Cathepsin S Is Activated During Colitis and Causes Visceral Hyperalgesia by a PAR2-Dependent Mechanism in Mice

FIORE CATTARUZZA,* VICTORIA LYO,* ELLA JONES,5 DAVID PHAM,5 JAMES HAWKINS,9 KIMBERLEY KIRKWOOD,* EDUARDO VALDEZ–MORALES,3 CHARLES IBEAKANMA,1 STEPHEN J. VANNER,1 MATTHEW BOGYO,5 and NIGEL W. BUNNETT8

Departments of *Surgery, and §Radiology, University of California, San Francisco, California; 1Gastrointestinal Diseases Research Unit, Division of Gastroenterology, Queen’s University, Kingston, Ontario, Canada; 3Department of Pathology, Stanford University School of Medicine, California, and §NHMRC Australia Fellow, Professor of Pharmacology and Medicine, Monash Institute of Pharmaceutical Sciences, Parkville, Australia

BACKGROUND & AIMS: Although proteases control inflammation and pain, the identity, cellular origin, mechanism of action, and causative role of proteases that are activated during disease are not defined. We investigated the activation and function of cysteine cathepsins (Cat) in colitis. METHODS: Because protease activity, rather than expression, is regulated, we treated mice with fluorescent activity-based probes that covalently modify activated cathepsins. Activated proteases were localized by tomographic imaging of intact mice and confocal imaging of tissues, and were identified by electrophoresis and immunoprecipitation. We examined the effects of activated cathepsins on excitability of colonic nociceptors and on colonic pain, and determined their role in colonic inflammatory pain by gene deletion. RESULTS: Tomography and magnetic resonance imaging localized activated cathepsins to the inflamed colon of piroxicam-treated il10−/− mice. Confocal imaging detected activated cathepsins in colonic macrophages and spinal neurons and microglial cells of mice with colitis. Gel electrophoresis and immunoprecipitation identified activated Cat-B, Cat-L, and Cat-S in colon and spinal cord, and Cat-S was preferentially secreted into the colonic lumen. Intraluminal Cat-S amplified visceromotor responses to colorectal distension and induced hyperexcitability of colonic nociceptive neurons that promotes neurogenic inflammation and pain in the skin and intestine.

CONCLUSIONS: Activity-based probes enable noninvasive detection, cellular localization, and proteomic identification of proteases activated during colitis and are potential diagnostic tools for detection of predictive disease biomarkers. Macrophage cathepsins are activated during colitis, and Cat-S activates nociceptors to induce visceral pain via protease-activated receptor-2. Cat-S mediates colitis pain and is a potential therapeutic target.

Keywords: Activity-Based Probes; Proteases; Protease-Activated Receptors; Inflammation; Pain.
concentration, and a tag for detection. We used ABPs with an acyloxymethylketone warhead that binds to cysteine cathepsins, and a near-infrared reporter for optical imaging of cathepsin activities.\cite{11,12} We examined whether Cat-S, which was activated and secreted, excites colonic nociceptors and induces colonic pain, and determined the contribution of PAR2 by gene deletion. By studying Cat-S–deficient mice, we defined the causative role of Cat-S in colonic inflammatory pain.

**Methods**

See Supplementary Materials for detailed methods.

**Mice**

C57BL/6 mice, il10−/− mice, par2−/− and par2+/+ mice,\cite{13} and cat-s−/− and cat-s+/+ mice\cite{14} were studied. Institutional Animal Care Use Committees approved all procedures.

**ABPs**

ABPs with an acyloxymethylketone warhead included GB123, a nonquenched probe labeled with Cy5; GB138, a similar probe labeled with IR Dye 800; and GB137, a quenched probe with a dimethyl benzoic acid-based linker and a Cy5 fluorophore.\cite{11,12} Whereas GB123 and GB138 fluoresce whether or not they are bound to proteases, GB137 fluoresces only after proteolytic attack. These probes label Cat-B, Cat-L and Cat-S, and are serum-stable, cell-permeant, and are suitable for administration to animals and for optical imaging.

**Induction of Colitis**

Piroxicam-induced colitis in il10−/− mice. This model was selected because mice develop a chronic colitis that resembles Crohn’s disease. il10−/− mice (5–6 weeks) were fed piroxicam in nonfluorescent food for 2 weeks (week 1: 180 mg · kg−1 food; week 2: 260 mg · kg−1 food), followed by piroxicam-free food for 8–10 days before study. Control mice were age- and sex-matched wild-type mice that did not receive piroxicam.

**Trinitrobenzene sulfonic acid–induced colitis in C57BL6, cat-s−/− and cat-s+/+.** This model was selected because trinitrobenzene sulfonic acid (TNBS)-induced colitis is associated with colonic hyperalgesia, and the model allows convenient study of genetically modified mice. Mice were fasted overnight and sedated with isoflurane. TNBS (2 mg/mouse, 50% ethanol/saline, 50 µL) or vehicle (control) was injected via a PE10 catheter inserted 4 cm from the rectum. Mice were studied after 3 days.

**Administration of activity-based probes.** GB123 (250 µM, 66% dimethyl sulfoxide/phosphate-buffered saline, 100 µL, intravenous) was administered 24 h before study. GB137 (31 µM, 8% dimethyl sulfoxide/phosphate-buffered saline, 10 µL) was injected intratraherically and 3 h later spinal cord (T13–L5) was collected for analysis. To identify proteases in the colon, mice were anesthetized with isoflurane and a 2 cm length of proximal colon was ligated to form a closed loop. GB123 (1 µM, 20 mM sodium acetate [pH 7.4], 5 mM EDTA, 5 mM DTT), 250 µL was injected into the loop and 5 min later the loop fluid was collected and centrifuged (16,100g, 5 min, 4°C). Samples (100 µg protein) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel fluorescence.

**Noninvasive imaging.** Sequential fluorescence tomography (FT), magnetic resonance, and x-ray computerized tomography images were acquired from mice immediately after death. The same FT imaging parameters were used for all mice, and the images are shown with the same fluorescence gating and are quantified as the relative increase in signal above baseline. GB123 signals were quantified in the excised colon by reflectance imaging.

**Immunofluorescence and cellular confocal imaging.** Tissues were fixed in 4% paraformaldehyde, 0.1 M phosphate-buffered saline (pH 7.4) (2 h, room temperature). Frozen sections were processed for indirect immunofluorescence, and were observed by laser scanning confocal microscopy. Identical parameters were used to acquire images of control and inflamed tissues.

**SDS-PAGE, Western blotting, and immunoprecipitation.** Tissue homogenates (35–50 µg) were analyzed by SDS-PAGE (15%), and ABP-bound proteins were detected by in-gel fluorescence. Signals were normalized to β-actin, detected by Western blotting. For immunoprecipitation, homogenates (100 µg protein) were incubated with Cat-B, Cat-L, or Cat-S antibodies, followed by protein A/G beads. Immunoprecipitates were analyzed by SDS-PAGE and in-gel fluorescence.

**In vitro reactions with ABPs.** Homogenates (100 µg protein) were incubated with GB123 or GB138 (1 µM, 20 mM potassium phosphate [pH 7.4], 5 mM EDTA, 5 mM DTT, 1 h, room temperature). Human Cat-B, Cat-L or Cat-S (50 ng) were incubated GB123 (1 µM, 400 mM sodium acetate [pH 5.5]), 4 mM EDTA, 8 mM DTT [Cat-B, Cat-L] or 20 mM potassium phosphate [pH 7.4], 5 mM EDTA, 5 mM DTT [Cat S], 1 h, room temperature). Samples were analyzed by SDS PAGE and in-gel fluorescence.

**Cat-S-induced colonic inflammation and pain.** Human Cat-S (5 µg, 50 µL) or vehicle (0.9% saline) was injected via a catheter inserted 3 cm from the rectum. Some mice were pretreated with the irreversible Cat-S inhibitor morpholinurea-lucine-homophenylalanine-vinyl phenyl sulfone3 (250 nM, 50 µL intracolonic injection) 30 min before Cat-S. At 15 min after Cat-S, ethanol (35%, 50 µL) was similarly administered to promote mucosal permeability. At 1 h after Cat-S, visceromotor responses (VMR) were recorded by electromyography of abdominal muscles.\cite{15,16} Colorectal distension (CRD) was induced by distension of a colonic balloon (15–60 mm Hg). To assess activation of nociceptive neurons in the spinal cord, c-fos was localized in the spinal cord collected 2 h post–Cat-S.\cite{17} To assess inflammation, myeloperoxidase activity was measured in colonic extracts at 1.5 h post-Cat-S.\cite{17}

**Electrophysiological recordings from colonic dorsal root ganglia neurons.** Dorsal root ganglia (DRG) neurons innervating the colon of C57BL/6 mice were identified by retrograde tracing.\cite{18} DRG (T9–T13) were dissociated and cultured overnight.\cite{18} Patch clamp recordings were made in current clamp mode at room temperature from small neurons with a capacitance of ≤40 pF (putative nociceptive neurons). Intrinsic excitability was assessed using 500-ms duration current injections to establish the rheobase (firing threshold) and the number of action potentials at twice the rheobase during the 500-ms pulse. Neurons were exposed to human Cat-S (500 nM) or vehicle (control) for 60 min prior to patch clamping.

**Statistical Analysis**

Data are mean ± standard error of mean from 4–6 mice per group. Differences were examined using analysis of variance and Dunnett’s post-hoc test or using Student’s t test. P < .05 was considered significant.
Results

Noninvasive Optical Imaging of Activated Cathepsins in the Inflamed Intestine

To localize activated cathepsins in the intestine, we administered GB123 to control and piroxicam-treated *il10*−/− mice. GB123 was detected by FT imaging after 24 h, when excess unbound probe was excreted, and sequential magnetic resonance and computerized tomography images were obtained to define the location of GB123 signals. In control mice without colitis, GB123 fluorescence in the abdomen was minimal after 24 h, consistent with excretion of unbound probe (Figure 1A). In piroxicam-treated *il10*−/− mice, with histologically documented inflammation of colon and cecum (not shown), GB123 signals were detected throughout the intestine, indicated by coronal, transverse and lateral FT images of the abdomen (Figure 1A). Total abdominal GB123 fluorescence was 32-fold increased in piroxicam-treated *il10*−/− mice compared to control mice (Figure 1B). Analysis of co-registered FT, magnetic resonance, and computerized tomography images of the abdomen in the coronal and transverse plane revealed a low GB123 signal in the large intestine of control mice that was markedly increased in mice with colitis, although GB123 fluorescence was also increased in other regions of the bowel and the liver (Figure 1C). GB123 accumulation in the inflamed colon was confirmed by reflectance imaging of excised colon (Figure 1D).

Cellular Confocal Imaging of Activated Cathepsins in the Inflamed Intestine

To confirm activation and to identify the cellular source of cathepsins, we localized GB123-bound proteases in the colon by confocal microscopy. In control animals, there was minimal detectable GB123 fluorescence in sections of colon collected 24 h after administration of GB123 (Figure 2A), consistent with results from FT. In contrast, GB123 was detected in multiple discrete cells in the lamina propria and submucosa of the colon of piroxicam-treated *il10*−/− mice (Figure 2A). Most GB123-stained cells expressed F4/80-immunoreactive (IR), which identifies macrophages that were markedly up-regulated in the inflamed colon (Figure 2A, arrows). GB123 was also detected in macrophages in the mucosal vasculature, consistent with infiltration of macrophages into the inflamed colon (Figure 2A, arrow heads). High-magnification images indicated that GB123 was localized to discrete vesicles of macrophages, which probably represent lysosomes or endosomes (Figure 2A′). Because GB123 covalently binds Cat-B, Cat-L, and Cat-S, we simultaneously localized

---

**Figure 1.** Optical imaging of activated cathepsins in colitis. Piroxicam-treated *il10-ko* mice with colitis or control mice received intravenous GB123 and were imaged 24 h later. (A) FT abdominal images. (B) Quantification of abdominal GB123 FT images (purple box denotes region of interest). *P < .05. (C) Co-registered computed tomography, magnetic resonance, and FT images indicating GB123 accumulation in the intestine (arrows). (D) Representative reflectance images of excised colon indicating GB123 accumulation during inflammation.
GB123 with cathepsins. Cat-B-IR and Cat-L-IR partially colocalized with GB123 in macrophages (Figure 2B, arrows), but were also detected in vesicles in colonocytes (Figure 2B, arrow heads). Cat-S-IR colocalized with GB123 in macrophages (Figure 2B, arrows).

Proteomic Identification of Activated Cathepsins in the Inflamed Intestine

Because ABPs covalently modify activated proteases, probe-bound proteases can be fractionated by gel electrophoresis and identified immunochemically.11,12 To characterize activated cathepsins in the inflamed intestine, we fractionated intestine from mice treated with GB123 by SDS-PAGE and detected probe-bound proteases by in-gel fluorescence. GB123-bound proteases were detected in extracts of proximal colon and cecum of piroxicam-treated il10-ko mice corresponding in size to Cat-B (31 kDa), Cat-S (28 kDa), and Cat-L (25 kDa) (Figure 3Ai and Bi). When compared to control mice, signals in the proximal colon of piroxicam-treated il10-/- mice were up-regulated by 2.0-fold for Cat-B, 1.7-fold for Cat-L, and 1.7-fold for Cat-S (all P < .05 to control) (Figure 3Ai). In the cecum, signals were increased by 3.0-fold for Cat-B, 2.2-fold for Cat-L, and 3.0-fold for Cat-S (Cat-S, Cat-L; P < .05 to control) (Figure 3Bi). To confirm the identity of GB123-bound proteases, Cat-B, Cat-L, and Cat-S were immunoprecipitated from extracts of inflamed proximal colon and cecum, and immunoprecipitates were analyzed by in-gel fluorescence. This analysis identified Cat-B, Cat-L, and Cat-S in the proximal colon and cecum of piroxicam-treated il10-/- mice (Figure 3C). Western blotting of purified proteases confirmed that antibodies were selective for Cat-B, Cat-L, and Cat-S (not shown). GB123 also labeled purified proteases (Figure 3D). To determine whether these proteases are secreted into the intestinal lumen, we injected GB123 into a closed loop of proximal colon from control and piroxicam-treated il10-/- mice and after 5 min analyzed luminal fluid by SDS-PAGE and in-gel fluorescence. GB123-bound proteases corresponding to Cat-B and Cat-S were detected in the lumen of control and inflamed colon (Figure 3Ei). However, Cat-S alone was activated by 4.7-fold in lumen of the inflamed colon. These results confirm activation of Cat-B, Cat-L, and Cat-S in colitis, and reveal secretion of activated Cat-S.

Figure 2. Confocal cellular localization of activated cathepsins in the colon. Piroxicam-treated il10-ko mice with colitis or control mice received intravenous GB123 and colon was collected 24 h later. (A) Localization of GB123 and F4/80, which identifies macrophages, indicating GB123 accumulation in infiltrated macrophages of inflamed colon (arrows). Arrowhead indicates macrophage in vasculature. (A’) High-power views of regions denoted by white boxes. (B) Localization of GB123, F4/80, and Cat-B-IR, Cat-L-IR, and Cat-S-IR in inflamed colon. GB123 signals partially colocalized with Cat-B, Cat-L, and Cat-S in macrophages (arrows). All cathepsins were detected in macrophages, but Cat-B and Cat-L were also found in colonocytes. LP, lamina propria; SM, submucosa. Scale bar = 10 μm.
Figure 3. Identification of activated cathepsins. Piroxicam-treated *il10-ko* mice with colitis or control mice received intravenous GB123 and tissue was collected 24 h later. (A, B) Analysis of colon (A) and cecum (B) by SDS-PAGE and in-gel fluorescence identified GB123-bound proteases corresponding to Cat-B, Cat-L, and Cat-S in inflamed tissues. Each lane is an individual mouse. Quantification (Aii, Bii) revealed cathepsin activation (bar graphs). *P* < .05. (C) Immunoprecipitation confirmed identification of Cat-B, Cat-L, and Cat-S in inflamed colon and cecum. (D) GB123 labeled purified human Cat-B, Cat-L, and Cat-S. (Ei) GB123 was injected into a closed loop of colon of *il10-ko* mice with colitis or control mice and luminal fluid was collected 5 min later. Analysis by SDS-PAGE and in-gel fluorescence identified Cat-B and Cat-S, but only Cat-S activity was increased in colitis (Eii). *P* < .05.
**Intracolonic Cat-S Causes Visceral Hyperalgesia and Activates Spinal Nociceptors via PAR2**

Subsequent studies focused on Cat-S, which was robustly activated in the inflamed intestine and selectively secreted into the colonic lumen, and which remains active at extracellular pH. To determine whether Cat-S causes visceral pain, we administered Cat-S (5 μg) or vehicle into the colonic lumen of C57/BL6 mice. Pain was assessed by determining VMR to graded CRD, and activation of spinal nociceptive neurons was evaluated by quantifying c-fos-IR in nuclei within laminae I/II of the spinal cord. Within 1 h of administration, Cat-S significantly increased the VMR to all CRD pressures (15, 30, 45, 60 mm Hg) compared to vehicle or to basal measures (Figure 4A). The greatest difference between vehicle (0.14 ± 0.03 mV · s) and Cat-S (0.34 ± 0.08 mV · s) (2.4-fold; P < .05) was observed at 30 mm Hg. Pretreatment with the Cat-S–selective inhibitor morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone abolished the pronociceptive actions of Cat-S, indicating a requirement for protease activity (Figure 4A). Cat-S can activate PAR2, an established mediator of visceral hyperalgesia, but the role of PAR2 in Cat-S–mediated pain has not been examined. To determine whether PAR2 mediates Cat-S–induced visceral pain, we administered Cat-S into the colonic lumen of par2+/+ and par2−/− mice. In par2+/+ mice, Cat-S amplified the VMR to CRD in a similar manner to C57/BL6 mice (Figure 4B). Cat-S also increased the number of c-fos-IR nuclei by 2.2-fold over vehicle (vehicle, 9.1 ± 0.8; Cat-S, 20.3 ± 2.3; P < .05) in laminae I/II of the dorsal horn (Figure 4C and C'). However, Cat-S neither increased the VMR to CRD (Figure 4B) nor increased the number of c-fos-IR nuclei in the dorsal horn (Figure C and C') of par2−/− mice. Moreover, Cat-S did not affect myeloperoxidase activity in the colon of par2+/+ mice (Figure 4D). Thus, luminal Cat-S amplifies VMR to CRD and activates spinal nociceptive neurons by a PAR2-dependent mechanism.

**Cat-S Induces Hyperexcitability of Colonic Nociceptors**

To determine whether Cat-S can directly excite colonic nociceptors, we examined the effects of Cat-S on membrane currents of DRG neurons innervating the colon. Using whole cell perforated patch techniques, the rheobase and action potential discharge at 2 times rheo-

---

**Figure 4.** Effects of luminal Cat-S on pain and inflammation. Cat-S or vehicle (Veh.) was injected into the colonic lumen. (A, B) VMR to CRD basally (all groups combined) and at 1 h after Cat-S or vehicle. Cat-S caused hyperalgesia in C57/BL6 mice (A) and par2+/+ but not in par2−/− (B) mice. Pretreatment with the Cat-S inhibitor morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone (LVHS) abolished Cat-S–induced hyperalgesia (A). **P < .01, *P < .05 to basal. (C, C') c-Fos-IR neurons in laminae I/II of spinal cord (T12/L2) at 1.5 h after Cat-S or vehicle. Cat-S increased nuclear c-fos-IR in par2+/+ but not in par2−/− mice. *P < .05 to vehicle. (D) Colonic myeloperoxidase (MPO) at 1.5 h after Cat-S or vehicle. Cat-S did not significantly increase colonic MPO.
Cat-S is activated in spinal microglial cells after nerve injury and contributes to neuropathic pain. It is not known whether visceral inflammation affects cathepsin activity in the spinal cord. To determine whether colitis results in activation of spinal cathepsins, we administered GB137 by intrathecal injection to mice. A quenched probe, which fluoresces only after proteolytic attack, was selected to avoid the requirement for clearance of unbound probe prior to analysis, which may be slow from spinal fluid. Confocal imaging of sections of spinal cord that receive input from colonic sensory nerves (T13–L2) collected 3 h after probe injection revealed a low level of GB137 fluorescence in control animals, but GB137 accumulated throughout the spinal cord of piroxicam-treated *il10−/−* mice (Figure 7A). GB137 colocalized with Lamp1-IR in spinal neurons, identified by NeuN-IR, and was also detected in microglial cells that were identified in the spinal cord in response to GB137 administration.

**Cathepsins Are Activated in the Spinal Cord During Colitis**

Cat-S is activated in spinal microglial cells after nerve injury and contributes to neuropathic pain. It is not known whether visceral inflammation affects cathepsin activity in the spinal cord. To determine whether colitis results in activation of spinal cathepsins, we administered GB137 by intrathecal injection to mice. A quenched probe, which fluoresces only after proteolytic attack, was selected to avoid the requirement for clearance of unbound probe prior to analysis, which may be slow from spinal fluid. Confocal imaging of sections of spinal cord that receive input from colonic sensory nerves (T13–L2) collected 3 h after probe injection revealed a low level of GB137 fluorescence in control animals, but GB137 accumulated throughout the spinal cord of piroxicam-treated *il10−/−* mice (Figure 7A). GB137 colocalized with Lamp1-IR in spinal neurons, identified by NeuN-IR, and was also detected in microglial cells that were identified in the spinal cord in response to GB137 administration.

**Cathepsins Are Activated in the Spinal Cord During Colitis**

Cat-S is activated in spinal microglial cells after nerve injury and contributes to neuropathic pain. It is not known whether visceral inflammation affects cathepsin activity in the spinal cord. To determine whether colitis results in activation of spinal cathepsins, we administered GB137 by intrathecal injection to mice. A quenched probe, which fluoresces only after proteolytic attack, was selected to avoid the requirement for clearance of unbound probe prior to analysis, which may be slow from spinal fluid. Confocal imaging of sections of spinal cord that receive input from colonic sensory nerves (T13–L2) collected 3 h after probe injection revealed a low level of GB137 fluorescence in control animals, but GB137 accumulated throughout the spinal cord of piroxicam-treated *il10−/−* mice (Figure 7A). GB137 colocalized with Lamp1-IR in spinal neurons, identified by NeuN-IR, and was also detected in microglial cells that were identified in the spinal cord in response to GB137 administration.

**Cathepsins Are Activated in the Spinal Cord During Colitis**

Cat-S is activated in spinal microglial cells after nerve injury and contributes to neuropathic pain. It is not known whether visceral inflammation affects cathepsin activity in the spinal cord. To determine whether colitis results in activation of spinal cathepsins, we administered GB137 by intrathecal injection to mice. A quenched probe, which fluoresces only after proteolytic attack, was selected to avoid the requirement for clearance of unbound probe prior to analysis, which may be slow from spinal fluid. Confocal imaging of sections of spinal cord that receive input from colonic sensory nerves (T13–L2) collected 3 h after probe injection revealed a low level of GB137 fluorescence in control animals, but GB137 accumulated throughout the spinal cord of piroxicam-treated *il10−/−* mice (Figure 7A). GB137 colocalized with Lamp1-IR in spinal neurons, identified by NeuN-IR, and was also detected in microglial cells that were identified in the spinal cord in response to GB137 administration.
tified by Ox42-IR. Notably, GB137 partially colocalized with Cat-S-IR in microglial cells. Analysis of extracts of spinal cord from GB137-treated mice by SDS-PAGE and in-gel fluorescence did not reveal signals that were sufficiently strong to quantify. However, Cat-B, Cat-L, and Cat-S were identified in homogenates of spinal cord that were incubated with GB138 (Figure 7B). Quantification revealed that Cat-B, Cat-S, and Cat-L were up-regulated in the spinal cord of mice with colitis (Figure 7Bi). Cat-B and Cat-S were identified by immunoprecipitation (Figure 7C).

**Discussion**

Despite the importance of proteases and PARs for inflammation and pain, the spectrum of proteases that are activated in inflammatory diseases is unclear, and their identity, cellular origin, mechanism of action, and causative roles of specific activated proteases are not defined. By administering near-infrared ABPs to mice with chronic colitis, we detected increased cysteine cathepsin activity in the colon by noninvasive imaging, and localized this activity to macrophages by confocal imaging. Proteomic analysis identified activated Cat-B, Cat-L, and Cat-S. Cat-S activity was selectively increased in the lumen during colitis, indicating secretion, and luminal Cat-S caused colonic pain and increased excitability of colonic nociceptive neurons by a PAR2-dependent mechanisms. Cat-S deletion attenuated inflammatory pain of the colon. Our results reveal activation of cathepsins in macrophages of the inflamed colon, and identify Cat-S as a new mediator of colonic pain. ABPs offer a powerful approach to detect and identify the spectrum of proteases that are activated during disease, and may represent a diagnostic approach that identifies causative biomarkers of disease.

**Inflammation-Induced Activation of Cathepsins in the Colon and Spinal Cord**

Our results show that Cat-S is activated in macrophages and that Cat-B and Cat-L are activated in macro-
phages and colonocytes in the colon of piroxicam-treated il10-ko mice, which develop colitis that resembles Crohn’s disease. Noninvasive FT imaging detected accumulation of GB123 in the colon of mice with colitis, and confocal imaging showed that GB123 mainly accumulates in infiltrating macrophages of the lamina propria, although GB123 was also detected in epithelial cells. Although it is not possible to unequivocally determine which activated cathepsins bind to GB123 in these cells, Cat-S-IR was confined to macrophages, and Cat-B-IR and Cat-L-IR were detected in macrophages and colonocytes. Analysis of colonic extracts by electrophoresis revealed that GB123 was bound to proteases corresponding in mass to Cat-B, Cat-L, and Cat-S, which were identified by immunoprecipitation. Our results confirm a major role for macrophages and cathepsins in intestinal inflammation. Cat-B, Cat-D, and Cat-L are up-regulated in macrophages in the colon of patients with inflammatory bowel disease and mice with colitis. Cat-K is expressed by granulomas of patients with Crohn’s disease, and Cat-G is up-regulated in biopsies from patients with ulcerative colitis. Although these studies observed up-regulation of cathepsin mRNA and protein during colitis, they did not assess enzymatic activity, the key determinant of protease function. Activity assays usually rely on use of substrates and inhibitors that lack absolute specificity, and are unsuitable for localization of activated proteases by noninvasive or cellular imaging. By using near-infrared ABPs that covalently label only active proteases, we were able to localize activity and identify activated Cat-B, Cat-L, and Cat-S. Of these, Cat-S was selectively activated in the lumen during colitis, suggesting secretion from macrophages. Although other cathepsins may also be secreted, Cat-S is unusual in that it retains full activity at normal extracellular pH. Cat-B is also released from intestinal segments after injury, and activity of Cat-G, a neutrophil serine protease, is also increased in feces of ulcerative colitis patients.

A drawback of unquenched probes such as GB123 is that they fluoresce whether or not they are bound to proteases, necessitating imaging 24 h after systemic administration, when unbound probe is cleared. To determine whether cysteine cathepsins are also activated in the spinal cord during colitis, we intrathecally administered GB137, a quenched ABP that fluoresces only after protease attack. This approach enabled localization of proteases in tissues where clearance of unbound probe could be delayed. Colitis induced accumulation of GB137, indicative of cysteine cathepsin activation, in neurons and microglial cells throughout the spinal cord. To our knowledge, the activation of spinal cathepsins during colitis has not been reported previously. Peripheral nerve injury results in up-regulation, activation, and release of Cat-S from spinal microglial cells. Given the extensive activation colonic sensory nerves during colitis, it is likely that activated Cat-S contributes to the GB137 signal, particularly in microglial cells. Indeed, GB137 accumulated in Cat-S expressing microglial cells.

**Causative Role of Cysteine Cathepsins in Colonic Inflammation and Pain**

The administration of Cat-S into the colonic lumen, to mimic the increased activity observed during colitis, enhanced the nocifensive response of mice to colorectal distension, suggesting mechanical hyperalgesia, and induced c-fos expression in neurons in superficial laminae of the spinal cord, consistent with activation of spinal nociceptive neurons. These changes occurred without obvious signs of inflammation, assessed by measurement of granulocyte infiltration. Cat-S deletion did not affect the response to colorectal distension under basal conditions, suggesting that Cat-S does not contribute to mechanotransduction in the colon. However, in mice with TNBS colitis, Cat-S deletion decreased the VMR to 60 mm Hg distending pressure by 32%, implicating Cat-S as a mediator of inflammatory hyperalgesia in the colon. Given the robust activation of colonic Cat-S during colitis, it is likely that Cat-S causes pain by peripheral mechanisms, and further work is required to examine the role of spinal Cat-S in visceral pain. Inhibitors of Cat-B, Cat-L, and Cat-D also ameliorate colitis in mice, although the role of these proteases in visceral pain has not been examined.

**Mechanisms of Cathepsin-Induced Inflammation and Pain in the Colon**

In addition to their physiological roles in intracellular antigen presentation, zymogen activation and hormone processing, secreted cysteine cathepsins contribute to inflammatory diseases of multiple systems. During chronic inflammation, macrophages destroy extracellular matrix by secreting Cat-B, Cat-L, and Cat-S, which may aggravate colitis by promoting paracellular permeability and influx of inflammatory cells. Inflammatory mediators stimulate Cat-S secretion from macrophages and microglial cells, and secreted Cat-S is active at normal extracellular pH and may have widespread extracellular actions. Because Cat-S derives from macrophages and spinal microglial cells, peripheral and central neuroimmune mechanisms could mediate its effects on inflammation and pain (Supplementary Figure 1). We observed that par2 deletion attenuated the effects of intracolonic Cat-S on visceromotor responses and c-fos expression in spinal neurons, and abolished Cat-S-induced hyperexcitability of nociceptive neurons. These result is consistent with the report that Cat-S can activate heterologously expressed PAR2. Activation of PAR2 on primary spinal afferent neurons innervating the colon induces neurogenic inflammation and pain, and PAR2 activation on colonocytes increases paracellular permeability. Thus, Cat-S may induce colonic pain and inflammation by activating PAR2 on several cell types. Although our results show that expression of PAR2 is necessary for Cat-S—induced hyper-
algesia, we did not directly examine whether Cat-S cleaves PAR$_2$, and thus cannot exclude the possibility that Cat-S activates other proteases that in turn activate PAR$_2$. Furthermore, Cat-S released from spinal microglial cells during nerve injury liberates fractalkine from dorsal horn neurons, thereby contributing to the amplification and maintenance of chronic pain, and similar mechanisms may occur during colitis (Supplementary Figure 1).

Conclusions

We conclude that Cat-B, Cat-L, and Cat-S are activated in macrophages during colitis, and that secreted Cat-S causes colonic pain and hyperexcitability of colonic nociceptive neurons via PAR$_2$. Cysteine cathepsin inhibitors may be used to treat colonic pain and inflammation. ABPs enable identification of activated proteases during colitis. Given the recent advances in fluorescence endoscopy, near-infrared ABPs and imaging may facilitate early diagnosis and provide mechanistic insights into colonic disease.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi:10.1053/j.gastro.2011.07.035.

References

Acknowledgments

We thank S. Coughlin and H. Chapman for par2 and cat-s knockout mice, Erik Lindström for providing Cat-S and Cat-S inhibitor, and E. Grady for help with establishing the colitis models.

Conflicts of interest
The authors disclose no conflicts.

Funding
Supported by National Institutes of Health (NIH) grants DK43207, DK57850, DK39957 (NWB), Crohn’s and Colitis Foundation of Canada (SJV), and NIH grant EB005011 (MB).
**Supplementary Methods**

**Mice**
C57BL/6 mice were from Charles River Laboratories (Hollister, CA and Montreal, QB). II10-/- mice (B6.129P2-II10m1Cgr/J, stock 002251) were from Jackson Laboratory (Sacramento, CA). Par2-/- and par2 +/- mice (C57BL/6) were from S. Coughlin (UCSF).1 and catS-/- and catS +/- mice (C57BL/6) were from H. Chapman (UCSF).2 These mice were maintained as heterozygotes and littersmates were compared. Mice were maintained under temperature- (22 ± 4°C) and light- (12-h light/dark cycle) controlled conditions.

**Proteases**
Human Cat-B, Cat-L, and Cat-S were from EMD Biosciences (La Jolla, CA) and Calbiochem (Merck KGaA, Darmstadt, Germany). Recombinant human Cat-S and the irreversible Cat-S-selective inhibitor morpholinurea-leucine-homophenylalanine-vinyl phenyl sulfone3 were gifts from Erik Lindtsröm, Medivir (Sweden).

**Antibodies**
Sources and dilutions of primary antibodies are shown in Supplementary Table 1. Secondary antibodies conjugated to fluorescein isothiocyanate or Rhodamine RedX were from Jackson Immunoresearch (West Grove, PA).

**Noninvasive Imaging**
Mice were sacrificed with sodium pentobarbital (200 mg/kg, intraperitoneal). The abdomen was shaved and mice were secured in a nonmagnetic imaging cassette (Perkin Elmer, Waltham, MA) with the optical path set at 13 mm. FT images were acquired using the FMT2500 Quantitative Tomography Imaging System (Perkin Elmer) with the 680 nm channel that was precalibrated for GB123. The same imaging parameters were used for all mice, and the images are shown with the same fluorescence gating and are quantified as the relative increase in signal above baseline. Magnetic resonance (MR) images were acquired immediately after FT imaging using a 7T (300 mHz) narrow-bore scanner (7T/310 DDR System; Varian Inc., Palo Alto, CA). The imaging cassette was fitted into a home-built external bed for consistent positioning. The cassette was locked into place inside a quadrature volume coil, and a 3-dimensional gradient echo sequence was used to image (TR: 30 ms, TE: 4.2 ms, Flip Angle: 35 degrees), with an acquisition matrix of 256 × 128 × 128, giving voxels measuring 187.5 μm × 312.4 μm × 250 μm. For anatomical co-registration of FT and MR images, x-ray computerized tomography (CT) images were acquired at 75 kVp and 315 μA, and volumetric CT images were reconstructed in a 512 × 512 × 512 format with voxel dimensions of 170 μm3 using a generalized cone beam Feldkamp algorithm provided by the manufacturer. All images were exported to standard DICOM format and processed using AMIDE software. Coregistration transformation matrices for the 3 imaging modalities were determined using an anisotropic 3-well calibration phantom filled with a solution of 0.1 mM sodium iodide for MR and CT contrast. To ensure that mouse cassette was positioned at the exact same place within the MR bore for each scan, five 3-dimensional gradient echo scans were taken of the phantom. The phantom centroid was calculated for each scan, with a standard deviation of 0.28 mm, 1.18 mm, and 0.27 mm. Similarly, 5 scans of the phantom were repeated on the FM imaging station and the CT system. GB123 signals were quantified in the excised colon by reflectance imaging (Xenogen IVIS100; Caliper Life Sciences, Hopkinton, MA) using the Cy5.5 filter.

**Immunofluorescence and Cellular Confocal Imaging**
Tissues were immersion-fixed in 4% paraformaldehyde, 0.1 M PBS (pH 7.4) (2 h, room temperature), and were cryoprotected in 30% sucrose in PBS (overnight, 4°C). Tissues were embedded in optimal cutting temperature compound, and frozen sections (20 μm) were prepared. Sections were incubated with primary antibodies (Supplementary Table 1) in 100 mM PBS (pH 7.4), 10% normal goat serum, 0.1% Triton X-100. Sections were washed and incubated with fluorescent secondary antibodies (1:200, 2 h, room temperature). Specimens were observed using a Zeiss LSM510 Meta confocal microscope (Carl Zeiss, Thornwood, NY) with a Fluar Plan Apo NA 0.8) and Plan Neofluor 63× (NA 1.4) objectives. Images were acquired with 1024 × 1024 pixel resolution and an iris of <2.5. Images are of 3–6 optical sections at 1-μm intervals. Identical parameters were used to acquire images of control or inflamed tissues.

**SDS-PAGE, Western Blotting, and Immunoprecipitation**
Tissues were homogenized and sonicated in Hank’s balanced salt solution, 10 mM HEPES pH 7.4, and centrifuged (16,100g, 30 min, 4°C). Supernatant proteins (35–50 μg) were denatured by boiling (5 min) in 250 mM Tris (pH 6.8), 8% SDS, 40% glycerol, 0.08% bromophenol blue, 50 mM DTT. Samples were analyzed by SDS-PAGE (15% acrylamide). ABP-bound proteins were detected by in-gel fluorescence using the Odyssey Infrared Imaging System (LiCOR Bioscience, Lincoln, NE). Signals were normalized to β-actin, which was detected by Western blotting. For immunoprecipitation, tissue homogenates (100 μg protein) from ABP-treated mice were incubated with Cat-B, Cat-L, or Cat-S antibodies (1 μg, 50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.5% deoxycholate, 0.1% SDS, rotation, 16 h, 4°C). Samples were incubated with protein A/G PLUS beads (Santa Cruz Biotechnology, Santa Cruz, CA, overnight, 4°C). Immunoprecipitates were pelleted, washed with
RIPA buffer, and analyzed by SDS-PAGE and in-gel fluorescence.

**Cat-S—Induced Colonic Inflammation and Pain**

Mice were sedated with isoflurane. Human Cat-S (5 μg, 50 μL) or vehicle (0.9% saline) was injected via a PE10 catheter inserted 3 cm from the rectum. Mice were held inverted to prevent leakage. One group of mice were treated with the irreversible Cat-S inhibitor morpholinourealeucine-homophenylalanine-vinyl phenyl sulfone (LHVS)³ (250 nM, 1% dimethyl sulfoxide, 10% Tween80, 89% saline, 50 μL intracolic injection), 30 min before Cat-S. After 15 min after Cat-S administration, ethanol (35%, 50 μL) was similarly administered to promote mucosal permeability. At 1 h after Cat-S administration, VMR were recorded by electromyography of abdominal muscles.⁴ ⁵ CRD was induced by graded distensions of a colonic balloon using helium (15, 30, 45, 60 mm Hg, three 10-s trials at each pressure, 2 min recovery between each distention). Electromyography records were quantified by integrating rectified signals obtained during the stimulus and are expressed as mV · s. To assess activation of nociceptive neurons in the spinal cord, c-fos was localized in the spinal cord collected 2 h after Cat-S administration.⁴ c-Fos-IR nuclei in laminae I and II of the dorsal horn (T12–L2) were counted in 5 sections per segment per mouse using a computer-assisted image analysis system (NIH Image). To assess inflammation, myeloperoxidase activity was measured by enzymatic assay of colon collected 1.5 h after Cat-S administration.⁶ Data are expressed as mU myeloperoxidase · mg⁻¹ colonic tissue.
Electrophysiological Recordings From Colonic DRG Neurons

DRG neurons innervating the colon in C57BL/6 mice were identified by retrograde tracing injecting the fluorescent retrograde tracer Fast Blue (Cedarlane Laboratories; Homby, ON, Canada) into the wall of the colon, as described. To isolate DRG neurons for patch clamp studies, mice were anesthetized with ketamine and xylazine, sacrificed by transcardial perfusion, and DRG (T9–T13) were dissociated. Dispersed neurons were suspended in Dulbecco’s modified Eagle medium (pH 7.2–7.3) containing 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM glutamine, plated on poly-D-lysine/laminin-coated cover slips and incubated overnight in a humidified incubator (95% O2, 5% CO2, 37°C). Amphotericin-perforated patch clamp experiments were performed in current clamp mode at room temperature. Recordings were made from small neurons with a capacitance of ~40 pF, which are putative nociceptive neurons. Signals were acquired using an Axopatch 200B amplifier and digitized with a Digidata 1322A A/D converter (Axon Instruments, Sunnyvale, CA). Signals were low-pass filtered at 5 kHz, acquired at 20 kHz, stored and analyzed using Clampfit 9 (Axon Instruments). Inclusion criteria included resting membrane potential more negative than ~45 mV and overshooting action potentials with a hump on the falling phase. Solutions (mM) were the extracellular solution: 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, 10 D-glucose (pH 7.4) and pipette solution: 110 potassium gluconate, 30 KCl, 10 HEPES, 1 MgCl2, 2 CaCl2 (pH 7.25). The liquid junction potential was taken to be 12 mV, which was used for correction. Intrinsic excitability of neurons was assessed using 500-ms duration current injections to establish the rheobase (firing threshold) and the number of action potentials at twice the rheobase during the 500-ms pulse. Neurons were exposed to human Cat-S (500 nM) or vehicle (control) for 60 min prior to patch clamping.

References


Supplementary Table 1. Primary Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Conditions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Cat-B (AF965)</td>
<td>Goat</td>
<td>IP: 1.0 μg, overnight, 4°C</td>
<td>R&amp;D Systems (Minneapolis, MN)</td>
</tr>
<tr>
<td>Mouse Cat-L (AF1515)</td>
<td>Goat</td>
<td>IP: 1.0 μg, overnight, 4°C</td>
<td>R&amp;D Systems (Minneapolis, MN)</td>
</tr>
<tr>
<td>Human Cat-S (AF1183)</td>
<td>Goat</td>
<td>IF: 1:300, overnight, 4°C</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
</tr>
<tr>
<td>Cat-B (S-12 sc-6493)</td>
<td>Goat</td>
<td>IF: 1:300, overnight, 4°C</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
</tr>
<tr>
<td>Cat-L (D-20 sc-6501)</td>
<td>Goat</td>
<td>IF: 1:300, overnight, 4°C</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
</tr>
<tr>
<td>Cat-S (M-19 sc-6505)</td>
<td>Goat</td>
<td>IF: 1:300, overnight, 4°C</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
</tr>
<tr>
<td>F4/80</td>
<td>Rat</td>
<td>IF: 1:400, overnight, 4°C</td>
<td>BMA Biomedicals (Augst, Switzerland)</td>
</tr>
<tr>
<td>NeuN</td>
<td>Mouse</td>
<td>IF: 1:500, overnight, 4°C</td>
<td>Millipore (Billerica, MA)</td>
</tr>
<tr>
<td>Ox42 (M1/70)</td>
<td>Rat</td>
<td>IF: 1:200, overnight, 4°C</td>
<td>BD Pharmigen (San Diego, CA)</td>
</tr>
<tr>
<td>Lamp1</td>
<td>Rat</td>
<td>IF: 1:300, overnight, 4°C</td>
<td>ABR Affinity Bioreagents (Golden, CO)</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse</td>
<td>WB: 1:10,000, overnight, 4°C</td>
<td>Sigma-Aldrich (St Louis, MO)</td>
</tr>
<tr>
<td>c-fos</td>
<td>Rabbit</td>
<td>IH: 1:20,000, overnight, 4°C</td>
<td>Chemicon (Temecula, CA)</td>
</tr>
</tbody>
</table>

IF, immunofluorescence; IH, immunohistochemistry; IP, immunoprecipitation; WB, Western blotting.