Up-regulation of Cathepsin G in the Development of Chronic Postsurgical Pain

An Experimental and Clinical Genetic Study

Xiaodong Liu, B.Sc., M.Eng., Ph.D., Yuanyuan Tian, B.Med., M.Med., Ph.D.,
Zhaoyu Meng, B.Sc., M.Phil., Ph.D., Yan Chen, B.Sc., M.Sc., Ph.D., Idy H. T. Ho, B.Sc.,
Kwong Wai Choy, B.Sc., M.Sc., Ph.D., Peter Lichtner, Ph.D., Sunny H. Wong, M.B.Ch.B., D.Phil., M.R.C.P.,
Jun Yu, M.D., Ph.D., Tony Gin, M.D., F.R.C.A., F.A.N.Z.C.A., F.H.K.C.A.,
William K. K. Wu, M.Phil., Ph.D., F.R.C.Path., Christopher H. K. Cheng, B.Sc., Ph.D.,
Matthew T. V. Chan, M.B.B.S., Ph.D., F.A.N.Z.C.A., F.H.K.C.A.

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ABSTRACT

Background: Proteases have been shown to modulate pain signaling in the spinal cord and may contribute to the development of chronic postsurgical pain. By using peripheral inflammation in rats as a chronic pain model, the authors identified the deregulation of proteases and their inhibitors as a hallmark of chronic pain development using a genome-wide screening approach.

Methods: A microarray analysis was performed and identified spinal cathepsin G (*CTSG*) as the most up-regulated gene in rats with persistent hyperalgesia after intraplantar injection of complete Freund's adjuvant (n = 4). Further experiments were performed to elucidate the mechanisms of CTSG-induced hyperalgesia by intrathecally applying specific CTSG inhibitor (n = 10). The authors also evaluated the association between *CTSG* gene polymorphisms and the risk of chronic postsurgical pain in 1,152 surgical patients.

Results: CTSG blockade reduced heat hyperalgesia, accompanied by a reduction in neutrophil infiltration and interleukin 1β levels in the dorsal horns. In the gene association study, 246 patients (21.4%) reported chronic postsurgical pain at 12-month follow-up. Patients with AA genotypes at polymorphisms *rs2070697* (AA-15.3%, GA-24.1%, and GG-22.3%) or *rs2236742* (AA-6.4%, GA-20.4%, and GG-22.6%) in the *CTSG* gene had lower risk for chronic postsurgical pain compared with wild-types. The adjusted odds ratios were 0.67 (95% CI, 0.26 to 0.99) and 0.34 (95% CI, 0.21 to 0.98), respectively.

Conclusions: This study demonstrated that CTSG is a pronociceptive mediator in both animal model and human study. CTSG represents a new target for pain control and a potential marker to predict patients who are prone to develop chronic pain after surgery. **(Anesthesiology 2015; 123:838-50)**

CHRONIC postsurgical pain is a well-recognized complication affecting at least 10% of patients undergoing common surgical operations, such as hernia repair, mastectomy, and joint replacement surgery.[1–6] Among those who experienced chronic postsurgical pain, a substantial proportion of patients reported severe pain that adversely affected their quality of life.[2] Despite the magnitude of this problem, there is no consensus for managing chronic postsurgical pain.

Central sensitization, a phenomenon when nociceptive neurons in the spinal dorsal horns become sensitized by peripheral tissue inflammation, is important in the development of chronic pain. Further study on the molecular mechanisms of central sensitization is therefore highly warranted to identify the potential targets for the development of new biomarkers and analgesics that may be useful to predict and to treat chronic postsurgical pain, respectively.[7] Maintenance of inflammatory milieu within the dorsal horns contributes to central sensitization. Emerging evidence suggests that inflammatory mediators act as modulators of pain signal transmission in the spinal dorsal

What We Already Know about This Topic

- Central sensitization plays an essential role in the development of chronic pain in patients who undergo surgery. Management of central sensitization therefore is of substantial importance in the management of postsurgical pain.
- The development of inflammation within the spinal cord after injury has been shown to contribute to central sensitization. Of interest, a variety of proteases facilitate inflammatory cell influx into the cord.
- The authors used a genome-wide screen to identify the contribution of cathepsin G to the development of chronic pain in rodents and then identified the polymorphisms of the cathepsin G gene in humans undergoing surgery that modulated postsurgical nociception.

What This Article Tells Us That Is New

- Cathepsin G blockade reduced inflammation in the spinal cord and reduced pain behavior in rodents.
- In humans, two specific polymorphisms were associated with a lower risk for the development of chronic postsurgical pain.
- The data suggest that cathepsin G is a pronociceptive mediator in experimental subjects and humans; as such, it offers a potential therapeutic target for prevention of chronic postsurgical pain.

This article is featured in "This Month in Anesthesiology," page 1A. Corresponding article on page 745. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). The findings of

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horns.[8] The proinflammatory cytokines, such as interleukin (IL) 1β and tumor necrosis factor-α, may sensitize the ascending nociceptive pathway with or without glial cell activation.[8] For instance, the chemokine (C-X3-C motif) ligand 1 was up-regulated in spinal astrocytes after peripheral nerve injury,[9] leading to the release of IL-1β from activated microglia and thus sensitization of nociceptive neurons.[10] These processes are mediated through the signal transducer and activator of transcription 3 (Stat3)–dependent pathway. In this respect, selective Stat3 inhibition reduces the chemokine production and the associated mechanical allodynia.[11]

Proteases modulate peripheral and central inflammation by regulating chemotaxis of immune cells and production of cytokines and chemokines.[12,13] Recently, several proteases have been identified as pain modulators in the spinal cord. Inhibition of spinal matrix metalloproteinase 2 and 9 reduced neuropathic pain after peripheral nerve injury.[14] Similarly, reversal of neuropathic pain in rats could be achieved by selective inhibition of spinal microglial cathepsin S.[15] These results suggest that proteases function as modulators of pain-related cytokines and chemokines. Targeting proteases may therefore represent a new approach to regulate nociceptive transmission in the spinal cord.

In this study, using a genome-wide screening approach, we identified cathepsin G (CTSG) as an up-regulated protease in the dorsal horns. We hypothesized that an increase in CTSG activity contributed to pain hypersensitivity *via* central sensitization in a rat model of chronic inflammatory pain. To establish the clinical relevance of these findings, we evaluated the novel association between *CTSG* gene polymorphisms and the risk of chronic postsurgical pain in a large cohort of surgical patients.

Materials and Methods

The experimental protocols and surgical procedures were approved by the Animal Experimentation Ethics Committee, the Chinese University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China. Male Sprague–Dawley rats, weighting 200 to 250 g, were used in all experiments.

Reagents

We used CTSG inhibitors—tosyl phenylalanyl chloromethyl ketone (TPCK; Sigma-Aldrich, USA)—and CTSG-specific inhibitor (CatGI; Merck KGaA, Germany) to block the activity of CTSG in our experiments.[16,17] Both reagents were dissolved in dimethyl sulfoxide (Sigma-Aldrich) at concentrations of 50 mM and were stored at −20°C before use. Purified human neutrophil CTSG protein was obtained from EMD4Biosciences (Merck KGaA). CTSG protein was dissolved in a solution (2 mU/μl) containing 100 mM sodium chloride, 50 mM sodium acetate (pH 5.5), and diluted with normal saline for administration in animal (8 mU per injection) and cell experiments (2.5 mU/ml).

Induction of Peripheral Inflammation

Peripheral inflammation was induced by intraplantar injection of complete Freund's adjuvant (CFA; Sigma-Aldrich) into the left hind paw during isoflurane anesthesia. Rats in the control group were injected with 100 μl of normal saline under the same experimental conditions.

Intrathecal Catheterization and Injection

Intrathecal catheterization was performed as previously described.[11] In brief, during isoflurane anesthesia, an 18-gauge guiding needle was inserted into the intrathecal space between lumbar vertebrae 5 and 6. A PE10-polyethylene catheter was then implanted through the guiding needle until it reached the lumbar enlargement of the spinal cord. The rats were housed individually after surgery and allowed to recover for 7 days before further treatment. Drugs (CatGI, CTSG protein, or saline, 10 μl per injection) were injected through the indwelling catheter over 5 min.

Pain Behavioral Test

Thermal hyperalgesia was determined by using the plantar analgesia meter (Model 390G; IITC Life Science, USA). Each rat was placed in a Plexiglas chamber on a glass plate located above a light box. A beam of nonnoxious light was aimed onto the mid-plantar surface of left hind paw. Innocuous heat, generated by increasing light power was then applied to the hind paw. The light beam was immediately switched off when hind paw withdrawal was observed. The duration between the start of the noxious heating and paw withdrawal was defined as paw withdrawal latency (PWL). We set a maximum time limit of 20 s to prevent inadvertent tissue damage. PWL for each animal at any time point was averaged from at least three tests separated by an interval of 5 min. Investigators who determined pain behavioral response were blinded to group assignment.

Microarray Analysis

Total RNA was extracted with RNeasy lipid tissue mini kit (QIAGEN, Germany). Ten microgram of RNA extracted from each tissue was used as template for the synthesis of complementary DNA (cDNA). *In vitro* transcription (cRNA synthesis and labeling) was performed using low RNA input fluorescent linear

this study were partially presented at Anesthesiology™ 2014 annual meeting, American Society of Anesthesiologists, New Orleans, Louisiana, October 11–15, 2014, and at the 42nd Annual meeting of the Society of Neuroscience in Anesthesiology and Critical Care, New Orleans, Louisiana, October 10, 2014. Drs. Wu, Cheng, and Chan share senior authorship.

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amplification kit (Agilent Technologies, USA) in the presence of cyanine 5-cytosine triphosphate. Synthesized fluorescence-labeled cRNA was then used for oligomicroarray hybridization. The hybridization solution was prepared according to *in situ* hybridization kit plus (Agilent Technologies). Hybridization was performed using Agilent 4×44 k whole rat genome microarray (Agilent Technologies) in a hybridization oven at 60°C for 18h. The microarray scanner system (Agilent Technologies) was used for data analysis. The expression level of specific genes was determined by signal intensities. Fold change of specific gene was made between the average expression levels of two rats from the CFA group and two rats in the control group. *A priori* cutoff was set to filter out the differential genes according to the average expression levels of each data set. Accordingly, the differential gene list was selected based on the fold change (≥ 1.5) and P value (< 0.05) of unadjusted Student t test. Hierarchical cluster analyses for the whole data sets and the differential gene list were performed with GeneSpring software (Agilent Technologies). Cluster on both entities and condition and Pearson centered distance metric was considered for all the data sets during clustering.

Cell Culture

Primary astrocyte culture was performed as previously described.[11] In brief, the cortex of neonatal (1 to 2 days old) rats was collected and used for single-cell isolation. Cells were seeded onto 75 cm^2 flasks and grew to confluency. To remove the contamination of other glial cells, flasks were shaken (speed at 225 rpm) at 37°C for 8 h. Enriched astrocyte cultures were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (Life Technologies, USA) and 1% antimycotic at 37°C.

Immunofluorescence

Fresh lumbar spinal cords were harvested and fixed with 4% paraformaldehyde overnight at 4°C. Spinal cords were dehydrated in 30% sucrose solutions for 2 days (or until the tissues sink). The spinal cords were then embedded in optimal cutting temperature compound (Sakura Finetek, USA), and 20-µm sections were cut on a cryostat (Leica, Germany). A free-floating section staining method was adopted. All the sections were blocked for unspecific antibody binding sites using 5% normal calf serum in 0.01 M phosphate-buffered saline with 0.3% Triton x-100 (Sigma-Aldrich). After blocking for 1 h at room temperature, sections were incubated with the primary antibody solution overnight at 4°C, followed by incubation with the secondary antibody at room temperature for 1 h. Then, sections were transferred to the glass slides and mounted in ProLong Gold Antifade Reagent (Life Technologies). All the antibodies including antiglial fibrillary acidic protein (anti-GFAP, 1 µg/ml; Santa Cruz, USA), antimyeloperoxidase (1:300 dilution; Santa Cruz), anti-S100A9 (1:300; Santa Cruz), anti-IL-1β (1:300; R&D System, USA), and the fluorochrome-conjugated secondary antibody (1:1,000; Molecular Probes, USA) were prepared in 1% bovine serum albumin—phosphate-buffered saline with Triton x-100. All images were collected on a Zeiss laser scanning microscope (Carl Zeiss, Germany) using 20X or 40X objective with the same microscope acquisition parameters.

Quantitative Real-time Polymerase Chain Reaction

Total RNA was extracted using trizol/phenol/chloroform following a standard protocol. cDNA was prepared from 2 µg of total RNA using the high-capacity cDNA reverse transcription kit (Life Technologies). Gene levels were determined by SYBR green master mixture on an HT7900 system (Applied Biosystems, USA), with β-actin as the internal control.

Western Blots

Tissues or cells were lysed in lysis buffer (50 mM Tris, 150 nM NaCl, 1% NP-40, 1 mM EDTA, pH 7.6) containing 10% protease inhibitor cocktail. After blocking with 5% skim milk for 1 h, membranes were incubated with primary antibodies against phospho-inhibitor of κB (IκB) (1:1,000; Santa Cruz), phospho-P38 mitogen–activated protein kinases (p38MAPK) (1:1,000; Cell Signaling Technology, USA), p38MAPK (1:1,000; Cell Signaling Technology), phospho-Stat3 (1:1,000; Cell Signaling Technology), Stat3 (1:2,000; Santa Cruz), IL-1β (1:300; R&D System), or β-actin (1:3,000; Santa Cruz) overnight at 4°C. The membranes were incubated with horseradish peroxidase–conjugated anti-mouse or rabbit secondary antibody (1:3,000; Cell signaling Technology) for 1 h at room temperature. The protein bands were visualized using the enhanced chemiluminescence detection system (GE Healthcare Biosciences, USA).

Statistical Analyses for Animal Experiments

Paw withdrawal latency from behavioral assessment were analyzed among groups using ANOVA with repeated measures. Intergroup difference was tested using unpaired t test. Data from the reporter assay and quantitative reverse transcription polymerase chain reaction were compared between groups using unpaired t test. Multiple comparisons were adjusted with Bonferroni correction. All reported P values were two sided, and a P value less than 0.05 was considered statistically significant.

CTSG Gene Association Study

We collected blood samples before surgery from patients who were enrolled in an observational cohort study of chronic postsurgical pain. The details of the study objectives, design, and methods are reported at the Chinese clinical trial registry (ChiCTR-ONC-10001099). We recruited adult patients, aged 18 yr old or older who were scheduled for a wide variety of surgical procedures that include a skin incision. Patients were not eligible if they only required a pure endoscopic or radiologic procedure. Patients were also excluded if they were previously enrolled in the study or who were not expected to be available for, or to cooperate with, postoperative interviews. Patients were contacted at 12 months after surgery and were asked to rate their experience of pain over the surgical site using the brief pain inventory.[18] In addition, patients

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also reported their current health status based on the EQ-5D scale.[19] The primary outcome was pain over the surgical site that has persisted for 12 months after the index surgery.[20] We genotyped single-nucleotide polymorphisms (SNPs) with predicted minor allele frequency greater than 5% in Chinese Han population. Genotype frequencies were analyzed using the Hardy–Weinberg equilibrium analysis. We performed a multivariable logistic regression analysis to determine the effect of *CTSG* gene polymorphism on chronic postsurgical pain. The prespecified independent variables included patient's age, sex, smoking habits, history of education and employment, and pain syndrome before the index surgery. In addition, we tested the effects of anesthetic techniques (regional block, combined general and regional anesthesia *vs.* general anesthesia alone), severity of acute postoperative pain, and opioid consumption during early postoperative period on the development of chronic postsurgical pain. Given that the reported incidence of chronic postsurgical pain was at least 10%,[1–6] we determined that a cohort of more than 1,100 patients would ensure a stable regression model for evaluation of 10 variables.[21,22] Similarly, the same sample size would provide 81% power to identify SNPs with minor allele frequency 20% or greater for an odds ratio 1.4 or greater. The collection of genomic DNA and genetic analysis were approved by the Clinical Research Ethics Committee. All patients gave written informed consent.

Results

Spinal CTSG Was Up-regulated upon Induction of Chronic Inflammatory Pain in Rats: A Microarray Analysis

We evaluated the gene expression in a chronic inflammatory pain model by intraplantar injection of CFA (100 μl per rat). In this model, PWL was significantly reduced after CFA injection (P = 0.0013, ANOVA), confirming the development of chronic pain (fig. 1A). Ipsilateral lumbar dorsal

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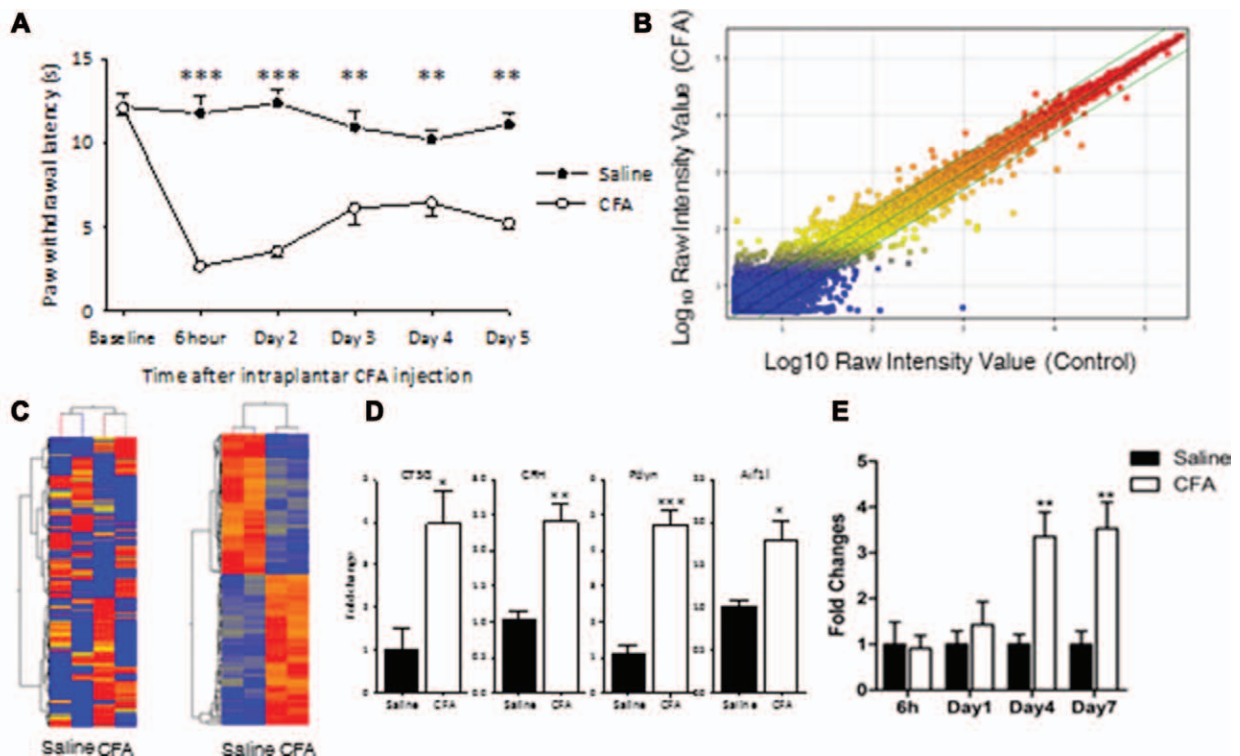

Fig. 1. Microarray analysis of lumbar dorsal horn in complete Freud's adjuvant (CFA)–induced chronic inflammatory pain in rats. (*A*) Intraplantar CFA injection induced heat hyperalgesia, n = 10 in each group. P = 0.0001, repeated-measures ANOVA, **P < 0.01, ***P < 0.001, compared with saline controls, Bonferroni-adjusted t test. (*B*) After behavioral testing, lumbar dorsal horns from two animals in each group were harvested for microarray analysis. The x-axis indicates gene expression level of control (saline treated) rats. The y-axis indicates the gene expression level of CFA-treated rats. The *color* indicates the level of expression for a specific gene. *Red* shows genes that are highly expressed, whereas *blue* indicates low level of expression. The *green lines* indicate the threshold for gene selection (≥1.5 fold change). Genes outside of the *green lines* were selected as the differential genes, unadjusted P < 0.05. (*C*) Hierarchical cluster analysis for the whole data set (*left*) and the list of differential genes (*right*). (*D*) Validation of differential gene expression using quantitative reverse transcription polymerase chain reaction from harvested lumbar dorsal horns. Spinal expression of prodynorphin (Pdyn), corticotropin-releasing hormone (CRH), allograft inflammatory factor 1-like (Aif1l), and cathepsin G (CTSG) was confirmed to be up-regulated in response to CFA-induced chronic inflammatory pain, n = 4, *P < 0.05, **P < 0.01, ***P < 0.001. (*E*) Changes of *CTSG* gene expression over time in the spinal dorsal horn. Up-regulation of CTSG mRNA was observed on day 4 after CFA treatment and persisted on day 7, n = 4 at each time point, **Bonferroni-adjusted P < 0.01. *Error bars* represent SEM.

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horn at L4 to L6 was then harvested for microarray analysis. To preserve the migrated blood-borne cells, perfusion was avoided before tissue harvest.[23] Total tissue RNA was then extracted, and the expression level of specific genes was determined according to the average signal intensities (fig. 1B). A total of 169 annotated genes exhibited 1.5 or greater fold change between groups. Hierarchical cluster analysis demonstrated distinct gene expression patterns in dorsal horn tissues harvested from CFA- and saline-injected rats (fig. 1C). Among these, 87 genes were up-regulated and 82 genes were down-regulated after CFA injection. Ontology analysis revealed that 9 of the 169 genes (5.3%) were proteases or protease inhibitors (Supplemental Digital Content 1, http://links.lww.com/ALN/B188). Although none of the candidate genes had reached statistical significance in a Bonferroni-adjusted analysis, *CTSG* expression exhibited the highest fold change (6.17 fold), and further experiments were conducted to determine its role in spinal cord pain modulation.

As an independent platform to validate the findings from our microarray analysis, the mRNA expression of four selected genes, including *CTSG*, was measured by reverse transcription polymerase chain reaction (fig. 1D). *CTSG* gene was up-regulated (4.7 fold, $P < 0.001$, Bonferroni-adjusted *t* test) in the dorsal horns of CFA-injected rats (n = 4) compared with controls (n = 4). Furthermore, experiment was performed to explore the changes of *CTSG* gene expression over time (n = 4 in each time point). Figure 1E shows that *CTSG* expression was only increased 4 days after CFA injection and persisted on day 7. This result suggested that up-regulation of *CTSG* in the spinal cord contributed to the maintenance of chronic inflammatory pain. Interestingly, *CTSG* expression was not altered in a rat neuropathic pain model with chronic constriction injury to the sciatic nerve (Supplemental Digital Content 2, http://links.lww.com/ALN/B189). Finally, up-regulation of genes that are known to be associated with chronic pain, namely prodynorphin (4.0 fold, $P = 0.027$), corticotropin-releasing hormone (2.4 fold, $P = 0.009$), and allograft inflammatory factor 1-like (1.8 fold, $P = 0.021$), was confirmed in our CFA model.

CTSG Inhibition Reduced CFA-induced Chronic Inflammatory Pain in Rats

We then evaluated the effect of CTSG inhibition on the development of chronic inflammatory pain. Rats were randomly assigned to receive subcutaneous injection of either vehicle or CTSG inhibitor TPCK[16,17] 1 h before CFA injection (n = 12 in each group). Heat hyperalgesia was detected within 30 min after intraplantar CFA injection and persisted for the subsequent 5 days. Pretreatment with a single subcutaneous injection of TPCK reduced hyperalgesia compared with vehicle (fig. 2A). The data suggested that CTSG contributed to pain hypersensitivity in CFA-induced chronic inflammation.

Similarly, we tested whether CTSG inhibition might reverse chronic inflammatory pain in a posttreatment model. Rats (n = 14 in each group) were randomly assigned to receive vehicle or TPCK 4 days after intraplantar CFA injection. Treatment was given twice daily with a subcutaneous injection 1 h before behavioral testing and 12 h after the first injection. Figure 2B shows that PWL was similar between groups before treatment on day 4. After TPCK injections, heat hyperalgesia was significantly reduced on subsequent days. The data confirmed the therapeutic effect of CTSG inhibition on chronic inflammatory pain.

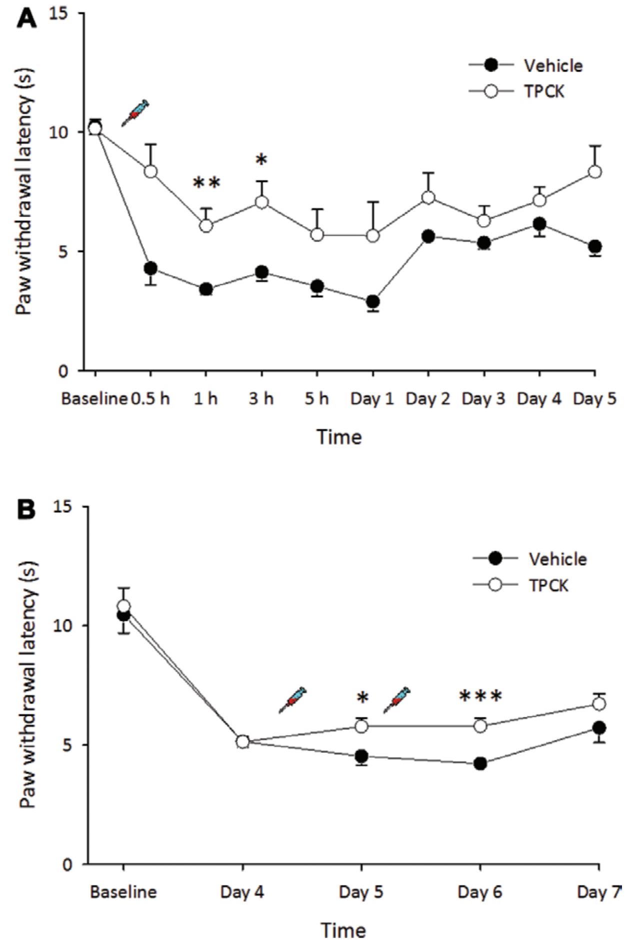

Fig. 2. Inhibition of cathepsin G attenuated complete Freud's adjuvant (CFA)–induced heat hyperalgesia in rats. (*A*) Pretreatment with subcutaneous injection of cathepsin G inhibitor tosyl phenylalanyl chloromethyl ketone (TPCK) attenuated CFA-induced heat hyperalgesia compared with controls using dimethyl sulfoxide as vehicle, n = 12, $P < 0.0001$, repeated-measures ANOVA. (*B*) Postadministration of TPCK on day 4 after intraplantar CFA injection reversed heat hyperalgesia, n = 14, $P < 0.05$, repeated-measures ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Bonferroni-adjusted *t* test. *Error bars* represent SEM.

Potential Mechanisms: CTSG Inhibition Reduced Neutrophil Infiltration to the Spinal Cord after CFA Injection

Because CTSG has been implicated in neutrophil chemotaxis,[24] we hypothesized that CTSG contributes to the development of pain hypersensitivity by promoting neutrophil-mediated inflammation in the spinal cord. In this experiment, we evaluated the effects of CTSG inhibition on

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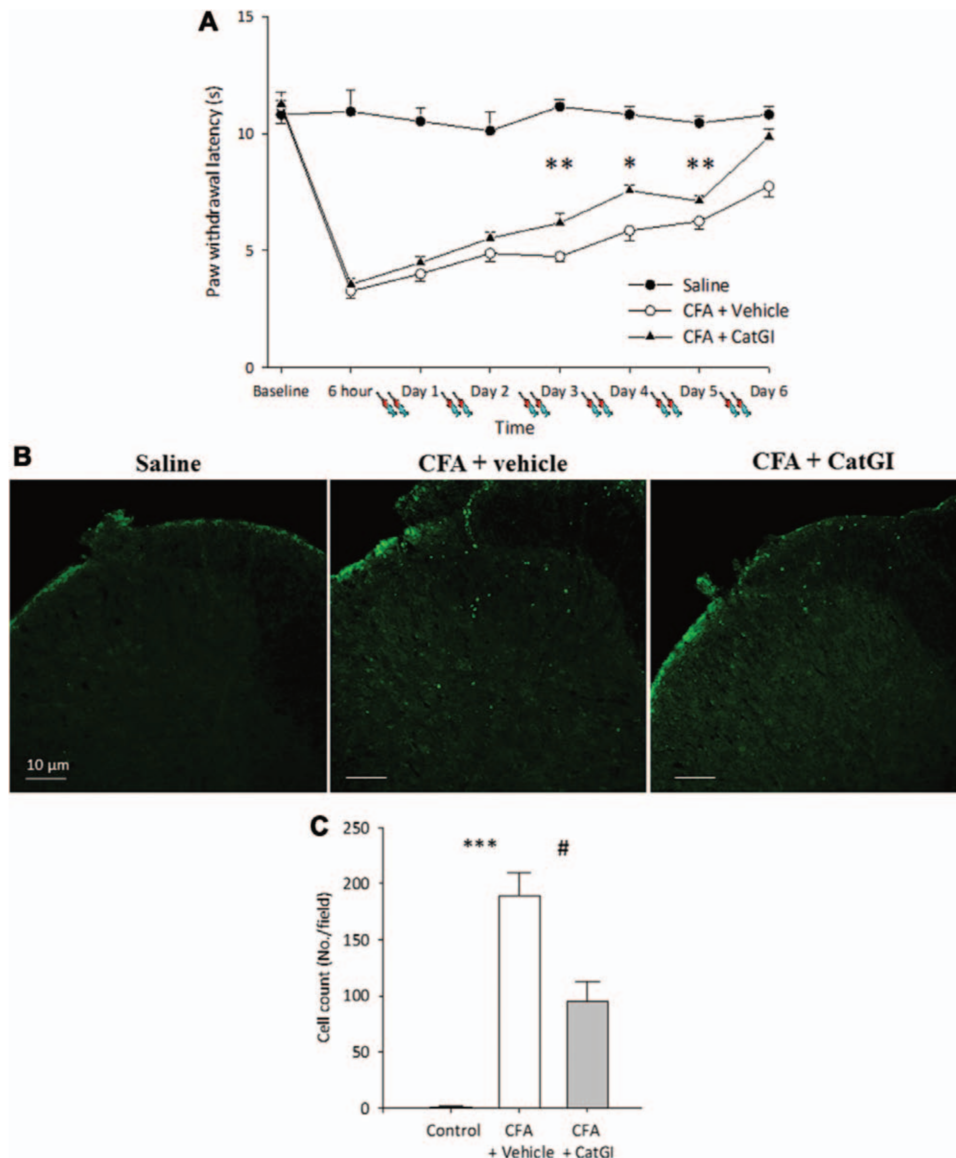

Fig. 3. Cathepsin G (CTSG) inhibition reduced heat hyperalgesia by limiting neutrophil infiltration to spinal cord. (*A*) Daily intrathecal injection of CTSG-specific inhibitor (CatGI) attenuated complete Freud's adjuvant (CFA)–induced persistent pain compared with vehicle controls. Intraplantar saline injection as negative controls, n = 10 in each group, $P < 0.001$, repeated-measures ANOVA. *$P < 0.05$, **$P < 0.01$, Bonferroni-adjusted *t* tests. (*B*) CTSG contributed to neutrophil infiltration into the spinal cord. Spinal cord sections were stained for myeloperoxidase (*green*). Four days after CFA injection, neutrophil (myeloperoxidase-positive cells) infiltration was increased in the spinal cord. Administration of CatGI reduced the amount of neutrophil infiltration. (*C*) Neutrophil (myeloperoxidase-positive cells) cell counts in the spinal cord. The number of neutrophils in the spinal cord dorsal horn was significantly increased after intraplantar CFA injection, $P < 0.001$, ANOVA. Daily intrathecal injection of CatGI significantly decreased neutrophil counts. A total of nine sections from three rats in each group were counted. ***$P < 0.001$ compared with control, *$P < 0.05$ compared with CFA and vehicle group, Bonferroni-adjusted *t* tests. *Error bars* represent SEM.

heat hyperalgesia (fig. 3A) and neutrophil trafficking (fig. 3, B and C) in the spinal dorsal horns. Using an implanted intrathecal microcatheter, CatGI (a specific CTSG inhibitor) or saline (vehicle) was injected twice daily to rats with CFA-induced chronic inflammatory pain (n = 10 in each group). In addition, rats with intraplantar injection of saline was used as negative controls (n = 10). CatGI instead of TPCK was used for intrathecal injection because of its higher water solubility.

Compared with negative controls, the number of neutrophils in the dorsal horns, as revealed by myeloperoxidase immunofluorescence staining, was significantly increased after intraplantar CFA injection (fig. 3, B and C). The changes corresponded to the induction of heat hyperalgesia (fig. 3A). Under fluorescence microscopy, neutrophils were distributed over both the superficial and deep layers of the spinal dorsal horn (fig. 3B). In addition, we found a substantial proportion of neutrophils

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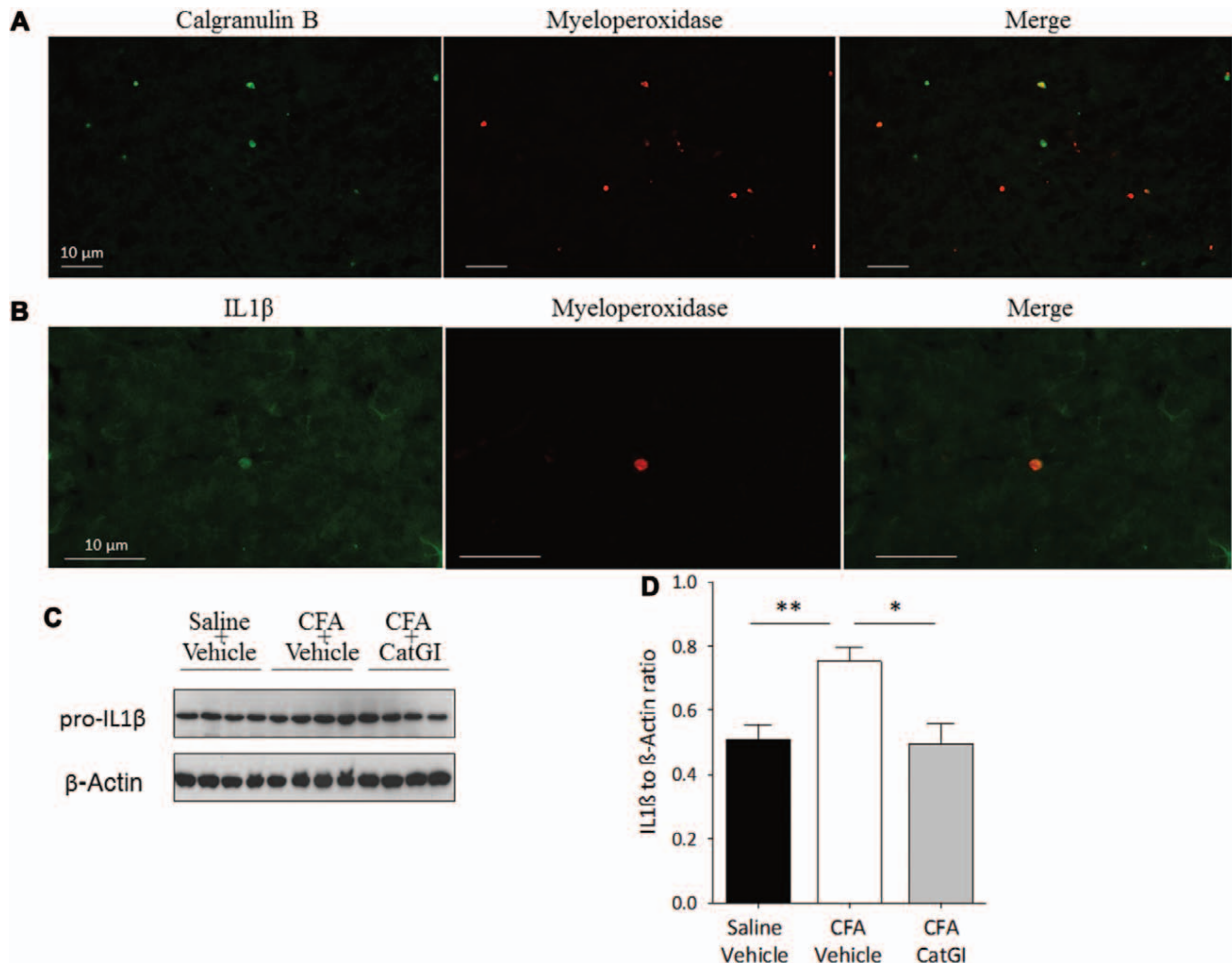

Fig. 4. Complete Freud's adjuvant (CFA)–induced chronic inflammatory pain increased the number of S100A9-immunoreactive cells in the rat spinal cord. (*A*) A subpopulation of myeloperoxidase (*red*)–positive cells was colabeled with calgranulin B (S100A9, *green*), indicating that these cells were involved in the production of proinflammatory mediators. (*B*) Interleukin (IL)-1β (*green*) was colabeled with myeloperoxidase (*red*). This finding showed that neutrophil was a source of IL-1β in the spinal cord. (*C*) Intrathecal injection of cathepsin-G-specific inhibitor (CatGI) reduced IL-1β expression. CatGI = 100 μM in 10 μl, n = 10 in each group. (*D*) Densitometric analysis of Western blots. $^{**}P < 0.01$ compared with saline with vehicle group, and $^{*}P < 0.05$ compared with CFA with vehicle group, Bonferroni-adjusted *t* tests. *Error bars* represent SEM.

expressed calgranulin B (S100A9; fig. 4A), indicating that these neutrophils were involved in the production of proinflammatory mediators.

In contrast, heat hyperalgesia was significantly decreased with CTSG inhibition (fig. 3A). Similarly, the number of neutrophils was reduced in the spinal dorsal horns after intrathecal injection of CatGI (fig. 3, B and C; n = 10). Using Western blot analysis, we demonstrated that total IL-1β level in the spinal dorsal horns was reduced (fig. 4, B–D; n = 10). These data suggested that CTSG is important in the modulation of neutrophilic infiltration to the spinal dorsal horns after peripheral inflammation. These neutrophils act as a source of inflammatory molecules such as IL-1β in the spinal cord that is essential to the maintenance of chronic inflammatory pain (fig. 4B and Supplemental Digital Content 3, http://links.lww.com/ALN/B190).

CTSG Aggravated the Activation of Spinal Astrocytes by Peripheral Inflammation

Previous studies have demonstrated the role of astrocytes in maintaining an inflammatory milieu during the development of chronic pain.[25,26] In this experiment, we tested whether CTSG could activate spinal astrocytes to release proinflammatory mediators. Using cultured spinal astrocytes, treatment with CTSG protein increased phosphorylation of IκB, p38MAPK, and Stat3 in a time-dependent manner (fig. 5A). All of these are important inflammatory mediators necessary for the induction and maintenance of chronic pain.[8]

We next tested whether CTSG treatment could activate astrocytes *in vivo*. After implantation of an intrathecal microcatheter, rats were randomly allocated to receive purified CTSG protein or an equivalent volume of saline (n = 10 per group). Four days later, rats were sacrificed

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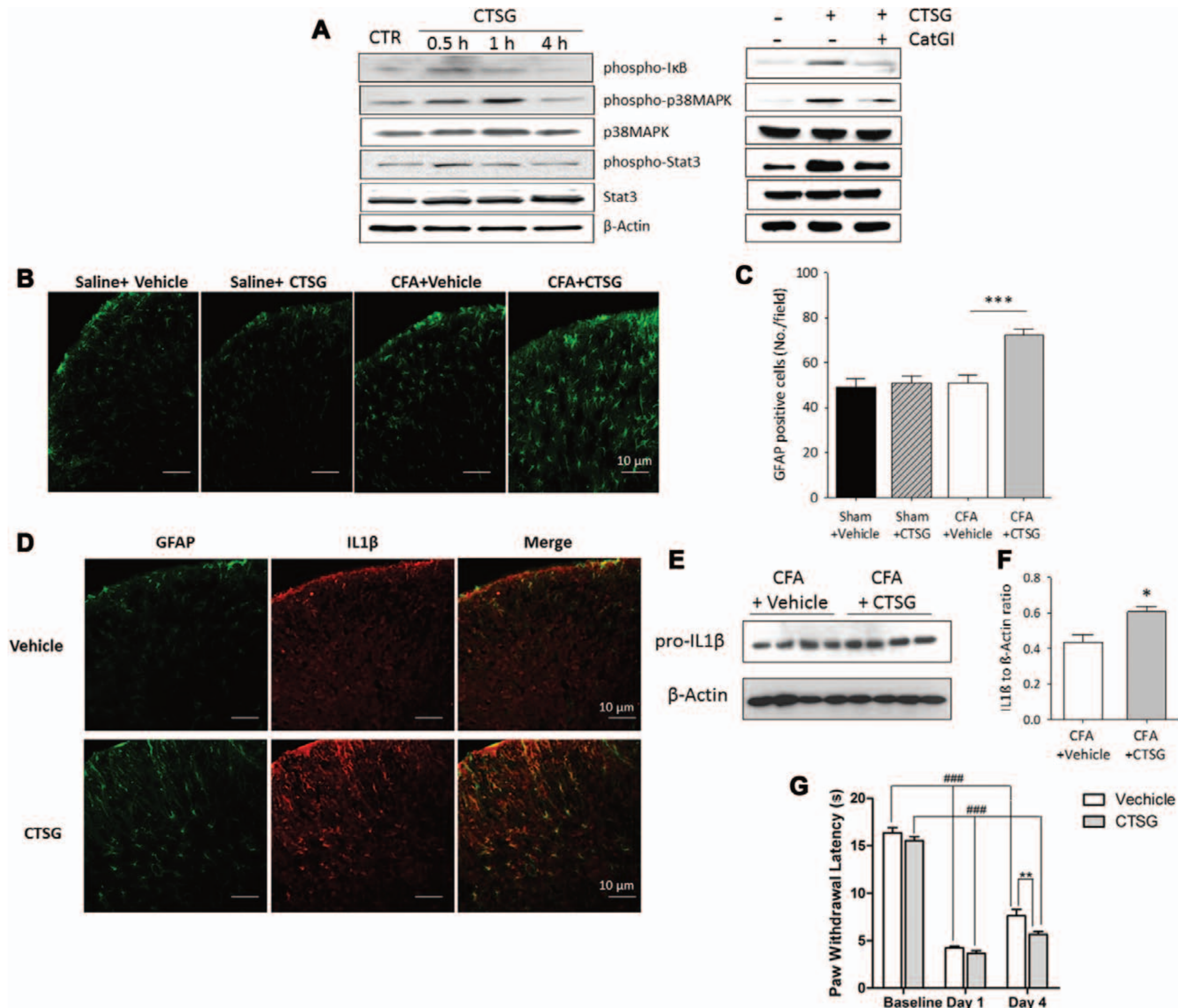

Fig. 5. Cathepsin G (CTSG) and astrocyte reactivation. (*A*) CTSG treatment activated signaling proteins in cultured astrocytes, with > 97% antiglial fibrillary acidic protein (GFAP)–positive cells. CTSG-specific inhibitor (CatGI) pretreatment reversed signaling activation by CTSG (CTR = control). (*B*) Microphotographs of GFAP staining of spinal cord sections. Compared with vehicle, intrathecal injection of purified CTSG protein had no effect on GFAP density in the spinal dorsal horn. However, when CTSG protein was injected to rats that were previously treated with intraplantar complete Freud's adjuvant (CFA) injection, GFAP density in the spinal cord was significantly increased (n = 10 in each group). (*C*) The number of GFAP-positive cells in the spinal cord after intrathecal CTSG treatment was only increased in rats previously treated with intraplantar CFA injection, suggesting that CTSG alone is insufficient to reactivate the spinal astrocytes, $P < 0.001$, repeated-measures ANOVA, n = 10 in each group, ***$P < 0.001$ compared with saline, Bonferroni-adjusted *t* tests. (*D*) Compared with vehicle, CTSG treatment increased interleukin (IL)-1β expression in spinal astrocytes, n = 10 in each group. (*E*) Western blot confirmed the up-regulation of IL-1β in spinal cord after CatGI treatment. n = 10 in each group. (*F*) Densitometric analysis of Western blots. *$P < 0.05$ compared with CFA with vehicle group, unpaired *t* test. (*G*) Intrathecal injection of purified CTSG protein increased CFA-induced heat hyperalgesia on day 4. ###$P < 0.0001$ compared with baseline, repeated-measures ANOVA, n = 10 in each group; **$P < 0.01$ compared with CFA vehicle group on day 4, Bonferroni-adjusted *t* test. *Error bars* represent SEM. p38MAPK = P38 mitogen–activated protein kinase.

and spinal sections were harvested for GFAP immunofluorescence staining. Concordant with our *in vitro* data, intrathecal injection of CTSG protein aggravated the increases in the number and cell size of spinal astrocytes in rats previously injected with CFA (fig. 5, B and C). However, it is noteworthy that intrathecal administration of CTSG protein *per se* had no effect on GFAP density in the spinal dorsal horn in the absence of CFA injection (fig. 5, B and C). In CFA-injected rats, intrathecal administration of CTSG protein was also associated with an increase in IL-1β mRNA expression (data not shown). The protein expression of IL-1β in the spinal dorsal horns was also increased and exhibited colocalization with GFAP (fig. 5, D–G). Overall, the data suggested that CTSG aggravated the activation of spinal astrocytes by peripheral inflammation.

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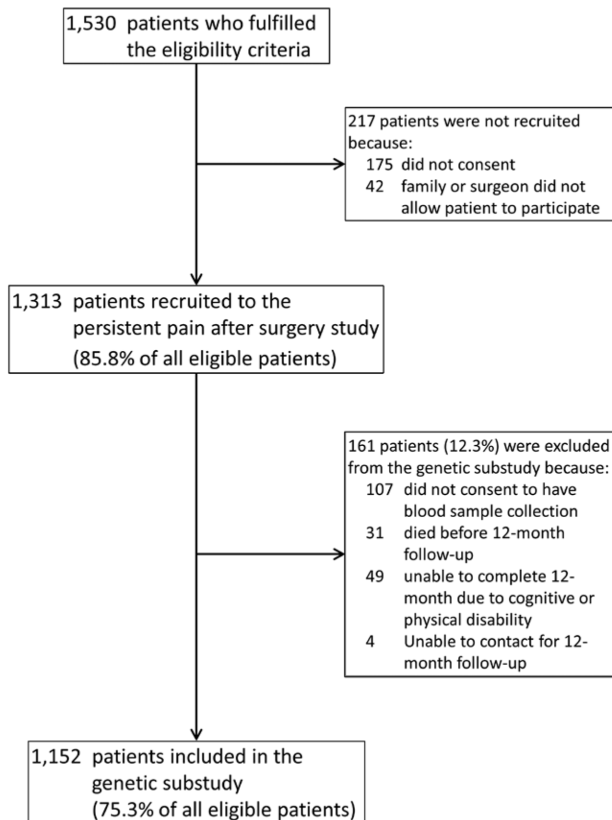

Fig. 6. Study flow chart of persistent pain after surgery.

CTSG Polymorphisms Were Associated with Lower Risks of Chronic Postsurgical Pain

Given that CTSG acted as a pronociceptive mediator in our animal experiments, we conducted a candidate gene association study to determine whether variants in the *CTSG* gene were associated with an altered risk of chronic pain after surgery. Between January 2011 and November 2012, we recruited a total of 1,313 patients to an observational cohort study on the characteristics and epidemiology of chronic postsurgical pain. Among these patients, 1,152 were also enrolled to a genetic substudy (fig. 6). Patient characteristics, surgical details, severity of acute pain, and opioid consumption during the 3 days after surgery are summarized in table 1. A total of 246 patients (21.4%) reported persistent wound pain since the index surgery. At 12-month follow-up, the average (± SD) pain score was 3.2 ± 2.0 (0 = pain free, 10 = worst imaginable pain). Chronic postsurgical pain produced significant interference to all attributes of daily activities (table 2), and this was associated with poorer quality of life (table 3). All chronic pain patients were taking gabanoids, tricyclic antidepressants, nonsteroidal antiinflammatory drugs, and tramadol regularly. Eighty-five patients (34.6%) were undergoing cognitive behavioral therapy training.

Genotyping was performed for all SNPs in the *CTSG* gene with reported minor allele frequency greater than 5% in the Chinese Han population. Only two SNPs (*rs2070697* and *rs2236742*) fulfilled the inclusion criteria. Both SNPs were significantly associated with chronic postsurgical pain. Patients who were homozygous carriers of the A allele for either of the SNPs had a lower rate of chronic postsurgical pain compared with wild-type (table 4). In a multivariable regression model, the protective effect of both SNPs remained significant when adjusted for age, sex, smoking habit, employment history, education level, prior pain syndrome, and severity of acute pain after index surgery. The area under the receiver operating characteristic curve for our multivariable regression model was 0.71 (95% CI, 0.67 to 0.85).

Discussion

In this study, we have identified CTSG as a pronociceptive mediator in the development of pain hypersensitivity in an animal model of chronic inflammatory pain. Upon peripheral inflammation, CTSG expression in the spinal dorsal horn was significantly up-regulated. Inhibition of CTSG attenuated chronic inflammation-associated hyperalgesia, and this was accompanied with a decrease in neutrophilic infiltration and a lower level of IL-1β in the spinal dorsal horn. We further demonstrated the relevance of CTSG gene polymorphisms in the development of chronic postsurgical pain. The risk of chronic postsurgical pain was significantly reduced in patients carrying homozygous Aalleles of SNP *rs2236742* or *rs2070697* in the *CTSG* gene. Taken together, our data suggest that CTSG modulates pain hypersensitivity and is a potential target for pain management.

We found an increase in spinal cord neutrophils after induction of peripheral inflammation. This is consistent with the large body of evidence showing infiltration of mast cells, lymphocytes, and neutrophils in the dorsal root ganglion and spinal dorsal horn during the development of chronic pain.[8,23,27,28] In addition, we observed a subpopulation of neutrophils expressing high levels of S100A9 and IL-1β, indicating that neutrophils constitute an important source of inflammatory mediators in the initiation and maintenance of central sensitization.[23] Our study suggests that inhibition of CTSG could reduce the pain signaling, manifested as a decrease in heat hyperalgesia, by lowering neutrophil trafficking and IL-1β levels in the spinal cord. In the current study, we did not evaluate the effects of CTSG on mechanical stimulation. Nevertheless, CTSG inhibition would be expected to decrease allodynia because of its suppressive effect on IL-1β production in the spinal cord, which in turn, has been reported as an important modulator of mechanical allodynia.[14] Previous studies have demonstrated the chemotactic properties of CTSG.[29–31] Interestingly, CTSG released from the spinal cord neutrophils has been shown to modulate the secretion of other chemokine, such as chemokine (C-X3-C motif) ligand 2, that are important for further neutrophil migration.[32] In this regard, it is highly likely that CTSG serves as a proinflammatory mediator for signal amplification during the development of central sensitization in the spinal cord. It should be noted that CTSG

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Table 1. Patient Characteristics, Surgical Details, and Early Postoperative Pain

Demographic Variables	All Patients	Patients without Pain	Patients with Chronic Postsurgical Pain	*P* Values
Number of patients	1,152	906	246	
Male sex	551 (47.8)	399 (44.0)	152 (61.8)	0.004
Age (yr)	61.3 ± 14.7	61.8 ± 14.9	60.3 ± 13.6	0.015
Weight (kg)	62.4 ± 13.5	62.2 ± 13.4	64.8 ± 11.7	0.065
Unemployed	829 (72.0)	664 (73.3)	165 (67.1)	0.060
Years of education completed				0.059
Never went to school	130 (11.3)	92 (10.2)	38 (15.4)	
0–6 yr	284 (24.7)	224 (24.7)	60 (24.4)	
7–12 yr	420 (36.5)	345 (38.1)	75 (30.5)	
>12 yr	318 (27.6)	245 (27.0)	73 (29.7)	
Preexisting comorbidity				
Cardiovascular disorder	57 (4.9)	44 (4.9)	13 (5.3)	0.913
Hypertension	277 (24.0)	221 (24.4)	56 (22.8)	0.655
Diabetes mellitus	159 (13.8)	130 (14.3)	29 (11.8)	0.353
Current smoker	312 (27.1)	247 (27.3)	65 (26.4)	0.790
Pain elsewhere before surgery	121 (10.5)	96 (10.6)	25 (10.2)	0.840
Surgical types				
Vascular surgery	230 (20.0)	171 (18.9)	59 (24.0)	0.091
Thoracic surgery	105 (9.1)	79 (8.7)	26 (10.6)	0.441
Orthopedic surgery	178 (15.5)	146 (16.1)	32 (13.0)	0.273
General surgery	245 (21.3)	204 (22.5)	41 (16.7)	0.057
Urologic or gynecologic surgery	178 (15.5)	141 (15.6)	37 (15.0)	0.919
Neurosurgery/spine surgery	127 (11.1)	110 (12.1)	27 (11.0)	0.930
Others	89 (7.5)	70 (7.7)	19 (7.7)	0.894
Anesthetic techniques				0.540
General anesthesia	968 (84.0)	765 (84.6)	203 (81.9)	
Neuraxial and regional blocks	61 (5.3)	47 (5.2)	14 (5.6)	
Combined general and regional anesthesia	123 (10.7)	92 (10.2)	31 (12.5)	
Cumulative opioid consumption during the first 3 days after surgery, morphine equivalent (mg)				
Median (IQR)	14.5 (11.5–17.5)	14.8 (12.0–17.5)	14.5 (10.0–17.5)	0.940
Mean ± SD	17.8 ± 17.1	17.9 ± 15.2	17.8 ± 21.2	
Average pain score during the first 3 days after surgery				
Median (IQR)	2 (0–4)	2 (0–4)	3 (0–6)	<0.001
Mean ± SD	2.4 ± 2.3	2.2 ± 2.1	3.1 ± 2.5	
Patients with severe acute postoperative pain*	243 (21.1)	180 (19.8)	66 (27.3)	0.005

Values are number (%), mean ± SD, or median (IQR).
* Average pain score > 5 (out of 10) during the first 3 days after surgery.
IQR = interquartile range.

Table 2. Impact of Chronic Postsurgical Pain

Interference Items	Number of Patients Reporting Interference	Interference Score
Normal work	106 (43.2)	1.87 ± 2.70
Walk	140 (56.8)	2.34 ± 2.76
Relation with others	62 (25.5)	0.83 ± 1.82
Enjoyment of life	123 (50.0)	2.11 ± 2.77
General activity	131 (53.4)	2.48 ± 3.00
Mood	146 (59.3)	2.46 ± 2.71
Sleep	117 (47.9)	2.19 ± 2.86

Interference of daily activities was scored by modified brief pain inventory. Scores ranges from 0 = no interference to 10 = completely interfered. Values are number (%) or mean ± SDs.

expression was not changed in dorsal root ganglion after intraplantar CFA injection. Furthermore, although local subcutaneous administration of CatGI reduced paw edema after CFA injection, intrathecal injection of CatGI did not produce the same effect (Supplemental Digital Content 4, http://links.lww.com/ALN/B191), despite significant attenuation of heat hyperalgesia (fig. 3A). Taken together, these findings suggest that CTSG act as a mediator of central sensitization in chronic inflammatory pain.

Reactivation of spinal astrocytes is one of the hallmarks in the development of chronic pain.[8,25,26] Our *in vitro* study in astrocytes showed that treatment with CTSG increased IκB phosphorylation—a marker of astrocyte reactivation. In these astrocytes, CTSG also increased the phosphorylation levels of Stat3 and p38MAPK in a time-dependent manner. It is commonly observed that activation of transmembrane receptor signaling results in Stat3 and p38MAPK phosphorylation.[11,33] Because previous studies have shown that

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Table 3. General Health Status Measured by EQ-5D in Patients with and without Chronic Postsurgical Pain at 12 Months after Index Surgery

	Proportion of Patients with Some Difficulty in EQ-5D Item		
EQ-5D Items	Patients with Chronic Postsurgical Pain	Patients without Chronic Postsurgical Pain	*P* Values
Total number of patients	246	906	
Motion	56 (22.7)	109 (12.0)	0.010
Self-care	32 (12.8)	70 (7.7)	0.200
Usual activities	46 (18.7)	92 (10.1)	0.010
Pain/discomfort	56 (22.9)	94 (10.4)	<0.001
Anxiety/depression	71 (28.9)	186 (20.5)	0.120
EQ-5D visual analog scale*	69 ± 19	76 ± 47	0.028

Values are proportion (%) or mean ± SD.
* EQ-5D visual analog scale was measured by a 100-mm linear scale, where 0 = worst imaginable health state and 100 = best possible health state.

Table 4. Factors Associated with Chronic Postsurgical Pain

			Univariate		Multivariate	
Factors	Total No. of Patients	Patients with Chronic Postsurgical Pain	Odds Ratio (95% CI)	*P* Values	Odds Ratio (95% CI)	*P* Values
Age (yr)						
≥65	455	80 (17.6)	Reference			
<65	697	166 (23.8)	1.46 (1.09–1.97)	0.011	1.48 (1.01–2.22)	0.041
Sex						
Female	601	94 (15.6)	Reference			
Male	551	152 (27.6)	2.05 (1.54–2.74)	0.004	1.17 (1.02–3.02)	0.039
Education						
Never went to school	130	38 (29.5)	Reference			
0–6 yr	284	60 (21.1)	0.64 (0.40–1.04)	0.070		
7–12 yr	420	75 (17.9)	0.53 (0.23–1.18)	0.421		
>12 yr	318	73 (23.0)	0.72 (0.46–1.14)	0.160		
Pain history						
No	1,031	221 (21.4)	Reference			
Yes	121	25 (20.7)	0.96 (0.60–1.54)	0.840		
Employment						
No	829	165 (19.9)	Reference			
Yes	323	81 (25.1)	1.34 (0.99–1.82)	0.061		
Smoking history						
No	840	181 (21.5)	Reference			
Yes	312	65 (20.8)	0.96 (0.70–1.32)	0.789		
Anesthetic technique						
General anesthesia	968	203 (21.0)	Reference			
Region anesthesia	61	14 (22.9)	1.12 (0.73–1.69)	0.836		
Combined technique	123	31 (25.2)	1.27 (0.56–2.85)	0.337		
Cumulative opioid consumption, morphine equivalent (mg)	1,152	17.8 ± 21.2	1.12 (0.81–1.53)	0.940		
Severe acute postoperative pain						
No	909	180 (19.8)	Reference		Reference	0.052
Yes	243	66 (27.1)	1.51 (1.02–2.21)	0.016	1.43 (0.63–3.21)	
rs2236742						
GG	792	179 (22.6)	Reference			
GA	318	65 (20.4)	0.87 (0.64–1.21)	0.467		
AA	31	2 (6.4)	0.24 (0.05–0.99)	0.030	0.34 (021–0.98)	0.043
rs2070697						
GG	372	83 (22.3)	Reference			
GA	536	129 (24.1)	1.10 (0.80–1.51)	0.539		
AA	222	34 (15.3)	0.63 (0.41–0.98)	0.038	0.67 (0.26–0.99)	0.044

Values are number (%) or mean ± SDs.
AA = homozygous variant; GA = heterozygous; GG = wild type.

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CTSG is capable of transactivating transmembrane receptors including formyl peptide receptor and epidermal growth factor receptor,[34,35] we believe that CTSG may activate spinal astrocytes by a similar mechanism to initiate the Stat3 and p38MAPK signaling pathway. In this regard, administration of purified CTSG aggravated astrocyte activation induced by peripheral inflammation. Because CTSG administration in the absence of CFA injection had negligible effect on astrocyte activation, it is highly possible that other cofactors are involved in this process.

Chronic postsurgical pain occurred in 21.4% of patients in our cohort. This finding is comparable with previous reports showing that 10 to 30% of patients experienced persistent pain after surgery.[1,2,5] We have shown that patients carrying homozygous A allele of SNPs *rs2070697* and *rs2236742* in the *CTSG* gene were associated with a lower risk for chronic postsurgical pain. These results are consistent with our animal data. Our finding, however, cannot precisely locate the SNPs that regulate the transcription of CTSG because both SNPs are found in the introns of the gene (Supplemental Digital Content 5, http://links.lww.com/ALN/B192). Therefore, they may merely represent positional markers of the underlying functional SNP. Resequencing of the exon regions is currently underway to reveal the locus that may interfere with functional characteristics of the CTSG protein. In addition to *CTSG* polymorphisms, our multivariate regression model showed that male patients and patients younger than 65 yr were at a higher risk for chronic postsurgical pain. This is consistent with some studies but not with others.[4,36] Patients reporting severe pain early after surgery also tended to develop chronic postsurgical pain, but this did not reach statistical significance in our multivariate model. Both unemployment and low level of education were thought to be risk factors in chronic pain syndrome.[37] However, we were unable to demonstrate the impact of these factors on chronic postsurgical pain, and this may be related to a cultural issue where the local Chinese community is less dependent on unemployment benefits.

In summary, we have shown that CTSG was up-regulated in spinal dorsal horns after peripheral inflammation. CTSG contributed to the development of pain hypersensitivity owing to its chemotactic properties, leading to an increase in neutrophils and release of inflammatory mediators in the spinal cord. CTSG also promoted astrocyte activation upon peripheral inflammation. Variations in the *CTSG* gene were associated with reduced risks of chronic postsurgical pain in two independent groups of patients undergoing surgical procedures. CTSG represents a potential target for chronic pain intervention. Furthermore, preoperative determination of CTSG gene polymorphisms may facilitate perioperative physicians to formulate an appropriate plan to prevent chronic postsurgical pain.

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Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Chan: Department of Anaesthesia and Intensive Care, The Chinese University of Hong Kong, 4/F Main Clinical Block and Trauma Centre, Prince of Wales Hospital, Hong Kong Special Administrative Region, People's Republic of China. mtvchan@cuhk.edu.hk. This article may be accessed for personal use at no charge through the Journal Web site, www.anesthesiology.org.

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