Matrilysin (Matrix Metalloproteinase-7) Mediates E-Cadherin Ectodomain Shedding in Injured Lung Epithelium

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Matrilysin (matrix metalloproteinase-7) is highly expressed in lungs of patients with pulmonary fibrosis and other conditions associated with airway and alveolar injury. Although matrilysin is required for closure of epithelial wounds *ex vivo*, the mechanism of its action in repair is unknown. We demonstrate that matrilysin mediates shedding of E-cadherin ectodomain from injured lung epithelium both *in vitro* and *in vivo*. In alveolar-like epithelial cells, transfection of activated matrilysin resulted in shedding of E-cadherin and accelerated cell migration. *In vivo*, matrilysin co-localized with E-cadherin at the basolateral surfaces of migrating tracheal epithelium, and the reorganization of cell-cell junctions seen in wild-type injured tissue was absent in matrilysin-null samples. E-cadherin ectodomain was shed into the bronchoalveolar lavage fluid of bleomycin-injured wild-type mice, but was not shed in matrilysin-null mice. These findings identify E-cadherin as a novel substrate for matrilysin and indicate that shedding of E-cadherin ectodomain is required for epithelial repair. (Am J Pathol 2003, 162:1831–1843)

Epithelial damage is a prominent feature of several pulmonary diseases. For example, in conducting airways, exfoliated epithelial cells are found in sputum and bronchoalveolar lavage fluid from patients with asthma, and the epithelium is severely damaged in cystic fibrosis and bronchiolitis obliterans.^{1–5} Alveolar epithelium is conspicuously damaged in acute lung injury/acute respiratory distress syndrome (ALI/ARDS), desquamative interstitial pneumonitis, and cystic fibrosis, among many other acute and chronic lung diseases.^{6,7} An immediate response to epithelial loss is the spreading and migration of intact cells to cover the denuded basement membrane.^{2,8,9} Numerous proteins and peptides, including cell adhesion molecules and proteolytic enzymes, regulate the repair process, and failure to restore a functional

epithelial barrier contributes to progressive injury, inflammation, and fibrosis.

The matrix metalloproteinases (MMPs) are a family of zinc-containing enzymes with proteolytic activity against a wide range of extracellular proteins.¹⁰ MMP expression is typically limited to tissue remodeling associated with normal and abnormal biological processes, such as development, involution, inflammation, tumor growth, and repair. Matrilysin (MMP-7), unlike many MMPs, is expressed by noninjured, noninflamed mucosal epithelia in most adult human tissues.^{11,12} In the human lung, matrilysin is constitutively expressed in tracheal glands and in tracheobronchial epithelium, and expression is acutely up-regulated by injury or exposure to bacteria.^{13,14} Work in our laboratory demonstrated that matrilysin is prominently expressed in injured airways and is necessary for airway mucosal repair,¹³ indicating that this a critical extracellular proteinase in repair processes.

Matrilysin is also prominently expressed by alveolar epithelium in cystic fibrosis and pulmonary fibrosis.^{15,16} Work in our laboratory found that matrilysin is induced in alveolar epithelium in mice after lung injury with bleomycin and expression increases and continues as fibrosis progresses.¹⁷ In this model, matrilysin was shown to regulate neutrophil influx by controlling chemokine compartmentalization during the first days after injury. However, the extended production of matrilysin indicates that this MMP serves multiple, distinct roles in repair of lung epithelium. In other words, matrilysin acts on different protein substrates involved in different processes at different stages of repair.

Epithelial (E)-cadherin, a transmembrane glycoprotein localized at adherens junctions, mediates calcium-dependent cell-cell adhesion in most epithelia. In cooperation with cytoskeletal structures, E-cadherin is thought to regulate cell differentiation and morphogenesis.¹⁸ Reduction or loss of E-cadherin correlates with increased malignancy in tumors and invasiveness in carcinoma cell lines *in vitro*.^{19,20} Although the regulation of cadherin ad-

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hesive activity is not completely understood, the level of cadherin gene expression correlates with the strength of adhesion, and the type of cadherin expressed affects the specificity of cell interaction.²¹ Posttranscriptional mechanisms regulating cadherin adhesion include modulation of cadherin clustering at the cell surface, changes in cadherin interaction with other proteins, and proteolysis of cadherin ectodomains.^{21–23}

In addition to cleaving extracellular matrix proteins, MMPs mediate shedding of transmembrane and membrane-associated proteins, including E-cadherin.²⁴ For example, exogenous matrilysin and stromelysin-1 (MMP-3) can release an 80-kd E-cadherin fragment from various cancer cell lines^{25,26} and ectopic expression of stromelysin-1 leads to cleavage of E-cadherin in mammary epithelial cells.²⁷ Although proteolytic cleavage of E-cadherin ectodomains has been suggested to enhance epithelial repair, the proteinase responsible for this process has not been identified. Here, we provide evidence that matrilysin mediates shedding of E-cadherin from airway epithelium during repair and from alveolar epithelium during progression of bleomycin-induced pulmonary fibrosis.

Materials and Methods

Immunohistochemistry

Archival blocks of formalin-fixed, paraffin-embedded normal and diseased human lung tissue were obtained from the Department of Pathology, Washington University School of Medicine. We analyzed specimens from ARDS with diffuse alveolar damage ($n = 4$), emphysema ($n = 5$), and desquamative interstitial pneumonitis ($n = 3$). Specimens from the tumor-free margins from lung adenocarcinoma resections were used as normal controls ($n = 5$). Sections were stained for matrilysin as described.¹³ For mouse lung and tracheal tissues, 5- μ m sections were cut from blocks of frozen lung tissue, air-dried for 5 minutes, and fixed in acetone for 10 minutes at -20°C and methanol for 6 minutes at -20°C . Sections were blocked for 1 hour at room temperature with TBST (25 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20) containing 1% bovine serum albumin and 2% goat serum and then incubated with primary antibodies at 37°C . For E-cadherin immunofluorescence on mouse tissues, sections were fixed as above and permeabilized with 1% Triton X-100 in Ca/Mg-phosphate-buffered saline (PBS) (0.01 mol/L phosphate, 0.138 mol/L NaCl, 0.27 mmol/L KCl, 1 mmol/L CaCl_2 , 0.5 mmol/L MgCl_2) for 15 minutes before adding primary antibody. Cells were grown on Nunc Lab-Tek II glass chamber slides (Fisher Scientific, Pittsburgh, PA), washed with Ca/Mg-PBS, fixed with 3.7% formaldehyde in Ca/Mg-PBS for 10 minutes, and permeabilized with 1% Triton X-100 in Ca/Mg-PBS for 15 minutes. Fixed cells were blocked with 1% bovine serum albumin in Ca/Mg-PBS for 1 hour at room temperature, incubated with antibodies at 37°C . Immunofluorescence of tissues and cells was imaged by indirect immunofluorescence microscopy and confocal laser-scanning microscopy.

Antibodies and Other Reagents

Polyclonal antibodies against the catalytic domain of human matrilysin¹² and against full-length mouse matrilysin²⁸ were previously generated in our laboratory. Antibodies against the extracellular domain of human (HECD-1) and mouse (ECCD-2) E-cadherin (Zymed, South San Francisco, CA), and against the cytoplasmic domain of human E-cadherin (Transduction Laboratories, San Diego, CA) were used at the manufacturers' recommended concentrations. Mouse anti-human actin antibody was obtained from Sigma Chemical Co., St. Louis, MO. Fluorescent Alexa 488- and Alexa 568-conjugated antibodies (Molecular Probes, Eugene, OR) were used at the manufacturer's recommended concentrations for indirect immunofluorescence and confocal laser-scanning microscopy.

Cell Culture

The human lung adenocarcinoma cell lines A549 and Calu3, the human colon carcinoma cell line HT29, and the canine kidney cell line MDCK were obtained from American Type Culture Collection (Manassas, VA). Cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 $\mu\text{g}/\text{ml}$ of gentamicin. A construct coding for a full-length human matrilysin cDNA carrying an autoactivating mutation (a gift from Lynn Matrisian, Ph.D., Vanderbilt University, Nashville, TN) was stably expressed in A549 cells under the control of a constitutive CMV promoter. The construct was cloned into pCIneo (Promega, Madison, WI), which contains a neomycin resistance gene, and was transfected into cells using Superfect reagent (Qiagen, Valencia, CA). Neomycin-resistant clones were selected with G418 at 800 mg/ml and were analyzed for matrilysin mRNA expression by Northern blot analysis.

Immunoprecipitation

Selected clones were screened for matrilysin protein secretion by [^{35}S] labeling. Briefly, cells were cultured to 60 to 70% confluence in basal culture medium then switched to labeling medium (L-methionine and L-cysteine free-Dulbecco's modified Eagle's medium, 5% fetal bovine serum dialyzed against 0.05 mol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl). After 12 hours, depleted cells were switched to labeling medium supplemented with 50 $\mu\text{Ci}/\text{ml}$ of [^{35}S]-methionine-cysteine TRAN³⁵S-LABEL (ICN, Costa Mesa, CA). Cells were pulsed overnight, and conditioned medium was collected and centrifuged at $5000 \times g$ to remove cellular debris. Aliquots (600 μl) of conditioned medium were mixed with an equal volume of immunoprecipitation buffer (0.01 mol/L Na_3PO_4 , 0.138 mol/L NaCl, 0.27 mmol/L KCl, 0.8% Triton X-100, 20 mmol/L ethylenediaminetetraacetic acid, 100 mg/ml bovine serum albumin) and precleared with 25 μl of protein A-Sepharose (Zymed) for 1 hour at 4°C . Protein A-Sepharose was removed by centrifugation, and supernatants were incubated with 5 μl per sample of rabbit anti-human

matrilysin antiserum overnight at 4°C with gentle agitation. Antibody-antigen complexes were precipitated with protein A-Sepharose, washed three times with immunoprecipitation buffer, and eluted by boiling in 40 μ l of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer. The precipitating reagents were removed by centrifugation, and supernatants were resolved by SDS-PAGE. Gels were dried, and [³⁵S]-labeled matrilysin was visualized by autoradiography. For inhibitor experiments, the hydroxamate MMP inhibitor SC68180A (Pharmacia, St. Louis, MO) was used at 25 μ mol/L concentration in culture medium, and the matrilysin selective inhibitor RS-101625 (Roche Bioscience, Palo Alto, CA) was used at 40 nmol/L concentration. All cell culture medium components were purchased from Bio-Whittaker (Walkersville, MD).

Cell Migration

Uniform 1-mm wounds were made in confluent A549 epithelial monolayers grown on plastic six-well plates. Cultures were rinsed three times with warm PBS, and cells were allowed to heal the wounds throughout 48 hours in regular culture medium. Cultures were photographed at time of wounding and 24 and 48 hours later. Wound area was calculated by measuring average width of the wound and multiplying by wound length. Wound closure was expressed as a percentage of initial wound area. For some experiments, hydroxyurea (Sigma), an inhibitor of cell proliferation, was dissolved in culture medium at 250 μ mol/L final concentration, and added at time of wounding.

Tracheal Explant Cultures

Tracheas from wild-type and matrilysin-null mice were explanted, placed in sterile medium (Dulbecco's modified Eagle's medium) with 5% fetal bovine serum and 50 μ g/ml gentamicin, cut, and splayed open along the long axis. Tracheas were wounded by cutting the splayed tracheas into uniform 3-mm pieces, rinsed with sterile PBS to remove cellular debris, and immediately placed into tissue culture wells (four tracheas/well) with just enough medium added to cover the tracheal tissue. Cultures were maintained for 24 hours. We tracked tracheal epithelial migration, or epiboly, along the cut edges of the tissue pieces. At 24 hours, conditioned medium was collected, cleared of cellular debris by centrifugation at 600 $\times$ g for 5 minutes, snap-frozen on dry ice, and stored for future analysis. Tracheal tissue was rinsed in PBS and frozen in OCT tissue-freezing medium for immunohistochemical analysis.

Transmission Electron Microscopy

Tracheal tissues were fixed for 24 hours at 4°C in 2.5% glutaraldehyde/0.1 mol/L Na cacodylate, rinsed for 20 minutes three times in 0.1 mol/L Na cacodylate, and secondarily fixed in 1.25% osmium tetroxide/0.1 mol/L Na cacodylate for 1 hour at room temperature. Fixed tissues

were rinsed twice in 0.1 mol/L Na cacodylate and twice in 15% ethanol for 20 minutes each, followed by dehydration in graded ethanols. Tissues were washed twice for 20 minutes in propylene oxide, incubated for 48 hours in 1:1 propylene oxide: Polybed 812 on a rotating shaker, 4 hours in pure Polybed 812 with rotation, and embedded in Polybed 812 overnight in the dessicator, followed by baking at 60°C for 48 hours. Blocks were thin-sectioned and stained with alkaline toluidine blue and observed with a light microscope. Appropriate areas were trimmed for ultra thin sections, which were poststained with 4% uranyl acetate and lead citrate and examined with a Zeiss 902 transmission electron microscope and recorded on electron microscopy film.

Bleomycin Lung Injury Model

C57BL6 wild-type and C57BL6 mice carrying a targeted deletion of the matrilysin gene²⁹ were anesthetized by intraperitoneal injection with 0.1 ml/20 g body weight of mouse cocktail (86.98 mg/kg ketamine/13.4 mg/kg xylazine in 0.9% sterile saline). The MMP-7^{-/-} mice were initially derived on a 129 background and backcrossed >10 generations onto the C57BL6 background. Wild-type mice were age- and strain-matched littermate controls. A small neck incision was made, and mice were administered 0.08 U of bleomycin by direct intratracheal instillation in 50 μ l of sterile saline with a 25-gauge needle. Control animals received an equal volume of saline.

Incisions were closed with silk suture, and animals were allowed to recover from anesthesia and take food and water *ad libitum*. Animals were sacrificed 10 days after injury by intraperitoneal injection of pentobarbital (100 mg/kg). Tracheas were cannulated with a 22-gauge 1-inch angiocatheter (Becton-Dickinson, San Diego, CA), and bronchoalveolar lavage (BAL) fluid was collected with 1 ml of sterile 0.9% saline solution. BAL fluid was concentrated 10-fold with Microcon centrifugation columns (Millipore, Bedford, MA), and 20 μ l of each sample was boiled in an equal volume of 2× SDS-loading buffer, resolved by SDS-PAGE, and analyzed by Western blot as described below. Similar experiments were performed in C57BL6 mice with a targeted deletion of the macrophage elastase (MMP-12) gene (obtained from Dr. Steven Shapiro, Harvard University, Boston, MA).³⁰ Bronchoalveolar lavage samples from bleomycin-treated C57BL6 gelatinase B (MMP-9)-null mice were obtained from Dr. Robert Senior, Washington University, St. Louis, MO.³¹ Lungs were removed and either homogenized in RNA STAT-60 reagent from Tel-Test, Inc. (Friendswood, TX) for RNA isolation or snap-frozen in OCT tissue-freezing medium for immunohistochemical analysis. All experiments were performed with the approval of the Washington University School of Medicine animal studies committee.

Western Blot Analysis

Cultured cells were lysed in 30 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L ethylenediaminetetraacetic acid, 1% Igepal CA630, 1% deoxycholic acid, 0.1% SDS,

Figure 1. Matrilysin expression in injured human alveolar epithelium. Sections of human lung specimens were stained for matrilysin protein (peroxidase). **A:** Matrilysin protein was not detected in alveolar epithelium in normal lung. **B:** Specimen from emphysema with interstitial fibrosis shows intense matrilysin staining in alveolar type II epithelial cells (**arrows**), particularly bordering sites of epithelial desquamation (**arrowheads**). Specimens of desquamative interstitial pneumonitis (**C**) and ARDS (**E**) show matrilysin staining localized to alveolar type II cells (**arrows**). Interstitial cells and inflammatory cells were negative for matrilysin staining. **D:** Serial section of lung from ARDS specimen incubated with preimmune serum. Scale bars, 50 μ m.

1 mmol/L phenylmethyl sulfonyl fluoride, and 20 μ g/ml aprotinin; and protein content in lysates quantified by the Bradford assay. Equal quantities (40 μ g) of protein/sample were resolved by SDS-PAGE, transferred to Hybond nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), and blocked overnight with 5% milk in TBST. Blots were probed with above antibodies at the manufacturers' recommended concentrations and visualized by enhanced chemiluminescence system (Amersham Pharmacia Biotech). For conditioned medium experiments, cells were cultured in serum-free medium for 24 hours. Medium was collected, cellular debris removed by centrifugation at $600 \times g$ for 5 minutes, and 500 μ l of medium was concentrated 10-fold as for BAL fluid and analyzed as above.

RNA Analysis

For Northern blotting studies, cultured cells were washed twice with PBS, and total RNA was extracted with RNA STAT-60 reagent according to the manufacturer's protocol. Total RNA (10 μ g) was denatured in loading buffer containing ethidium bromide and resolved by electrophoresis through 1.5% agarose gels containing formaldehyde. RNA was transferred to Hybond+ nylon membranes (Amersham Pharmacia Biotech) following the manufacturer's specifications and UV cross-linked. Equivalent loading and integrity of RNA was confirmed by comparing ethidium bromide staining of 18S and 28S rRNA bands. Gel-purified cDNA probes for human matrilysin were labeled by random priming, hybridized to membranes overnight, and visualized by autoradiography.

Results

Matrilysin Expression at Sites of Alveolar Injury

Matrilysin is prominently expressed in cystic fibrosis,¹³ a disease characterized by both fibrosis and chronic infection, and in idiopathic pulmonary fibrosis.¹⁶ To assess if matrilysin expression is a general feature of alveolar epithelial injury, regardless of the underlying condition, we examined a variety of human lung diseases. Consistent with our previous observations, matrilysin was not expressed in the alveolar epithelium of uninjured human lungs (Figure 1A). However, in specimens of emphysema, desquamative interstitial pneumonitis, and ARDS, strong immunoreactive signal for matrilysin protein was seen in epithelial cells lining damaged alveoli, particularly in cells bordering denuded epithelium (Figure 1; B, C, and E). Based on their morphology and location, matrilysin-positive cells were identified as alveolar type II cells. As in other tissues, matrilysin immunostaining was restricted to epithelial cells and was not seen in interstitial cells and only rarely in inflammatory cells. No immunoreactivity was seen in specimens processed with preimmune serum (Figure 1D).

Matrilysin Mediated E-Cadherin Shedding and Enhanced Alveolar Epithelial Migration in Vitro

Because it is prominently expressed by injured alveolar epithelium and promotes airway epithelial migration, we evaluated if matrilysin promotes alveolar cell migration *in vitro*. A549 human lung adenocarcinoma cells have fea-

Figure 2. Activated-matrilysin expression in A549 lung epithelial cells. Autoactivating human matrilysin cDNA was stably expressed in A549 cells. **A:** Northern analysis showed that control cells (C) did not express matrilysin mRNA. Clones 8 and 16 expressed high levels of matrilysin mRNA, whereas clones 13, 14, and 15 expressed low levels. HT29 colon carcinoma cells, which constitutively express matrilysin mRNA, were used as a positive control. The ethidium bromide-stained membrane shows equivalent loading and transfer. **B:** Matrilysin mRNA-positive clones 8 and 16 were labeled with [³⁵S]-L-methionine-L-cysteine, and secreted matrilysin was immunoprecipitated from conditioned medium. Clones aM8 and aM16 secreted matrilysin and both 29-kD promatrilysin and 19-kD active matrilysin forms were detected. **C:** Compared to vector-transfected cells (A549-V1), activated-matrilysin-expressing cells (aMat8) were more spindle-shaped and detached from each other.

tures of alveolar type II cells and have been used in models of alveolar repair *in vitro*.^{32,33} This cell line does not express matrilysin under basal conditions or in response to stimuli that induce matrilysin expression in other mucosal epithelial cell lines (data not shown). We stably expressed an autoactivating mutant of human promatrilysin (aMat) in A549 cells. This construct has a Val-Gly mutation in the prodomain sequence PRCGVDP that destabilizes the interaction of the cysteine residue with the active site zinc.³⁴ Two clones, aMat8 and aMat16, were identified that expressed high levels of the matrilysin mRNA and secreted activated matrilysin (Figure 2, A and B). Because the cDNA codes for full-length prepromatrilysin, both the 29-kD promatrilysin and 19-kD active forms were detected in the culture medium. Compared to vector-transfected cells, cells expressing activated-matrilysin appeared more spindle-shaped and were more detached from one another (Figure 2C), morphological changes characteristic of a migratory phenotype.³⁵ Expression of native full-length human promatrilysin cDNA in A549 cells showed no conversion to the active form of the enzyme and did not induce changes in cell shape or migration (data not shown).

Figure 3. Matrilysin-mediated E-cadherin ectodomain shedding *in vitro*. **A:** Immunofluorescence with a monoclonal antibody specific for the human E-cadherin ectodomain showed that E-cadherin localized to cell-cell junctions in vector-transfected cells (A549-V1) but was redistributed to the cytoplasm in aMat8 cells, correlating with decreased cell-cell attachment. **B:** Western blot for E-cadherin ectodomain (E-cad ecto) showed a reduction in intensity of band for 120-kD full-length E-cadherin in the lysate of aMat expressing cells. **C:** Increased shedding of an ~80-kD E-cad ecto-reactive band into the medium (arrowhead). Actin is shown as protein loading control for cell lysates. Calu3 lysate is shown as a control for full-length E-cadherin (arrow). **D:** Treatment of cells with the hydroxamate MMP inhibitors, SC68180A and RS101625 (MMP-7-specific), inhibited the shedding of the 80-kD fragment into the medium. **E:** Western blotting of cell lysates with antibody specific for the cytoplasmic domain of human E-cadherin identified an ~38-kD fragment (arrowhead) in the cell lysate of aMat-expressing cells that was reduced by treatment with SC68180A and was not seen in vector-transfected cells (V1). Full-length E-cadherin is indicated by arrow. Original magnification, $\times 400$ (A).

Because exogenous matrilysin can cleave E-cadherin from the surface of cultured cells^{25,26} and because release or reorganization of E-cadherin junctions is associated with epithelial migration, we evaluated E-cadherin expression in vector-transfected and aMat-transfected cells. Immunofluorescence microscopy using a monoclonal antibody specific for an epitope in the human E-cadherin extracellular ectodomain showed a decrease in E-cadherin at cell-cell junctions (Figure 3A). In the vector-transfected cells, E-cadherin was seen at points of contact between neighboring cells. In contrast, aMat-expressing cells showed a reduction in the intensity of E-cadherin ectodomain immunofluorescence and a loss of signal at points of cell-cell contact.

Western blot analysis for E-cadherin ectodomain showed that the 120-kD full-length E-cadherin in cell lysates from matrilysin-expressing cells was decreased compared to nontransfected or vector-transfected cells (Figure 3B). In addition, shed soluble E-cadherin ectodomain was detected in the conditioned medium from matrilysin-express-

Figure 4. Effect of activated-matrilysin expression on migration. **A:** Uniform 1-mm wounds were made in confluent aMat-transfected (aMat8) and vector-transfected cells (A549-V1), and wounds were allowed to heal by migration. aMat8 cells closed wounds faster than vector-transfected cells. **B:** Wound area was calculated at 24 hours and 48 hours after wounding in the presence or absence of 250 $\mu\text{mol/L}$ of hydroxyurea (H). Data represent the average of four experiments $\pm$ SD. **C:** Cells at the wound edge of aMat8 cells were detached (small arrowheads) from the migrating front, whereas control cells (V1) migrated as an intact sheet.

ing cells but not in medium from control cells (Figure 3C). Shedding of the E-cadherin ectodomain was markedly decreased in the presence of the broad-spectrum hydroxamate MMP inhibitor, SC-68180A, and was nearly completely inhibited by the matrilysin selective inhibitor, RS-101625 (Figure 3D). Using a monoclonal antibody targeted to the C-terminal cytoplasmic domain of E-cadherin, we detected an E-cadherin fragment of ~ 38 kd in lysates from matrilysin-expressing cells. This fragment was not present in lysates from vector-transfected cells and was reduced in cells cultured in the presence of the MMP inhibitor (Figure 3E). These data demonstrate that shedding of E-cadherin ectodomain was dependent on the catalytic activity of matrilysin.

We used an *in vitro* wound-healing assay to evaluate if E-cadherin shedding altered cell migration. Uniform 1-mm-wide wounds were created with a sterile pipette tip in confluent cultures of vector-transfected and aMat-transfected A549 cells. By 24 hours after wounding, $\sim 50\%$ of the wound area was closed in the aMat-expressing cells, but only 25% closure was observed in vector-transfected cells (Figure 4, A and B). By 48 hours, aMat cells had closed nearly 80% of the wound area, whereas the vector-transfected cells showed only 40% closure. The cell proliferation inhibitor, hydroxyurea, only slightly decreased the rate of wound closure in matrilysin-

expressing cells (Figure 4B) indicating that proliferation was not a major factor in promoting wound closure. Furthermore, many cells at the wound margin in the aMat-expressing cultures were separated from the migrating front; in contrast, vector-transfected cells migrated as a contiguous epithelial sheet (Figure 4C). Migration of matrilysin-expressing cells did not differ between cells grown on plastic or on various other extracellular matrix proteins, including type I collagen, gelatin, fibronectin, or Matrigel (data not shown).

Matrilysin Co-Localization with E-Cadherin in Migrating Lung Epithelium

To link a specific proteinase to a proteolytic process, the substrate and the enzyme should co-localize to the same pericellular compartment. To assess if matrilysin is specifically released and anchored to cell-cell junctions at the wound edge, we examined tracheal explants as a physiologically relevant model of epithelial repair. In this model, airway epithelial cells move from the cut edges over the basement membrane in an attempt to heal the wounded tissue (Figure 5E), and cells at the leading edge specifically express matrilysin.¹³

Figure 5. Matrilysin co-localization with E-cadherin in migrating tracheal epithelium. Tracheal explants from wild-type C57BL/6 mice were wounded, and confocal laser-scanning immunofluorescence microscopy superimposed with differential interference contrast images localized matrilysin and E-cadherin at 0 hours and 24 hours after wounding. **A:** Unwounded tracheal explant stained with anti-mouse matrilysin antibody and Alexa 568-conjugated secondary antibody. Cut edge of trachea is marked by **arrow**. **B and G:** Tracheal explant at 24 hours after wounding showed matrilysin expression (red) at basolateral surfaces of cells just behind leading edge of migrating epithelial front (**small arrows**). **Large arrowhead** indicates cilia. **C and H:** Unwounded trachea stained with anti-mouse E-cadherin ectodomain antibody and Alexa 488-conjugated secondary antibody. E-cadherin expression (green) was present at basolateral surfaces of tracheal epithelium with intense staining at apicolateral adherens junctions (**small arrows**). **D:** At 24 hours after wounding, E-cadherin expression remained apicolateral at cells back from wound edge (**small arrow**), was decreased in cells just behind wound edge (**asterisk**), and was redistributed evenly around surfaces in cells at leading edge of migrating epithelium (**large arrowhead**). **E:** Light microscopic image showed flattened migrating epithelial cell front with loss of cilia. Cells back from cut edge of trachea (**arrow**) retained cilia (**large arrowhead**). **F:** In merged images, matrilysin and E-cadherin were co-localized at basolateral surfaces of cells just behind leading edge of migrating cell front and at the apicolateral surfaces of ciliated cells. **I:** Close-up of **inset** in **F** showing co-localization of matrilysin and E-cadherin (**arrows**). Original magnifications, $\times 400$.

Matrilysin is not expressed in unwounded mouse trachea (Figure 5A). At 24 hours after wounding, matrilysin was induced and was seen in cells at the wound edge. In cells at the leading edge of the migrating cell front, matrilysin was distributed around the cell surface (Figure 5, B and G), as we have seen in injured alveoli.¹⁷ In contrast, in cells just behind the leading edge, the protein localized to the basolateral surfaces. Cells further back from the migrating front remained differentiated, as indicated by retained cilia, and showed a diffuse staining pattern for matrilysin throughout their cytoplasm.

In unwounded trachea, E-cadherin ectodomain localized to the basolateral surfaces of the pseudostratified airway

epithelium, with particularly intense staining at the apicolateral surfaces where adherens junctions are located (Figure 5C). At 24 hours after wounding, E-cadherin staining was still present at the apicolateral junctions of cells away from the wound edge, but in cells just behind the leading edge, E-cadherin staining was reduced (Figure 5, D and H). In cells at the leading edge of the migrating epithelial cell front, E-cadherin immunofluorescence was present but was distributed evenly around the cell surfaces of the flattened epithelium. Merged images showed that matrilysin and E-cadherin co-localized in the migrating cells just behind the leading edge of tracheal wounds and at the apicolateral surfaces of the ciliated cells (Figure 5, F and I).

Figure 6. Matrilysin-mediated E-cadherin shedding from wounded tracheal epithelium. Tissue lysates from wild-type and matrilysin-null mice and conditioned media (CM) collected at 24 hours from intact and wounded tracheal explant cultures were analyzed by Western blot with antibody targeted to E-cadherin ectodomain. Equivalent expression of full-length 120-kD E-cadherin is seen in intact tracheal tissue from wild-type (WT) and matrilysin-null (Mat^{-/-}) mice. Immunoreactivity for 80-kD E-cadherin ectodomain is prominently increased in conditioned medium from wounded wild-type tracheas but not in medium from wounded matrilysin-null tracheas.

Matrilysin-Mediated Shedding of E-Cadherin Ectodomain from Wounded Tracheal Epithelium

To evaluate if matrilysin sheds E-cadherin from migrating airway epithelium in tracheal tissue, we assessed the levels of E-cadherin ectodomain in the conditioned medium of wounded tracheal explant cultures. In conditioned medium from intact, unwounded tracheas, low levels of E-cadherin were detected in the conditioned medium of both wild-type and matrilysin-null samples (Figure 6). In contrast, E-cadherin was prominently shed into the culture medium of wounded wild-type tracheas, but only trace levels were seen in medium from matrilysin-null tracheas. Because other proteases, including MMPs, can cleave E-cadherin^{25,27} the low level of E-cadherin shedding into tracheal-conditioned medium from unwounded tracheas and from wounded matrilysin-null tracheas may reflect the activity of other proteinases.

Reorganization of Cell-Cell Contacts in Matrilysin-Dependent Epithelial Migration

To determine whether matrilysin-mediated E-cadherin shedding correlated with ultrastructural changes in cell-cell junctions in epithelial cells migrating at the wound edge, we used transmission electron microscopy to compare wounds made in tracheal explant cultures from wild-type and matrilysin-null mice. Because the differences in airway mucosal wound repair appeared to be because of a defect in initiation of migration in matrilysin-null tracheas, we made comparisons between wild-type and matrilysin-null mice early after wounding. In unwounded trachea from both wild-type and matrilysin-null mice, basal epithelial cells showed the characteristic membrane interdigitations that are rich in cell-cell junctions (Figure 7, A and B).^{36,37} By 6 hours after wounding, cells at the wound edge of tracheas from wild-type mice lost their basal membrane interdigitations, whereas in tracheas from matrilysin-null mice, the interdigitations persisted (Figure 7, C and D). By 12 hours after injury, the tracheal epithelial front on wild-type tracheas became flattened, migrating cells lost their differentiated phenotype, and cells at the wound edge began to spread over the basement membrane into the wound (Figure 8, A and C). In contrast, at 12 hours after wounding, the epithelium

in wounded tracheas from matrilysin-null mice still showed persistent basal interdigitations, the cell front retained the thickness of unwounded epithelium, and only cells at the very front edge of wound began to spread (Figure 8, B and D).

Matrilysin-Dependent E-Cadherin Shedding in Pulmonary Fibrosis

To determine whether matrilysin mediates E-cadherin ectodomain shedding from injured alveolar epithelium *in vivo*, we used the bleomycin model of lung injury in the mouse. Intratracheal administration of bleomycin induces an acute alveolar injury with lung inflammation followed by development of pulmonary fibrosis. Wild-type and matrilysin-null mice were instilled intratracheally with 0.08 U of bleomycin. This dose causes a severe lung injury without the mortality seen with higher doses and mediates induction of matrilysin expression, which peaks at 10 to 15 days after bleomycin instillation.¹⁷

Immunoblotting of BAL fluid collected from lungs of uninjured wild-type mice revealed a faint band at ~80 kD, likely representing the basal level of E-cadherin ectodomain shedding. In the first 3 days after injury, E-cadherin ectodomain immunoreactivity in BAL fluid was not different from that seen in BAL fluid from uninjured mice. In contrast, at 10 and 15 days after bleomycin treatment, when matrilysin expression peaks, the levels of E-cadherin ectodomain detected in BAL fluid were markedly increased (Figure 9A). However, in both uninjured and bleomycin-treated matrilysin-null mice, this 80-kD band was barely detected. To further demonstrate that E-cadherin shedding in response to bleomycin-induced lung injury was specifically dependent on matrilysin, BAL fluid of bleomycin-treated C57BL6 mice deficient for gelatinase A (MMP-9) or macrophage elastase (MMP-12), both of which are increased in the lungs of mice after inflammatory injury,^{30,31} was evaluated by Western blotting, and no differences in E-cadherin shedding were seen as compared to wild-type mice (Figure 9B). As was seen in the tracheal explants, the low level of basal E-cadherin shedding into BAL fluid from uninjured wild-type and matrilysin-null mice may reflect the activity of proteinases other than matrilysin. However, these data indicate that shedding of the E-cadherin ectodomain in response to lung injury was a matrilysin-dependent process.

We used immunofluorescence microscopy to determine whether E-cadherin shedding into BAL fluid correlated with a change in E-cadherin ectodomain expression in bleomycin-injured lungs. Bleomycin causes a patchy injury, and, as expected, there were no observed differences in E-cadherin ectodomain immunofluorescence in unaffected, non-lesional areas of lung in bleomycin-treated wild-type and matrilysin null mice. However, at 10 days after bleomycin treatment, the intensity of the immunofluorescence signal for E-cadherin ectodomain was reduced in the alveolar compartment of damaged lung (lesional areas) in bleomycin-treated wild-type mice as compared to matrilysin-null mice (Figure 9C). In the airways, no appreciable difference between ectodomain immunofluorescence intensity was

Figure 7. Reorganization of cell-cell contacts in migrating tracheal epithelium. Transmission electron microscopic analysis of wild-type and matrilysin-null tracheal explant cultures at times 0 hours and 6 hours after wounding (uranyl acetate and lead citrate). **A** and **B**: Unwounded wild-type trachea showed basalateral membrane interdigitations (**arrows**). **C**: By 6 hours after wounding, basalateral interdigitations are lost (**arrowheads**) from cells at epithelial wound edge of wild-type trachea. **D**: Basolateral interdigitations persisted (**arrows**) in cells at wound edge of matrilysin-null trachea. **E** and **F**: Higher magnification views of areas indicated by **rectangles** in **C** and **D**. Original magnifications: $\times 3500$ (**A**); $\times 10,000$ (**B**); $\times 7100$ (**C**, **D**); $\times 12,500$ (**E**, **F**).

Figure 8. Transmission electron microscopic analysis of wild-type and matrilysin-null tracheal explants at 12 hours after wounding. **A:** Tracheal epithelial front in wild-type trachea was flattened, and migrating cells spread over basement membrane (BM) into wound. **B:** Tracheal epithelium in matrilysin-null trachea retained thickness of uninjured epithelial and only cells at very front edge of wound began to spread. **C** and **D:** Higher magnification views of areas indicated by rectangles in **A** and **B** showed reorganization of cell-cell contacts in wild-type trachea (arrowheads), and persistent membrane interdigitations in matrilysin-null trachea (arrows). Original magnifications: $\times 3500$ (**A**); $\times 4500$ (**B**); $\times 8500$ (**C**); $\times 11,000$ (**D**).

observed in wild-type or matrilysin-null mice (data not shown), suggesting that the E-cadherin shedding into the BAL fluid was primarily from the alveolar compartment. Because bleomycin causes focal injuries mainly in the alveolar compartment, we did not expect to see a conspicuous repair response in the airway.

Because matrilysin co-localized with E-cadherin in migrating tracheal epithelium, we evaluated matrilysin expression in relation to E-cadherin in alveolar epithelium of bleomycin-injured wild-type mice. At 10 days after bleomycin-treatment, matrilysin was seen at the surfaces of damaged alveolar cells (Figure 9D). Merged image indicated that matrilysin localized with E-cadherin in damaged alveolar epithelium.

Discussion

Several MMPs are expressed in the injured lung,^{38–40} however, the specific role that most MMPs play in lung injury and repair remains largely unknown. The current results show that matrilysin protein expression is up-regulated in injured alveolar epithelium in a variety of human lung diseases. Although, it has been proposed that MMPs degrade extracellular matrix proteins during tissue injury and inflammation, the expression of certain enzymes in healthy tissues, such as matrilysin, indicates a role in epithelial homeostasis and repair. Previously, we reported that matrilysin is required for closure of large wounds in mouse tracheal explants, but the mechanism

Figure 9. Matrilysin mediates E-cadherin ectodomain shedding *in vivo*. Wild-type and matrilysin-null mice were instilled with 0.08 U of bleomycin, and bronchoalveolar lavage (BAL) fluid and lung tissue were harvested 3 to 15 days later. **A:** Western blot for E-cadherin in BAL fluid showed no significant difference in E-cadherin ectodomain immunoreactivity in the first 3 days after bleomycin treatment. At 10 and 15 days, E-cadherin immunoreactivity is increased in BAL fluid from wild-type mice but is not seen in BAL fluid from matrilysin-null mice. **B:** E-cadherin ectodomain is shed into BAL fluid of bleomycin-treated MMP-9 null and MMP-12 null mice. **C:** E-cadherin ectodomain immunofluorescence was reduced in alveolar epithelium in lesional areas of bleomycin-injured wild-type mice compared to that in matrilysin-null mice. Signal for E-cadherin is prominent in alveolar epithelium in nonlesional areas of both wild-type and matrilysin-null mice. **D:** At 10 days after bleomycin, immunofluorescence for E-cadherin and matrilysin were co-localized in damaged alveolar epithelium of wild-type mice (arrows). Original magnifications: $\times 200$ (**B**); 400 (**D**).

of its action was not determined.¹³ We show here that matrilysin facilitates migration by mediating E-cadherin ectodomain shedding and reorganization of cell-cell contacts in migrating airway epithelium. Furthermore, our previously reported findings that other MMPs, including gelatinase B, collagenase-1, and stromelysin-1 (MMP-3) were not expressed in wounded airway epithelium,¹³ and the lack of increase in E-cadherin shedding from matrilysin-null tracheal tissues suggest that E-cadherin shedding from migrating epithelium is specifically regulated by matrilysin. Although E-cadherin is highly expressed in normal human airways,⁴¹ the integrity of human airway mucosal epithelium is not compromised by constitutive matrilysin expression. This is supported by our data showing that the basolateral secretion of matrilysin in wounded epithelium is distinct from the constitutive, apical secretion seen in uninjured human airways and in glandular epithelium. In intact tissue, matrilysin is likely targeted to specific substrates, but after epithelial injury, it is directed to different proteins. Our results suggest that although matrilysin-mediated E-cadherin shedding is necessary for normal epithelial repair, it may be associ-

ated with a pathological process, such as progression of pulmonary fibrosis.

Re-epithelialization begins with the spreading and migration of surviving cells over a remodeled matrix, followed by proliferation and differentiation to restore the epithelial surface. Although other MMPs, such as gelatinase B (MMP-9), may function in lung repair by cleaving extracellular matrix proteins,^{31,42} our data suggest that matrilysin facilitates migration of surviving cells by promoting reorganization of cell-cell junctions. Matrilysin can degrade many extracellular matrix proteins *in vitro*, including fibronectin,⁴³ a prominent component of the provisional matrix in injured lungs. However, in our studies, matrilysin enhanced the migration of cells whether they were grown on plastic or on defined protein substrata, including fibronectin. Thus, the function of matrilysin in migration and repair does not appear to involve cleavage of an extracellular matrix protein.

The specific location of release of MMPs is a key factor in restricting substrate specificity. MMPs can be compartmentalized with their substrates by anchoring to the cell surface such as the binding of gelatinase A (MMP-2)

to the integrin $\alpha_5\beta_3$,⁴⁴ gelatinase B (MMP-9) to CD44,⁴⁵ and matrilysin to surface heparan sulfate proteoglycans,^{46,47} or by localized co-expression, such as the packaging of matrilysin with its prodefensin substrate in Paneth cell granules.²⁸ Likewise, we found that matrilysin was secreted specifically to locations of E-cadherin redistribution in migrating epithelium at the edge of mucosal wounds. These examples demonstrate that expression and activity of specific MMPs can be confined to specific locations and to a specific stage of repair.

Regulation of cadherin adhesive activity is not completely understood. Based on our data and other recent studies,^{25,26} MMP-mediated E-cadherin cleavage leads to enhanced migration and increased invasive capacity *in vitro*. In support of this idea, MMP inhibitors increase cadherin levels, stabilize cadherin-mediated cell-cell contacts, and augment adhesion in cultured fibroblasts.⁴⁸ An important distinction between the cell-based systems and the tracheal explant model is that E-cadherin is not completely lost from cell-cell contacts in migrating tracheal epithelium, but rather is redistributed. Therefore, the consequence of E-cadherin cleavage in normal repair may be a reorganization of E-cadherin-based cell-cell adhesion, rather than a loss of adhesion, as was suggested for tumor cell invasion.¹⁹ Indeed, E-cadherin may mediate cell motility and suppress cancer cell invasion by distinct mechanisms.²⁰ Thus, we describe a physiological role for matrilysin-mediated E-cadherin shedding in mucosal epithelial repair, and identify E-cadherin as a potential *in vivo* matrilysin substrate.

In addition to forming homophilic complexes between cells, the cytoplasmic domains of E-cadherin are coupled to the actin cytoskeleton by association with β -catenin.⁴⁹ β -catenin serves as a structural component of adherens junctions and as a noncadherin-dependent signaling factor. In concert with activation of the Wnt signaling pathway, free cytoplasmic β -catenin translocates to the nucleus and binds to transcription factors of the lymphocyte enhancer-binding factor-1/T-cell factor pathway to regulate expression of downstream target genes including *c-myc*, *WISP*, and *cyclin D1*.⁵⁰ Therefore, matrilysin-mediated E-cadherin shedding may lead to increased β -catenin translocation to the nucleus and altered gene expression. Interestingly, matrilysin is also a target gene for the Wnt/ β -catenin signaling pathway,⁵¹ suggesting a positive feedback loop regulating matrilysin gene expression in injured epithelium.

Matrilysin expression was recently reported to be a highly associated with pulmonary fibrosis in humans.^{15,16} Bleomycin-induced fibrosis is reduced in matrilysin-null mice compared to wild-type mice,¹⁶ but the more prominent phenotype is reduced neutrophil influx, which is linked to syndecan-1 ectodomain shedding.¹⁷ Distinct from the regulation of acute neutrophil influx, which occurs during the first few days after bleomycin, matrilysin-mediated E-cadherin ectodomain shedding into the BAL fluid was not seen until 10 days after injury, when matrilysin expression levels peaked and fibrosis progressed. These findings support the idea that matrilysin serves distinct functions and acts on different substrates mediating different repair processes.

The temporal link between matrilysin expression and fibrosis in the bleomycin model suggests that aberrant production of this MMP may contribute to pathology, as well. The cellular processes involved in progression of pulmonary fibrosis include chronic epithelial damage, fibroblast migration and proliferation, apoptosis, extracellular matrix remodeling, and enhanced local coagulation.⁵² Matrilysin can regulate apoptosis by cleaving Fas ligand, as shown during prostate involution,⁵³ and it may promote local coagulation by cleaving tissue factor pathway inhibitor.⁵⁴ Based on our results, we propose that matrilysin initially functions to promote cell migration during repair after acute epithelial damage. However, if the stimulus for lung damage is ongoing, such as in persistent inflammation or fibrosis, matrilysin may contribute to ongoing epithelial activation by mediating persistent shedding of the E-cadherin ectodomain, altering cell-cell interactions, and perhaps changing gene expression by altering β -catenin signaling pathways. We are now exploring how matrilysin and E-cadherin may regulate these processes.

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