells (150,000 cells per coverslip) were incubated for 2 hours on the coverslips in serum-free medium in a humidified CO₂ incubator at 37°C. Unattached cells were gently removed with PBS, and the remaining attached cells were fixed with 3% paraformaldehyde in PBS.

Figure 1. Cleaved Ln-5 is present in remodeling but not in quiescent mammary gland. Rat mammary glands were collected at different stages of maturation as indicated. Tissues were pulverized and examined by western blot analysis for the presence of Ln-5. The $\gamma 2$ subunit migrates as a 135-kd band and a 100-kd band ($\gamma 2'$). The latter is a biosynthetic maturation product of the 135-kd form, which occurs by proteolysis of the amino terminus.¹ The $\gamma 2x$ band at 80 kd was used to indicate the presence of MMP2-cleaved Ln-5. The $\gamma 2x$ band was absent in sexually immature (less than 2 weeks old) rat but was present in the sexually mature (8 weeks old) rat, as well as during pregnancy. During lactation $\gamma 2x$ was absent, but it reappeared during involution. Tissue of involuting mammary gland is from mice.

Cells were photographed with phase-contrast optics on a Zeiss Axiophot microscope. Cell areas were measured using a Bio-Rad MRC600 confocal microscope and CoMOS software, which calculates areas based on manual outlining of individual cells. Typically, areas of 80 cells were measured and averaged.

Results

Cleaved Ln-5 Is Present Only in Remodeling Mammary Gland

To determine whether cleavage of Ln-5 is associated with tissue remodeling, we examined by Western blot analysis rat mammary gland tissue at various stages of sexual maturation for the presence of $\gamma 2x$, an 80-kd proteolytic fragment of Ln-5 generated by MMP2 digestion.⁴ As shown in Figure 1, $\gamma 2x$ was present in sexually mature, pregnant, and involuting (ie, postlactating) rat mammary gland tissue. In contrast, the $\gamma 2x$ fragment was not detected in sexually immature or in lactating animals. Therefore, the presence of $\gamma 2x$ correlates well with stages of active remodeling of the gland, both when the duct network is actively expanding (ie, during sexual maturation

Figure 2. MMP2 and cleaved Ln-5 are present in remodeling mammary glands from sexually immature rats treated with estrogen (E) and progesterone (P). Animals were treated with sex steroids (E+P) or vehicle (control) as described under Materials and Methods. Increased arborization of the mammary gland was observed in hormone-treated compared to control animals (A, B) by whole-mount mammary glands. Frozen sections were immunostained with a MMP2 polyclonal antiserum (C, D) or as a control with secondary antibody alone (E). Western blots showed uncleaved Ln-5 in tissue extracts from control animals and cleaved Ln-5 in hormone-treated animals. (Each lane represents a different animal.) The mapping of the $\gamma 2x$ fragment by using the polyclonal antiserum 2794 (H) demonstrates that this fragment is the same fragment we found and investigated previously *in vitro*.⁴ The band at 120 kd (*) was consistently observed with both antisera in the hormone-treated animals, but its nature is unknown. Scale bar, 0.05 mm (A); 100 μ m (C, D, E).

and pregnancy) and the end buds are invading the stroma, and when they are involuting (ie, postweaning) and reduction of branches and alveoli occurs.

Sex Steroids Induce MMP2 Expression and Cleaved Ln-5 in Remodeling Mammary Gland Tissue

To determine whether sex hormones may induce ECM proteolysis in the mammary gland, we injected sexually immature female rats with estrogen and progesterone.

The results shown in Figure 2 are representative of four identically treated animals. As expected, mammary glands in hormone-treated rats (Figure 2A) were more developed than in control rats (Figure 2B). Several new side branches in the ductal arborization were observed, and new end buds with a larger diameter penetrated extensively the fat pad tissue. The glandular parenchyma of treated glands occupied a larger volume in the fat pad than controls (Figure 2).

Immunohistochemical analysis revealed that MMP2 was readily detectable in mammary gland specimens from hormone-treated animals (Figure 2C), while it was completely absent in untreated animals (Figure 2D). The staining was localized at the periphery of the lobules in the BM zone, mostly concentrated around the myoepithelial cells, and it was occasionally present in some luminal cells. Some stromal cells also showed positive MMP2 staining. In adjacent sections, the secondary an-

tibody alone (Figure 2E) showed no staining, indicating specificity of the MMP2 staining.

In the same rats, the presence of Ln-5 in mammary tissue samples from hormone-treated and control animals was investigated by Western blot analysis. In the sexually immature untreated rats, Ln-5 was present in the intact form (Figure 2F). In contrast, in the hormone-treated animals the $\gamma 2x$ chain (Figure 2, G and H) was easily detectable, indicating the presence of cleaved Ln-5. The polyclonal antiserum 2794 directed against the carboxyl terminal end of the Ln-5 γ chain was used to confirm the identification of the $\gamma 2x$ fragment in the E+P treated animals (Figure 2H).

These data suggest that sex steroids may induce MMP2 cleavage of Ln-5 in remodeling mammary tissue.

MMP2-Cleaved Ln-5 Induces Mammary Epithelial Reorganization in an ex Vivo Mammary Gland Culture

To better visualize the effects of MMP2 on the epithelial architecture of the mammary gland, we developed an *ex vivo* assay in which fragments of explanted mammary glands were maintained in culture in the presence of MMP2, MMP9, plasmin, or control medium. Histological and scanning microscopic analyses revealed dramatic changes in the epithelial organization of samples incubated with MMP2 (Figure 3B). No substantial changes were detected, however, in control medium specimens

Figure 3. MMP2 alters the cellular architecture of the breast epithelium. Explanted mammary glands from sexually immature rodents were cultured *in vitro* (see under Materials and Methods). In control samples (A), the cells of the mammary epithelium were in close contact with each other showing typical quiescent epithelial organization. In the presence of MMP2 (B), some of the cells from the epithelial breast sheet lost contact with the BM and gathered in the center of the lumen, while other cells still in contact with the BM had a dramatically altered morphology. They had lost contact with each other and were rounded or elongated in shape. This effect was blocked by BB94, an inhibitor of MMPs (C). Scale bar, 18 μ m.

(Figure 3A) or in specimens treated with MMP9 or plasmin (data not shown). In the MMP2-treated explants, some luminal epithelial cells were detached from the BM and grouped within the lumen of the mammary ducts.

Figure 4. MMP2 cleaves Ln-5 in explanted mammary gland tissue but does not dissolve the BM. Cultured fragments of mammary glands from sexually immature rats (Figure 3) were processed for either Western blot analysis (A) or immunohistochemistry (B). Western blot analysis (A) using polyclonal antibody 1963, which recognizes the γ chain of Ln-5, revealed the presence of the 80-kd γ 2x fragment band in cultures treated with MMP2 (C) but not in the control cultures (B) or in the cultures co-incubated with BB94 (D). By immunohistochemistry, Ln-5 was localized evenly along the BM in the samples treated with MMP2 (B) as well as in the control cultures (A) and those co-treated with MMP2 and BB94 (C). Scale bar, 60 μ m.

Moreover, cells still in contact with the BM had changed their morphology, some rounder and others more elongated compared to the control samples. This effect of MMP2 was dose-dependent and was completely inhibited by the presence of the MMP inhibitor BB94 (Figure 3C).

Tissues were processed in parallel for Western blot analysis and examined for the presence of the γ 2x proteolytic fragment. As shown in Figure 4A, γ 2x was detected only in those specimens treated with MMP2 and not in control tissue or in tissue cultured with MMP2 and BB94.

We also localized Ln-5 in the BM of the MMP2-treated mammary gland explants immunohistochemically using a polyclonal antibody to the γ subunit of Ln-5 (Figure 4B). Ln-5 staining was evenly distributed in the BM of ducts and alveoli, where it appeared as a continuous ring in control and in MMP2-treated tissue. This observation suggests that cleaved Ln-5 remains localized in the BM, that is, in the vicinity of the epithelial cells and is not removed by MMP2 digestion. Taken together, these results indicate that exogenously applied MMP2 cleaves Ln-5 *in situ*, resulting in a rearrangement of the epithelial organization of the mammary gland.

MMP2-Treated Human Ln-5 Induces Epithelial Reorganization in Vitro

To extend these findings to the human breast gland, we investigated the epithelial reorganization induced by

Figure 5. Changes in morphology of MCF-10 cells on MMP2-cleaved Ln-5. By scanning electron microscopy, MCF-10 cells plated on uncleaved human Ln-5 (A) appeared flat and large, a morphology typical of an immobile cell. In contrast, cells grown on cleaved human Ln-5 (B) were either elongated with filopodia and lamellipodia or rounded, having the appearance of a motile phenotype. MCF-10 cells were plated on rat (C, D) or human (E, F) Ln-5, either cleaved or uncleaved, for 90 minutes. MCF-10 cells were large and in contact with each other on uncleaved rat or human Ln-5 (C, E), where they assumed a typical epithelial-like polygonal shape. In contrast, cells on MMP2-cleaved rat and human Ln-5 (D, F) had lost contact with each other and appeared round or elongated with a ruffling in the membrane. The difference in cell size was quantified by measuring the cell surface area using a computerized analysis system. The cell surface area was similar whether MCF-10 cells were cultured on human or rat Ln-5 (G), but there was a large reduction in cell size in cultures grown on cleaved human or rat Ln-5. This size difference reflects the morphological changes we observed and suggests that rat and human Ln-5 have similar functional roles in epithelial reorganization. Scale bars, 6 μm (A, B) and 10 μm (B, D, E, F).

MMP2 in a normal human mammary epithelial cell line, MCF-10, which secretes and deposits Ln-5³⁰ and which under appropriate culture conditions grows as an organized epithelial monolayer

MCF-10 cells plated on human Ln-5 appeared flattened (Figure 5, A and E), while on MMP2-treated Ln-5 they assumed a morphology typical of a motile pheno-

type (Figure 5, B and F). The changes in morphology observed were similar to those seen on MMP2-treated rat Ln-5 (Figure 5, C and D). Cells plated on either rat or human Ln-5 were large, organized in close contact with each other, and formed epithelium-like structures (Figure 5, C and E). In contrast, cells plated on MMP2-treated human or rat Ln-5 (Figure 5, D and F) were smaller, some rounder, others more elongated with filopodia and lamellipodia. Overall, cells plated on either human or rat intact Ln-5 had an average cell area three-fold greater than those plated on cleaved Ln-5 (Figure 5G).

To test whether or not MMP2-induced morphology changes were dependent on Ln-5, glass coverslips were coated with 804G spent medium, which contains soluble Ln-5, and, in parallel, with 804G medium depleted of Ln-5 with a specific antibody (see under Materials and Methods). Within 2 hours, cells were completely spread on coverslips coated with spent medium (Figure 6A) but not with depleted medium (Figure 6C), indicating that Ln-5 had been removed by the depletion procedures. Furthermore, on addition of MMP2, a change in morphology, with appearance of filopodia and cell polarization, was observed in coverslips coated with Ln-5 containing medium, but not with the depleted medium (Figure 6, B and D). Those results suggest that MMP2 induces changes in morphology only in the presence of Ln-5.

Discussion

We present evidence that proteolytic cleavage of Ln-5 by MMP2 occurs during the remodeling of breast gland tissue induced by sex steroids. To summarize: 1) In mammary gland tissue, Ln-5 was consistently cleaved during stages of remodeling (eg, in sexually mature, pregnant, and involuting rats), but was intact in quiescent tissue (eg, before puberty or during lactation). 2) Ln-5 was cleaved and MMP2 was detectable during mammary gland expansion induced by the injection of sex steroids in sexually immature rats. 3) In *ex vivo* assays, the mammary epithelium was rearranged and Ln-5 was cleaved when mammary gland specimens were incubated with MMP2. 4) The remodeling of the epithelial architecture induced by MMP2 was dose-dependent and protease-specific, since no effect was observed with other proteases (eg, MMP9 or plasmin). 5) Finally, morphological changes observed in the presence of cleaved Ln-5 were reproducible in a human breast cell line, MCF-10, *in vitro*. Together, our data suggest an interaction between MMP2 and Ln-5 may occur during the remodeling of the mammary gland induced by sex steroids.

The ECM likely plays an important role in tissue development and differentiation. For instance, specific differentiated functions of mammary cells⁹ are supported by contact with the ECM. A role for the ECM in morphogenesis has also been proposed and is supported by some experimental evidence.³⁵ Epithelial-mesenchymal interactions, mediated at least in part at the BM interface, are critical in regulating branching morphogenesis.^{15,16} Interestingly, the structure of the BM may vary in the mammary gland. At quiescent sites, next to end buds, the BM

Figure 6. MCF-10 cell morphology is dependent on the presence of Ln-5 and cleaved Ln-5. MCF-10 cells plated on coverslips coated with Ln-5-enriched ECM (A) appear well spread, while they are still poorly spread on coverslips coated with ECM depleted of Ln-5 by absorption with a specific antibody (C). MCF-10 cells become elongated in the presence of MMP2 on Ln-5 enriched ECM (B) but not on Ln-5 depleted ECM (D). Scale bar, 10 μ m.

is 14-fold thicker than at the tip of end buds, where actively branching occurs or invading epithelial cap cells are located.¹² These cells penetrate into the fat tissue until they completely fill it.¹² Our results show that an important component of the BM, Ln-5, is proteolytically cleaved during remodeling of the mammary gland, and that its cleavage correlates with reorganization of the gland induced by sex steroid treatment. It remains to be determined whether Ln-5 cleavage plays a direct role in the subsequent reorganization of the gland.

Consistent with other observations,³⁶ in our experiments MMP2 was detected immunohistochemically in stromal cells and occasionally in luminal cells, but it was mostly present in the cytoplasm or in the proximity of the myoepithelial cells. This is the site where new branches are generated³⁷ and where ECM remodeling occurs.^{36,38} It is not known, however, how ECM remodeling affects cell behavior. Our data suggest that the cleavage of Ln-5 may represent a structural change in the ECM that causes induction and guidance of cell migration during tissue reorganization. Since cultured cells plated on Ln-5 alone can be induced to migrate in the presence of

MMP2, the presence of MMP2-cleaved Ln-5 may itself be sufficient for migration.⁴ We cannot rule out, however, that additional mechanisms for MMP2-induced remodeling exist, or that proteases other than MMP2, such as MMP3, may cleave Ln-5 to produce a migratory substrate. More studies are necessary to clarify these points.

MMP2 and cleaved Ln-5 were detected both during branching morphogenesis and involuting phases of breast gland remodeling. Additional studies are necessary to pinpoint the exact locations of MMP2 and cleaved Ln-5 in the mammary gland tree with respect to areas of branching or reductive morphogenesis. Unfortunately, at this time, we do not have reagents that can distinguish between intact and cleaved Ln-5, which would be critical for such studies.

Our experiments with tissue explants indicate that although MMP2 can have a disruptive effect on mammary epithelial architecture, it does not degrade the BM or affect other tissue types. The changes we observed, however, do not resemble branching morphogenesis, which presumably requires several additional signals in a coordinated fashion. Nevertheless, even though it does

not faithfully reproduce the *in vivo* process, the tissue explant technique may be useful to test factors that affect epithelial organization and dissect their mechanism of action.

How mammary epithelial cells acquire motility on MMP2-cleaved Ln-5 is not yet known; however, it is possible that integrins might play a role in interpreting ECM cues. Both $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins can interact with Ln-5.³⁹ Yet while $\alpha_3\beta_1$ integrins are involved in adhesion and migration via focal adhesions, $\alpha_6\beta_4$ supports the formation of hemidesmosomes which link the intermediate filament cytoskeleton to the BM.⁴⁰ It is likely that these integrins are affected differentially by MMP2 actions on Ln-5. For instance, on cleaved Ln-5 it is expected that the anchoring functions of hemidesmosomes may be lost, whereas the migratory functions of $\alpha_3\beta_1$ may be enhanced. In addition, we cannot rule out the possibility that also $\alpha_6\beta_4$ may have a role on cleaved Ln-5, since it has been reported that it is involved in migration.⁴¹ These issues remain to be elucidated at the molecular level.

At the functional level, both rat and human Ln-5 behaved similarly. That is, on treatment with MMP2 they supported changes in cell morphology. However, we have been unable to characterize the structural consequences of MMP2 treatment on Ln-5 because of the scarcity of that material.

In a more general sense, our results point to the possibility that, during breast branching morphogenesis, proteases may unveil ECM cues that guide the morphogenetic process itself. Their role, then, may be more intricate than simple degradation of ECM barriers. Direct links still remain to be established, however, between sites of ECM proteolysis and the presence of putative morphogenetic cues. Other factors, such as sex hormones, may also directly or indirectly activate ECM proteolysis and *vice versa*, ECM proteolysis might influence cellular responsiveness to sex steroids, eg, via integrin-mediated signaling.⁴² Model systems, such as the *ex vivo* explants we describe here, may help discover the molecular mechanism underlying the complex events of tissue remodeling.

Acknowledgments

We thank Zaverio Ruggeri, Daniel Solomon, and Anthony Pelletier for helpful discussions.

References

- Burgeson RE, Chiquet M, Deutzmann R, Ekblom P, Engel J, Kleinman H, Martin GR, Meneguzzi G, Paulsson M, Sanes J, Timpl R, Tryggvason K, Yamada Y, Yurchenco PD: A new nomenclature for the laminins. *Matrix Biol* 1994, 14:209–211
- Xia Y, Gil SG, Carter WG: Anchorage mediated by integrin $\alpha_6\beta_4$ to laminin 5 (epiligrin) regulates tyrosine phosphorylation of a membrane-associated 80-kD protein. *J Cell Biol* 1996, 132:727–740
- Baker SE, Hopkinson SB, Fitchmun M, Andreason GL, Frasier F, Plopper G, Quaranta V, Jones JCR: Laminin-5 and hemidesmosomes: role of the α_3 chain subunit in hemidesmosome stability and assembly. *J Cell Sci* 1996, 109:2509–2520
- Giannelli G, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V: Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science* 1997, 277:225–228
- Miyazaki K, Kikkawa Y, Nakamura A, Yasumitsu H, Umeda M: A large cell-adhesive scatter factor secreted by human gastric carcinoma cells. *Proc Natl Acad Sci USA* 1993, 90:11767–11771
- Meneguzzi G, Marinkovich MP, Aberdam D, Pisani A, Burgeson R, Ortonne JP: Kalinin is abnormally expressed in epithelial basement membranes of Herlitz's junctional epidermolysis bullosa patients. *Exp Dermatol* 1992, 1:221–229
- Baudoin C, Miquel C, Blanchet-Bardon C, Gambini C, Meneguzzi G: Herlitz junctional epidermolysis bullosa keratinocytes display heterogeneous defects of nicein/kalinin gene expression. *J Clin Invest* 1994, 93:862–869
- Schwartz MA, Schaller MA, Ginsberg MH: Integrins: emerging paradigms of signal transduction. *Annu Rev Cell Dev Biol* 1995, 11:549–599
- Roskelley CD, Srebrow A, Bissell MJ: A hierarchy of ECM-mediated signalling regulates tissue-specific gene expression. *Curr Opin Cell Biol* 1995, 7:736–747
- Werb Z: ECM and cell surface proteolysis: regulating cellular ecology. *Cell* 1997, 91:439–442
- Cunha GR, Hom YK: Role of mesenchymal-epithelial interactions in mammary gland development. *Gland Biology Neoplasia* 1996, 1:21–35
- Williams JM, Daniel CW: Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. *Dev Biol* 1983, 97:274–290
- Silberstein GB, Van Horn K, Shyamala G, Daniel CW: Progesterone receptors in the mouse mammary duct: distribution and developmental regulation. *Cell Growth Differ* 1996, 7:945–952
- Humphreys RC, Lydon J, O'Malley BW, Rosen JM: Mammary gland development is mediated by both stromal and epithelial progesterone receptors. *Mol Endocrinol* 1997, 9:801–811
- Talhok RS, Chin JR, Unemori EN, Werb Z, Bissell MJ: Proteinases of the mammary gland: developmental regulation *in vivo* and vectorial secretion in culture. *Development* 1991, 112:439–449
- Witty JP, Wright JH, Matrisian LM: Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. *Mol Biol Cell* 1995, 6:1287–1303
- Chen WT: Membrane proteases: roles in tissue remodeling and tumour invasion. (Review). *Curr Opin Cell Biol* 1992, 4:802–809
- Duffy MJ, McCarthy K: Matrix metalloproteinases in cancer: prognostic markers and targets for therapy. (Review). *Int J Oncol* 1998, 12:1343–1348
- Symposium CJ, Talhok RS, Alexander CM, Chin JR, Clift SM, Bissell MJ, Werb Z: Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J Cell Biol* 1994, 125:681–693 (Note: Published erratum appears in *J Cell Biol* 1996, 132 following 752)
- Lochter A, Galosy S, Muschler J, Freedman N, Werb Z, Bissell MJ: Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J Cell Biol* 1997, 139:1861–1872
- Risek B, Klier FG, Phillips A, Hahn DW, Gilula NB: Gap junction regulation in the uterus and ovaries of immature rats by estrogen and progesterone. *J Cell Sci* 1995, 108:1017–1032
- Corcoran ML, Stetler-Stevenson WG, Brown PD, Wahl LM: Interleukin 4 inhibition of prostaglandin E2 synthesis blocks interstitial collagenase, and 92-kd type IV collagenase/gelatinase production by human monocytes. *J Biol Chem* 1992, 267:515–519
- Fridman R, Bird RE, Hoyhtya M, Oelkelt M, Komarek D, Liang CM, Liotta LA, Stetler-Stevenson WG, Fuerst TR: Expression of human recombinant 72 kd gelatinase and tissue inhibitor of metalloproteinase-2 (TIMP-2): characterization of complex and free enzyme. *Biochem J* 1993, 289:411–416
- Itoh Y, Binner S, Nagase H: Steps involved in activation of the complex of pro-matrix metalloproteinase 2 (progelatinase A) and tissue inhibitor of metalloproteinases (TIMP)-2 by 4-aminophenylmercuric acetate. *Biochem J* 1995, 308:645–651
- Wilhelm SM, Collier IE, Marner BL, Eisen AZ, Grant GA, Goldberg GL:

- SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J Biol Chem* 1989, 264:17213-17221
26. Wang X, Fu X, Brown PD, Crimmin MJ, Hoffman RM: Matrix metalloproteinase inhibitor BB-94 (batimastat) inhibits human colon tumor growth and spread in a patient-like orthotopic model in nude mice. *Cancer Res* 1994, 54:4726-4728
27. Giannelli G, Savoia P, Schiraldi O, DeLuca M, Marchisio PC, Quaranta V: Psoriatic lesions in patients with chronic liver disease are distinct from psoriasis vulgaris lesions based on integrin adhesion receptors. *Hepatology* 1994, 20:56-65
28. Tait L, Soule HD, Russo J: Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10. *Cancer Res* 1990, 50:6087-6094
29. Band V, Sager R: Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *Proc Natl Acad Sci USA* 1989, 86:1249-1253
30. Stahl S, Weitzman S, Jones JCR: The role of laminin-5 and its receptors in mammary epithelial cell branching morphogenesis. *J Cell Sci* 1997, 110:55-63
31. Langhofer M, Hopkinson SB, Jones JCR: The matrix secreted by 804G cells contains laminin related components that participate in hemidesmosome assembly in vitro. *J Cell Sci* 1993, 105:753-764
32. Giannelli G, Brassard J, Foti C, Stetler-Stevenson WG, Falk-Marzillier J, Zamboni Zallone A, Schiraldi O, Quaranta V: Altered expression of basement membrane proteins and their integrin receptors in Lichen planus: possible pathogenetic role of gelatinases A and B. *Lab Invest* 1996, 74:1091-1104
33. Gospodarowicz D: In *Methods for Preparation of Media: Supplements and Substrata*. Edited by DW Barnes, DA Sirbasku, GH Stao. New York, Alan R. Liss, 1984, pp 275-293
34. Falk-Marzillier J, Domanico SZ, Pelletier AJ, Mullen L, Quaranta V: Characterization of a tight molecular complex between integrin $\alpha 6 \beta 4$ and laminin-5 extracellular matrix. *Biochem Biophys Res Commun* 1998, 251:49-55
35. Chuong CM, Edelman GM: Expression of cell-adhesion molecules in embryonic induction. I. Morphogenesis of nestling feathers. *J Cell Biol* 1985, 101:1009-1026
36. Monteagudo C, Merino MJ, San-Juan J, Liotta LA, Stetler-Stevenson WG: Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. *Am J Pathol* 1990, 136:585-592
37. Daniel CW, Siberstein GB: In *The Mammary Gland Development, Regulation, and Function*. Edited ME Lipmann, RB Dickson. New York, Plenum Publishing Corp, 1987, pp 8-36
38. Andersson LM, Dundas SR, O'Hare MJ, Gusterson BA, Warburton MJ: Synthesis of gelatinases by rat mammary epithelial and myoepithelial cell lines. *Exp Cell Res* 1994, 212:389-392
39. Hynes RO: Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992, 69:11-25
40. Sonnenberg A, Calafat J, Janssen H, Daams H, van der Raaij-Helmer LM, Falcioni R, Kennel SJ, Aplin JD, Baker J, Loizidou M, Garrod D: Integrin $\alpha 6 \beta 4$ complex is located in hemidesmosomes suggesting major role in epidermal cell-basement membrane adhesion. *J Cell Biol* 1991, 113:907-917
41. Rabinovitz I, Mercurio AM: The Integrin $\alpha 6 \beta 4$ functions in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containing motility structures. *J Cell Biol* 1997, 139:1873-1884
42. Fendrick JL, Raafat AM, Haslam SZ: Mammary gland growth and development from the postnatal period to postmenopause: ovarian steroid receptor ontogeny and regulation in the mouse. *J Mammary Gland Biol Neoplasia* 1998, 3:7-22