

Role of Spinal CXCL1 (GRO α) in Opioid Tolerance

A Human-to-rodent Translational Study

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ABSTRACT

Background: The pivotal role of glial activation and up-regulated inflammatory mediators in the opioid tolerance has been confirmed in rodents but not yet in humans. Here, the authors investigated the intraspinal cytokine and chemokine profiles of opioid-tolerant cancer patients; and to determine if up-regulated chemokines could modify opioid tolerance in rats.

Methods: Cerebrospinal fluid samples from opioid-tolerant cancer patients and opioid-naïve subjects were compared. The cerebrospinal fluid levels of tumor necrosis factor- α , CXCL1, CXCL10, CCL2, and CX3CL1 were assayed. The rat tail flick test was utilized to assess the effects of intrathecal CXCL1 on morphine-induced acute antinociception and analgesic tolerance.

Results: CXCL1 level in cerebrospinal fluid was significantly up-regulated in the opioid-tolerant group ($n = 30$, 18.8 pg/ml *vs.* 13.2 pg/ml, $P = 0.02$) and was positively correlated ($r^2 = 0.49$, $P < 0.01$) with opioid dosage. In rat experiment, after induction of tolerance by morphine infusion, the spinal cord CXCL1 messenger RNA was up-regulated to 32.5 ± 11.9 -fold. Although CXCL1 infusion alone did not affect baseline tail-flick latency, the analgesic efficacy of a single intraperitoneal injection of morphine dropped significantly on day 1 to day 3 after intrathecal infusion of CXCL1. After establishing tolerance by intrathecal continuous infusion of morphine, its development was accelerated by coadministration of CXCL1 and attenuated by coadministration of CXCL1-neutralizing antibody or CXCR2 antagonist.

Conclusions: CXCL1 is up-regulated in both opioid-tolerant patients and rodents. The onset and extent of opioid tolerance was affected by antagonizing intrathecal CXCL1/CXCR2 signaling. Therefore, the CXCL1/CXCR2 signal pathway may be a novel target for the treatment of opioid tolerance. (**ANESTHESIOLOGY 2015; 122:666-76**)

OPIOID analgesics are the mainstay of treatment for severe pain, especially cancer pain.¹ However, the efficacy of long-term opioid analgesics is progressively attenuated by tolerance. Classical neuron-centered concepts such as internalization of opioid receptors,² up-regulation of *N*-methyl-D-aspartate receptor function,³ or down-regulation of glutamate transporter activity⁴ can only partially explain the phenomenon. Recent evidence showing glial activation and up-regulated inflammatory mediators in the rodent central nervous system (CNS) has confirmed the pivotal role of neuroinflammation in neuropathic pain or opioid tolerance, or both.^{5,6} Microglia are derived from bone marrow during the perinatal period and are reported to participate in neuropathic and postoperative pain and opioid tolerance.⁷ Intrathecally administered microglial inhibitor minocycline can prevent the development but fails to attenuate established morphine tolerance.⁸ Astrocytes not only metabolically support neurons in the CNS but also have active roles in multiple pathological conditions such as stroke, seizure,

What We Already Know about This Topic

- Opioid tolerance limits the utility of chronic opioid therapy
- Chemokines produced by spinal cord tissue may contribute to opioid tolerance

What This Article Tells Us That Is New

- In human cancer patients as well as rats, chronic opioid administration causes the enhanced expression of CXCL1
- The spinal administration of CXCL1 to rats speeds the onset and increases the severity of opioid tolerance

pathological pain, and opioid tolerance.⁹ Astrocytes as well as microglia also participate in acute postoperative pain.¹⁰ In response to chronic morphine and peripheral nerve injury-related pain, astrocytes produce proinflammatory cytokines that enhance neuropathic pain behavior and offset the analgesic efficacy of morphine.¹¹ By suppressing astroglial activation, both neuropathic pain and morphine tolerance are attenuated.^{12,13}

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Proinflammatory cytokines (*e.g.*, tumor necrosis factor- α [TNF α]) have a pivotal role in neuroinflammation related to nerve injury-induced pain and chronic morphine exposure.^{14,15} Gene transfer of tumor necrosis factor soluble receptor can inhibit spinal TNF α production, thus preventing the development of morphine tolerance.¹⁶ TNF α subsequently induces rapid expression of CCL2 (MCP-1), CXCL10 (IP-10), and CXCL1 (GRO α) in primary astroglial cell culture¹⁷ and may contribute to the consolidation of morphine tolerance. CCL2 has an important role in the development of neuropathic pain,^{18,19} but its role in morphine tolerance is based on limited information.²⁰ CXCL1 is involved in neutrophil chemotaxis and degranulation at the early phase of inflammation in peripheral tissue. In the nervous system, CXCL1 can also modulate neuronal excitability.^{21,22} A recent study showed that CXCL1 is up-regulated in the spinal nerve ligation model of neuropathic pain and is colocalized with spinal astrocyte markers. Intraspinal applied lentiviral vectors of CXCL1 short hairpin RNA can persistently attenuate neuropathic pain behavior. This evidence implies the involvement of CXCL1/CXCR2 in nerve injury-induced neuropathic pain.²³ The release of CX3CL1 (fractalkine) by neurons and expression of its only receptor, CX3CR1, primarily by microglia suggests the critical involvement of CX3CL1/CX3CR1 signaling in neuron-to-microglia cross-talk.²⁴ Moreover, animal studies have implicated CX3CL1/CX3CR1 signaling in the pathogenesis of neuropathic pain, morphine tolerance,^{25,26} and chronic visceral pain.²⁷

Although evidence in animals shows that neuroinflammation participates in the pathogenesis of morphine tolerance, there is no human evidence to support. In this reverse translational study, we explored the possibility that TNF α , CXCL1, CXCL10, CCL2, and CX3CL1 play roles in human opioid tolerance. We found that CXCL1 levels were significantly higher in the cerebrospinal fluid (CSF) of a group of 30 opioid-tolerant patients compared to a control group of 10 age-matched opioid-naive patients. The CSF level of CXCL1 was positively correlated to opioid dosage. Last, we conducted proof-of-concept animal studies to confirm the relationship between CXCL1 and morphine tolerance.

Materials and Methods

Part 1: Human Study

Setting and Consent Process. After obtaining Institutional Research Ethics Committee (National Taiwan University Hospital Research Ethics Committee, Taipei, Taiwan) approval, this prospective case-control study was conducted at a tertiary medical center in accordance with the Helsinki Declaration and the International Association for the Study of Pain's *Guidelines for Pain Research in Animals and Humans*. Participant recruitment and sample collections were carried out from June 2010 to August 2013. All participants were

informed by the investigators about the aims of the study and that the study would not affect any of their ongoing therapies. Informed consents were obtained before CSF samples were collected.

Opioid-tolerant Patients. Opioid tolerance was defined as daily dosage of intravenous morphine greater than 100 mg (or other opioids given at equipotent doses or by other routes of administration, *e.g.*, transdermal fentanyl and orally- or intraspinally delivered opioids). We recruited advanced stage cancer patients suffering from cancer-related pain under strong opioids and optimal adjuvant drugs. In general, we followed National Comprehensive Cancer Network guidelines to titrate opioid dosage and to manage breakthrough pain. The goal of pain management was to keep patients from having background pain (or only mild pain, *i.e.*, numeric rating scale less than 3). The breakthrough pain was managed by proper dose of immediate release oral morphine when at home or intravenous morphine when hospitalized. The duration of regular opioids usage was longer than 1 month. Patients with evidence of CNS involvement were excluded. CSF samples (3 ml) were collected immediately after intrathecal catheterization or immediately before a scheduled refilling of an implanted intrathecal pump. We made certain that the pain score is less than 3 at the time of CSF sampling.

Opioid-naive Control Subjects. Opioid naive was defined as not taking any opioids in the last 3 months. Opioid-naive control subjects had no chronic pain or ongoing acute pain at the time of CSF sampling. Patients scheduled for surgical removal (under spinal anesthesia) of implants used to treat lower extremity bone fractures were recruited. Lumbar puncture was performed at the L3–4 or L4–5 interlaminar space with a 27G spinal needle, without traumatic tapping or repeated puncture attempts. CSF (3 ml) was collected immediately before injection of bupivacaine for anesthesia.

CSF Sample Processing and Cytokine/Chemokine Analysis.

All CSF samples were centrifuged immediately after collection at 3,000 rpm for 5 min at 4°C and aliquots were stored at –80°C until assayed. The Procarta cytokine profiling kit (Panomics-Affymetrix, Santa Clara, CA) was used to quantify TNF α , CXCL1, CXCL10, CCL2, and CX3CL1 in CSF according to the manufacturer's protocol. Briefly, a 96-well filter plate was prewet with reading buffer. The reagents (in the order of addition to the plate) were as follows: premixed antibody beads before buffer removal by vacuum filtration; CSF samples with incubation on a shaker at 600 rpm for 60 min at room temperature and then washing; premixed detection antibodies with incubation on a shaker at 600 rpm for 30 min at room temperature; streptavidin phycoerythrin with incubation on a shaker at 600 rpm for 30 min at room temperature and then washing, and finally reading buffer. The plate was read by a Luminex (Austin, TX) instrument and the data were analyzed by the designated Luminex acquisition software.

Part 2: Animal Study

Chemicals and Reagents. Morphine hydrochloride was purchased from the National Bureau of Controlled Drugs, National Health Administration (Taipei, Taiwan). CXCL1 and CXCL1-neutralizing antibody (CXCL1-Ab) were purchased from R&D Systems (Minneapolis, MN). Antileukinate hexapeptide (RRWWCR, with an acetylated N terminus and amidated C terminus) was purchased from Yao-Hong Biotechnology (Taipei, Taiwan).

Generation of Fab Fragments of CXCL1-Ab. To exclude the possible interaction of Fc fragment with Fc receptors on glia cells, Fab fragments of antibody were prepared. Fab fragments were prepared with immobilized papain (Pierce, Rockford, IL) according to the manufacturer's protocol. Briefly, the antibodies were dialyzed against 20 mM sodium phosphate buffer at pH 7.0 containing 10 mM EDTA. The digestion buffer (20 mM sodium phosphate, 20 mM cysteine-HCl, 10 mM EDTA at pH 7.0) was freshly prepared before the digestion reaction. Immobilized papain slurry was prewashed with the digestion buffer. The dialyzed antibodies were mixed with immobilized papain slurry in a 1:10 v/v ratio and incubated at 37°C for 6 h. The digestion was stopped by adding 10 mM Tris-HCl at pH 7.5 and the immobilized enzyme was separated from IgG fragments by centrifugation. The Fab fragments were then separated from undigested IgG and Fc fragments using an immobilized protein A resin (GE Healthcare Life Sciences, Piscataway, NJ). IgG fragment mixture was dialyzed against phosphate buffer saline and incubated with immobilized protein A at room temperature for 30 min. After centrifugation, the supernatant, which contains the Fab fragments, was collected. The immobilized protein A resin was washed and supernatant was combined to Fab fraction. The Fab fraction was ready for downstream experiments.

Experimental Animals

All experiments were performed in accordance with the International Association for the Study of Pain and the National Institutes of Health *Guidelines on Laboratory Animal Welfare* and the recommendations of National Taiwan University Animal Care and Use Committee. Adult male Sprague-Dawley rats ($n = 3$ to 7 per protocol; weight, 250 to 275 g) were purchased from BioLASCO Taiwan Co. Ltd. (I-Lan, Taiwan). Rats were housed individually and maintained in a controlled environment (12-h light/dark cycle) with food and water freely available. The rats were randomly allocated to different experimental conditions. The behavior test (tail-flick response) was performed in a blinded manner.

Intrathecal Catheterization and Osmotic Pump Implantation

Intrathecal catheters (polyethylene PE10 tubing, 5 cm; Becton Dickinson, Franklin Lakes, NJ) were inserted into the upper thoracic spine by laminotomy during anesthesia with chlorhydrate through the dura mater into the subarachnoid space. The catheter was advanced caudally so that the tip

rested on the lumbar enlargement. The rostral end of the catheter was firmly fixed to the thoracic spine and hidden in the interscapular soft tissue. Rats showing signs of motor dysfunction (*e.g.*, paralysis) were excluded from the study. After recovery for 7 days, the catheter was connected to primed Alzet osmotic minipumps (Durect Corp., Cupertino, CA) for the delivery of drugs for 5 days.

Evaluation of the Tail-flick Response and Antinociceptive Effect of Morphine

The analgesic effect of morphine was evaluated by using the tail-flick assay (tail-flick analgesia meter, Columbus Instruments, Columbus, OH). Latency was measured with a 0.1 s precision. A 15-s cut-off time was used to prevent permanent tissue damage. Three measurements were made at each time point. For the assessment of the antinociceptive effect of morphine, the percent maximum possible antinociceptive effect (%MPE) was calculated by comparing the test latency before (baseline [BL]) and after drug injection (TL) using the equation: $\%MPE = ([TL - BL] / [cut-off\ time - BL]) \times 100$.

Morphine Tolerance Paradigms

Two paradigms were used to assess the effects of CXCL1 signaling on morphine tolerance. The first was the daily intraperitoneal morphine injection paradigm. After establishing intrathecal saline or CXCL1 (1.2 ng/h) continuous infusion by osmotic minipumps for 24 h, morphine (10 mg/kg) was injected intraperitoneally. Morphine antinociceptive effects were assessed at 15, 30, 45, 60, 75, and 90 min and %MPEs were calculated. The same procedure was done for 3 consecutive days. The area under the curve (AUC) for time-response was considered an index of the antinociceptive effect at each dose of intraperitoneal morphine. The study design is shown in figure 1A.

The second paradigm was intrathecal continuous infusion of morphine. After establishing intrathecal continuous infusion of morphine (15 μ g/h) using osmotic minipumps (with or without CXCL1, CXCL1 neutralizing antibody [CXCL1-Ab], or CXCL1 receptor [CXCR2] blocker-antileukinate hexapeptide), antinociceptive effects of morphine were assessed at 16, 24, 48, 72, 96, and 120 h. CXCL1, CXCL1-Ab, and the antileukinate hexapeptide were coinfused at the rate of 1.2 ng/h, 3.6 ng/h, and 5 μ g/h, respectively. The study design is illustrated in figure 1B.

Total RNA Extraction, Reverse Transcription-polymerase Chain Reaction (PCR), and Real-time Quantitative PCR

Rats were sacrificed after continuous intrathecal of infusion of morphine or saline by osmotic minipump for 48 h. The spinal cord L4–L5 dorsal horn regions were identified and isolated for the total RNA extraction. Single-strand complementary DNA was synthesized using SSIII reverse transcription reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All complementary DNA samples were stored at -20°C . Real-time PCR was performed using a

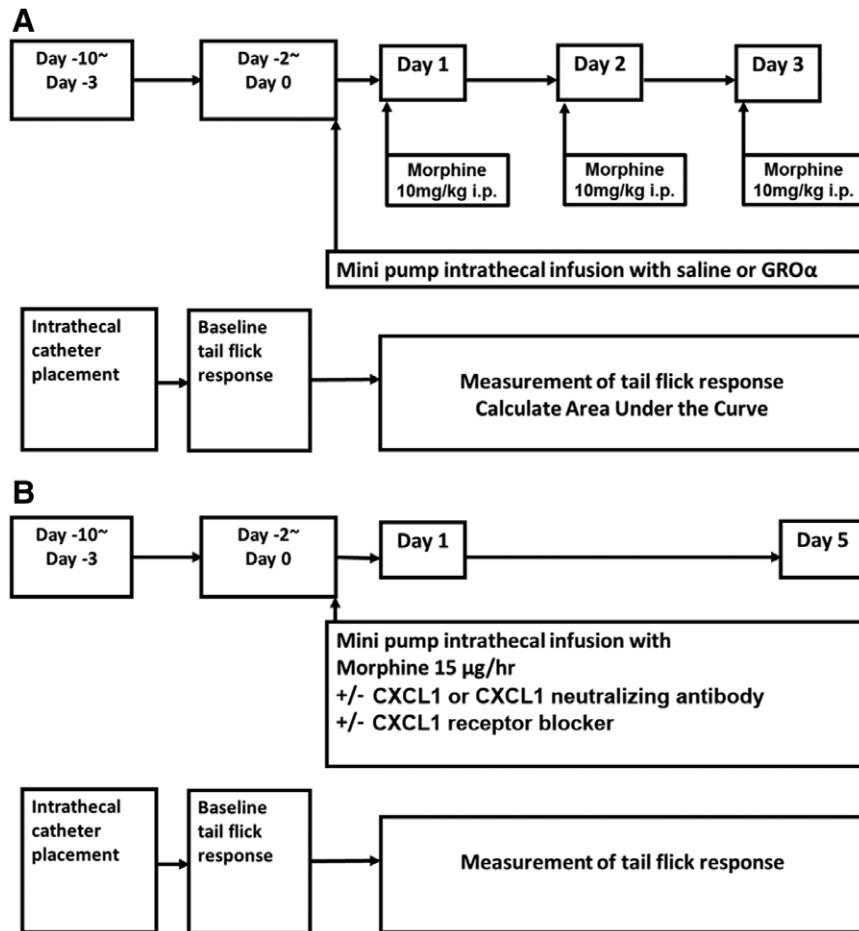


Fig. 1. Experimental design for morphine tolerance. (A) Intraperitoneal morphine tolerance paradigm: CXCL1 was intrathecally infused using an osmotic pump (1.2 ng/h) 24 h before the first dose of morphine. Morphine was administered (10 mg/kg per day) via intraperitoneal injection. (B) Intrathecal morphine tolerance paradigm: Morphine with or without CXCL1, CXCL1-neutralizing antibody, or CXCR2 antagonist was intrathecally infused using an osmotic minipump. i.p. = intraperitoneal.

StepOnePlus Real-Time PCR system (Invitrogen). Relative messenger RNA (mRNA) levels were calculated according to the $2^{-\Delta\Delta CT}$ method. All ΔCT values were normalized to glyceraldehyde 3-phosphate dehydrogenase. Oligonucleotide primers were used as follows: Rat CXCL1 forward 5'-AGA ACA TCC AGA GTT TGA AGG TGA-3' and reverse 5'-GTG GCT ATG ACT TCG GTT TGG-3'; Rat glyceraldehyde 3-phosphate dehydrogenase forward 5'-GGC AAG TTC AAT GGC ACA GT-3' and reverse 5'-TGG TGA AGA CGC CAG TAG ACT C-3'.

Statistical Analysis

The CSF cytokine/chemokine concentrations are presented as medians (25th; 75th percentiles). Comparison between the two groups was done by using the Mann-Whitney test. Linear regression was used to illustrate the possible relationship between daily morphine equivalent dose and the selected chemokine. The difference was considered statistical significant when $P < 0.05$.

As for animal studies, results are presented as means \pm SEM. The data were tested using two-way repeated measures

ANOVA. *Post hoc* comparisons at different time points were done with Bonferroni correction. The difference was considered statistically significant when $P < 0.05$.

The data were analyzed using GraphPad Prism, version 5.0 for Windows (GraphPad Software, Inc., San Diego, CA).

Results

Cytokine and Chemokine Analysis in Opioid-tolerant Patients

Patient Demographics. Thirty patients with lung carcinoma ($n = 6$), colorectal carcinoma ($n = 12$), pancreatic carcinoma ($n = 6$), hepatobiliary carcinoma ($n = 2$), breast carcinoma ($n = 2$) and sarcoma ($n = 2$), and ongoing cancer-related pain controlled by strong opioids were recruited as the opioid-tolerant group. All the recruited patients were stage 4 but no CNS involvement and had their cancer pain well controlled at the time of CSF sampling. Ten age-matched opioid-naïve patients were also recruited as control subjects. The age, gender, and cancer diagnosis are summarized in table 1.

Table 1. Subject Characteristics

	Opioid Tolerant	Naive Control
Age	52.8 (13)	51.5 (13.5)
Gender		
Male	20	7
Female	10	3
Diagnosis	Lung cancer (6) Colorectal cancer (12) Pancreatic cancer (6) Hepatobiliary cancer (2) Breast cancer (2) Sarcoma (2)	Spinal anesthesia (10)

Cerebrospinal fluid was collected from opioid-naive control subject during scheduled spinal anesthesia before injecting local anesthetics.

Cytokine/Chemokine Measurement in CSF Samples. The median CSF concentration of CXCL1 was significantly higher among opioid-tolerant patients than among opioid-naive patients (18.8 pg/ml *vs.* 13.2 pg/ml, $P = 0.02$). There was no significant between-group difference in CSF concentrations of TNF α , CXCL10, CCL2, and CX3CL1. The concentration distributions for CXCL1, CXCL10, CCL2, and CX3CL1 were shown in figure 2. Reanalysis of this relationship after logarithmic transformation of the concentration values (fig. 3A) identified two populations within the opioid-tolerant patients with different CSF CXCL1 levels. Interestingly, CSF CXCL1 concentration was positively correlated with daily morphine equivalent dose ($r^2 = 0.49$, $P < 0.01$) (fig. 3B).

Effects of CXCL1/CXCR2 Signaling on Rat Morphine Tolerance

Effects of Intrathecal Morphine, CXCL1, CXCL1-Ab, and Hexapeptide on Baseline Tail-flick Latency. To examine whether intrathecal CXCL1 (1.2 ng/h), CXCL1-Ab (3.6 ng/h), and CXCR2 antagonist-antileukine hexapeptide (5 μ g/h) affected baseline thermal response, these substances were administered *via* intrathecal continuous infusion using osmotic pump. The tail-flick responses (presented in seconds) were examined at 0, 4, 16, 24, 48, 72, 96, and 120 h. We found that administration with tested dose of CXCL1, CXCL1-Ab, and antileukine hexapeptide did not affect the tail-flick latency compared with saline group. Morphine analgesic effect reached maximal at 16 h then gradually declined (fig. 4).

Increase of CXCL1 mRNA Expression after Intrathecal Continuous Morphine Infusion. Continuous intrathecal infusion of morphine (15 μ g/h) or saline was administered using osmotic pump for 48 h. The rat spinal cord L4–L5 dorsal horn region was identified and isolated for the expression of CXCL1 mRNA by real-time PCR. It was found that intrathecal infusion with morphine increased CXCL1 mRNA levels to 32.5 ± 11.9 -fold of saline control ($n = 4$ for each treatment) (fig. 5).

Effects of Exogenous CXCL1 on Morphine Antinociception and Development of Tolerance in Rats. Based on our human study finding which suggested that CXCL1 had a potential

role in the development of opioid tolerance, we tested whether this phenomenon could be verified experimentally in an animal model. Continuous intrathecal infusion of CXCL1 (1.2 ng/h) was administered using an osmotic pump for 24 h before the first dose of intraperitoneal morphine. Exogenous CXCL1 significantly decreased the antinociceptive efficacy of morphine (fig. 6). On day 1, the analgesic efficacy expressed by AUC of 10 mg intraperitoneal morphine in CXCL1-treated rats was only 66% of the AUC in saline-infused control rats. On day 2, the AUC was 45% for CXCL1-treated rats which was significantly lower than saline-infused control rats (86%, compared with day 1). On day 3, the AUC was 15% for CXCL1-treated rats while saline-infused control rats still retained 50% efficacy. Therefore, intrathecally delivered CXCL1 decreased morphine analgesic efficacy and accelerated the development of morphine tolerance.

Modulating Morphine Tolerance by Intervening CXCL1/CXCR2 Signaling. Since intrathecal exogenous CXCL1 infusion accelerated the development of tolerance to intraperitoneally administered morphine, we then coinfused morphine with CXCL1, CXCL1-Ab, or CXCL1 receptor (CXCR2) antagonist intrathecally using osmotic minipumps to mimic intrathecal morphine infusion in clinical setting. As shown in figure 7, analgesic efficacy peaked after 16 h of intrathecal continuous infusion of morphine (15 μ g/h), then declined gradually. MPE decreased to $43.8 \pm 7.1\%$, $18.8 \pm 2.5\%$, and $7.1 \pm 4.4\%$ at 24, 48, and 72 h, respectively. Coadministration of morphine with CXCL1 further accelerated the development of morphine tolerance ($P = 0.02$). The MPE in CXCL1 plus morphine coinfusion rapidly declined to $4.8 \pm 2.7\%$ at 24 h, which was significantly lower than morphine alone infusion ($P < 0.001$). On the other hand, coinfusion of CXCL1-neutralizing antibody partially preserved morphine analgesic efficacy ($P = 0.02$). *Post hoc* tests showed the significantly higher MPE among CXCL1-Ab plus morphine coinfusion than morphine alone infusion at 48 h ($58.1 \pm 8.0\%$ *vs.* $18.8 \pm 2.5\%$, $P < 0.001$), 72 h ($34.6 \pm 2.9\%$ *vs.* $7.1 \pm 4.4\%$, $P < 0.05$), and 96 h ($30.0 \pm 2.7\%$ *vs.* $2.0 \pm 2.5\%$, $P < 0.05$) (fig. 7A). The analgesic efficacy of intrathecal morphine was also preserved by coadministration with CXCR2 antagonist-antileukine hexapeptide at 24 h ($92.1 \pm 6.4\%$, $P < 0.001$), 48 h ($52.1 \pm 7.7\%$, $P < 0.001$), 72 h ($32.7 \pm 4.4\%$, $P < 0.05$), and 96 h ($24.2 \pm 3.4\%$, $P < 0.05$), respectively (fig. 7B).

Discussion

Herein, we documented evidence that CXCL1 might be implicated in the pathogenesis of opioid tolerance in both humans and rodents.

CXCL1 (also known as growth-related oncogene [GRO] or keratinocyte-derived chemokine) is a chemokine in the CXC family. CXCL1 was first purified from human malignant melanoma cells and is reported to play an important role in inflammation and cancer.^{28,29} Among the three isoforms of GRO (GRO α /CXCL1, GRO β /CXCL2, and GRO γ /CXCL3), GRO α /CXCL1 possesses the highest

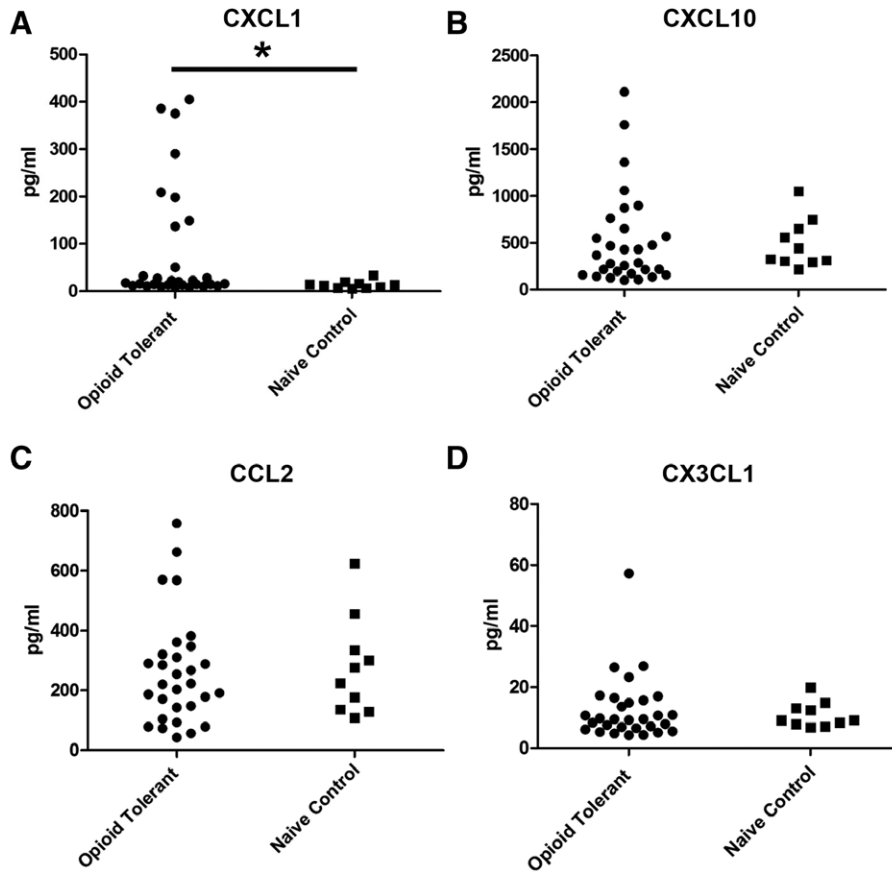


Fig. 2. CXCL1 concentration is higher in cerebrospinal fluid of opioid tolerant patients than in naive control subjects. Cerebrospinal fluid samples were collected from 30 opioid tolerant patients and 10 naive control subjects. Note that CXCL1 was significantly increased in opioid-tolerant patients (A). However, levels of CXCL10 (B), CCL2 (C), and CX3CL1 (D) were not significantly different between opioid-tolerant patients and naive controls. * $P < 0.05$ as compared with naive controls.

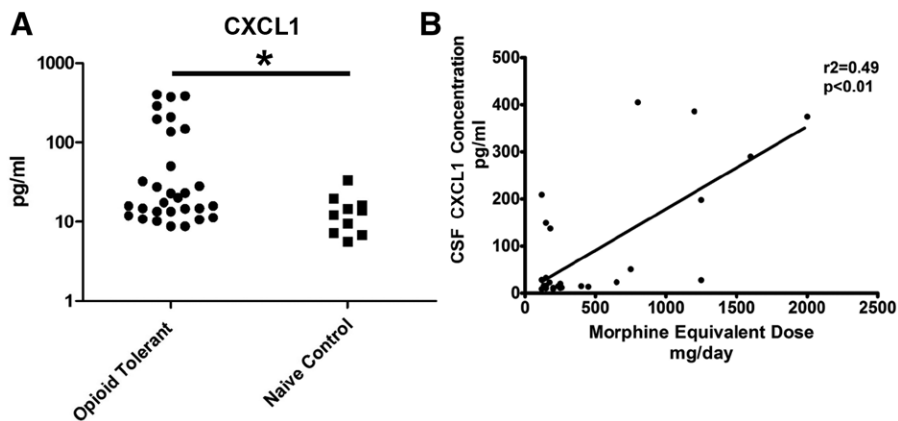


Fig. 3. CXCL1 is positively correlated with daily morphine equivalent dose. (A) Log scale was used to reanalyze the differences in cerebrospinal fluid concentration between opioid tolerant patients and naive controls. (B) Among opioid-tolerant patients, cerebrospinal fluid CXCL1 level was shown to be positively correlated with the daily morphine equivalent dose. * $P < 0.05$ as compared with naive controls.

affinity to their shared receptor, CXCR2.^{30,31} CXCL1 is reported to attract neutrophils, stimulate endothelial cell-mediated angiogenesis, and activate macrophages or T cells.^{29,32,33} An animal study showed that CXCL1 also plays a crucial role in neuroinflammation. One study showed that

CXCL1 (when activated *via* NF- κ B signaling) can recruit neutrophils to sites of inflammation in traumatic spinal cord injury.³⁴ Up-regulation of CXCL1 is also involved in brain injury.^{35,36} Long-term opioid administration activates spinal cord glial cells and neuroinflammation, which

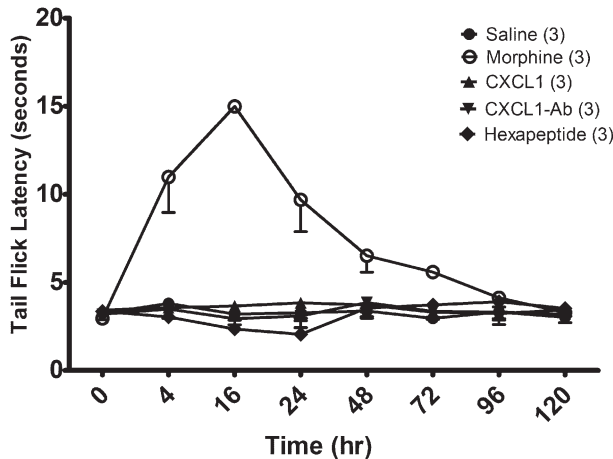


Fig. 4. CXCL1, CXCL1-Ab or Hexapeptide alone does not affect rat tail-flick latency throughout 120h infusion. CXCL1 (1.2 ng/h), CXCL1-Ab (3.6 ng/h), hexapeptide (5 μ g/h), morphine (15 μ g/h), and saline control were individually infused intrathecally *via* osmotic minipump. Tail-flick latency responses (s) were observed at 0, 4, 16, 24, 48, 72, 96, and 120 h. Morphine analgesic efficacy peaked at 16 h and gradually declined. None of the other infused substances affected tail-flick latency.

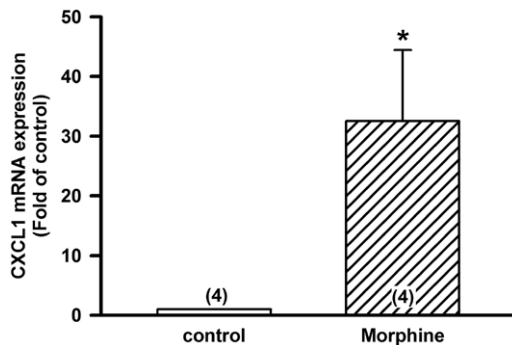


Fig. 5. Intrathecal morphine infusion increases the expression of CXCL1 in rat. Morphine was administered *via* osmotic pump at an infusion rate of 15 μ g/h. After 48 h, spinal L4–L5 dorsal horn was isolated for real-time polymerase chain reaction analysis. Note that treatment with morphine increased the expression of CXCL1 in spinal cord dorsal horn. Data were presented as mean \pm SEM. * $P < 0.05$ compared with control (saline infusion). mRNA = messenger RNA.

is considered to be one of the mechanisms leading to morphine tolerance.^{25,37}

While the involvement of CXCL1 in neuroinflammation has already been demonstrated, the relationship between morphine tolerance and CXCL1 is unknown at the start of our studies. Our study in humans found a significant increase in CSF CXCL1 in opioid-tolerant cancer patients and a strong positive correlation between CSF CXCL1 level and daily opioid dosage. CXCL1 has been detected in humans in a variety of neurological diseases.^{38–40} For example, CXCL1 is markedly up-regulated in bacterial meningitis but not in aseptic meningitis and healthy controls,³⁸ and up-regulated

in neuroinflammatory diseases such as multiple sclerosis, acute disseminated encephalomyelitis,³⁹ and opsoclonus-myoclonus syndrome.⁴⁰ Of note, the CSF CXCL1 level in our opioid-naïve control group was also comparable with levels reported in healthy control subjects of the above-mentioned neurological disease studies. Our findings suggested that increase in CXCL1 may be related to opioid tolerance, since our opioid-tolerant patients had neither neurological comorbidity nor cancer with CNS involvement.

Parallel to evidence in humans, we also found a rapid and significant up-regulation of CXCL1 mRNA in the rat spinal cord after the induction of tolerance by intrathecal morphine infusion for 48 h. Although CXCL1 alone infused intrathecally did not affect tail-flick latency throughout the study period for 5 days, exogenous CXCL1 can markedly decrease morphine antinociceptive efficacy and accelerate the development of morphine tolerance. By using intrathecal coinfusion technique, we found that morphine analgesic efficacy dropped to nearly undetectable within 24 h among CXCL1 coinfused rats. On the contrary, by blocking CXCL1/CXCR2 signaling with coinfused CXCL1 neutralizing antibody or receptor antagonist, morphine analgesic efficacy could be at least partially preserved. Thus, morphine tolerance might be attenuated by CXCL1/CXCR2 signaling interventions. Although the antileukinate hexapeptide (a potent inhibitor of CXCR2) has been reported to suppress inflammatory injury in acute pancreatitis or lung injury,^{41,42} it has never been reported to suppress the development of morphine tolerance.

Although CXCL1 has been implicated in both pain^{21,23} and cancer progression,^{43,44} we recruited only opioid-tolerant cancer patients, not chronic noncancer pain patients, in our human study. Opioid dose was typically titrated to effect, but was generally greater in patients with more advanced disease. Based on our study design, it is therefore difficult to interpret whether the up-regulation of CXCL1 is related to cancer disease progression *per se* or related to long-term opioid use that causes tolerance. In this study, we tried to minimize confounding effect from cancer progression by recruiting relatively homogenous patients. They were all stage 4 cancer patients with distant metastasis but none of them had CNS involvement. Thus, we could rule out the possibility that changes of CSF CXCL1 were resulted from CNS metastasis. Although all the participants were in similar disease status, their opioid dosage range was very wide. We found a strong positive correlation between CSF CXCL1 level and daily opioid dosage, which further implied that up-regulated CXCL1 might be related to long-term use of high dose opioids. Although we recruited only patients with advanced stage cancer with relatively stable dosage of opioids and disease status, the underlying cancer diagnosis would still be an inevitable confounding covariate. We could not recruit chronic noncancer pain patients as study subjects; because in our society, nonsteroidal antiinflammatory drugs and weak opioids are widely used to control most neuropathic pain and chronic musculoskeletal pain. However, strong opioid

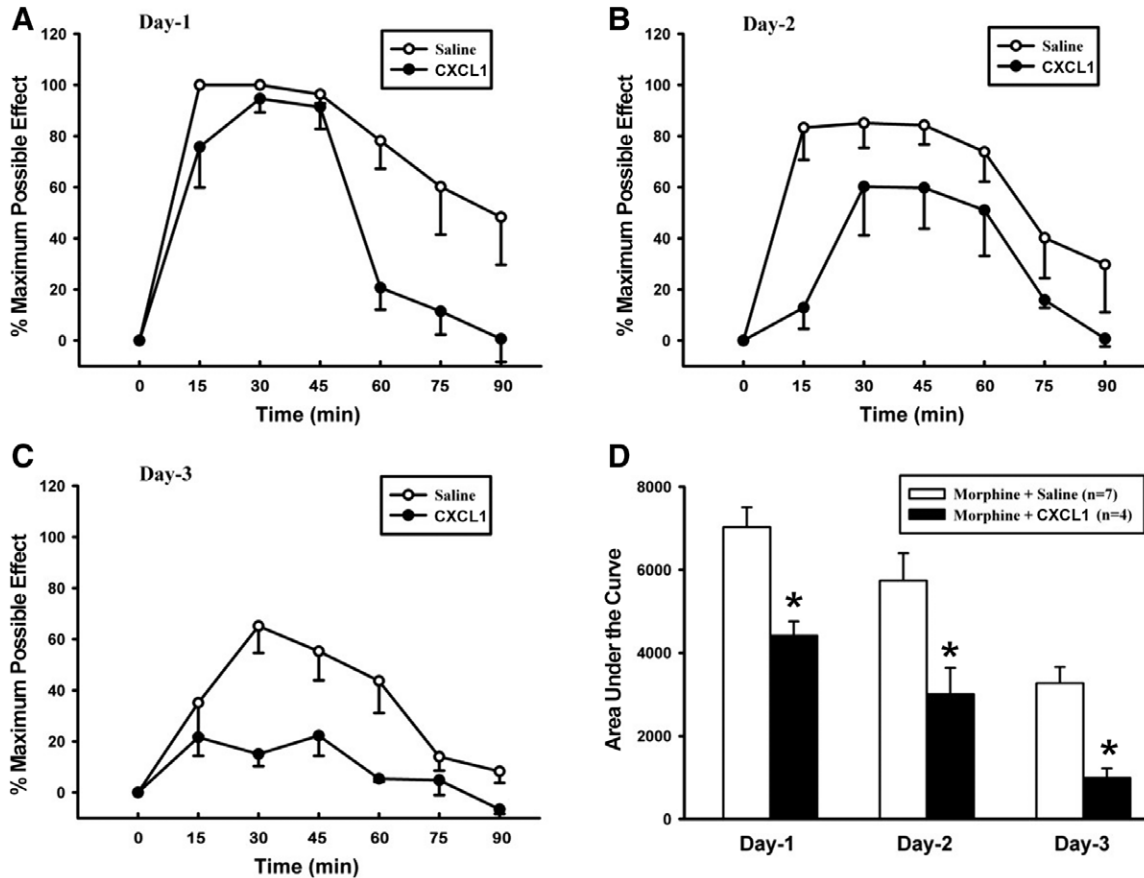


Fig. 6. Exogenous CXCL1 decreases morphine analgesic efficacy and accelerates development of morphine tolerance in daily intraperitoneal morphine paradigm. CXCL1 was intrathecally administered via an osmotic pump at an infusion rate of 1.2 ng/h. Morphine was intraperitoneally injected at 10 mg kg⁻¹ day⁻¹. The time-course of analgesic action of acute morphine treatment was evaluated by assessment of the latency of the tail-flick response and the calculated % maximum possible effects on day 1 through day 3 (A–C). The area under the curve was summarized in (D). Note that intrathecal administration of CXCL1 accelerated tolerance induced by intraperitoneal injection of morphine in rats. **P* < 0.05 as compared with morphine + saline control group at different time points tested by Bonferroni correction.

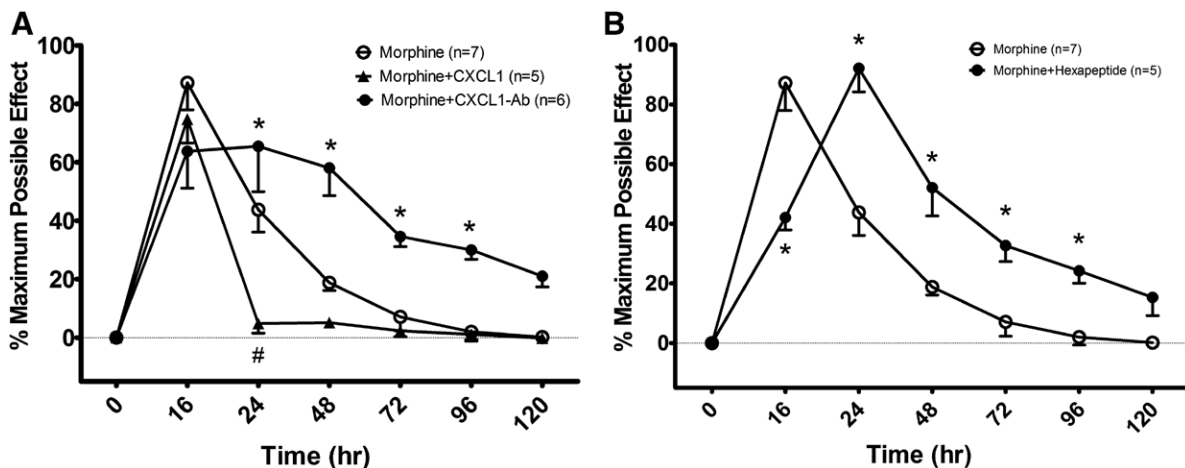


Fig. 7. The effect of intervening CXCL1/CXCR2 signaling on morphine tolerance. All the drugs were administered intrathecally using an osmotic pump. The infusion rate was as follows: morphine 15 μg/h, CXCL1 1.2 ng/h, CXCL1-Ab 3.6 ng/h, antileukinate hexapeptide (CXCR2 receptor blocker) 5 μg/h. Note that exogenous CXCL1 markedly accelerated the development of morphine tolerance (A), whereas CXCL1 neutralizing antibody (A) or antileukinate hexapeptide (B) inhibited the induction of morphine tolerance and partially restored morphine analgesic efficacy. **P* < 0.05 as compared with morphine group at different time points tested by Bonferroni correction.

use for chronic noncancer pain in our society is very limited and it is difficult to recruit enough noncancer patients using high dose opioids.⁴⁵ Furthermore, chronic pain *per se* would also be another inevitable confounding covariate. In human research setting, it is unethical to conduct study by inducing opioid tolerance in healthy subjects without pain. Therefore, we conducted subsequent translational animal studies to illustrate that not only CXCL1 was up-regulated in morphine tolerant rat but also exogenous CXCL1 decreased morphine analgesic efficacy and blocking CXCL1/CXCR2 signaling will restore morphine analgesic efficacy.

In our human study, the subjects were prospectively recruited through a convenience sample. The number of participants in each group was designed to exceed the lower bound of large sample interference for clinical research, 30 patients. However, only subjects in the opioid-tolerant group met a sample size of 30. We barely recruited 10 age-compatible subjects in the opioid-naive group for the following reasons: (1) Most surgeries for the removal of an implant for healed fracture were done under intravenous general anesthesia, not spinal anesthesia, in our institute. (2) Most of our citizens believe lumbar puncture with CSF sampling is bad for their spine health and will cause low back pain.

Zhang *et al.* have recently noted in a spinal nerve ligation model that CXCL1 up-regulation occurred primarily in reactive astrocytes and paralleled neuropathic pain behaviors such as mechanical allodynia and heat hyperalgesia.²³ Knockdown of CXCL1 mRNA by intrathecal short hairpin RNA lentiviral vector is shown to persistently attenuate spinal nerve ligation-induced pain hypersensitivity. Since peripheral nerve injury and long-term opioid exposure both turn on neuroinflammation manifested by sustained astrocyte activation,^{11,12,25} it would be reasonable to hypothesize that modulating CXCL1–CXCR2 signaling could be a promising therapeutic approach to attenuate opioid tolerance. Directly suppressing astrocyte activation using commercially available Ibudilast (a phosphodiesterase inhibitor used for asthma) (Kyorin Pharmaceutical, Tokyo, Japan) restores the antinociceptive effect of morphine in opioid-tolerant lab animals.¹³ This finding further illustrates the potential for control of neuropathic pain and opioid tolerance by novel drugs targeting astrocytes.

To our surprise, we could not find a difference in CSF TNF α level between opioid-naive and tolerant subjects despite abundant lab animal data suggesting a difference.¹⁵ Although the assay was very sensitive, the level of CSF TNF α in both the naive and tolerant groups was very low (and even below detection limits in some patients), and was comparable to the level reported in patients with lumbar stenosis-related radicular pain and complex regional pain syndrome.^{46,47} This finding implies that, just as in the pathogenesis of nerve injury-induced neuropathic pain, the pathogenesis of opioid tolerance might involve TNF α at the initial stage but not the well-established stage as in our patient group.¹⁴ Although

the involvement of CCL2 and CX3CL1 in neuropathic pain-associated neuroinflammation has been shown in lab animals,^{48,49} we could not detect a statistically significant difference in CSF levels of CCL2 and CX3CL1 between age-compatible opioid-naive and tolerant human subjects.

In conclusion, our investigation of the levels of various cytokines and chemokines in the CSF of opioid-tolerant cancer patients suggests that CXCL1 may be involved in the pathogenesis of opioid tolerance. Our animal studies showed that blockade of CXCL1/CXCR2 signaling can inhibit the development of morphine tolerance. Therefore, CXCL1/CXCR2 may be a new target for developing drugs that attenuate morphine tolerance and may be especially useful for treating patients requiring high dose opioids.

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Competing Interests

The authors declare no competing interests.

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ANESTHESIOLOGY REFLECTIONS FROM THE WOOD LIBRARY-MUSEUM

The Pentothal Tubing Bag of Frank L. Faust, M.D.



Measuring 13 × 4.5 in (33 × 11 cm), this cloth bag (*high*) sheltered rubber tubing dedicated to intravenous administration of sodium thiopental (branded as “Pentothal”) during the mid- to late-1940s by “F. L. Faust” (*low*), better known as Frank Leo Faust, M.D. (surgeon and anesthesiologist, New Orleans, Louisiana) (1916–2013). Perhaps a bag like this one held the “Pentothal tubing” used by Dr. Oral Crawford in the September 1949 *ANESTHESIOLOGY* article, “A Simplified Inexpensive Method for Holding Syringes of Pentothal Sodium for Continuous Injection.” Dr. Faust had trained initially in surgery under Dr. Rudolf Matas and alongside Drs. Michael DeBakey and Alton Ochsner. Following his 1944 “short course” in anesthesiology at the Lahey Clinic, Dr. Faust joined his lifelong colleague, Dr. John Adriani, in transforming the practice of anesthesiology in New Orleans. (Copyright © the American Society of Anesthesiologists, Inc.)

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