Mechanical Induction of an Epithelial Cell Chymase Associated with Wound Edge Migration

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Chymase is a chymotrypsin-like serine protease predominantly produced by mast cells. In this study, human cutaneous and gingival keratinocytes, ovary surface epithelia, and a porcine epithelial cell line were assayed by homology-based cloning, and the amplified DNA fragment was identified as a chymase. In vitro, chymase could not be induced by serum or cytokine treatment alone. Chymase was activated 3-fold within 60 min in basal media by scratch wounding cultured monolayers and further potentiated over 10-fold at 18 h by additional serum and cytokine treatment. Chymase activity was cell-associated and found to peak within 24 h of wounding and then steadily decreased as cultures healed, reaching baseline levels before confluence was reestablished. Affinity column purified enzyme effectively degraded fibronectin and was found by Western blot analysis using a human chymase antibody to be of about 30 kDa. Immunostaining revealed chymase activation at the wound edge colocalizing with reactive oxygen species generation. Specifically, chymase activation was attenuated by inhibition of nitric oxide, superoxide, and peroxynitrite. Exogenous peroxynitrite but not hydrogen peroxide also resulted in chymase activation in unwounded monolayers. Disruption of cytoskeletal stress fibers by cytochalasin D attenuated both chymase activation and the amplified DNA fragment was identified as a chymase. In vitro, chymase could not be induced by serum or cytokine treatment alone. Chymase was activated 3-fold within 60 min in basal media by scratch wounding cultured monolayers and further potentiated over 10-fold at 18 h by additional serum and cytokine treatment. Chymase activity was cell-associated and found to peak within 24 h of wounding and then steadily decreased as cultures healed, reaching baseline levels before confluence was reestablished. Affinity column purified enzyme effectively degraded fibronectin and was found by Western blot analysis using a human chymase antibody to be of about 30 kDa. Immunostaining revealed chymase activation at the wound edge colocalizing with reactive oxygen species generation. Specifically, chymase activation was attenuated by inhibition of nitric oxide, superoxide, and peroxynitrite. Exogenous peroxynitrite but not hydrogen peroxide also resulted in chymase activation in unwounded monolayers. Disruption of cytoskeletal stress fibers by cytochalasin D attenuated both wound-activated chymase and reactive oxygen species generation. Chymase inhibitor chymostatin reduced the loss of cell-cell contacts and the onset of porcine and human skin epithelial cell migration at the wound edge. This shows that an epithelial chymase is rapidly activated by a ligand-independent mechanism following mechanical stress via cytoskeletal and reactive oxygen species signaling and is associated with the onset of epithelial cell migration.

Chymase is a chymotrypsin-like serine protease, predominantly found in mast cells where it is stored within secretory granules as a zymogen complexed with heparin proteoglycan. It can be activated and released into the interstitial tissues following mechanical wounding such as vascular injury caused by balloon catheterization or vein grafting (1). Mast cell activation and subsequent degranulation can be induced by both ligand-dependent and -independent pathways. The ligand-dependent pathway operates through activation of the high affinity IgE receptor (FceRI) on the cell surface by IgE antigen triggering a cascade of signal transduction pathways (2). The ligand-independent pathway involves, at least in part, the generation of ROS, including H$_2$O$_2$ and O$_2^-$. Conversely, chemical scavengers of peroxides and peroxynitrites almost completely abrogate ligand-independent mast cell activation. Also, treatment of mast cells with DPI, an inhibitor of NAD(P)H oxidase production of O$_2^-$ greatly reduced mast cell activation (3). Chymase has several actions that could be involved in both normal and pathological wound healing such as angiostatin II formation (4), degradation of extracellular matrix (5), and activation of matrix metalloproteinases (6) and cytokines, including TGF-$eta_1$ (7) and antibacterial peptides (8). The presence of mast cell chymase has been noted in skin wounds (9), although it is not clear that the enzyme can diffuse to the zone of reepithelialization (10).

A few studies have reported non-mast cell sources of chymase. A human heart chymase cDNA was cloned from ventricular mRNA preparations. The purified protein from the same tissue was used to synthesize a polyclonal antibody, which then detected chymase localized to the interstitium of the myocardium, the granules of mast cells, endothelial cells, and cells described as “mesenchymal” but not myocytes (11). Human heart chymase was also found by Western immunoblot to be distributed in other tissues, including uterus, tonsil, stomach, esophagus, coronary artery, and aorta, although the cellular origin within these tissues was not determined (12). Also, homology-based cloning has been used to generate a full-length cDNA of a novel chymase from rat vascular smooth muscle

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2 The abbreviations used are: ROS, reactive oxygen species; SAAPNA, N-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide; SAAVNA, N-succinyl-L-alanyl-L-alanyl-L-valyl-p-nitroanilide; SAAAA, N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide; GPNA, N-glycyl-L-phenyl-p-nitroanilide; TLCK, 1-chloro-3-tosylamido-7-amino-L-2-heptanone; TPCK, tosyl-L-phenylalanyl chloromethyl ketone; BAPNA, N-$\alpha$-benzoyl-L-arginine-p-nitroanilide; PBS, fetal bovine serum; O$_2^-$, superoxide; NO, nitric oxide; H$_2$O$_2$, hydrogen peroxide; ONOO$^-$, peroxynitrite; DPI, diphenyl-iodonium; L-NAME, N$^\omega$-nitro-L-arginine methyl ester; NAC, N-acetyl cysteine; PLE, porcine periodontal epithelial cell; NHEK, normal human epithelial keratinocyte; PBS, phosphate-buffered saline; DTT, dithiothreitol; TGF, transforming growth factor; RT, reverse transcription; TBS, Tris-buffered saline; DCFDA, 2',7'-dichlorodihydrofluorescein diacetate.

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cells. DNA sequencing showed the clone to have homology to the five known rat mast cell chymes of between 59 and 81%. Southern blot analysis showed the smooth muscle chymase gene to reside at a chromosomal location distinct from any of the mast cell chymes. Northern blot analysis and in situ hybridization detected mRNA expression in pulmonary artery and aortic smooth muscle cells. Its expression in these two tissues was elevated 8- and 11-fold, respectively, in hypertrophied tissues. Furthermore, substrate and protease inhibitor assays detected chymase activity in primary smooth muscle cell cultures from rat, porcine, and human aorta and pulmonary artery (4). Immunostaining of human renal tissue using a mast cell chymase antibody was able to localize chymase also to vascular smooth muscle cells, endothelial cells, and perivascular myofibroblasts. However, the expression of chymase was absent in glomerular and tubular epithelial cells (13).

To date, there are no reports of epithelial chymotrypsin-like proteases being involved in wound healing. Stratum corneum chymotryptic enzyme (kallikrein 7) is located in high suprabasal keratinocytes of skin epidermis and mucosal epithelial cells of the hard palate where it plays a central role in epidermal homeostasis (14) and may also be involved in skin inflammatory reactions (15). Previously, we have shown in the expression in human normal ovarian surface epithelial cells of a chymotryptic activity that could be up-regulated by culturing on fibrin clots (16). We also showed the production of a chymotryptic protease in cultured normal human epithelial keratinocytes (NHEK), human gingival, and porcine periodontal ligament epithelium (PLE) cell lines. This activity was released in very low amounts into the media, which was detectable after affinity column purification of conditioned medium (17). Further characterization was required to identify the chymotryptic activity and elucidate its activation mechanism and physiological function. Therefore, in this study we first identified epithelial chymotryptic activity from four normal epithelial cell types by homology-based cloning and DNA sequencing. Second, using PLE we investigated chymase activation using an in vitro scratch wound model of dense cell monolayers in either basal media (ligand-independent) or supplemented media (ligand-dependent) conditions. Third, chymase expression was localized relative to the wound edge. Fourth, we studied the role of mechanical wound-induced ROS and cytoskeleton organization associated with the rapid ligand-independent activation of the epithelial chymase. Finally, we studied possible physiological functions by assaying the degradation of fibronectin, and using both PLE and NHEK correlated the activation of epithelial chymase at the wound edge with the onset of epithelial cell migration.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Commercial normal human epithelial keratinocytes were cultured in KGM (Lonza, Allendale, NJ) according to the supplier’s directions. Human gingival keratinocytes were a gift from Dr. D. Oda (University of Washington, Seattle) and were also cultured in KGM. Porcine ligament epithelial cells were isolated as described previously (17) and cultured in α-minimal essential medium supplemented with 10% FBS (Flow Laboratories, McLean, VA). Human ovary surface epithelia, a gift from Dr. P. Kruk (University of South Florida, Tampa), were cultured in Medium 199:MCDB 105 with 10% FBS. All media contained 100 IU/ml penicillin G, 50 μg/ml gentamycin, and 50 ng/ml amphotericin B (Invitrogen). All other reagents were purchased from Sigma unless otherwise stated.

**Homology-based Cloning**—GenBank™ sequences of several chymotrypsin-like proteases were aligned using ClustalW as follows: 1) human neutrophil cathepsin G (hCAT-G); 2) rat mast cell proteinase II (rMCPII); 3) mouse granzyme B (GranB); 4) human chymase (hCMA); 5) mouse mast cell protease II (mMCprt2); 6) dog mast cell chymase (dMC); and 7) rat smooth muscle chymotryptic enzyme (hSMCCE). PCR primers (forward, gtccac/actt/gggg/agecctt/caa; reverse, ccca/ggatc/tctcttggaat) were used for RT-PCR to amplify from quiescent epithelial cultures, and the product was analyzed on a 1.5% agarose gel. Gel fragments were subject to PCR-based two-pass DNA sequencing (Invitrogen).

**Protease Induction**—Confluent monolayers of PLE were cultured to quiescence in basal α-minimum Eagle’s medium for 18 h. Monolayers were then treated with H2O2, AgNO3, NH4Cl, or ONOO−, at various concentrations for 1 h at 4 °C, and then washed with prewarmed medium and incubated at 37 °C for an additional 1 h. In wound experiments confluent quiescent cultures were scratched with a 1-cm fragment of a fine tooth plastic comb covering the entire surface as described previously (18). Control and wounded cultures were washed one time with basal media and then further cultured in basal media up to 18 h. Some cultures were supplemented with FBS, TGF-β, epidermal growth factor, KGF-1, or platelet-derived growth factor (Upstate Biotechnology, Inc., Lake Placid, NY). The time course protease expression was assayed in parallel experiments of wounded monolayers and nonwounded, low density cultures supplemented with 10% FBS for up to 5 days. At 24-h intervals, cultures from both groups were stored intact at −20 °C. At the conclusion of experiments substrate was added directly to samples and incubated for 18 h. The changes in absorbance at 405 nm were recorded spectrophotometrically. Cultures were then fixed in 4% formaldehyde, 5% sucrose, PBS and stained with 0.5% crystal violet in 20% methanol. Images were digitally recorded, and relative surface area was calculated using NIH Image 1.62 (NIH, Bethesda), and enzyme activity was expressed on a per-cell basis.

**Substrate and Inhibitor Specificity**—Cell or media samples were assayed for substrate specificity using the synthetic chromogenic substrates N-succinyl-l-alanyl-l-alanyl-l-prolyl-l-phenylalanine-p-nitroanilide (SAAPNA) for chymotrypsin-like, N-succinyl-l-alanyl-l-alanyl-l-valyl-l-p-nitroanilide (SAAVNA) for elastase, N-succinyl-l-alanyl-l-alanyl-p-nitroanilide (SAAAPA) for proteosomic chymotrypsin, N-glycyl-l-phenyl-p-nitroanilide (GPNA) for dipetidyl peptidase I, and N-(alpha-benzoyl-l-arginine-p-nitroanilide (BAPNA) for trypsin-like activity. Assays were done at a final substrate concentration of 1 mm in PBS at 37 °C for 18 h. Enzyme inhibition was tested by incubating samples with 1-chloro-3-tosylamido-7-amino-1-2-heptanone (TLCK; trypsin-like), tosyl-l-phenylalanine chloromethyl ketone (TPCK), and chymostatin (chymotrypsin-like) or epoxymycin (proteosomic chymotrypsin).
sin-like) at concentrations up 50 μM in the presence of SAAPNA.

**Immunostaining**—Experiments were performed on 12-mm diameter glass coverslips sterilized by glow discharge. Quiescent culture surfaces were scraped twice in an x-shape with a pipette tip and then further cultured for various times. Samples were fixed (2% paraformaldehyde, 5% sucrose, PBS), permeabilized (0.5% Triton X-100, PBS, 4 min), and then washed five times in PBS. Cells were quenched (fresh 0.5% NaBH₄, PBS) and blocked (3.0 mg per ml bovine serum albumin, 1 mg per ml glycerine, PBS, 30 min). Samples were then incubated in the blocking solution with primary antibody to human chymase 1 (CMA1-Chemicon, Temecula, CA) for 1 h at room temperature. Samples were then washed five times (0.1 mg per ml bovine serum albumin, PBS), incubated in block solution with Alexa 488 secondary antibody (Molecular Probes, Eugene, OR) and 4′,6-diamidino-2-phenylindole nuclear stain for 1 h in the dark. Samples were washed three times with PBS and then mounted in Vectashield (Vector Laboratories, Burlington, CA). Images were digitally recorded under laser epifluorescence at 488 nm. A parallel set of samples was processed for detection of ROS by preincubation with 0.5 mg/ml 3,3-diaminobenzidine, 0.1 mM H₂O₂, pH 7.6, alone, or including 0.1 mg per ml of MnCl₂ for 5 min before to 10 min after each time point. At each time point samples were then washed three times with PBS, counterstained with hematoxylin, washed five times with PBS again, then fixed (2% paraformaldehyde, 5% sucrose, PBS), and mounted. Images were digitally recorded under tungsten illumination. Alternatively, ROS was assayed by pretreatment with 10 μM DCFDA for 30 min prior to incubation with phallacidin or cytochalasin D and wounding and quantitated using NIH Image 1.62.

**Zymography**—Cells were resuspended in nonreducing Laemmli buffer and loaded onto an SDS-12% polyacrylamide gel containing 0.5 mg/ml β-casein. After electrophoresis, gels were washed two times for 10 min with 100 ml of 2.5% (v/v) Triton X-100, 50 mM Tris-HCl at 22 °C, and then three times for 10 min with H₂O. The gel was incubated in 50 mM Tris-HCl, 200 mM NaCl, 20 mM CaCl₂, and in some experiments 100 μM TPCK or TLCK, pH 7.4, at 37 °C overnight and then stained with 0.5% Coomassie Brilliant Blue R-250 for 30 min in 40% methanol, 5% acetic acid and destained in H₂O. The molecular weights of the lysis bands were determined by reference to wide range molecular mass markers (Invitrogen).

**Affinity Column Purification**—One step purification of chymotrypsin-like activity was carried out as described previously (17). A 1-ml aprotinin-Sepharose (Amersham Biosciences) column was equilibrated by running through 20 ml of equilibration buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM CaCl₂). Media or cell lysate was passed through the column, which was then washed with equilibration buffer until the absorbance at 280 nm reached a base line. Bound protease was eluted (100 mM HCl, pH 1.0, 1 mM CaCl₂) and collected in 100-μl aliquots into 50 μl of 2 mM Tris-HCl, pH 7.5. The protein elution profile was determined spectrophotometrically. Aliquots from each peak were pooled then concentrated with a 5-kDa molecular weight cutoff spin concentrator (Amicon, Beverly, MA).

**Silver Stain Gel Analysis**—An aliquot of column purified material in reducing loading buffer was fractionated on 12% PAGE. Gels were fixed for 1 h (40% EtOH, 10% acetic acid), washed three times (30% EtOH) for 20 min, and then washed in distilled water for 20 min. Gels were then sensitized in 0.02% Na₂S₂O₃ for 1 min and then washed in H₂O three times for 20 s. Gels were then incubated in fresh cold 0.1% AgNO₃ for 20 min at 4 °C and then washed three times for 20 s in H₂O, transferred to a fresh container, and washed once more in H₂O. Gels were then developed in 3% Na₂S₂O₃, 0.05% formalin until bands were visible. Development was then terminated by first washing in distilled water for 20 s and then washing in 5% acetic acid for 5 min. Gels were finally washed in H₂O three times for 10 min and then digitally recorded under tungsten light.

**Western Blot Analysis**—Wound crude lysates and column-purified material were fractionated by 12% PAGE and electroblotted to Immobilon-P membranes (Millipore, Bedford, MA). The filter was blocked at room temperature for 1 h (5% skim milk powder, 0.1% Tween 20, TBS) and then membranes incubated with anti-human chymase antibody for 2 h at 22 °C. After washing the membranes three times with TBS, 0.1% Tween 20 membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Molecular Probes) in TBS, 0.1% Tween 20. After 1 h of incubation at 22 °C, the membranes were extensively washed with TBS, 0.1% Tween 20 followed by washing with TBS alone. The membranes were developed with the ECL system (Amersham Biosciences) according to the manufacturer’s protocol.

**Migration Studies**—PLE and NHEK cultured as described above were wounded in the presence of increasing concentrations of chymostatin. At 6 and 18 h after wounding fixed and crystal violet-stained cultures were analyzed by measuring cell migration distance past the marked wound origin (0 h) as described previously (19) of five randomly selected fields of treated and control cultures in three independent experiments.

**Statistical Analysis**—Analysis of variance and Student’s t test were used to compare means of two or more different treatment groups. The level of significance was set to p < 0.05 unless otherwise stated. Data are expressed as mean ± S.E.

**RESULTS**

**Epithelial Cells Express Chymase**—PCR primers were designed for homology cloning of a segment of the highly conserved chymotrypsin-like substrate binding region around consensus sequences spanning the first (amino acids 52–57) and sixth (amino acids 192–198) catalytic residues (Fig. 1A). Four normal epithelial lines were cultured to quiescence at semiconfluence and then processed for RT-PCR. Amplified bands analyzed on 1.5% agarose gels showed a single band of the 396-bp predicted molecular weight (Fig. 1B). Excised bands were then processed for two-pass PCR-based dideoxy DNA sequencing reactions and analyzed chromogenically. Resulting sequence data were BLAST analyzed using the EMBL human data base. All four DNA sequences were found to be highly conserved over the area of interest and to show closest homology to human heart chymase (>90%). Sequence alignment for PLE and NHEK fragments are provided as supplemental material.
Epithelial Chymase Activation Requires Mechanical Wounding—PLE were used in an in vitro scratch wounding model. Dense, quiescent cultures were scratch-wounded on a 1-mm grid (Fig. 2A, inset), as described previously (18), and then further cultured for 4 h in either basal media or media supplemented with 10% FBS. All groups were then cultured in fresh basal media for an additional 18 h. Media and cells were assayed with chromogenic substrates for chymotrypsin-like (SAAPNA), proteosomic chymotrypsin-like (SAAANA), elastase-like (SAAVNA), trypsin-like (BAPNA), and dipeptidyl peptidase I (GPNA) activity. None of the substrates revealed any detectable levels of these proteases in unconcentrated raw media samples as reported previously (17) or in 5-fold concentrated media (data not shown). Also, neither elastase nor proteosomic chymotrypsin-like activity was detected in any samples (data not shown). Chymase, trypsinic and dipeptidyl peptidase I activity was detected in intact cell monolayers, and chymase and dipeptidyl peptidase I activities increased about 3-fold in wound cultures maintained in basal media. Wounded cultures pulse-supplemented with 10% FBS showed a 10-fold increase in chymase activity at 18 h. Tryptic and dipeptidyl peptidase I activities were not further increased by FBS treatment (Fig. 2A). Ligand-dependent induction was further studied by supplementing basal media-treated wounded cultures with cytokines characteristic of wound sites. It was found that there was a dose-dependent induction of cell-associated chymotryptic activity in response to TGF-β1, KGF-1, epidermal growth factor, and platelet-derived growth factor (Fig. 2B). The time course expression profile of chymotryptic activity in the

![FIGURE 1. Homology cloning of a chymotrypsin-like sequence from four normal epithelial cell types. A, amino acid sequences of various chymotrypsin-like enzymes were aligned using ClustalW and degenerate PCR primers deduced around consensus sequences between the first (amino acids 52–57) and sixth (amino acids 192–198) catalytic residues. B, quiescent epithelial cells from NHEK (lane 1), human gingiva (lane 2), human ovary surface (lane 3), and porcine periodontal ligament (PLE) (lane 4) were processed for RT-PCR. Amplified product analyzed by 1.5% agarose gels revealed a single band of the predicted 396 bp molecular weight.](image1)

![FIGURE 2. Induction of cell-associated epithelial chymotrypsin-like enzyme. A, dense quiescent monolayers of PLE were scratch-wounded on a 1-mm grid (inset). At 18 h post-wounding with or without the presence of 10% FBS, cultures were assayed for enzyme activity using chromogenic substrates for chymotrypsin-like (SAAPNA), proteosomic chymotrypsin-like (SAAANA), elastase-like (SAAVNA), trypsin-like (BAPNA), and dipeptidyl peptidase I (GPNA). B, wounded monolayers were cultured in media supplemented with various concentrations of cytokines for 18 h and then assayed for chymotrypsin-like activity. C, wounded monolayers and low density nonwounded cultures in the presence of 10% FBS were assayed for chymotrypsin-like activity (SAAPNA) at 24-h intervals until both groups reached confluence at day 5. Values are ± S.E. from three independent experiments done in quadruplicate. Significant difference from controls is indicated by *, p < 0.05; **, p < 0.01.](image2)
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Chymase Is Cell Membrane-associated and Not Heparin-associated—Cell extracts were analyzed by β-casein zymography that is sensitive for chymotrypsin-like activity (Fig. 3A). Extracts from intact monolayers revealed two bands of 32 and 27 kDa, consistent with the molecular weight of chymase and a commonly observed degradation product, respectively (Fig. 3A, lane 1). Both of these bands were markedly stronger in the wounded cell fractions (Fig. 3A, lanes 2, 3, and 5). No activity was found in media samples (data not shown). Zymograms processed in the presence of chymotrypsin-like inhibitor TPCK showed almost complete inhibition of chymotrypsin-like activity of cell extracts and pancreatic α-chymotrypsin control (Fig. 3A, lanes 5 and 6), whereas trypsin-like inhibitor TLCK had a limited effect (lanes 3 and 4). Inhibition specificity was quantified by SAAPNA degradation using cells from wounded cultures in the presence of chymotrypsin-like inhibitors chymostatin and TPCK, proteosomic chymotrypsin-like inhibitor epoxymycin, and trypsin inhibitor TLCK. Both chymostatin and TPCK strongly reduced chymase activity in a concentration-dependent manner, whereas epoxymycin had no effect. Consistent with the zymograms, the trypsin inhibitor had only a small effect (Fig. 3B). The inhibitor α-antitrypsin is known to be ineffective against chymase bound to heparin but highly inhibitory against unbound chymase (20). Concentration-dependent inhibition of chymase activity by α-antitrypsin (Fig. 3C) is in agreement with the previous report for rat vascular smooth muscle chymase (4) and consistent with the enzyme not being complexed with heparin upon activation. Exogenous intact heparin is thought to be cell-impermeant and known to modulate chymase specificity (21). Exogenous heparin was found to inhibit activated chymase of intact cells indicating that it was, in part, cell membrane-associated. Cell lysates showed greater heparin inhibition consistent with intracellular chymase activation (Fig. 3D).

Ligand-independent Activation Does Not Involve Chymase Synthesis—Chymase was subjected to aprotinin-Sepharose affinity column purification, and two elution peaks were detected as reported previously (17). The elution profile of intact and basal media wounded samples was virtually identical. However, the magnitude of peaks was found to greatly increase in cultures wounded in the presence of FBS. Hence ligand-independent chymase induction does not involve synthesis, whereas ligand-dependent induction does (Fig. 4A). Samples were pooled from each peak for intact and FBS-treated wound groups (Fig. 4B) and then fractionated by SDS-PAGE followed by silver staining. A predominant peak at 30 kDa was observed, and relative protein amounts in the dominant band agreed with column elution peak sizes (Fig. 4B, lanes 1 and 2). Cell lysate and purified chymase were subject to zymography and showed a single dominant band of activity in both (Fig. 4B, lanes 3 and 4). Cell lysate and purified chymase were subject to Western analysis using an antibody to human chymase (CMA1). Again, a dominant band of 30 kDa was seen in both (Fig. 4B, lanes 5 and 6). Because fibronectin is a major physiological target of chymase (5), purified protease was incubated in the presence of commercial human fibronectin. Epithelial chymase totally degraded fibronectin (Fig. 4C).
Chymase Activation at the Wound Edge Is Associated with ROS—Because wounding was found to be required for chymase activation, we focused on its possible mechanism via ligand-independent signaling. Epithelial wounding has been reported previously to induce rapid generation of ROS at the wound edge (22), and ROS has been implicated in mast cell chymase activation (3). Here the time frame of ROS generation was correlated to chymase activation. Monolayers pretreated (23) to detect the presence of ROS species H$_2$O$_2$ (diaminobenzidine) and O$_2^*$ (diaminobenzidine/MnCl$_2$) were subsequently wounded and cultured for increasing periods. Wound-induced H$_2$O$_2$ was not detected in any cultures (data not shown). However, after wounding a staining pattern for O$_2^*$ started in the cells at the wound edge within 10 min and then progressed at subsequent time points away from the wound edge (Fig. 5A). Ligand-independent chymase activation in wounded cell monolayers as described above was detected in parallel experiments using the CMA1 antibody. After wounding chymase staining was first detected within 30 min localized to the wound edge, well after ROS detection was widespread. At later time points the staining increased in intensity and in cell distance away from the wound edge, peaking between 1 and 2 h (Fig. 5B).

Chemical ROS Induction Activates Chymase—To confirm that the ligand-independent mechanism of chymase activation involved ROS, chemical mediators of ROS generation were used. The optimal time frame for activation was first confirmed by scratch-wounding cultures and showed ligand-independent chymase activation by SAAPNA degradation reaching an activation plateau within 2 h (Fig. 6A) in agreement with the immunostaining results. Subsequent experiments tested chemical induction of unwounded monolayers at 2 h. To test whether Ca$^{2+}$ influx was involved, monolayers were treated with ionomycin (B), H$_2$O$_2$ (C), AgNO$_3$ (D), NH$_4$Cl (E), and ONOO$^-$ (F) and then assayed for chymase activity by colorimetric substrate degradation (SAAPNA).

**FIGURE 4.** Purification of epithelial chymase. A, PLE cell extracts from intact and wounded cultures in basal media or media pulse-supplemented with 10% FBS were collected at 18 h and subject to aprotinin-Sepharose affinity column purification. B, intact (lane 1) and 10% FBS-wounded (lane 2) purified material was analyzed by 12% PAGE followed by silver staining. Cell extracts before (lane 3) and after (lane 4) column purification were analyzed by 12% $\beta$-casein zymography. Cell extracts before (lane 5) and after (lane 6) purification were analyzed by Western analysis using an antibody to human chymase 1. C, column-purified PLE chymase was incubated with human fibronectin, and degradation at time points was assayed by 12% PAGE.

**FIGURE 5.** Reactive oxygen species production and coincident epithelial chymase expression at epithelial wound sites. A, PLE monolayers in basal media were preincubated with DAB + MnCl$_2$ for the colorimetric detection of reactive oxygen species as brown precipitate near the wound edge (black arrows) at various time points after wounding. Scale bar, 50 $\mu$m. B, in parallel experiments wounded cultures were fixed at various time points and stained with chymase antibody (green) and 4',6-diamidino-2-phenylindole nuclear stain (blue). Wound edge origin is indicated by white arrow. Scale bar, 100 $\mu$m. Images are representative of results from three independent experiments.

**FIGURE 6.** Modulated epithelial chymase activation in association with chemically induced reactive oxygen species. A, PLE monolayers in basal media were wounded and then assayed for chymase activity at time points up to 2 h. Unwounded monolayers were treated for 2 h with ionomycin (B), H$_2$O$_2$ (C), AgNO$_3$ (D), NH$_4$Cl (E), and ONOO$^-$ (F) and then assayed for chymase activity by colorimetric substrate degradation (SAAPNA).
involvement of membrane-localized NAD(P)H oxidase production of $O_2^-$ was further tested with DPI, a specific inhibitor of the nox subunit of NAD(P)H oxidase. Pretreatment of cultures with increasing concentrations of DPI showed about 65% reduction of chymase activation by wounding (Fig. 7D). The role of nitric oxide was studied by pretreating cultures with the NO synthase-specific inhibitor l-NAME. Treatment with l-NAME was found to decrease wound activation of chymase by about 65% (Fig. 7E). Because inhibition by l-NAME and DPI was consistent with the involvement of ONOO$^-$, the downstream product of $O_2^-$ and NO, ONOO$^-$ scavenger uric acid was tested. It was found to completely inhibit chymase activity to levels that were slightly below the unwounded controls (Fig. 7F). These findings are consistent with those above showing that exogenous ONOO$^-$-mediated chymase activation.

Cytoskeleton Integrity Is Required for Wound Induction of Chymase—Previous studies have reported that perturbation of the cytoskeleton mediates ROS production. Measuring DCFDA fluorescence showed that cultures pretreated with cytochalasin D but not phallacidin blocked wound-induced ROS (Fig. 8A). Stabilizing the cytoskeleton with phallacidin prior to wounding did not affect the dynamics of chymase activation by wounding, suggesting that cytoskeletal remodeling was not required (Fig. 8B). Conversely, disrupting the cytoskeleton by pretreatment with cytochalasin D was found to diminish wound-induced chymase activation in a time- and concentration-dependent manner (Fig. 8C). Immunostaining wound edge cells with chymase antibody showed strong association of the enzyme with the cytoskeleton. However, there was a progressive loss of activated chymase staining associated with disruption of the cytoskeleton (Fig. 8D–F).

Epithelial Chymase Is Associated with the Onset of Cell Migration at the Wound Edge—Migration distance past the wound edge origin was assayed (19) with quiescent monolayers of PLE and NHEK in the presence of chymostatin. Preliminary experiments ruled out use of TPCK and TLCK in live cell experiments because they were both found to be toxic at low (2.5 μM) concentrations (supplemental material) in general agreement with previous studies on airway epithelia (26). Relative to the untreated control wound cultures, chymostatin at 25 μM and above significantly inhibited cell migration at 6–18 h ($p < 0.05$) in both the porcine (Fig. 9A) and human (Fig. 9B) epithelial lines. Furthermore, at 50 μM chymostatin treatment reduced the loss of cell–cell contacts of both PLE (Fig. 9C) and NHEK (Fig. 9D) nonmigrating cells. A chymase activation mechanism associated with membrane perturbation via cytoskeletal and ROS signaling is proposed (Fig. 10).

DISCUSSION

We report that chymase expression is characteristic of epithelial cells. Induction of the epithelial chymase required mechanical wounding, which was itself sufficient to activate the chymase, yet could be further potentiated by ligand binding. Mechanical force may be a common control of chymase activation. Previously, rat vascular smooth muscle chymase could not be induced by cytokine treatment of cultured cells but was up-regulated by induced hypertension in rats (4). Similarly, human
chymase expression in vascular smooth muscle cells was up-regulated in hypertensive patients at levels that correlated significantly with the increase in blood pressure (13). Also, chymase is released into the extracellular matrix of vascular tissues after mast cells have been activated following balloon catheterization or vessel grafting (1). ROS is known to be rapidly induced at the epithelial wound edge and to effect ligand-independent signaling cascades in epithelia (22). Also, ROS, including H$_2$O$_2$ and O$_2^-$, lead to mast cell activation, a precursor of chymase activation, by a ligand-independent mechanism (27). ROS also seem to mediate store-independent Ca$^{2+}$/H$^+$ influx in mast cells (3).

In mechanically wounded epithelia wound-induced H$_2$O$_2$ levels were not detected, whereas O$_2^-$ levels were rapidly and strongly elevated in wound edge cells and before colocalizing chymase activation was detectable. Detectable O$_2^-$ was progressively observed in cells distal to the wound edge, colocalizing with chymase activation, suggesting either O$_2^-$ diffusion or intercellular signal activation of ROS production. It was also found that in unwounded cell monolayers that H$_2$O$_2$ treatment inhibited chymase activation consistent with previous work which showed that treatment of mast cells with H$_2$O$_2$ results in transient intracellular acidification that was inhibitory to mast cell activation (28). Conversely, mast cell alkalization by NH$_4^+$/H$^+$ treatment is sufficient to cause general mast cell activation (29). Also, treatment of *Xenopus laevis* oocytes with NH$_4^+$/H$^+$ perturbed cell membrane conductance and generated intracellular ROS associated with a rapid intracellular alkalization (24). Consistent with this we found that treatment of intact monolayers with either exogenous NH$_4^+$ or ONOO$^-$ activated epithelial chymase. NAD(P)H oxidases, which are the predominant source of O$_2^-$, have been detected in a wide range of tissue, including several epithelia types (30). When NAD(P)H oxidase activity is measured at different pH values, profound inhibition at low pH (31) but increased activity above pH 7.4 has been reported (32).

FIGURE 8. Cytoskeleton involvement in wound activation of epithelial chymase. A, PLE were preincubated with DCFDA, and oxidation was measured by epifluorescence with or without the presence of 10 µM phallacidin or cytochalasin D in confluent and wounded cultures. Values are ± S.E. from three independent experiments done in quadruplicate. Significant difference from controls is indicated by **, *p < 0.01. B, monolayers preincubated with 10 µM phallacidin were compared with untreated cultures for chymase activation by SAAPNA degradation at time points up to 2 h. C, monolayers were treated with various concentrations of cytochalasin D for 0, 10, or 20 min and then wounded, incubated for 2 h, and then assayed for chymase activity by SAAPNA degradation relative to unwounded monolayers (dotted reference line). D–F, monolayers preincubated with cytochalasin D were stained for cytoskeleton (green), chymase (red), and nuclei (blue) 2 h after wounding; D, control; E, 50 nM, or F, 200 nM. Scale bar, 50 µm.

FIGURE 9. Epithelial chymase activity association with the onset of wound edge cell migration. Dense, quiescent monolayers of PLE (A) and NHEK (B) were wounded in the presence of increasing concentrations of chymostatin and then further cultured in serum-free conditions for 6 or 18 h. Cultures were fixed and stained, and five fields per sample were analyzed for cell migration distance beyond the original wound edge (0 h). Significant difference from controls is indicated by *, *p < 0.05. Representative images are shown of PLE (C) and NHEK (D) from three independent experiments with or without chymostatin (50 µM) with wound origins (0 h) indicated by arrows. White scale bar, 500 µm; black scale bar, 100 µm.
Perhaps paradoxically, some previous studies have suggested that disruption of the cytoskeleton can also trigger ROS production. Disruption of actin fibers at the plasma membrane enhances formylmethionylleucylphenylalanine-triggered oxidative burst in phagocytes (39). Also, in human pulmonary airway endothelial cells disruption or depolymerization of actin filaments by cytochalasin D or latrunculin A markedly increased basal and hyperoxia-mediated ROS production, whereas stabilization of actin filaments with phalloidin blocked the oxidative response (36). Also, cytochalasin D-induced disruption of the monocyte cytokinesis induces NAD(P)H oxidase to generate \( \text{O}_2^- \), whereas its inhibition significantly reduced cytochalasin D-induced NF-\( \kappa \)B activation (40). In contrast, this study found that in wounded epithelial monolayers cytochalasin D disruption of the cytokinesis eliminated both wound-induced ROS generation and chymase activation in a concentration-dependent manner, whereas stabilization by phallacidin had no discernible inhibition on chymase activation. This difference in ROS generation relative to cytoskeletal state may be due to the difference between chemical treatments of earlier studies and the mechanical induction used in this study.

It has been suggested that the actin cytokinesis may act as a mechanical linkage that induces and organizes the distribution of tenseness-type signals (41) in ways that vary according to the modality and cell type. Using an *in vitro* neuronal model it was previously found that sublethal mechanical stretch produced large overproduction of ROS but not \( \text{ONOO}^- \) (42). In contrast, umbilical vein endothelial cells treated with steady laminar shear stress expressed increased NO and \( \text{ONOO}^- \) induced signaling via increased tyrosine nitration (43). Furthermore, shear stress-induced NO in endothelial cells was associated with cytoskeletal rearrangement and altered cell morphology (44). Mechanical force has been shown to activate Src in fetal rat lung cytoskeletal rearrangement and altered cell morphology (44). Mechanical force has been shown to activate Src in fetal rat lung mesenteric arteries (45). Using bovine coronary artery explants in a passive stretch model it was found that resulting \( \text{O}_2^- \) production was eliminated by NAD(P)H oxidase inhibition but not by inhibition of Src activation (46). Also, laminar flow stress-induced Src activation in SW620 cells could be blocked by direct inhibition by protein phosphatase 2 but not by cytoskeletal stabilization by phalloidin (47).

Taken together, the results of this study suggest that mechanical forces involved in wounding act through the plasma membrane at the intersection of constitutively localized NAD(P)H oxidase and/or nitric-oxide synthase complexed with cortical actin resulting in up-regulation of ROS generation and leading to chymase activation. The final steps of this activation pathway are yet to be determined. Dipeptidyl peptidase I can activate mast cell chymase proenzyme in cell-free systems and is sometimes cited as its physiological activator. However, when human mast cell-like HMC-1 cultures are treated with the dipeptidyl peptidase I inhibitor, chymotryptic activity in treated cells did not differ significantly from untreated cells (48). Likewise, in this study inhibition of dipeptidyl peptidase I had no inhibitory effect of epithelial chymase activation.

It is not known to what extent chymases may play a significant role in wound reepithelialization. Murine skin burn

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**FIGURE 10. Proposed mechanism of ROS mediation of ligand-independent wound induction of epithelial chymase.** Mechanical wounding perturbs the cell membrane-associated NAD(P)H and cortical cytoskeleton thereby activating NAD(P)H production of \( \text{O}_2^- \) which reacts with nitric-oxide synthase product NO to generate \( \text{ONOO}^- \). The observed inhibitors of ROS generation are indicated. The dotted arrows indicate \( \text{ONOO}^- \) activates chymase without synthesis by some, as yet, unknown mechanism.

Because \( \text{ONOO}^- \) was found to induce chymase activation, we studied the involvement of its upstream nitric-oxide synthase pathway. NAD(P)H oxidase product \( \text{O}_2^- \) can react with nitric-oxide synthase product NO to generate \( \text{ONOO}^- \) to effect ligand-independent intracellular signaling (25). We found that inhibition of NAD(P)H oxidase or nitric-oxide synthase or scavenging their respective products, \( \text{O}_2^- \) and NO, or their combined downstream product \( \text{ONOO}^- \) all strongly inhibited wound-induced chymase activation. Moreover, scavenging \( \text{ONOO}^- \) had the greatest inhibitory effect of all treatments on chymase activation, reducing it below control levels, suggesting it to be a key rate-limiting effector. Evidence from several sources supports NAD(P)H oxidase being located on the cell plasma membrane in association with elements of the cytoskeleton that may play a role in its activation (33, 34). In human pulmonary airway endothelial cells, the activation of NAD(P)H oxidase is regulated by Src-dependent tyrosine phosphorylation of and association with p47^Phox^ (35). In the same cell type hyperoxia-mediated ROS/\( \text{O}_2^- \) production was associated with increased tyrosine phosphorylation of cortactin and interaction between cortactin, actin, and p47^Phox^ (36). Likewise, nitric-oxide synthase has also been identified in several human epithelial types (37). Its association with the cytoskeleton enables optimal nitric oxide production, and some isoforms are also linked to the plasma membrane (38). Our finding that the cell-impermeant reducing agent DTT inhibiting chymase activation is consistent with the involvement of a membrane-associated source of ROS.
wounds show a rapid increase in mast cell density and chymase activity at the wound edges in the second half of the reepithelializing period (9). Full-thickness skin wounds in mast cell-deficient mice showed normal healing rates but abnormal collagen organization at the wound edges where the preponderance of wound mast cells were found in the late stages of normal healing (49). In contrast, in human experimental skin wounds chymase-positive mast cells decreased in number and were absent from the reepithelializing margin (50). Also, it is not known if epithelial cells themselves are exposed to mast cell chymase. Immuno localization in murine epithelial cells demonstrate limited diffusibility of mast cell chymase; thus, it remains in the vicinity of degranulating mast cells situated near the basement membrane (10).

Epithelial chymase may have multiple wound-associated functions. This study found that activated epithelial chymase was overwhelmingly cell-associated and rapidly degraded heparin, a major constituent of extracellular matrix. Inhibition of chymase activation in wounded human and porcine epithelial monolayers also strongly prevented the loss of cell-cell contacts and slowed the onset of epithelial migration for up to 18 h. Previously, exogenous chymase was found to promote the migration of vascular smooth muscle cells via the activation of promatrix metalloproteinase-2. Furthermore, oral administration of a chymase inhibitor to dogs reduced metalloproteinase-2 activity and was found to promote the migration of vascular smooth muscle cells (10).

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