Research Report

Regulation of spinal neuroimmune responses by prolonged morphine treatment in a rat model of cancer induced bone pain

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ABSTRACT

Cancer induced bone pain (CIBP) is a major clinical problem. Although opioids remain the principal axis in drug therapies for CIBP, their sustained application is known to induce cellular and molecular adaptations including enhanced neuroimmune reactivity. This is generally characterized by glial activation and proinflammatory cytokine production which frequently results in pharmacological tolerance. This research was performed to investigate spinal neuroimmune responses after prolonged systemic morphine treatment in a rat model of CIBP. The model was established using a unilateral intra-tibia injection of Walker 256 mammary gland carcinoma cells. Subcutaneous morphine was repeatedly administered from postoperative days 14 to 19. Mechanical allodynia to von Frey filaments and ambulatory pain scores were recorded to investigate changes of nociceptive behaviors. Spinal glial activation was detected by immunohistochemistry and real-time PCR; the production of proinflammatory cytokines (IL-1β and TNF-α) was examined through real-time PCR and ELISA. Results showed that chronic morphine use failed to elicit analgesic tolerance in the rat CIBP model. Moreover, the treatment had no significant influence on the activated spinal glia morphology, cell density and expression of special cytomembrane markers, whereas it significantly down-regulated the local proinflammatory cytokine production at the mRNA and protein level. Collectively, these data suggest that chronic morphine treatment in CIBP is not concomitant with pharmacological tolerance, at least partially because the treatment fails to amplify spinal neuroimmune responses.

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1. Introduction

Cancer induced bone pain (CIBP) remains a severe clinical issue, and it is a very complicated pain syndrome including localized ongoing background pain and spontaneous pain or movement-evoked (incident) pain (Mercadante and Arcuri, 1998; Portenoy and Frager, 1999; Urch, 2004). To date about 85% of patients with bone metastases suffer from this intolerable...
Pathologic pain, which is associated with increased morbidity, increased anxiety and depression, reduced performance status, and a reduced quality of life (Bruera and Kim, 2003). Although there have been several recent advances in the therapeutic management, the primary drugs remain mu opioid receptor analgesics so far, represented by such drugs as morphine (Cherny, 2000; Mercadante, 1999; Ruiz-Garcia and Lopez-Briz, 2008). The chronic nature of cancer pain often requires prolonged morphine application through controlled release tablets, repeated bolus injections, and so on (Clemens and Klaschik, 2007; Delaney et al., 2008; Quigley, 2005). Unfortunately, some evidence suggests that long-term morphine treatment may lead to cellular and molecular adaptations (Williams et al., 2001) that often result in pharmacological tolerance (i.e., a decreased analgesic effect with prolonged administration of a constant dose) (Bohn et al., 2000).

Most animal studies regarding opioid tolerance have only addressed neuronal mechanisms, and have ignored other potential modulators. Recent evidence has demonstrated that spinal neuroimmune responses, which are characterized by glial activation and proinflammatory cytokine production, make a great contribution to the generation and maintenance of opioid tolerance (Raghavendra et al., 2002, 2004a; Tawfik et al., 2005). Since the first study revealed that spinal neuroimmune reactivity was closely related to morphine tolerance, evidence rapidly accrues that chronic morphine treatment can directly activate both astrocytes and microglia (Cui et al., 2006; Raghavendra et al., 2002; Song and Zhao, 2001; Tai et al., 2006) and simultaneously stimulate the production of proinflammatory cytokines (Johnston et al., 2004; Raghavendra et al., 2002, 2003), both of which in turn play an active role in the development and maintenance of morphine tolerance. Moreover, that spinal neuroimmune responses are causal to, rather than simply associated with morphine tolerance, is further established by the finding that these responses are significantly abrogated by intrathecal injection of glial metabolism inhibitors and proinflammatory cytokine inhibitors (Raghavendra et al., 2002), or are accompanied with a down-regulation of glial GLAST and GLT-1 glutamate transporters (Tai et al., 2006). In sum, it is likely that opioid-induced/increased spinal neuroimmune activation undermines opioid-induced pain suppression (Watkins et al., 2007b).

However, most animal studies mentioned above were performed either in the absence of painful tissue injury which precluded extrapolation to the clinical situations (Zollner et al., 2008), or in neuropathic pain which demonstrated “naïve opioid tolerance” (Watkins et al., 2007b). Interestingly, some research claimed that opioid tolerance did not develop frequently in patients or animals with pathologic pain resulting from cancer (Portenoy and Lesage, 1999; Urch et al., 2005; Zech et al., 1995). Utilizing a rat model of inflammatory pain that mimics cancer associated pain, Zollner et al. (2008) indeed observed that chronic morphine application did not result in opioid tolerance, as the continuous availability of endogenous opioids increased recycling and preserved signaling of mu receptors in peripheral sensory neurons. As a unique pathological syndrome, CIBP possesses elements reminiscent of inflammatory, neuropathic, and cancer pain, but little is known about neuroimmune responses to chronic morphine administration in this particular pathological pain state. Thus, the aims of this study were to 1) investigate whether chronic systemic morphine treatment elicited pharmacological tolerance in a rat CIBP model induced by unilateral inoculation of Walker 256 mammary gland carcinoma cells into the tibia; and 2) examine the spinal neuroimmune activity after chronic morphine use in this model.

2. Results

2.1. Radiological evaluation of bone destruction

All of the radiographs taken from carcinoma cells, chronic saline, and chronic morphine treated rats showed severe deterioration with full thickness unicortical bone loss on the postoperative day 19. This in combination with results from nociceptive behavioral tests suggested that the CIBP model was successfully established. No evident radiological difference was observed in the contralateral tibia bone from all of carcinoma cell inoculated rats (including carcinoma cell group, chronic saline group, and chronic morphine group, data not shown). Representative radiographs are presented in Fig. 1.

Fig. 1 - Tibial radiographs of tested rats on day 19 after surgery. Radiographs of ipsilateral tibia from naïve rats (A) showed intact cortical bone, while tibia from carcinoma cell group (B), chronic saline group (C), and chronic morphine group (D) exhibited evident bone destruction.
2.2. Behavioral tests

The preoperative baseline of withdrawal thresholds to von Frey filaments or scores of ambulatory pain in all tested rats was similar (P>0.05, data not shown). A previous study using the same rat model showed that mechanical allodynia and ambulatory pain scores increased in a time-dependent manner after inoculation of carcinoma cells and reached a stable state commencing from postoperative day 14 (Mao-Ying et al., 2006). Thus, this steady-state period was chosen in this research to study the pharmacological action of sustained morphine on nociceptive behaviors. We observed that from postoperative days 14 to 19, carcinoma cell treated rats demonstrated a profound reduction in ipsilateral hindpaw withdrawal latency (P<0.05) and a significant increase in ambulatory pain scores (P<0.05) compared with naïve rats, while chronic morphine treated rats displayed a significant decrease of nociceptive hypersensitivity in both mechanical allodynia (P<0.05) and ambulatory pain scores (P<0.05) compared with carcinoma cell treated rats. The continuous exposure to subcutaneous morphine demonstrated the powerful inhibition of mechanical responses to von Frey filaments, but partly effective in controlling the ambulatory pain when compared with behavioral outcomes of naïve rats (P<0.05) This suggests that other mechanisms are associated with the development of breakthrough pain and that adjuvant therapies are necessary to reverse it completely (Colvin and Fallon, 2008; Urch et al., 2005). On the postoperative day 19, chronic morphine demonstrated the potent analgesic capability compared with non-treatment or chronic saline treatment (P<0.05), and there was no significant alteration of the analgesic efficacy for sustained morphine use between day 19 and other days (P>0.05), suggesting that chronic morphine use did not elicit notable pharmacological tolerance. Throughout the observation period, there was no significant behavioral difference between rats inoculated with PBS and naïve rats (P>0.05), or between carcinoma cell treated and chronic saline treated rats (P>0.05). Behavioral results are shown in Fig. 2.

2.3. Systemic morphine impacts on the activation of spinal glia

Immunohistochemistry for the detection of GFAP, a cytomembrane marker for normal and activated astrocytes, demonstrated baseline expression of GFAP in the lumbar enlargement of naïve rats (Fig. 3D). GFAP labeled resting astrocytes displayed round nuclei and slender processes and were distributed throughout the gray matter. In contrast, on postoperative day 19 astrocytes distributed throughout the spinal gray matter in the carcinoma cell treated rats were rounded with few, densely overlapping projections that displayed intense GFAP immunoreactivity (Fig. 3C). However, chronic morphine treatment did not provoke astrocytes further activated in terms of immunohistochemical scores (Figs. 3A and B, Table 1). There was no evident difference between astrocytic images of carcinoma cell treated and chronic saline treated rats. On the other hand, the antibody against OX42 showed moderate expression of resident microglia throughout the gray matter of spinal cord from naïve and carcinoma cell treated rats. These microglia exhibited quiescent or resting cell morphology: small compact somata bearing many long thin ramified processes (Figs. 4C and D). However, on postoperative day 19,

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Fig. 2 - The effects of chronic morphine treatment on ambulatory pain (left) and mechanical allodynia (right). Rats received twice-daily subcutaneous injection of saline or morphine on postoperative days 14 to 18 at 12-h intervals, with a final injection on the morning of day 19. The behavioral tests were accessed on days 1, 3, 5 and 6 of the treatment (i.e. postoperative days 14, 16, 18, and 19). During the whole tested periods, chronic morphine treatment significantly attenuated scores of ambulatory pain (P<0.05, versus rats from carcinoma cell group), but failed to revert them to the baseline completely (P>0.05). Meanwhile, sustained morphine use evidently controlled mechanical allodynia when compared with rats from carcinoma cell group (P<0.05). There was no significant behavioral difference between naïve rats and PBS-inoculated rats throughout the observation period (P>0.05). Data are expressed as mean ± SEM. *P<0.05, compared with naïve rats; #P<0.05, chronic morphine group compared with carcinoma cell group (n=9 each group).
microglia exposed to sustained morphine use remained in the quiescent or resting state (Figs. 4A and B). These immunohistochemistry results suggested that: 1) astrocytes might play a more active and important role than microglia in maintaining CIBP, similar to some other types of persistent pathological pain states (Watkins et al., 2007a,b); and 2) continued morphine use failed to further amplify spinal glial responses in terms of their morphology, cell density, and immunostaining of cytomembrane surface markers.

In order to further determine the regulation of morphine on the spinal glia, quantitative real-time PCR was performed to detect changes of spinal glia membrane markers, which had already been established to be a sensitive and reliable method to track the spinal glia responses in pathological pain states (Tanga et al., 2004). IGTAM, also known as MAC-1 or CR3/CD11b, is a trans-membrane protein with an extracellular domain that is expressed on the outer membrane of microglia. TLR4 is a trans-membrane receptor protein with extracellular leucine-rich repeat domains and a cytoplasmic signaling domain, and has already been established to be exclusively expressed by microglia in the CNS. CD14 is not expressed on parenchymal microglia, but is up-regulated on activated microglia. GFAP is a major intermediate filament protein expressed in mature astrocytes of the CNS and has widely been accepted as an astrocytic differentiation marker. These membrane surface markers were utilized extensively to reflect

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Note: Scoring is as follows: 0, normal unactivated tissues; +, mild activation; ++, moderate activation; +++ intense activation. DH, dorsal horn; VH, ventral horn. There was no significant difference for GFAP immunostaining both in dorsal and ventral horns between rats from carcinoma cell group and chronic morphine group.

Fig. 3 – GFAP immunostaining of the lumbar (L4-5) spinal cord on day 19 post surgery. The representative images were from chronic morphine group (A and B), carcinoma cell group (C) and naïve rats (D). A low-power image (A) showed that the up-regulation of GFAP was distributed throughout the entire ipsilateral spinal cord to the tibia with cancer. And activated astrocytes (B and C) demonstrated profoundly cell proliferation and hypertrophy, whereas the resting astrocytes (D) displayed small round nuclei and slender processes. Scale bars are 100 µm. Panels B, C, and D are high-power microphotographs of the identical yellow rectangle part (shown in A) from each group (n=3 each group).
glial reactivity (Raghavendra et al., 2004b; Tanga et al., 2004; Tawfik et al., 2005). Similar to the immunochemical results, GFAP mRNA expression in carcinoma cell treated rats was significantly higher than intact animals \((P<0.05, \text{Fig. 5})\), whereas chronic morphine treatment did not enhance GFAP mRNA expression in comparison with carcinoma cell treated rats \((P>0.05, \text{Fig. 5})\). There was no significant difference in GFAP mRNA expression between chronic saline and carcinoma cell treated rats \((P>0.05, \text{Fig. 5})\). In addition, no significant differences in mRNA expression between naïve rats and the rest of tested rats were detected using microglial membrane markers, i.e. IGTAM, CD14, and TLR4 \((P>0.05, \text{Fig. 5})\).

2.4. Systemic morphine impacts on the production of proinflammatory cytokines

To fully characterize the effects of chronic morphine administration on spinal neuroimmune responses, real-time PCR and ELISA were utilized to determine changes in proinflammatory cytokine production in the spinal cord parenchyma. The mRNA levels of IL-1\(\beta\) or TNF-\(\alpha\) within L4–5 spinal segments from carcinoma cell and chronic saline treated rats showed a significant increase compared with those from naïve rats \((P<0.05, \text{Fig. 6})\). Intriguingly, prolonged morphine treatment significantly decreased the mRNA levels of both IL-1\(\beta\) and TNF-\(\alpha\) in comparison with chronic saline or carcinoma cell treated rats \((P<0.05, \text{Fig. 6})\), but failed to completely revert their expression to the baseline \((P<0.05, \text{Fig. 6})\). Simultaneously, ELISA results regarding influences of chronic morphine on two cytokine expressions were similar to mRNA changes as well \((\text{Fig. 7})\). There was no evident difference in two cytokines at both mRNA and protein levels between naïve rats and PBS-inoculated rats, or between carcinoma cell and chronic saline treated rats \((P>0.05, \text{Figs. 6 and 7})\).

3. Discussion

The main findings of this study showed that prolonged morphine treatment failed to produce analgesic tolerance in this rat model of CIBP. Meanwhile, chronic morphine treatment had no significant influence on the spinal microglia and
Fig. 5 – Real-time PCR quantitation of the glial marker expression (astrocyte, GFAP; microglia, CD14, TLR-4 and IGTAM) in the lumbar spinal cord. The expression levels of GFAP from carcinoma cell group, chronic saline group and chronic morphine group were significantly increased compared to naïve rats ($P < 0.05$), but there were no difference of GFAP expression between carcinoma cell group and chronic morphine group ($P > 0.05$). Moreover, there was no significant difference for microglia markers (CD14, TLR-4 and IGTAM) between naïve rats and the rest of the tested rats ($P > 0.05$). Data are expressed as mean±SEM. *$P < 0.05$, versus naïve rats ($n=3$ each group).

Fig. 6 – Transcription regulation of IL-1β and TNF-α at the mRNA level by prolonged morphine treatment on day 19 after surgery. Both cytokines showed a significant increase in rats from carcinoma cell group and chronic morphine group when compared to naïve rats ($P < 0.05$), while sustained morphine use profoundly down-regulated two cytokine productions in comparison with non-treatment ($P < 0.05$). However, chronic morphine treatment failed to revert two cytokine productions to the baseline ($P < 0.05$). There was no significant difference for two cytokine expressions between naïve rats and PBS-inoculated rats ($P > 0.05$). Data are expressed as mean±SEM. *$P < 0.05$, compared with naïve rats; #$P < 0.05$, chronic morphine group compared with carcinoma cell group ($n=3$ each group).
astrocytes according to their morphology, density, and expression of specific membrane surface markers, whereas it significantly down-regulated the production of spinal IL-1β and TNF-α at both mRNA and protein levels.

The underlying mechanisms of CIBP appear very complex, having inflammatory, neuropathic and tumourigenic components. Recently, several animal models imitating human conditions have helped us explore potential mechanisms (Mantyh, 2006; Pacharinsak and Beitz, 2008). In both patients and animal models, the severity of cancer pain is closely correlated with the extent of bone destruction and ongoing osteoclast activity, and generally tends to increase as the disease progresses (Medhurst et al., 2002; Portenoy and Lesage, 1999). Current analgesic therapies for CIBP have not changed significantly for over a decade, and morphine does remain the pharmacological profile, and also supported the rationale for morphine tolerance with a relative lower dose in another rat model of CIBP. The authors attributed this phenomenon to the slight wearing off of drug effects between doses and a resetting of the nociceptive thresholds as a result. We hope to make the paradoxical phenomenon clear in terms of the action of spinal neuroimmune activity.

Central neuroimmune activation and neuroinflammation, which are characterized by glial activation and proinflammatory cytokine production, have been postulated to mediate and/or modulate a variety of pathological states, including neurodegenerative diseases (Skaper, 2007), persistent pain states, and opioid tolerance (DeLeo et al., 2004). Unlike neurons, the activation of glia is a multi-dimensional process, including morphological changes, cell proliferation, up-regulation of morphological changes, cellular activation, and proinflammatory cytokines, reactive oxygen, and nitrogen species that affect neuronal integrity or functions (Watkins and Maier, 2003). Our immunohistochemical results showed that, on postoperative day 19 the intratrabecular inoculation of carcinoma cells evoked extensive astrocytic activation from the dorsal to ventral horn, including cell hypertrophy, proliferation, and the enhanced immunostaining of GFAP (which was called spinal cord astrogliosis indicative of CIBP) (Gobährisch et al., 2006; Luger et al., 2005; Zhang et al., 2005), in parallel with the nociceptive behavioral hypersensitivity. In contrast, microglia remained in a resting state. Quantitative real-time PCR regarding glial membrane surface markers provided us the further evidence that astrocytes, not microglia, may mediate the maintenance of behavioral abnormality in our CIBP model. Because the exact role of spinal glia in CIBP was not the focus of this study, we did not make an extensive or further research about them. Inconsistent with previous studies, chronic morphine failed to exaggerate spinal glial responses in light of their characteristics in morphology, density, and membrane markers. Thus, we switched to investigate the local proinflammatory cytokine production.

Fig. 7 – Translation regulation of IL-1β and TNF-α at the protein level by prolonged morphine treatment on day 19 after surgery. Both cytokines showed a significant increase in rats from carcinoma cell group and chronic morphine group in comparison with naïve rats (P<0.05), while sustained morphine use profoundly down-regulated two cytokine productions in comparison with non-treatment (P<0.05). However, chronic morphine treatment failed to revert two cytokine productions to the baseline (P<0.05). There was no significant difference for two cytokine expressions between naïve rats and PBS-inoculated rats (P>0.05). Data are expressed as mean±SEM. *P<0.05, compared with naïve rats; #P<0.05, chronic morphine group compared with carcinoma cell group (n=3 each group).
The exact spinal cellular sources of the proinflammatory signal in chronic pain remain of interest so far and required further investigation. However, growing evidence documents that under different pathologic conditions these cytokines are derived mainly from spinal glia (microglia and astrocyte) (Milligan and Watkins, 2009) and to some extent from other cells, such as neurons, endothelial cells, fibroblasts and meningeal cells (Watkins et al., 2007a; Wieseler-Frank et al., 2007). Glial cells, responding to a variety of nociceptive signals, synthesize and release proinflammatory cytokines, which would further contribute to the elevation of proinflammatory cytokine levels known to sensitize dorsal spinal cord pain responsive neurons, thereby enhancing pain. Thus, both spinal glia and proinflammatory cytokines were extensively regarded as hallmarks of neuroimmune responses inducing the pathological pain. In this research, non-treated CIBP rats exhibited enhanced synthesis of both IL-1β and TNF-α at the mRNA or protein level, suggesting that increased neuroimmune responses play a vital role in CIBP, and down-regulation of neuroimmune action may be an alternative unexploited method to effectively control cancer pain. Of note, our studies demonstrated that chronic morphine treatment did significantly reduce the expression of both cytokines. In combination with influences on spinal glia, we think, sustained morphine exposure in this rat CIBP model relieved the enhanced spinal neuroimmune responses to some degree.

The underlying cellular and molecular mechanisms of opioid tolerance remain controversial. Several possible mechanisms have been proposed, including (1) down-regulation of opioid receptors; (2) uncoupling of G-protein from opioid receptor, causing internalization/endocytosis, which facilitates opioid receptor resensitization; and (4) activation of excitatory amino acid (EAA) receptors and neuroimmune cascades (Tai et al., 2006). Inconsistent with most of previous results, our study revealed that, chronic systemic morphine treatment (1) did not produce analgesic tolerance; (2) exerted no significant influence on the spinal glia in terms of cell morphology, density, and intensity of cellular membrane surface markers; and (3) down-regulated evidently the production of IL-1β and TNF-α. We attribute this paradoxical phenomenon to: (1) most of prior researches concerning morphine tolerance are induced from normal animals or animals with neuropathic pain, whose spinal glia are quiescent or partially activated. Thus, after sustained morphine treatment the spinal glia either begin to be activated (in naïve rats) (Song and Zhao, 2001) or are further amplified (in rats with neuropathic pain) (Raghavendra et al., 2002), then produce more proinflammatory cytokines. While in CIBP activated astrocytes are present throughout the entire ipsilateral spinal gray matter, which may mean that astrocytes are fully activated and achieve a saturated, activated state (Honore et al., 2000), so under this particular pathological condition chronic morphine treatment instead inhibits the activated glial functions and decreases the production of proinflammatory cytokines (Hansson et al., 2008); (2) chronic systemic morphine can decrease the driving force to peripheral or central nociceptive neurons through directly binding the mu opioid receptor, down-regulating the nociceptive hypersensitivity, and blocking the nociceptive signal transmitted to glia, ultimately inhibiting the spinal neuroimmune responses; (3) morphine exerts potent anti-immune activity peripherally, while the immune-competent cells, such as mast cells, macrophages, fibroblasts, and endothelial cells within or adjacent to bone tumor play a vital role to maintaining CIBP.

In conclusion, this study reveals that chronic morphine use in the rat model of CIBP failed to provoke analgesic tolerance with down-regulation of spinal neuroimmune responses. This further suggests that CIBP is a unique pain state and it is necessary for researchers to reconsider possible neurobiological influences after prolonged exposure to opiates in different pathological pain states. In addition, our results also give a possible explanation as to why sustained morphine is clinically effective for the management of CIBP without producing pharmacological tolerance.

4. **Experimental procedures**

4.1. **Preparation of animals**

All experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology and conducted in accordance with the NIH guide for the care and use of laboratory animals and Ethical Issue of the IASP (Zimmermann, 1983). Female Wistar rats, provided by the Institute of Laboratory Animal Science, Tongji Medical College, Huazhong University of Science and Technology, weighing 170–200 g, were kept under controlled conditions (24±0.5 °C, sawdust bedding, 12 h alternating light-dark cycle, food and water ad libitum).

4.2. **Preparation of cells**

Walker 256 rat mammary gland carcinoma cells (purchased from the Institute of Cancer Research, Chinese Academy of Medical Science and Peking Union Medical College) were injected into the abdominal cavity of female Wistar rats (2×10^6 cells/0.5 ml). After 6–7 days cancerous ascites was harvested steriley, and then carcinoma cells were washed briefly with PBS three times by centrifugation for 5 min at 800 rpm. With haemocytometer the pellet was re-suspended within PBS and adjusted to an appropriate concentration (4×10^6/ml). Finally, the cell suspension was maintained on ice until injection.
4.3. Surgical procedure

The method was modified from Mao-Ying et al. (2006). Briefly, rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) and placed abdominal side up. The right leg was shaved and disinfected with 7% iodine. About 1 cm rostral–caudal incision was made in the skin over the top medial half of the tibia. The tibia was carefully exposed with minimal damage to blood vessels and muscles. Utilizing a 23-gauge needle the bone was pierced around 0.5–1 cm below the knee joint distal to the epiphysial growth plate. Then the needle was removed and replaced with a 50 μl Hamilton microsyringe containing cancer cells or PBS. A volume of 10 μl carcinoma cells (4×10^3) was slowly injected into the bone cavity. No leakage of carcinoma cells occurred out of the tibia. The injection site was closed using bone wax as soon as the syringe was removed and the wound was closed and dusted with penicillin powder. Animals were placed in a heated pad until they had regained consciousness, and were returned to their home cages.

4.4. Drug administration

Morphine sulfate was purchased from Sigma (St. Louis, MO, USA) and dissolved in sterile 0.9% physiological saline at the appropriate concentration of 10 mg/ml. Morphine (10 mg/kg) was administered subcutaneously in the scrub at the back of the neck. A total of 45 rats were used in this study: 9 of these were naive rats (received no intervention), 9 received intratibial injection of vehicle (PBS group), and 27 received intratibial injection of cancer cells. Rats inoculated with cancer cells were further divided into 3 subgroups. Carcinoma cell group (n=9) did not receive any treatment after intratibial injection of cancer cells. Chronic morphine group (n=9) received bi-daily (8:00 AM/8:00 PM) subcutaneous injection of morphine (10 mg/kg) commencing on postoperative day 14 and continuing through day 18, with a single injection on the morning of postoperative day 19. During this period, the abnormal nociceptive behaviors in this model reached a relatively stable level (Mao-Ying et al., 2006). Meanwhile, chronic saline group (n=9) received bi-daily injection of physiological saline according to the same schedule. Repeated s.c. administration of morphine at the dose of 10 mg/kg for successive several days is a common approach to induce morphine tolerance in rodents with or without tissue damage (Bohn et al., 2000; Christensen and Kayser, 2000; Raghavendra et al., 2002, 2004a; Zollner et al., 2008). Development of analgesic and pharmacologic tolerance to chronic morphine was recorded on days 1, 3, 5 and 6 of the morphine treatment (i.e. postoperative days 14, 16, 18 and 19).

4.5. Behavioral tests

For all groups with pharmacological interventions, behavioral tests were performed 1 h after drug administration (Raghavendra et al., 2002; Urch et al., 2005). Animals were left to acclimatize to the area for 30 min before testing. Behavioral signs of ambulatory-evoked pain were assessed using the Ugo Basile model 7750 Rotarod (Ugo Basile, Italy). The apparatus was set to accelerate from 0 to 20 revolutions per minute (rpm) over 60 s and the time in seconds maintained on the beam before each rat fell was recorded (with a maximum cut-off of 150 s). Ambulation was observed and scored as follows: normal=0, slight limping=1, marked limping=2, and avoidance of use of limb=3. The use of the Rotarod test has been reported to be a reliable and consistent means of assessing ambulatory-evoked pain (Donovan-Rodriguez et al., 2005; Urch et al., 2003, 2005).

Behavioral measurements of mechanical allodynia were carried out immediately following the test of ambulatory-evoked pain. Rats were placed into a small plexiglass box with a wire mesh floor. After approximately 20 min habituation, mechanical allodynia was recorded when the hind paw made withdrawal responses to von Frey filaments according to the “up and down” method (Chaplan et al., 1994). An ascending series of von Frey filaments (Stoelting Company, Wood Dale, IL, USA) with logarithmically incremental stiffness (0.40, 0.60, 1.0, 2.0, 4.0, 6.0, 8.0 and 15.0 g) were applied perpendicular to the mid-plantar surface (avoiding the less sensitive tori) of each hind paw. Each filament was held for about 10 s at an interval of 10 min between each application. The trial began with application of the 2.0 g von Frey hair. The positive response was defined as a withdrawal of hind paw upon the stimulus. Whenever a positive response to a stimulus occurred, the next weaker von Frey filament was applied, and whenever a negative response occurred, the next stronger filament was applied. The testing was comprised of five more stimuli after the first change in response occurred, and values of responses were converted to a 50% withdrawal threshold using the formula given by Dixon (1980) and Chaplan et al. (1994).

4.6. Immunohistochemistry

On the postoperative day 19 immediately after behavioral tests, rats were deeply anesthetized with chloral hydrate (300 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde in 0.1 M PBS at pH 7.4. The lumbar spinal cord was removed, immersed in the same fixative for 2 h at 4 °C, and transferred to 30% sucrose (W/V) in PBS overnight for cryoprotection. The 30 μm free-floating sections were cut on a cryostat and rinsed briefly in PBS with 0.05% Triton X-100, and blocked in PBS with 10% goat serum for 30 min at room temperature (R.T.), then incubated with primary antibody for 24 h at 4 °C. A mouse anti-rat monoclonal antibody to OX-42 (1:50 working dilution from BD Bioscience, San Jose, CA) was used to label the expression of CD11b/c on microglia. A rabbit anti-rat polyclonal antibody to glial fibrillary acidic protein (GFAP) (1:5000 working dilution from Dako Co., Carpentaria, CA) was used to label astrocytes. After a three-minute washing in PBS, sections were incubated for 2 h at R.T. with TRITC-conjugated goat anti-mouse IgG (1:100, Pierce) or FITC-conjugated goat anti-rabbit IgG (1:100, Pierce). Finally, sections were rinsed three times for 3 min in PBS and mounted onto gelatin-coated slides. Control sections were processed similarly, except that primary antibodies were omitted. Following staining five or more sections from each animal were surveyed under low and medium magnifications to achieve a score based on the following scale: baseline staining (0), mild response (+), moderate response (++), and intense response (+++). The criteria for each score have been described in detail previously (Colburn et al., 1997). In brief,
“activation response” scoring is based on the observed cell morphology, local cell density, and intensity of OX42 and GFAP immunoreactivity.

4.7. RNA extraction and cDNA synthesis

Under chloral hydrate (300 mg/kg, i.p.) anesthesia immediately after behavioral tests on the postoperative day 19, L4–5 spinal segments were quickly removed, sagittally cut into ipsilateral and contralateral hemicords on the plane from the dorsal median sulcus to the ventral median fissure, and then the ipsilateral side was snap frozen in liquid nitrogen for analyses of mRNA. All samples were stored at −80 °C until the time of assays. Total RNA was extracted from about 25 mg of ipsilateral hemi-spinal cord tissues according to the instructions of TRI REAGENT (MRC Co., Cincinnati, USA). Then, the acquired RNA samples were treated with DNase to remove any trace of contaminating DNA using the DNA-Free kit (Ambion, TX, USA). Concentration and purity of the total RNA were determined by measuring absorbance at 260 and 280 nm by spectrophotometry. Total RNA was reversely transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas Inc, MD, USA). The first-strand cDNA was synthesized using 5 μg of the total RNA, 0.5 μg of Oligo(dT)18 primer, 2 μl of 10 mM dNTP mix, 4 μl of 5× reaction buffer, 20 U RiboLock™ Ribonuclease Inhibitor and 200 U RevertAid™ M-MuLV Reverse Transcriptase in a total final volume of 20 μl of 10 mM dNTP mix, 4 μl of 5× reaction buffer, 20 U RiboLock™ Ribonuclease Inhibitor and 200 U RevertAid™ M-MuLV Reverse Transcriptase in a total final volume of 20 μl. The reaction was carried out at 42 °C for 1 h and terminated by deactivation of the enzyme at 70 °C for 10 min. Control reactions lacking either reverse transcriptase or template RNA were included to assess genomic DNA and non-specific contamination (n=3 per group).

4.8. Real-time polymerase chain reaction (PCR)

PCR amplifications were conducted using SYBR Green Premix Ex Taq™ (TaKaRa Biotechnology Co., Ltd. Dalian, China) according to the manufacturer’s instruction. Primer sequences for the genes of interest (GFAP, ITGAM, CD14, CD11b, IL-1β, and TNF-α) were designed and synthesized by Invitrogen and shown in Table 2. Thermocycling was conducted using a LightCycler (Roche Diagnostics) initiated by a 60 s pre-incubation at 94 °C, followed by 40 cycles (94 °C, 5 s; 60 °C, 20 s) with a single fluorescent reading taken at the end of each cycle. The threshold cycle (Ct), the number of cycles to reach the threshold of detection) was determined for each reaction, and levels of the targeted mRNA were quantified relative to levels of the housekeeping gene GAPDH using the relative quantification 2−ΔΔCt method (Livak and Schmittgen, 2001). Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimmers. Each experiment was run three times and each sample was run in triplicate.

4.9. Protein estimation by ELISA

On the 19th postoperative day, L4–5 spinal segments were collected in the same procedure as for real-time PCR. Standard Sandwich ELISA was performed for quantitative determination of IL-1β and TNF-α at the protein level in the spinal cord parenchyma. The tissue homogenization was prepared as described previously (Gearhart et al., 2006). In brief, ipsilateral hemi-spinal cords were homogenized in RIPA including a protease inhibitor. Samples were spun at 16,000 g for 30 min at 4 °C. Supernatant was aliquoted and stored at −80 °C for future protein quantification. Total protein concentrations were determined by the Bradford assay (Bradford, 1976) and used to adjust results for sample size (Schoeniger-Skinner et al., 2007). IL-1β (BioSource International, Inc., Camarillo, CA) and TNF-α (Jingmei Biotech Co. Ltd., Shenzhen, China) protein quantification were acquired by comparing samples to the standard curve generated from the respective rat-specific kit according to the respective manufacturer’s protocol (n=3 per group).

4.10. Radiology

Ipsilateral hind limbs from cadavers were collected, placed on a clear plane plexiglass and exposed to an X-ray source to observe tibial destruction by tumor and determine whether the rat model was set up successfully. The Kodak Directview DR3000 system (Eastman Kodak Company, Rochester, NY, USA) was used to obtain tibial radiographs from all rats tested.

| Table 2 – Primer sequence for the rat genes characterized in this study. |
|-----------------------------|------------------|------------------|
| Gene          | Accession no. | Primer Sequence         |
| GAPDH         | NM_010008     | Sense 5′-CCCACATGATCCGGTTG-3′ |
|               |                | Antisense 5′-TAGCCCGGATGCTTTTAAGT-3′ |
| GFAP          | NM_017009     | Sense 5′-TGGCCACCAGTGATGGA-3′ |
|               |                | Antisense 5′-GATTGCTCAGACATGGCAGTTTC-3′ |
| CD14          | AF087943      | Sense 5′-GCCAACAGGTCGGGCTT-3′ |
|               |                | Antisense 5′-TGGCCACGCCTAATAGT-3′ |
| TLR-4         | NM_019178     | Sense 5′-GATGCGCTACGATGCAATGTT3′ |
|               |                | Antisense 5′-CACCTGGAGGTGTTCTGTGAA-3′ |
| IGTAM         | U59801        | Sense 5′-CTGGCTAGGATGTCCTTCTG-3′ |
|               |                | Antisense 5′-TGGCCACGAGGCCTATAC-3′ |
| IL-1β         | M98820        | Sense 5′-GATGCGCTACGATGCAATGTAAC-3′ |
|               |                | Antisense 5′-TGGCCACGAGGCCTATAC-3′ |
| TNF-α         | S40199        | Sense 5′-GATGCGCTACGATGCAATGTAAC-3′ |
|               |                | Antisense 5′-TGGCCACGAGGCCTATAC-3′ |
4.11. Data analysis

Raw data were presented as mean±SEM and subjected to statistic evaluation using two-way analysis of variance (two-way ANOVA) followed by post-hoc comparison (Student–Newman–Keuls test) to confirm significant differences between groups. P<0.05 was considered significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2010.02.039.

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