Extracellular Cleavage of E-Cadherin by Leukocyte Elastase During Acute Experimental Pancreatitis in Rats

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Background & Aims: **Cadherins play an important role in cell-cell contact formation at adherens junctions. During the course of acute pancreatitis, adherens junctions are known to dissociate—a requirement for the interstitial accumulation of fluid and inflammatory cells—but the underlying mechanism is unknown.** ***Methods:*** **Acute pancreatitis was induced in rats by supramaximal cerulein infusion. The pancreas and lungs were either homogenized for protein analysis or fixed for morphology. Protein sequencing was used to identify proteolytic cleavage sites and freshly prepared acini for ex vivo studies with recombinant proteases. Results were confirmed in vivo by treating experimental pancreatitis animals with specific protease inhibitors.** ***Results:*** **A 15-kilodalton smaller variant of E-cadherin was detected in the pancreas within 60 minutes of pancreatitis, was found to be the product of E-cadherin cleavage at amino acid 394 in the extracellular domain that controls cell-contact formation, and was consistent with E-cadherin cleavage by leukocyte elastase. Employing cell culture and ex vivo acini leukocyte elastase was confirmed to cleave E-cadherin at the identified position, followed by dissociation of cell contacts and the internalization of cleaved E-cadherin to the cytosol. Inhibition of leukocyte elastase in vivo prevented E-cadherin cleavage during pancreatitis and reduced leukocyte transmigration into the pancreas.** ***Conclusions:*** **These data provide evidence that polymorphonuclear leukocyte elastase is involved in, and required for, the dissociation of cell-cell contacts at adherens junctions, the extracellular cleavage of E-cadherin, and, ultimately, the transmigration of leukocytes into the epithelial tissue during the initial phase of experimental pancreatitis.**

Cadherins comprise a family of transmembrane proteins that are located at adherens junctions (for review see Troyanovsky[1]) and display calcium-binding motifs in their extracellular domain, which are essential for homophilic cell adhesion. In epithelial organs, E-cadherin is the most abundant regulator of adherens junctions, and its extracellular domain is composed of 5 subunits, EC-1 through EC-5, and the homophilic adhesion activity of this molecule has been mapped to the amino terminal EC-1 domain.[2] X-ray characterization of this domain revealed a 7-stranded (A-G) β-sandwich structure,[3] and adhesive interaction seems to be driven by the β-sandwich topology. This region does not, in itself, participate in Ca^{2+} binding but includes an HAV sequence (in single letter code for amino acids) that mediates adhesive interactions.[4,5] The intracellular domains serve as highly conserved linkers to the cytoskeleton via connecting α- and β-catenins.[6] These intracellularly located proteins are essential for cell-cell adhesion, and mutations in either the E-cadherin-binding site for α- and β-catenin or in the catenins themselves will disrupt cell contacts even in the presence of an intact extracellular E-cadherin domain.[7] Although there is extensive knowledge about the role of E-cadherin mutations, E-cadherin down-regulation, processing, and subcellular relocalization during tumor development or malignant growth,[8] there is only limited information on the role of E-cadherin in inflammation.

Epithelial cell-cell contacts at adherens junctions form a selective barrier and are involved in the active transport of fluids, ions, and small molecules. During inflammatory disorders, cell-cell contacts frequently dissolve, which permits an unregulated movement of fluids and electrolytes into the interstitial space, resulting in tissue edema. In a model system for an inflammatory disorder (experimental pancreatitis), we have shown that this edema formation is associated with a dissociation of adherens junctions between epithelial acinar cells and a

Abbreviations used in this paper: CCK, cholecystokinin; PMN, polymorphonuclear.

0016-5085/05/$30.00
doi:10.1053/j.gastro.2005.08.002

disappearance of E-cadherin from their basolateral membrane.[9]

In the present study, we characterized the early events involved in cell-cell contact dissociation, edema formation, and cell damage during pancreatitis and focussed on the mechanism that causes the disappearance of E-cadherin from the cell surface. In theory, the process involved in the dissociation of E-cadherin-mediated cell contacts should include cytotoxic or proteolytic agents that are released by either inflammatory cells[10–13] or originate from the pancreatic acinar cell itself.[14–16] Prior to the start of our project, the second hypothesis appeared more likely because (1) inflammatory cells were thought to play a role only in the later disease course but not the initial events of experimental pancreatitis, and (2) the early phase of pancreatitis is known to be associated with the generation of large amounts of cytotoxic compounds such as free oxygen radicals and an extensive activation of proteolytic digestive enzymes.[17–20] We were surprised to learn from our experiments that E-cadherin is cleaved very early in the disease course (within the first hour) by polymorphonuclear (PMN) granulocyte elastase, whereas pancreatic proteases are neither required nor involved in this process. Moreover, inhibition of PMN elastase not only prevented E-cadherin cleavage and cell-cell contact dissociation but also pancreatic inflammation and leukocyte infiltration during pancreatitis. These findings provide the first direct evidence for a role of inflammatory cells in the initial disease phase of pancreatitis and designates them a prospective target for future treatment strategies.

Materials and Methods

Materials

Cerulein was obtained from Pharmacia, Freiburg, Germany. Collagenase from *Clostridium histolyticum* (EC.3.4.24.3) was from SERVA (lot No. 14007, Heidelberg, Germany; collagenase activity, 1.50 PZ U/mg). Human neutrophil elastase was purchased from Calbiochem (San Diego, CA; catalog No. 324681; protein concentration, >20 U/mg protein specific activity; in 50 mmol/L Na-acetate, pH 5.5, and 200 mmol/L NaCl; purity >95%). Bovine pancreatic trypsin, human myeloperoxidase, porcine pancreatic elastase, bovine pancreatic chymotrypsin, and bovine α-amylase were obtained from Calbiochem (Schwalbach, Germany). The substrates rhodamine 110 (R110)-(CBZ-Ile-Pro-Arg)$_2$ and R110-(CBZ-Ala$_4$)$_2$ were purchased from Molecular Probes (Eugene, OR). The substrate 7-amino-4-methylcoumarin (AMC)-(Suc-Ala$_2$-Pro-Phe) was obtained from Bachem (Heidelberg, Gemany). The amylase quantification kit "Amyl" is commercially available from Roche (Ingelheim, Germany). Elastase inhibitor II was from Calbiochem (catalog No. 324744; San Diego, CA). The biologically active phosphorylated cholecystokinin (CCK) octapeptide [Tyr(SO3H)27]-cholecystokinin fragment was obtained from Sigma (Taufkirchen, Germany, catalog No. 2175). For the detection of E-cadherin, 2 different antibodies were used: monoclonal anti-E-cadherin clone 36 directed against the C-terminus (catalog No. 20820; Transduction Laboratories, San Diego, CA) and polyclonal rabbit anti-E-cadherin H108 directed against the N-terminus (catalog No. SC-7870; Santa Cruz, CA). For the detection of human neutrophil elastase, mouse monoclonal antibody clone AHN-10 (catalog No. MAB1056, lot 258CCD; Chemicon International, Temecula, CA) was used. Fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD45 (leukocyte common antigen) monoclonal antibody was used to label and quantitate inflammatory cells in paraffin sections during different intervals of pancreatitis (clone OX-1; BD Pharmingen, Heidelberg, Germany).

All other chemicals were of highest purity and were obtained either from Sigma-Aldrich (Eppelheim, Germany), Merck (Darmstadt, Germany), Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom), or Bio-Rad (Hercules, CA). Animals were bred at Charles River Breeding Laboratories (Sulzbach, Germany). All animal experiments were conducted according to the guidelines of the local animal use and care committee. PaTu-8988-S cells were purchased from DSZM (Braunschweig, Germany).

Induction of acute, cerulein-induced pancreatitis, male Wistar rats (140–250 g) were anesthetized with pentobarbital 30 mg/kg. A cannula was placed into the jugular vein, and the animals were infused with supramaximal concentrations of cerulein (10 μg/kg per hour) for up to 48 hours or treated with neutrophil elastase inhibitor II at a concentration of 50 μmol/L (Calbiochem, San Diego, CA) for 2 hours or a mixture of cerulein (10 μg/kg per hour) and neutrophil elastase inhibitor (50 μmol/L). Saline-infused animals served as controls. After exsanguination under ether anesthesia, the pancreas was rapidly removed and trimmed of fat, and tissue blocks were embedded in OCT (Tissue Tek, Sakura Finetek, Zoeterwoude, The Netherlands) for cryosections or fixed in 5% formaldehyde for electron microscopy (EM) cryolabelling or embedding in paraffin. The main part of the pancreas was frozen in liquid nitrogen and stored at −80°C for later protein analysis and detection of enzymatic activity.[21] Pancreatic tissue was homogenized with a Dounce S glass homogenizer in iced Triton X-100 lysis buffer containing protease inhibitors and subsequently immunoprecipitated and immunoblotted (Braun-Melsungen, Melsungen, Germany). Iced Triton X-100 lysis buffer contained protease inhibitors (1 mL/mg tissue, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 0.01 mol/L sodium pyrophosphate, 0.1 mol/L sodium fluoride, 1 mmol/L hydrogen peroxide, 1 mmol/L L-phenylmethylsulfonylfluoride [PMSF], and 0.02% soybean-trypsin inhibitor). Protein concentration was determined by a modified Bradford-assay (Bio-Rad, Unterhaching, Germany), and equal amounts of protein were used in subsequent experiments. DNA content of homogenates was determined with propidium iodine using a fluorescence reader (excitation 350 nm/emission 630 nm) and used to standardize cell homogenates to comparable cell numbers and corrected for

interstitial serum proteins in pancreatitis animals. For immunoprecipitation, a mixture of protein A and G-Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany) was preincubated with antibody in 20 mmol/L HEPES, pH 7.4. Lysates were precleared with rat nonimmune serum, added to the coupled antibody, and incubated for 1 hour at 4°C on a rotor wheel. Precipitates were washed with HNTG (washing buffer containing HEPES 50 mmol/L, NaCl 150 mmol/L, Triton-X 100 0.1%, Glycerin 10%) and boiled for 5 minutes in 2X SDS sample buffer. SDS polyacrylamide gel electrophoresis was performed in a discontinuous buffer system, and gels were blotted on nitrocellulose membranes (Hybond C, Amersham Pharmacia Biotech). After overnight blocking in NET-(0.2 %gelatine; washing buffer containing NaCl 1.5 M, EDTA 0.05 M, Tris-HCl 0.5 M [pH 7.5]), immunoblot analysis was performed, followed by enhanced chemoluminescence detection (Amersham Pharmacia Biotech) using horseradish peroxidase coupled sheep anti-mouse IgG (Amersham Pharmacia) or goat anti-rabbit IgG (Amersham, Pharmacia Biotech).[22,23] Densitometric analysis of the E-cadherin cleavage product in Figure 4 was undertaken using Western blots from 3 different experiments, and error bars represent mean values in percentage ± SEM.

Protein Sequencing

E-cadherin was immunoprecipitated using the monoclonal clone 36 antibody from Transduction Laboratories as described above. Samples were subjected to SDS-PAGE. Gels were equilibrated in 10 mmol/L CHAPS, pH 11, and methanol (10% vol/vol) for 30 minutes. Semidry transfer onto polyvinylidene difluoride membranes (PVDF) (catalog No. 162-0812, 0.2 μm, lot 149548A, Bio-Rad, Hercules, CA) was carried out at 1 mA/cm^2 constant current for 3 hours at 15°C in 10 mmol/L CHAPS, pH 11, and methanol (10% vol/vol). PVDF membranes were stained with Coomassie brilliant blue R-250 stain (Bio-Rad) 0.1% wt/vol and 50% vol/vol methanol for 15 minutes. Destaining was done in a solution containing 40% vol/vol methanol. Protein containing polyvinylidene difluoride membrane sections were excised and sequenced on a pulsed liquid phase sequencer (Applied Biosystems) according to the manufacturer's instructions.[24]

Assessment of Acinar Cell-Cell Contact Dissociation

Pancreatic acini were prepared by collagenase digestion (Collagenase Serva, Heidelberg, Germany) as previously described.[15] Acini were then washed and centrifuged at 50*g* for 1 minute in DMEM medium containing 0.2% BSA. Incubation buffer consisted of DMEM, 0.2% BSA, 0.02% soybean-trypsin inhibitor. To determine the extent of cell-cell contact dissociation, we used 2 independent methods. Living acini were incubated for up to 40 minutes with buffer alone or with buffer containing 2 μg/mL human neutrophil elastase or 5 μg/mL pancreatic elastase (Calbiochem, San Diego, CA) and then fixed for morphologic studies. Alternatively, the biovolume of living acini was determined. This assay is based on the disintegration of cell-cell contacts that permits individual acinar cells to dissociate from the acinus. Accordingly, the biovolume of intact acini (between 3 and 80 cells or a sphere with a diameter of 24–80 μm) decreases when cell contacts dissociate, whereas the biovolume proportion of individual single cells (mean diameter, 17μm; range, 11–23 μm) increases.[25] Suspensions of freshly prepared acini were diluted (1:200) in filtered (particle free) buffer, and the biovolume ratio was determined with a cell analyzing system (CASY I; Schärfe Systems, Reutlingen, Germany), which is based on resistance measurements with pulse-surface analysis. Measurements during incubation with the above reagents were made for single cells (11–23 μm) and intact acini (24–80 μm) and the results expressed as percentage of control incubations with buffer alone. Graphs indicate the means of 3 or more experiments in each group ± SD.[25]

Immunofluorescence microscopy cells of the human pancreatic ductal adenocarcinoma cell line PaTu-8988-S were cultured on chamber slides in DMEM (10% FCS, 1% L-glutamine) at 5% CO^2 to subconfluency and further incubated with either purified human neutrophil elastase (5 μg/mL) or purified porcine pancreatic elastase (5 μg/mL) for up to 120 minutes. Isolated rat pancreatic acini freshly prepared by collagenase digestion were treated with human neutrophil elastase for 120 minutes (20 μg/mL) at 37°C. Cells were washed twice in PBS and fixed in 4% PBS-buffered formaldehyde solution for 30 minutes, followed by membrane permeabilization in 0.1% Triton X-100/PBS buffer for 1 minute. Nonspecific antibody binding was blocked in PBS/BSA 1% wt/vol for 1 hour, followed by overnight incubation with the primary antibody at a concentration of 5 μg/mL at 4°C. Primary antibody binding was detected with an isospecies-specific secondary antibody conjugated to fluorescein isothiocyanate donkey anti-mouse IgG (1:100, lot 39656) or Cy 3-conjugated goat anti-rabbit IgG (1:200, lot 42925) (Dianova, Hamburg, Germany). Under otherwise identical conditions, controls were incubated with either species-specific nonimmune serum, purified IgG, or without primary antibody, and images were taken under the same exposure, brightness, and contrast settings. Fluorescence microscopy was performed on a high-resolution Nikon Improvison confocal imaging system (Nikon, Waxford, United Kingdom).

Sections (2–4 μm) of each paraffin-embedded tissue sample were deparaffinized with xylene and rehydrated through graded alcohol into distilled water. The sections were microwaved in 10 mmol/L citrate buffer (pH 6) for 5 minutes at 900 W and for 10 minutes at 500 W. Slides were then washed in Tris-buffered saline (5 mmol/L Tris-HCI, 0.3 mol/L $NaCI_2$, pH 7.4). All further steps were performed as described above. The primary antibody mouse anti-rat CD45 (5μg/mL) was precoupled to fluorescein isothiocyanate. For morphometric evaluation of the extent of pancreatic leukocyte infiltration, a minimum of 10 microscopic fields from each animal (n = 5) were randomly photographed at a fixed magnification (×63), and micrographs with positive evidence for CD45-positive cells (either intravascularly or in the interstitial space) were

used for further analysis. Results were expressed as number of CD45-positive cells per standard surface area ± SEM.

Electron Microscopy

Small blocks (2 mm in diameter) of rat pancreatic tissue from pancreatitis and control animals were fixed in 5% (wt/vol) paraformaldehyde/0.2 mol/L piperazine-N,N′-bis [2-ethanesulfonic acid], pH 7.0, cryoprotected with polyvinylpyrollidine/sucrose, and frozen in liquid nitrogen. Ultrathin frozen sections (60 nm) were prepared using a Leica (Bensheim, Germany) Cryo-ultramicrotome (block temperature, −110°C; knife temperature, −100°C). The sections on formvar-coated copper grids were blocked with PBS, 5% (wt/vol) fetal calf serum (FCS; Life Technologies, Rockville, MD), pH 7.4, and then incubated with mouse monoclonal anti-E-cadherin antibody (1:10–1:30; Transduction Laboratories, clone 36) for 45 minutes at room temperature. After washing with PBS, the sections were incubated with 10-nm gold-conjugated goat anti-mouse antibody (dilution 1:10; Dianova, Hamburg, Germany), washed again with PBS and water, and subsequently contrasted and embedded by incubation with methylcellulose/uranyl acetate on ice (9:1 mixture of 2% methylcellulose and 4% uranyl acetate). Samples were examined on a Philips 400 electron microscope (Hamburg, Germany). Pancreatic tissue was also fixed in 2% formaldehyde/2% glutaraldehyde for Epon embedding, and then osmium, uranyl, and lead-contrasted for EM of thin sections.[26]

Substrate and Inhibitor Specificity

Measurements of inhibitor specificity were initiated after adding 10 mU/mL porcine pancreatic elastase, human neutrophil elastase, bovine pancreatic trypsin, or bovine pancreatic chymotrypsin to the PMN elastase inhibitor. Enzyme activity was quantitated by substrate cleavage of 10 μmol/L neutrophil elastase substrate AMC-(MeOSuc-Ala_2-Pro-Val), elastase substrate R110-(CBZ-Ala_4)$_2$, trypsin substrate R110-(CBZ-Ile-Pro-Arg)$_2$, or chymotrypsin substrate AMC-(Suc-Ala_2-Pro-Phe). Measurements were performed in 100 mmol/L Tris, pH 8, and 5 mmol/L $CaCl_2$ buffer. The rise in fluorescence was monitored over 10 minutes at 37°C using a microplate fluorescence reader (SPECTRAmax GEMINI, Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 485 nm, a cut-off at 515 nm, and an emission of 530 nm. The wavelength combination 340 nm, 420 nm, 460 nm was used for measurements of the AMC-substrates. The inhibitory effect on 10 mU/mL myeloperoxidase was measured in the presence of 0.53 mmol/L of the substrate o-dianisidine and 0.15 mmol/L hydrogen peroxide over 10 minutes at 30°C at a wavelength of 460 nm and in 50 mmol/L potassium phosphate buffer at pH 6. The effect on 10 U/mL α-amylase was monitored using a commercial kit (Roche, Ingelheim, Germany) over 10 minutes at 37°C at 405 nm and in buffer containing sodium chloride (150 mmol/L). Enzyme activities were measured under the above conditions with concentrations of PMN elastase inhibitor from 5 pmol/L to 500 μmol/L diluted in DMSO. Background fluorescence or absorption of uncleaved substrates was subtracted, and results were shown as percentage activity in the presence or absence of the inhibitor.

Myeloperoxidase Activity in Lung and Pancreatic Homogenates

Tissue was homogenized on ice in 20 mmol/L potassium phosphate buffer (pH 7.4) and centrifuged for 10 minutes at 20,000*g* at 4°C. The pellet was resuspended in 50 mmol/L potassium phosphate buffer (pH 6.0) containing 0.5% cetyltrimethylammoniumbromide. The suspension was frozen/thawed 4 times, sonicated twice for 10 seconds, and centrifuged at 20,000*g* for 10 minutes at 4°C. Myeloperoxidase (MPO) activity was assayed after mixing 50 μL supernatant in 200 μL of 50 mmol/L potassium phosphate buffer (pH 6) containing 0.53 mmol/L O-dianisidine and 0.15 mmol/L H_2O_2. The initial increase in absorbance at 460 nm was measured at room temperature with a Dynatech MR 5000 Elisa reader (Eningen, Germany). The results are expressed in units of MPO activity on the basis of 1 unit being able to oxidize 1 μmol H_2O_2 per minute per milligram pancreatic protein.[27] Bars indicate mean values in mU MPO activity per milligram pancreatic protein ± SEM from 3 or more animals per time point.

Elastase Activity in Pancreatic Homogenates After PMN Elastase Inhibitor Treatment

Tissue was homogenized on ice in 100 mmol/L Tris, pH 8, and 5 mmol/L $CaCl_2$ and centrifuged for 10 minutes at 20,000*g* at 4°C. Protein content was determined according to the method of Bradford.

Ten micromolars R110-(CBZ-Ala_4)$_2$ substrate (final concentration) and 1 μg protein were incubated in 150 μL final volume, at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, at 37°C. Initial rates of substrate hydrolysis were measured in arbitrary fluorescence units per minute. Enzyme activity was calculated as units/milligrams with purified elastase as an internal standard, and activity was set in relation to cerulein-treated animals.

Determination of Pancreatic Water Content

The water content of the pancreas was quantified by comparing the weight of the freshly harvested organ (wet weight) with the weight of the same tissue after desiccation at 160°C for 24 hours (dry weight). The results were expressed as percentage water content.

Amylase Serum Levels

Blood was collected by aortic puncture during death of the animals. Following centrifugation, serum was stored at −20°C until assayed. Serum α-amylase was measured using 4,6-ethylidene-(G7)-1-4-nitrophenyl-(GI)-α, D-maltoheptaoside as substrate (EPS method) according to the manufacturer's protocol (Roche, Ingelheim, Germany).

Data Presentation and Statistical Analysis

Data in graphs were expressed as means ± SEM (or ±SD as stated in the text). Statistical comparison of groups at various time intervals was done by Student *t* test for independent samples as well as Mann-Whitney rank sum test if indicated using SigmaStat for Windows (SPSS Inc., Chicago, IL). Differences were considered significant at a level of $P < .05$. Data presentation was performed with SigmaPlot for Windows (SPSS Inc., Chicago, IL).

Results

Subcellular Localization and Redistribution of E-Cadherin During the Course of Acute Pancreatitis

Infusion of supramaximal concentrations of cerulein into male Wistar rats resulted in hyperamylasemia, interstitial pancreatic edema, and intracellular vacuolization with a maximum extent 6 hours after the start of the experiment and an almost complete resorption of the edema after 48 hours. We were interested in the fate of E-cadherin in the process of edema formation and performed electron microscopic analysis of control and cerulein-infused animals to see whether and how E-cadherin expression and cellular localization were altered in the early course of the disease process. Immunogold labeling revealed that E-cadherin expression was strictly membrane associated at adherens junctions and interdigitations of adjacent cells in control tissue (Figure 1*A–C*). Supramaximal secretagogue stimulation, however, resulted in a massive accumulation of interstitial fluid, a dissociation of cell-cell contacts, and redistribution of E-cadherin from cell contacts and the lateral cell membrane into the cytoplasm and interstitial space (Figure 1*D* and *E*). Twelve hours after the onset of acute pancreatitis, E-cadherin was found in the cytoplasm and in lysosomes (Figure 1*F* and *G*, asterisks), which indicates translocation and degradation. Furthermore, it was found in close proximity with endoplasmic reticulum (ER) elements, which may indicate production of newly synthesized E-cadherin (Figure 1*G*). Vacuolization, edema formation, and E-cadherin redistribution were not observed in saline-infused control animals.

E-Cadherin Cleavage During Pancreatitis

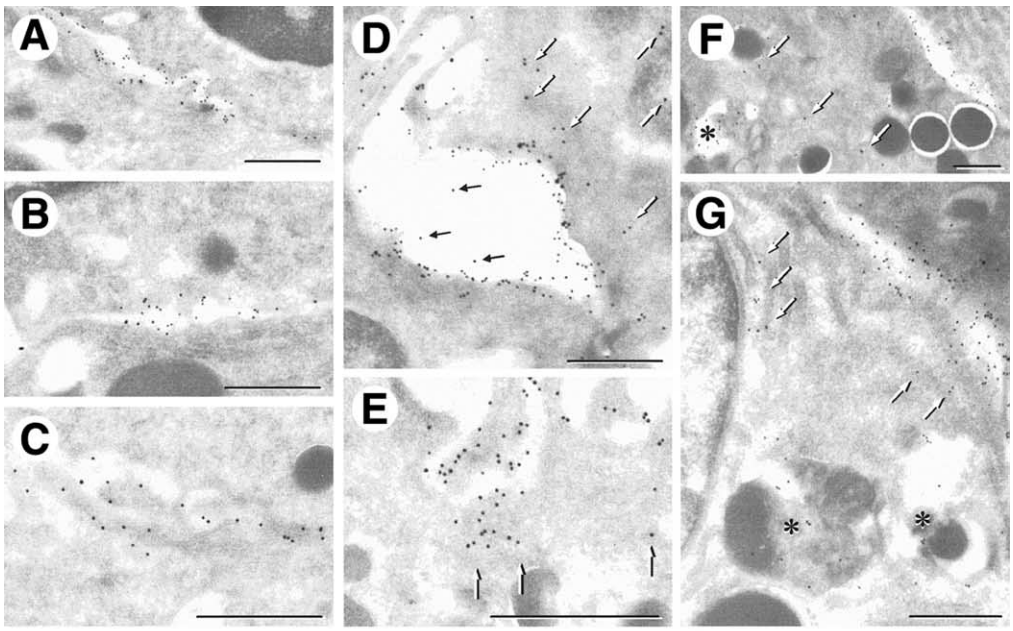
When pancreatic homogenates were assayed for E-cadherin expression by Western blot analysis using a monospecific C-terminal antibody, only a single 120-kilodalton band was detected in control animals (Figure 1*H*, 0 hours). As early as 1 hour after the onset of supramaximal cerulein stimulation, an approximately 15-kilodalton smaller variant of E-cadherin appeared. After 4 hours of supramaximal cerulein treatment, the antibody detected the newly synthesized precursor E-cadherin of approximately 130 kilodaltons, which was most prominent after 12 hours of supramaximal secretagogue stimulation. This was confirmed by semiquantitative RT-PCR in which an increase of the E-cadherin messenger RNA (mRNA) transcript was found after 4 hours of supramaximal cerulein stimulation (not shown). By subjecting immunoprecipitated E-cadherin to enzymatic deglycosylation, we ruled out that the smaller E-cadherin fragment of 105 kilodaltons represents deglycosylated E-cadherin (not shown). Immunoprecipitation of the 105-kilodalton E-cadherin fragment, using antibodies specifically recognizing either the C-terminus or the N-terminal peptide sequence, revealed that E-cadherin is cleaved at the extracellular region of the protein during early pancreatitis (Figure 1*H*).

Identification of the E-Cadherin Cleavage Site

To locate the cleavage site within the E-cadherin portion, we immunoprecipitated the fragment after 4 hours of cerulein stimulation from pancreatic homogenate and subjected the material to Edman peptide sequencing. The starting amino acid sequence of the fragment was GARIATLK, which matches amino acids 394 to 401 in the EC-3 domain of E-cadherin (protein bank accession number Q9R0T4; Figure 2). A scan of the E-cadherin sequence for the occurrence of patterns and profiles stored in the PABASE database (Omiga 2.0, Madison, Wisconsin) identified 11 proteases that could cleave E-cadherin in this position. Four of these proteases were known to be expressed in either pancreatic tissue or by inflammatory cells: (1) A carboxypeptidase, known as cathepsin IV, an exopeptidase that hydrolizes Z-Glu-Tyr and which has not previously been cloned or purifed[28]; (2) the metalloendoproteinase Meprin A, which is mainly expressed in the small intestine and known to degrade collagen IV[29]; (3) lysosomal cysteine peptidase cathepsin C[30]; and (4) polymorphonuclearelastase (leukocyte elastase E:C. 3.4.21.37). The latter belongs to the chymotrypsin family of serine proteases and is highly expressed in the azurophilic granules of PMN leukocytes (2 pg/cell),[31] which have been reported to accumulate in the pancreas during acute pancreatitis.[32]

E-Cadherin as Substrate for Leukocyte Elastase

In accordance with our initial hypothesis, we tested whether proteases released by inflammatory cells (now the most abundant and thus probable would be



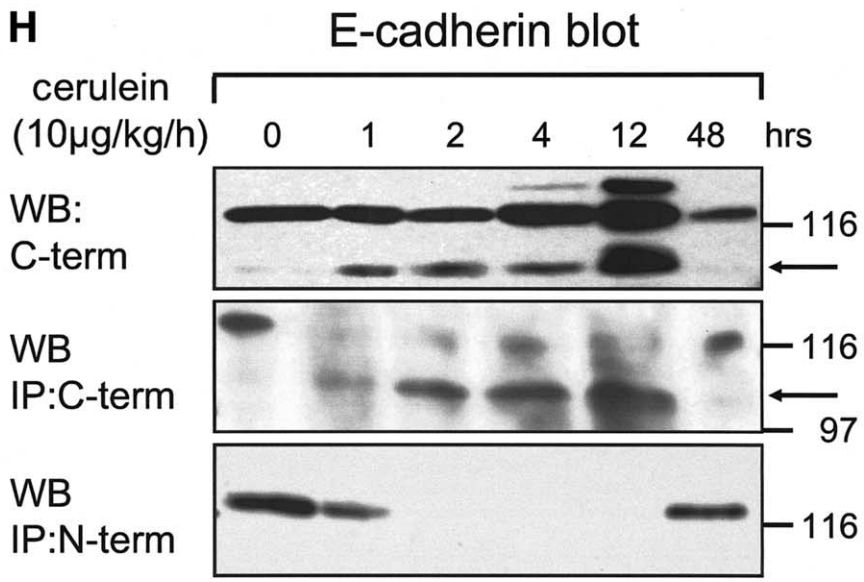


Figure 1. Ultrastructural localization of E-cadherin. Tissue blocks from rats treated with supramaximal concentrations of cerulein or saline for up to 12 hours were fixed, resin embedded, and labeled with antibodies directed against the intracelluar domain of E-cadherin (5-nm gold). Representative electron micrographs from control animals are shown in *A–C*, whereas *D* and *E* represent micrographs from animals infused with supramaximal concentrations of cerulein for 4 hours. In *F* and *G*, duration of cerulein infusion was 12 hours. *Bars* indicate 1 μm. In control tissue, E-cadherin is exclusively located at the cell membrane and at cell-cell contacts and interdigitations of epithelial cells (*A–C*). After 4 hours of supramaximal cerulein stimulation, E-cadherin label has separated from junctions and redistributed to the cytoplasm (*bold arrows* in *D* and *E*) or the widened interstitial space between acinar cells whose cell adhesions have dissociated (*fine arrows*). After 12 hours, E-cadherin label is detected in the cytoplasm, ER (*arrows* in *G*), and lysosomes (*asterisks*, *F* and *G*). Incubation of control sections with nonspecific IgG resulted in no background gold labeling (data not shown). (*H*) Western blots of E-cadherin in pancreatic homogenates between 0 and 48 hours of experimental pancreatitis using antibody directed against the E-cadherin C-terminus and, for immunoprecipitation (2 lower panels in *H*), against the C-terminus and the N-terminus. Note E-cadherin cleavage (105-kilodalton band) from the N-terminus as early as 1 hour after the start of the cerulein infusion and the E-cadherin precursor (130-kilodalton band) most prominent after 12 hours.

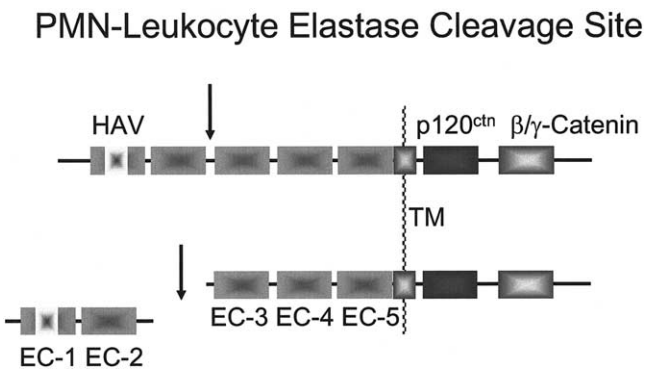


Figure 2. Schematic representation of E-cadherin domain structure and localization of the PMN-elastase cleavage site. The extracellular repeat units of E-cadherin EC1 through EC5 are preceded by a short, N-terminal signal sequence that is cleaved during maturation of E-cadherin. The HAV region (*light grey box*) of EC-1 is required for homotypic interactions. *TM* indicates the transmembrane region, whereas the proximal intracellular region (*dark grey box*) binds $p120^{ctn}$, and the distal region (*intermediate grey box*) binds either β-catenin or γ-catenin. Peptide sequencing of the cleaved E-cadherin detected after supramaximal cerulein stimulation identified amino acid Gly-394 at the N-terminus of the EC-3 domain. Proteolytic cleavage therefore must occur between Val-393 and Gly-394, suggesting PMN-elastase (leukocyte-elastase) as the most likely protease responsible for extracellular shedding.

PMN elastase) or generated by pancreatic exocrine cells (eg, activated digestive zymogens) cause E-cadherin processing at the identified cleavage site. We incubated cells of the epithelial pancreatic cancer cell line PaTu-8889-S, which do not express digestive protease zymogens, with PMN elastase. In cells treated with purified PMN elastase (2 μg/mL) for up to 120 minutes, an identical shortened E-cadherin fragment as seen during the course of cerulein pancreatitis was detected (Figure 3*A*). To exclude degradation of E-cadherin by endogenously expressed proteases, even in the presence of abundant protease inhibitors in the lysis buffer, cells were lysed in the presence (0+) and absence (0−) of PMN elastase. To ensure the specificity of the cleavage product found in the lysates, we performed immunoprecipitation experiments using homogenates from untreated control cells incubated with PMN elastase after immunoprecipitation as well as from cells treated with PMN elastase (2 μg/mL) before lysis (Figure 3*B*). Under both conditions, the smaller 105-kilodalton variant of E-cadherin was detectable, and, by Edman peptide sequencing, we confirmed that the fragment generated under PMN elastase treatment was identical to the one detected in acute pancreatitis.

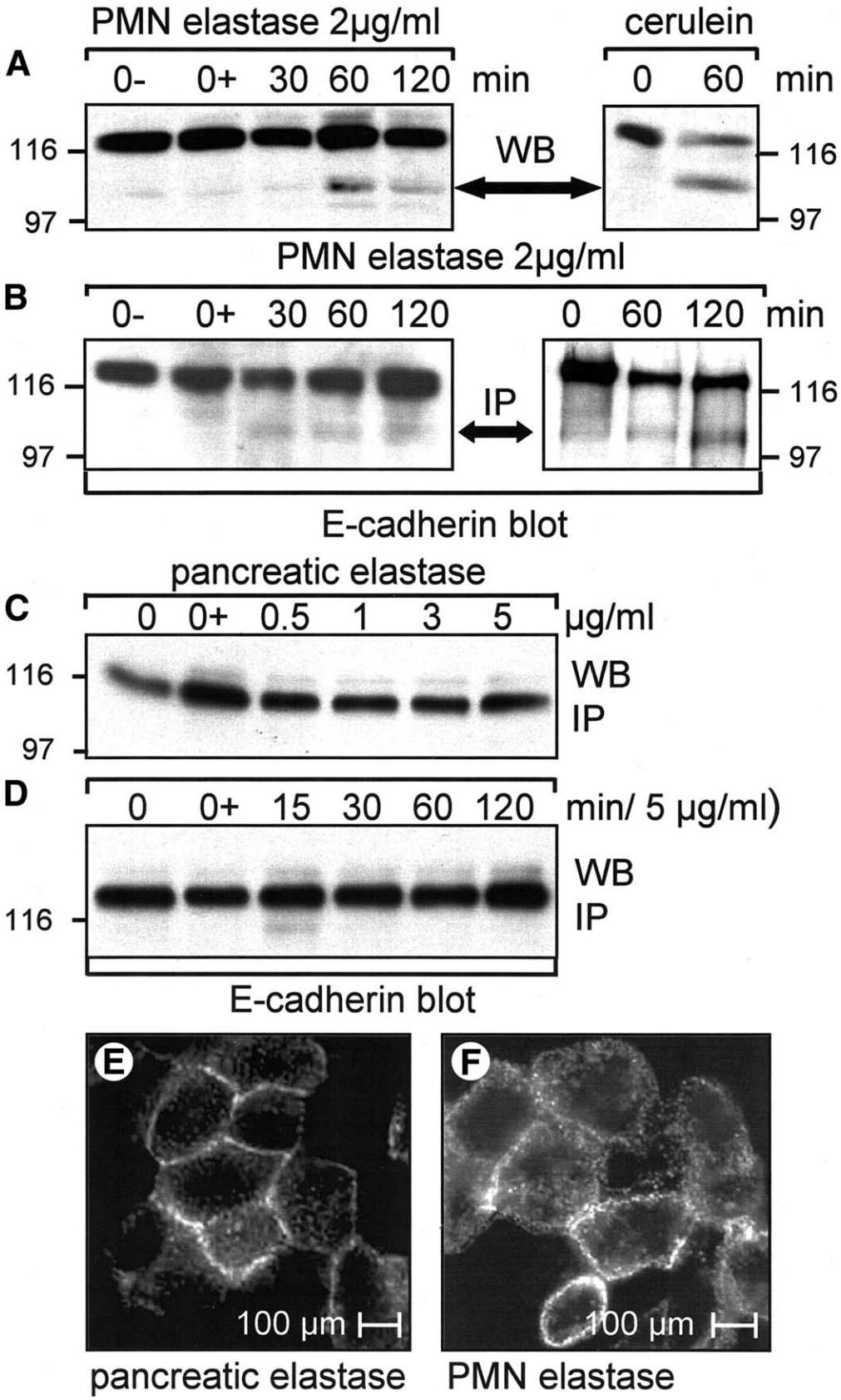
The substrate specificity of PMN elastase is very similar to that of pancreatic elastase, which is known to be activated intracellularly during pancreatitis and may subsequently be released into the interstitial space. Nevertheless, when we incubated PaTu-8889-S cells with purified pancreatic elastase (up to 5 μg/mL), we could not detect an E-cadherin cleavage product (Figure 3*C* and *D*). After treatment of PaTu-8889-S cells with pancreatic elastase (5 μg/mL), E-cadherin displayed a strong membrane localization on fluorescence microscopy (Figure 3*E*), resembling the distribution in untreated cells (not shown). Incubation with PMN elastase (2 μg/mL), however, induced a loss of membrane-bound E-cadherin and its redistribution to the cytoplasm (Figure 3*F*).

Ex Vivo Cleavage of E-Cadherin on Acinar Cells by PMN Elastase

Supramaximal stimulation of freshly isolated pancreatic acini, functional secretory units of 5 to 80 exocrine cells, with either cerulein or CCK (10^{-6} mol/L) leads to intracellular protease activation, cell injury, and release of activated proteases into the supernatant. This ex vivo model mimics several of the earliest changes in the onset of acute experimental pancreatitis. Therefore, to distinguish further between activities of exogenous (PMN elastase) and endogenous (activated digestive zymogens) proteases on the cadherin/catenin complex, pancreatic acini were freshly isolated and incubated with either CCK 10^{-6} mol/L, PMN elastase, or a combination of both. In addition, we incubated the cells with pancreatic elastase at a concentration of 5 μg/mL. Subsequently, immunoprecipitation studies were performed to identify the generation of the E-cadherin fragments. Interestingly, incubation with supramaximal concentrations of CCK alone, although followed by massive intracellular protease activation (not shown), did not cause E-cadherin cleavage (Figure 4*A* and *B*). Incubation with pancreatic elastase did also not result in E-cadherin cleavage. Incubation with PMN elastase alone, on the other hand, caused significant formation of the 105-kilodalton E-cadherin cleavage product accounting for up to 55% of the total E-cadherin content. Coincubation with CCK and PMN elastase did not further add to the cleavage of E-cadherin (Figure 4*A* and *B*). These results clearly demonstrate that endogenous pancreatic proteases in general—and pancreatic elastase in particular—are neither required nor involved in the cleavage of E-cadherin in pancreatic acinar cells upon supramaximal secretagogue stimulation.

The Role of PMN Elastase in the Dissociation of Cell-Cell Contacts and the Internalization of E-Cadherin

The extracellular EC-1 domain contains an HAV peptide sequence and has been suggested to be a prerequisite for the maintenance of intact cell-cell contacts at


PMN elastase 2µg/ml
cerulein
A
0- 0+ 30 60 120 min
0 60 min
116
97
WB
116
97
PMN elastase 2µg/ml
B
0- 0+ 30 60 120
0 60 120 min
116
97
IP
116
97
E-cadherin blot
pancreatic elastase
C
0 0+ 0.5 1 3 5 µg/ml
116
97
WB
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D
0 0+ 15 30 60 120 min/ 5 µg/ml)
116
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E
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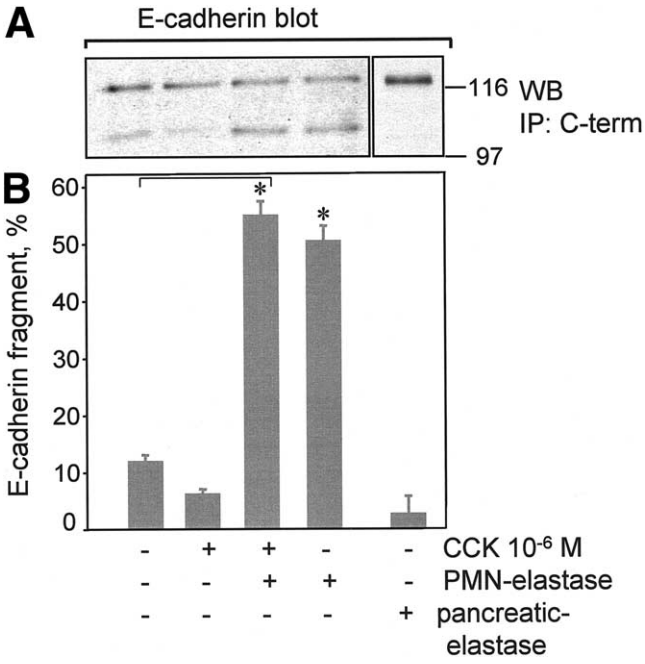

Figure 4. E-cadherin cleavage on pancreatic acini. Pancreatic acini were freshly isolated by collagenase digestion and incubated either in the presence of CCK 10^{-6} mol/L or with pancreatic elastase (5 μg/mL), with leukocyte elastase (5μg/mL), or with a combination of leukocyte elastase and cerulein for 120 minutes at 37°C. Saline incubation served as control. Subsequently, pancreatic acini were subjected to immunoprecipitation and analyzed for the presence of the E-cadherin fragment. Incubation of pancreatic acini with CCK alone or pancreatic elastase did not cause E-cadherin cleavage (*A* and *B*), whereas incubation with leukocyte elastase cleaved ~55% of E-cadherin. Incubation of pancreatic acini with a combination of leukocyte elastase and CCK did not increase E-cadherin cleavage over the extent obtained with leukocyte elastase alone. Densitometric analysis of the E-cadherin cleavage product was undertaken using Western blots from 3 different experiments, and *error bars* represent means in percentage ± SEM.

adherens junctions. In isolated pancreatic acini, which represent fully functional secretory units with intact stimulus-secretion coupling, cell-cell contacts remain intact for up to 4 hours in vitro. Over this period, their postisolation diameter and volume remain fairly stable. To study the effect of PMN elastase on acinar cell-cell contact integrity, we incubated freshly isolated rat pancreatic acini for up to 40 minutes with leukocyte elastase or pancreatic elastase. To determine the extent of cell contact dissociation, we measured the release of single cells (diameter, 11–23 μm) from intact acini vs the amount of remaining intact acini (diameter, 24–80 μm). An increase of single cells in parallel with a decline of the larger intact acini reflected a dissociation of cell contacts, which could be confirmed by confocal microscopy. During incubation with buffer alone serving as control, or with pancreatic elastase, acini remained stable throughout the entire experiment, and neither the proportion of single cells (Figure 5*A*) nor the number of intact acini (Figure 5*B*) changed. In immunofluorescence studies of these acini, E-cadherin displayed a clearly membrane-associated localization and nearly absent cytoplasmic expression (Figure 5*C*). Incubation with pancreatic elastase did not change the subcellular localization of E-cadherin (data not shown). Incubation with PMN elastase induced a rapid dissociation of adherens junctions between pancreatic acinar cells, resulting in the generation of single cells (Figure 5*A*) and a progressive decline of intact acini. The prominent cytoplasmic labeling of E-cadherin in these cells after PMN elastase incubation confirms its redistribution to the cytoplasm (Figure 5*D*). When we investigated homogenates of pancreatic acini for ubiquitinilation of E-cadherin and its sorting into the proteasome pathway, we found a rapid turnover of E-cadherin after PMN elastase incubation (not shown). These data give evidence that incubation of acinar cells with PMN elastase is entirely sufficient to dissociate cell-cell contacts and redistribute E-cadherin from the membrane to the cytoplasm.

Early Leukocyte Transmigration Into the Pancreas During Pancreatitis

To study whether leukocyte infiltration parallels E-cadherin cleavage, we infused animals with supramaximal concentrations of cerulein and measured PMN elastase protein expression (Figure 6*A*) as well as MPO activity (Figure 6*B*) as markers of leukocyte infiltration in pancreatic tissue homogenates at distinct intervals after pancreatitis onset. As early as 1 hour after the onset

Figure 3. N-terminal cleavage of E-cadherin by PMN-elastase in vivo. Incubation of PaTu-8889-S cells with PMN-elastase (2 μg/mL) for up to 120 minutes and subsequent Western blot analysis of total lysates (*A*, *left panel*) and immunoprecipitates (*B*, *left panel*) using E-cadherin antibody directed against the C-terminus revealed an E-cadherin fragment identical to the one observed after 60 minutes of supramaximal cerulein stimulation in vivo (*A*, *right panel*). Figure 3*B*, *right panel* shows the result of an incubation of immunoprecipitated E-cadherin with leukocyte elastase for up to 120 minutes. 0+ symbolizes lysis in the presence of leukocyte elastase (2 μg/mL). PaTu-8889-S cells were also incubated with pancreatic elastase in a time- and concentration-dependent manner (*C* and *D*), but no E-cadherin fragment was detected in immunoprecipitation studies and subsequent Western blotting for E-cadherin. Immunofluorescence analysis for E-cadherin of PaTu-8889-S cells treated with pancreatic elastase (5 μg/mL) for 120 minutes showed normal E-cadherin labeling at intact cell contacts and the plasma membrane (*E*), whereas incubation with leukocyte elastase (5 μg/mL for 120 minutes) resulted in cell-cell contact dissociation and progressive internalization (*F*) of E-cadherin.

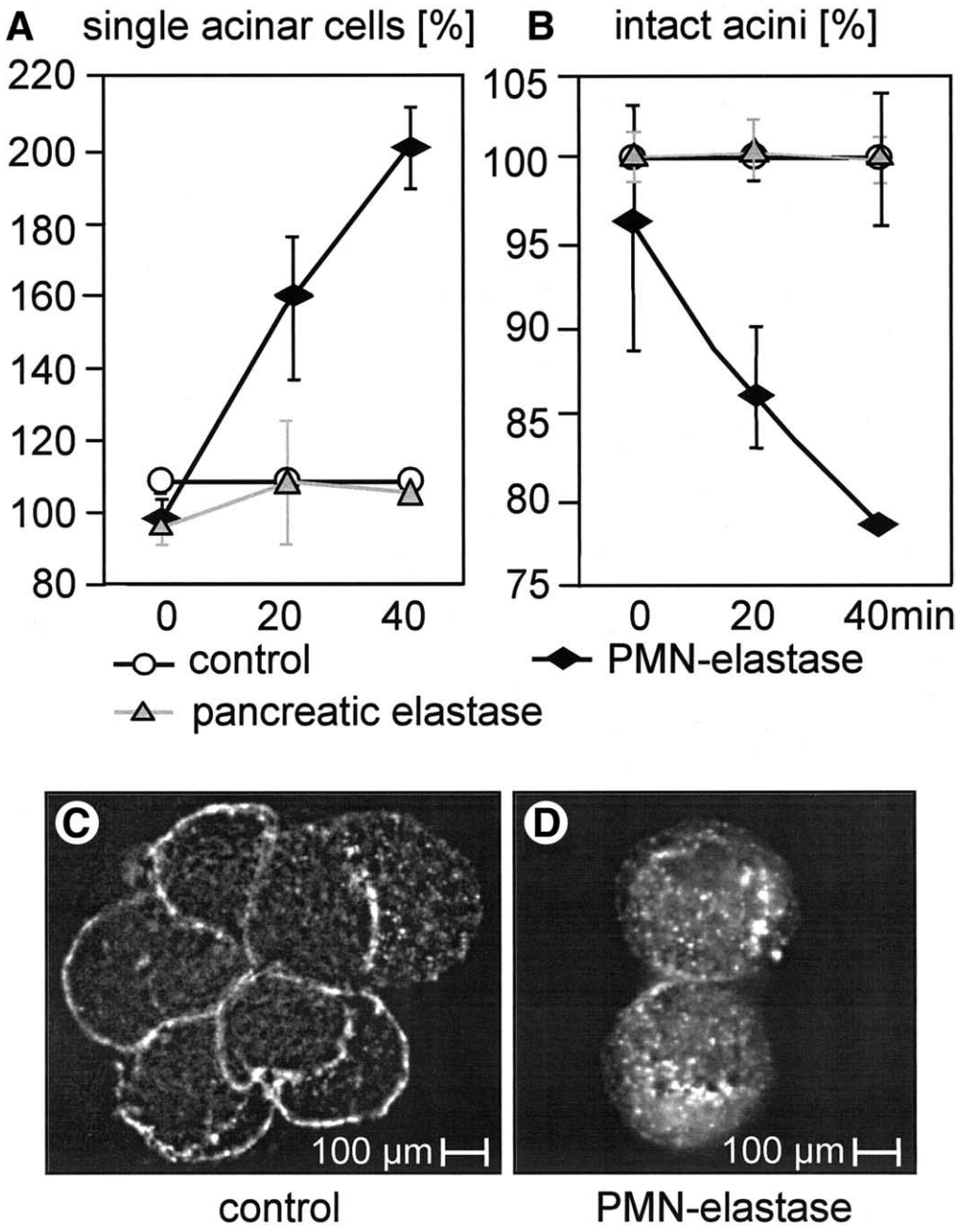

Figure 5. Cell contact dissociation after incubation with leukocyte elastase. We quantitated the biovolume of single cells (diameter, 11–23 μm; *A*) vs intact acini (diameter, 24–80 μm; *B*) after incubation with leukocyte elastase (2 μg/mL) or pancreatic elastase (5 μg/mL) for up to 40 minutes. Incubation with leukocyte elastase induced a rapid dissociation of cell-cell contacts within the acini as indicated by a progressive increase in the proportion of single cells (*A*, filled lozenges) and a parallel decline in the number of intact acini (*B*, filled lozenges). The biovolume of acini during incubation with buffer alone or with pancreatic elastase remained stable throughout the experiment, and neither the numbers of single cells (*A*) nor of intact acini (*B*) changed. We also studied paraformaldehyde-fixed acini for E-cadherin localization and change in morphology. Control acini and acini incubated with pancreatic elastase (not shown) displayed a clear membrane-bound E-cadherin staining and intact cell-cell contacs (*C*). After leukocyte elastase incubation (*D*), cell contacts were found largely dissociated, and E-cadherin was redistributed from the membrane to the cytosol.

of acute pancreatitis, a 10-fold increase of MPO activity compared with saline-infused control animals was found (Figure 6*B*). Maximal MPO activity was measured 12 hours after the onset of cerulein pancreatitis with a subsequent decline until 48 hours of supramaximal cerulein stimulation—a time point when cell-cell contacts have reassembled. In parallel to MPO activity, also PMN elastase, as determined by immunoprecipitation and Western blotting, peaked at 12 hours after the onset of pancreatitis but was already significantly increased after

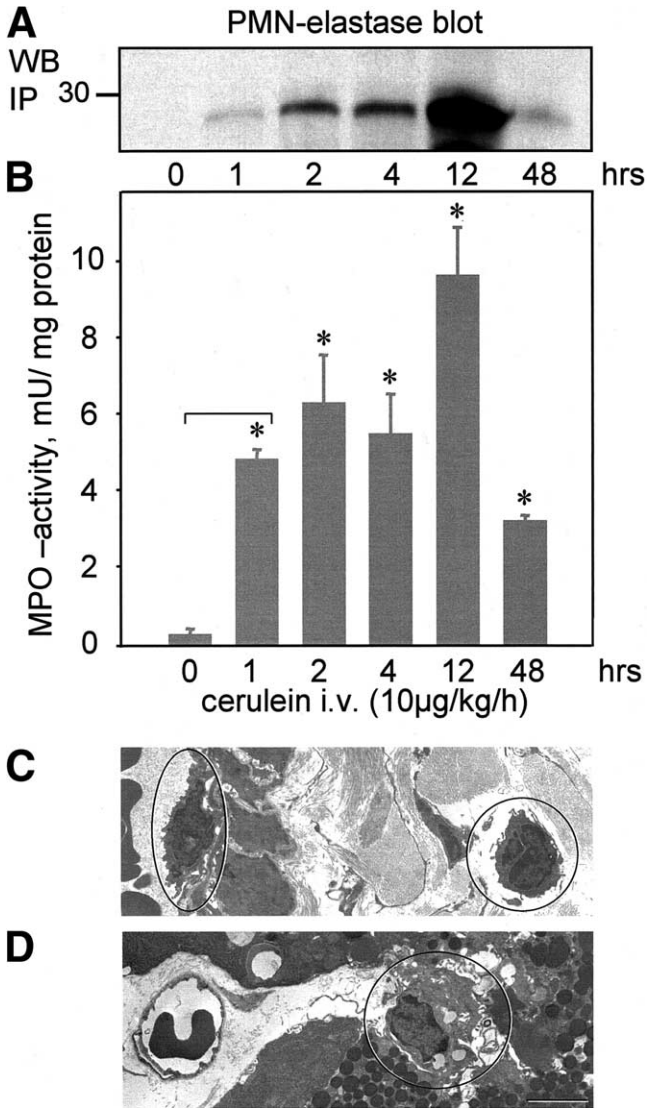

Figure 6. Leukocyte infiltration of pancreatic tissue during experimental pancreatitis. Protein amounts of PMN leukocyte elastase as determined by immunoprecipitation and Western blotting (*A*) as well as myeloperoxidase enzymatic activity (*B*) were already increased in pancreatic tissue after the first hour of experimental pancreatitis. The maximum increase of both enzymes was seen at 12 hours. Data shown are representative of 3 or more experiments (*B*, indicates means ± SEM). In parallel to the increased amounts of leukocyte marker enzymes in tissue homogenates, leukocyte migration from blood vessels into the interstitial space (*circles* in *C*) or in between pancreatic acinar cells (*circles* in *D*) is detectable in EM.

the first 60 minutes (Figure 6*A*). EM micrographs revealed a significant transmigration of inflammatory cells from small blood vessels into the interstitial space at that time (Figure 6*C* and *D*). Furthermore, when we quantitated leukocyte infiltration into pancreatic tissue during the course of acute pancreatitis, we found a significant increase in CD45-positive leukocytes, outside of blood vessels, that had transmigrated into the intercellular space (Figure 7*A* and *B*). As early as 1 hour after the onset of the disease, CD45 labeling revealed a 6-fold increased number of leukocytes in the space between pancreatic acini as illustrated in the right panel of Figure 7*B*. These results indicate that PMN elastase is capable of cleaving E-cadherin at the identified site in vivo and in vitro and that neutrophils, which secrete PMN elastase, appear at the site at which E-cadherin cleavage occurs.

Specificity of PMN Elastase Inhibition

To determine whether PMN elastase inhibition would prevent cell contact dissociation as well as E-

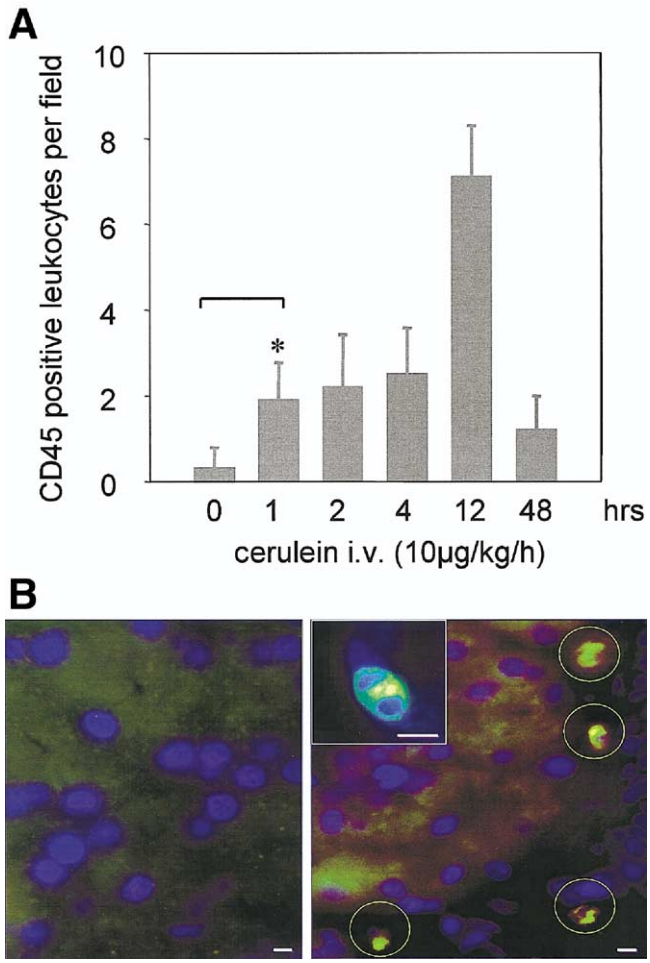

Figure 7. Quantitative leukocyte infiltration during experimental pancreatitis. Immunofluorescence labeling of common leukocyte antigen (CD45) was done in paraffin sections of pancreatic tissue over the entire time course of pancreatitis. Already, after 1 hour, a significant increase of CD45-positive leukocytes in the pancreatic tissue can be shown (*A*). The left micrograph (*B*) shows the labeling of untreated tissue, whereas the *right panel* is representative for 1 hour of cerulein treatment. CD45-positive fluorescent cells are marked with *circles*. The *insert* in the *right panel* shows at higher magnification that the cytoplasm, rather then the nuclei, of inflammatory cells are CD45 positive. For morphometric evaluation of the extent of leukocyte infiltration in the pancreas, 10 micrographs from each animal (n = 5) were randomly photographed at a fixed magnification (×63), and tissue-infiltrating CD45-positive cells were quantitated.

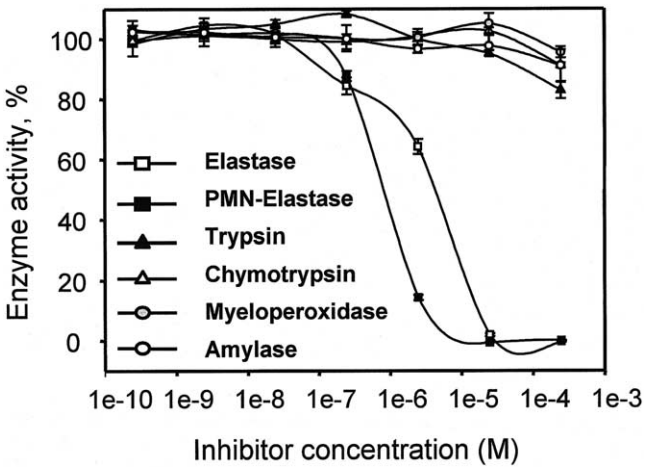

Figure 8. PMN-elastase inhibitor specificity in vitro. Myeloperoxidase (10 mU/mL), human neutrophil elastase (10 mU/mL), pancreatic elastase (10 mU/mL), trypsin (10 mU/mL), chymotrypsin (10 mU/mL), and amylase (10 U/mL) were incubated with various concentrations of inhibitor ranging from 5 nmol/L to 500 μmol/L, and enzyme activities were detected using specific fluorogenic or chromogenic substrates in a multiplate fluorescence or absorbance reader. Activity from 3 or more different experiments is expressed as the percentage of enzyme activity detected in the absence of the inhibitor. Human neutrophil elastase (PMN-elastase)—and to a somewhat lesser extent pancreatic elastase—was inhibited by the PMN-elastase inhibitor with 50% inhibition at 2.8 μmol/L (PMN-elastase). Trypsin, myeloperoxidase, chymotrypsin, and amylase were not significantly affected by incubation with the inhibitor.

cadherin cleavage and thus have a beneficial effect on the course of pancreatitis, we used a PMN elastase inhibitor in in vivo experiments. To determine the specificity of the inhibitor for PMN elastase, rather than for other pancreatic digestive enzymes, which are known to be activated or released during pancreatitis, we performed a series of in vitro experiments. We therefore compared the inhibitory capacity of a specific neutrophil elastase peptide inhibitor coupled to a chloro-methyl-coumarin residue (MeOSuc-Ala-Ala-Pro-Ala-CMK) on trypsin, chymotrypsin, pancreatic elastase, human neutrophil elastase, myeloperoxidase, and amylase. The inhibitor at concentrations ranging from 5 pmol/L to 500 μmol/L did not affect the activity of either trypsin, chymotrypsin, myeloperoxidase, or amylase. The inhibitory capacity for human neutrophil elastase was nearly half log higher than for pancreatic elastase. The IC 50 for PMN elastase was calculated to be 2.8 ± 1.7 μmol/L, whereas the IC 50 for pancreatic elastase was calculated to be 15.2 μmol/L ± 2.5 μmol/L (Figure 8).

PMN Elastase Inhibition Prevents E-Cadherin Cleavage and Reduces Leukocyte Infiltration During Pancreatitis

To investigate the role of PMN elastase-mediated E-cadherin cleavage and cell-cell contact dissociation, we inhibited PMN elastase in vivo in rats during experimental pancreatitis. To this end, we treated animals with the above neutrophil elastase peptide inhibitor coupled to a methyl-coumarin residue (MeOSuc-Ala-Ala-Pro-Ala-CMK), together with supramaximal concentrations of cerulein for 2 hours. E-cadherin cleavage products were identified from pancreatic tissue homogenates by immunoprecipitation and Western blotting. Although supramaximal concentrations of cerulein alone induced E-cadherin shedding, the intravenous administration of the inhibitor reduced E-cadherin cleavage almost completely (Figure 9*A*). MPO activity in pancreatic tissue

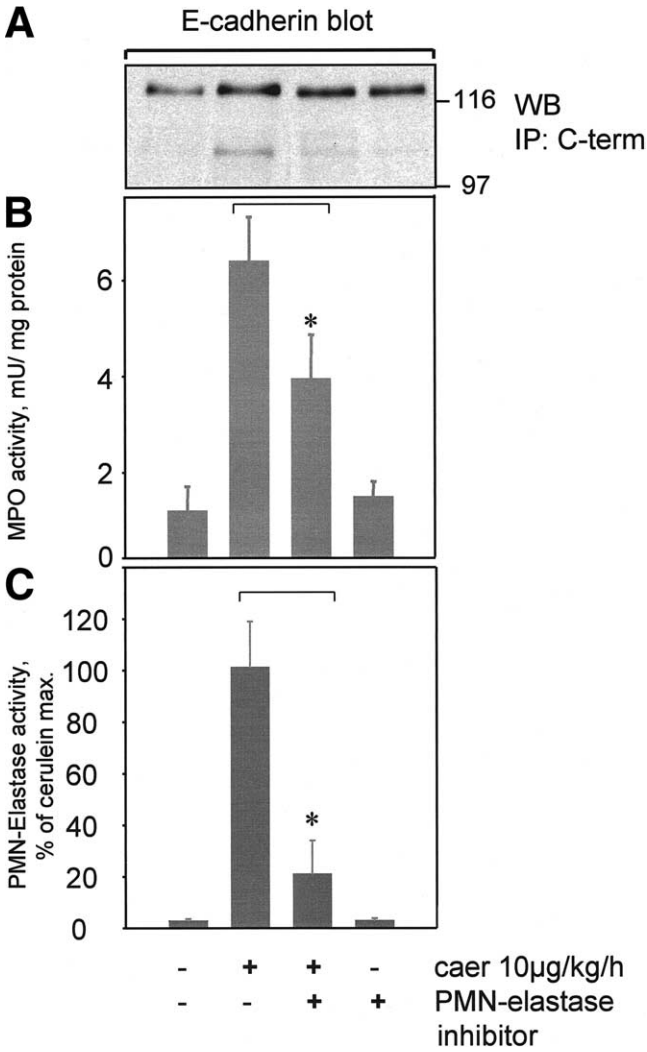

Figure 9. Inhibition of PMN-elastase activity prevents leukocyte transmigration during pancreatitis. Infusion of male Wistar rats with cerulein (10 μg/kg per hour) in the presence of the leukocyte elastase inhibitor (50 μmol/L) still resulted in the induction of pancreatitis but prevented cleavage of E-cadherin (*A*) and significantly reduced the translocation of leukocytes into pancreatic tissue as indicated by reduced MPO activity in pancreatic homogenates (*B*). *C* The equivalent inhibitor concentration reduced PMN elastase activity by 80% in pancreatic homogenates. Data shown are representative of 5 experiments (*B* and *C* represent means ± SEM).

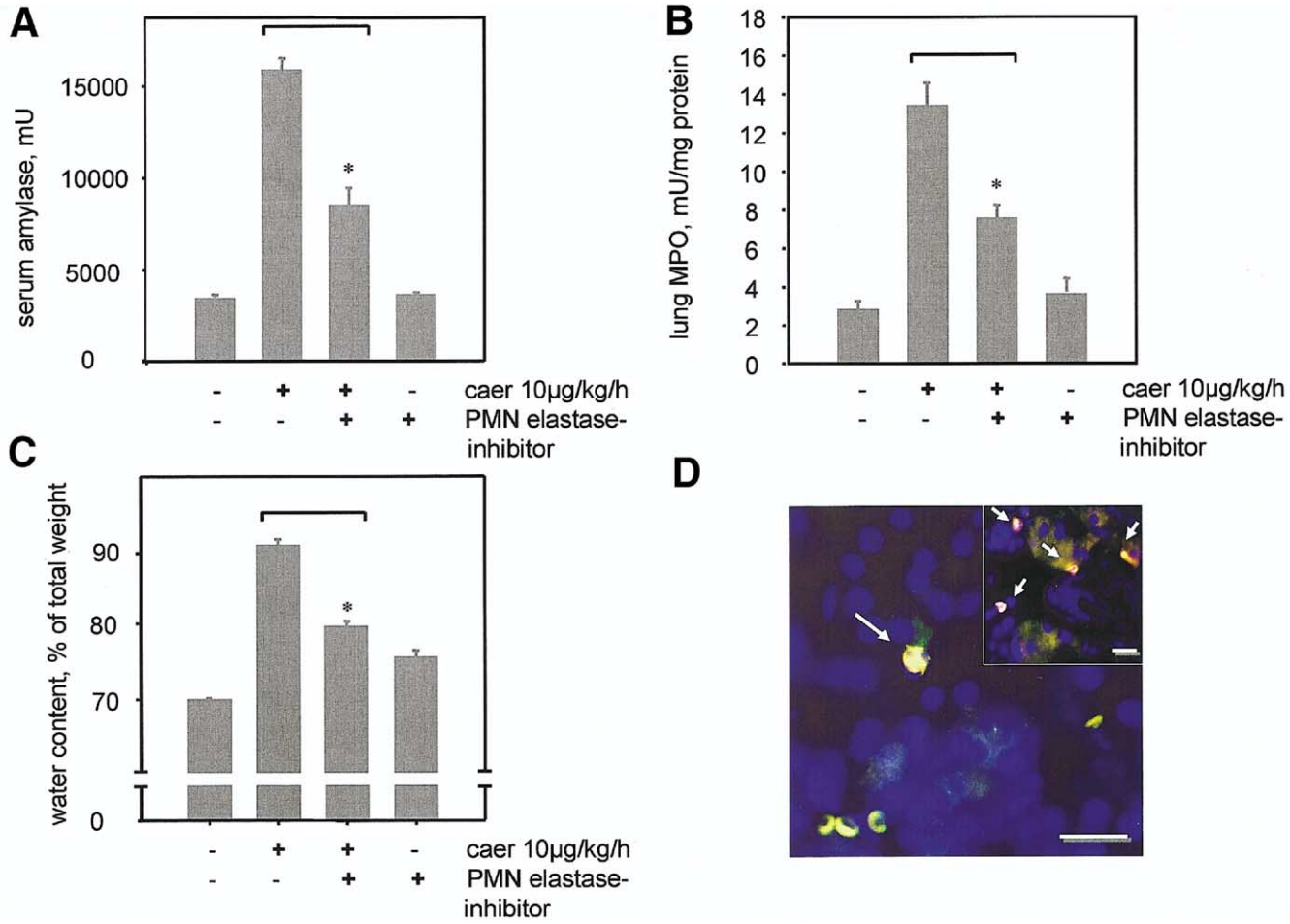

Figure 10. Inhibition of leukocyte elastase results in amelioration of acute pancreatitis. Infusion of male Wistar rats with cerulein (10 μg/kg per hour) in the presence of the leukocyte elastase inhibitor (50 μmol/L) for 2 hours led to a significant decrease in serum amylase (*A*), in the systemic inflammatory response as indicated by reduced MPO activity in the lungs (*B*), and in pancreatic edema (*C*). Immunolocalization of CD45 in *D* illustrates leukocyte extravasation into pancreatic tissue after cerulein administration for 2 hours (*insert*) and the combined administration of PMN elastase inhibitor and cerulein. *Size bars* indicate 200 μm. Data shown are representative of 5 experiments (*A–C* represent means ± SEM).

from animals treated with the PMN elastase inhibitor was found to be reduced, and thus the number of infiltrating inflammatory cells during pancreatitis (the mean MPO in cerulein treated animals was 6.1 ± 1.5 [SD] mU/mg protein vs 3.3 ± 1.6 [SD] mU/mg protein in animals treated with cerulein plus PMN inhibitor; 95% confidence interval for the difference of the means: 0.9 to 4.7 mU/mg protein, $P = .009$; n = 6; Figure 9*B*). Additionally, and as an internal control, we determined the inhibitor capacity for PMN elastase in vivo. PMN elastase-induced substrate cleavage of R110-$(CBZ\text{-}Ala_4)_2$ in pancreatic homogenates after cerulein application was reduced to 20% (Figure 9*C*). This clearly indicated that PMN elastase-mediated E-cadherin cleavage is a prerequisite for the dissociation of adherens junctions and leukocyte infiltration into the pancreatic tissue during the early course of pancreatitis. Inhibition of PMN elastase activity, on the other hand, can prevent inflammatory cells from entering the pancreas during an episode of acute pancreatitis.

Leukocyte Elastase Inhibition Ameliorates Local and Systemic Injury in Pancreatitis

To investigate whether inhibition of PMN elastase could ameliorate the course of acute pancreatitis, we determined serum amylase, pancreatic edema, and myeloperoxidase activity in the lungs after in vivo administration of the inhibitor (Figure 10). Serum amylase levels were significantly reduced after inhibitor treatment (amylase activity in cerulein-treated animals 16,962 mU ± 1257 [SD] mU vs 9177 mU ± 1638 [SD] mU in cerulein plus PMN elastase inhibitor-treated animals, n = 5; 95% confidence interval for the difference of the means: 4474–11,096, $P = .003$) (Figure 10*A*). Also, pancreatic edema was decreased (edema in cerulein

treated animals, 85.7% ± 0.9% [water content in percentage ±SD] vs 79.8% ± 0.5% in animals treated with cerulein plus PMN elastase inhibitor; n = 5; 95% confidence interval for the difference of the means: 4.9 to 7; $P < .001$; Figure 10*C*). The reduced myeloperoxidase activity in the lungs suggested that, besides local pancreatic damage, the systemic inflammatory response was beneficially affected (MPO in lung tissue in cerulein-treated animals was 13.4 ± 3.2 [SD] mU/mg protein vs 7.6 ± 1.9 mU/mg protein in animals treated with cerulein plus PMN elastase inhibitor; $P < .001$; 95% confidence intervals for the difference of the means: 3 to 8.7; n = 8; Figure 10*B*). To exclude a mere accumulation of leukocytes in pancreatic vessels to account for the increase in MPO activity in pancreatic tissue homogenates, we performed immunolabeling of CD45 (common leukocyte antigen) in paraffin sections. Already, after 2 hours, CD45 labelling revealed an increased leukocyte transmigration into pancreatic tissue as illustrated by Figure 10*D*. In summary, our data lead to the conclusion that administration of a PMN elastase inhibitor improves the clinical outcome of acute cerulein-induced pancreatitis.

Discussion

The regulatory mechanisms involved in cadherin-dependent cell adhesions are complex and not completely understood. Although it is well established that proteins of the cadherin/catenin complex play an important role in embryonic development, in tissue morphogenesis, and in malignant tumor invasion, information regarding the mechanisms that control the function of the cadherin/catenin complex in inflammation is sparse. We have previously shown that in vivo administration of secretagogues (cholecystokinin or acetylcholine) at concentrations that are in excess of those required for a maximal secretory response induces cellular changes in the rat or mouse pancreas that involve the dissociation of adherens junctions,[9] a complex cascade of tyrosine phosphorylation events regarding cell adhesion proteins,[25] a disassembly of the cytoskeleton,[33] and a disturbance of the vesicular transport within the acinar cell.[22] Because these changes are completely reversible within 48 hours in vivo, we used the animal model here to study the mechanisms involved in cell-cell contact dissociation and reassembly. When we studied E-cadherin expression in the pancreas over the first 48 hours of experimental pancreatitis, we detected an additional, unpredicted 105-kilodalton E-cadherin band, which appeared as early as 1 hour after disease induction. Because it is known that E-cadherin is highly *N*-glycosylated in the extracellular domain[34] and has an O-glycosylation site in the intracellular domain,[35] we tested deglycosylation as a possible cause for the smaller E-cadherin fragment but found no indication for any deglycosylation process. Immunoprecipitation experiments and peptide sequencing revealed that the newly formed molecule is a cleavage product of E-cadherin that lacks the extracellular EC-1 and EC-2 domains. Although classical cadherins consist of 5 repeated domains (EC-1 to EC-5) with great internal homology in their extracellular segment,[36,37] the only site involved in cell contact formation is in the cadherin EC-1 domain. This domain includes an HAV amino acid motif that directly mediates adhesive interactions. We therefore had to assume that the 105-kilodalton E-cadherin fragment identified during pancreatitis is nonadhesive. Our studies on pancreatic acini in which an increase in the shortened 105-kilodalton E-cadherin was associated with cell contact dissociation supported this notion. Mapping of the cleavage site to amino acid 394 and analysis of the cleavage motif ruled out caspases or metalloendoproteinases as possible candidates for the shedding of E-cadherin but identified PMN leukocyte elastase as most likely responsible for the observed cleavage. In previous cell culture experiments, proteolytic events had also been found to be involved in leukocyte transmigration through an endothelial layer,[38,39] although little was revealed about the underlying mechanisms and the resulting epithelial injury in vivo.[13] Proteolytic cleavage of E-cadherin as the mechanism by which the 105-kilodalton form is generated raised the question of whether the required proteolytic activity originated from invading inflammatory cells (eg, PMN leukocyte elastase) or from pancreatic acinar cells, which synthesize digestive protease zymogens in abundance and are known to activate them during pancreatitis.[15,18–20,27] To distinguish these, we followed several approaches: (1) In pancreatic epithelial tumor cells with intact adherens junctions but devoid of endogenous expression of any activatable digestive proteases, PMN elastase incubation produced the same 105-kilodalton E-cadherin cleavage product as seen in pancreatitis. Nevertheless, the efficacy of PMN elastase cleavage was not 100%, which could be explained by either a change in biochemical conformation because of detergents used for immunoprecipitation or may indicate that PMN elastase acts in concert with other proteases that are coinhibited in vivo by the PMN elastase inhibitor used here. (2) Incubation of pancreatic acini with PMN elastase resulted in E-cadherin cleavage, in redistribution of the intracellular part of the protein to the cytoplasm, and in a rapid dissociation of cell-cell contacts. On the other hand, supramaximal stimulation with a secretagogue alone, which is known to induce

immediate and abundant intracellular protease activation, did not induce E-cadherin cleavage nor did the combination of PMN elastase incubation with secretagogue stimulation increase E-cadherin cleavage observed with PMN elastase alone. (3) Pancreatic elastase, a digestive protease that is activated early in pancreatitis and can be released from acinar cells, had no effect on either the integrity of E-cadherin or on that of cell-cell contacts. Taken together, these data provide compelling evidence that PMN leukocyte elastase is, indeed, the enzyme that can cleave E-cadherin on adjacent pancreatic acinar cells to dissociate cell-cell contacts and permit inflammatory cells to enter the pancreatic tissue.

Tissue infiltration by inflammatory cells has traditionally been regarded as a late event and not as an early event in the course of pancreatitis.[21,26] In our study, we have compared the time course of E-cadherin cleavage with that of inflammatory cell infiltration and the presence of leukocyte products in the pancreas (CD45-positive leukocytes in the interstitial space, pancreatic tissue MPO activity, and pancreatic tissue PMN elastase expression). We were rather surprised to find inflammatory cell infiltration to occur as early as 1 hour after the start of pancreatitis and could confirm this observation by all 3 lines of evidence. Our data are, however, entirely consistent with previously published studies that used the same animal model and determined MPO activities from the third hour onward after the start of the cerulein infusion.[40] To our knowledge, no published reports exist in which pancreatic myeloperoxidase levels were determined in the rat at earlier time points during the course of experimental pancreatitis.

One line of evidence suggests that interleukin 8, tumor necrosis factor α, and MCP-1, which are released from macrophages, endothelial cells, and epithelial cells, are the mediators that attract neutrophils to the site of pancreatic injury.[41] In this context, it has been shown by others that a significant increase in the tumor necrosis factor α release from pancreatic acini can be detected as early as 30 minutes after the start of a supramaximal cerulein stimulation and can subsequently attract neutrophils to the site of inflammation.[42] In another line of evidence, the incubation of isolated neutrophils with purified pancreatic enzymes led to the degranulation of the neutrophils and to their transmigration in a matrigel chamber.[43] Because intracellular protease activation is a very early event in acute pancreatitis, it is thus feasible that activated digestive proteases directly induce the degranulation of neutrophils with a subsequent rise in extracellular PMN elastase. This scenario would be an alternative pathway to the classical, chemoattraction-mediated neutrophil infiltration.

Moreover, it has been demonstrated in different inflammatory models that E-selectin and P-selectin, the expression of both of which is a prerequisite for leukocyte transmigration, are up-regulated during the first 30 minutes of inflammation.[44,45] Real-time imaging studies of neutrophil transmigration through an endothelial layer have further shown that this process takes less than 10 minutes.[46]

Approximately 3 decades ago, Geokas et al[47] reported that pancreatic tissue obtained from patients who died of acute hemorrhagic pancreatitis showed substantially increased destruction of elastic tissue of intrapancreatic vessels and epithelial cells. This would suggest an important role for leukocyte elastase in the distinctive vascular injury and necrotic parenchyma of acute hemorrhagic pancreatitis in humans. Increased leukocyte elastase activity was also found in cerulein-induced pancreatitis.[48] Circulating levels of neutrophil elastase were shown to correlate with the severity of pancreatitis and were suggested as a reliable parameter to predict the severity of the disease.[49,50] From these data, one could conclude that inhibition of leukocyte elastase by specific inhibitors would ameliorate the severity of the disease, and, subsequently, several inhibitors were designed with different pharmacologic properties.[51,52] Song et al investigated the elastase inhibitor, guamerin-derived synthetic peptide (GDSP), which improved several parameters of cerulein-induced acute pancreatitis in the rat. They also reported effects of GDSP on superoxide formation by activated human neutrophils. This report thus indicated that neutrophil elastase inhibitors may ameliorate pancreatitis via effects on the endothelium, which prevent leukocyte migration from the vasculature into the interstitial space of the parenchyma.[53] Furthermore, treatment of experimental pancreatitis with antineutrophil serum did not only abolish signs of inflammatory response but also diminished protease activation and amylase serum levels.[54] All of this would be in accordance with our data concerning the question of whether inhibition of leukocyte elastase would be associated with reduced tissue damage and reduced leukocyte transmigration through epithelial cell-cell junctions and therefore of therapeutic benefit.

Our data must not only be regarded as direct evidence for a role of inflammatory cells in the initial phase of pancreatitis but also for a PMN elastase-mediated mechanism by which leukocytes dissociate cell contacts between epithelial acinar cells and transmigrate into the pancreatic tissue. In addition, our results point to a promising treatment strategy: PMN elastase inhibition as a therapy for pancreatitis. In patients with a severe course of pancreatitis, as well as those suffering from

inherited varieties of recurrent pancreatitis, a great extent of tissue damage is attributed to the action of inflammatory cells that have transmigrated into the pancreas.[55–61] Treatment with a PMN elastase inhibitor, which, according to our data, can greatly reduce pancreatic inflammation irrespective of the initiating triggering mechanism, seems like a promising therapeutic option for either limiting the extent of tissue injury or for preventing a disease recurrence.

We identified PMN elastase-dependent proteolytic cleavage of E-cadherin and subsequent dissociation of cell-cell contacts as a mechanism through which inflammatory cells transmigrate into the pancreas during an inflammatory process such as pancreatitis. Targeting this mechanism therapeutically seems like a feasible and promising treatment option for inflammatory disorders associated with extensive tissue damage.

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Received December 14, 2004. Accepted February 2, 2005.

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Supported by the Deutsche Forschungsgemeinschaft (to M.M.L. and B.K.), the BMBF (IZKF Münster to J.S. and M.M.L), and the Deutsche Krebshilfe Mildred-Scheel-Stiftung (to M.M.L. and J.M.).

The authors thank Dr. Elmar Wachter for his support in protein sequencing and C. Westermann, S. Agyemang, and U. Breite for expert technical assistance.