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EXTRACELLULAR SIGNAL-REGULATED KINASE ACTIVATION IN SPINAL ASTROCYTES AND MICROGLIA CONTRIBUTES TO CANCER-INDUCED BONE PAIN IN RATS

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Abstract—Cancer pain, especially cancer-induced bone pain, affects the quality of life of cancer patients, and current treatments for this pain are limited. The present study demonstrates that spinal extracellular signal-regulated kinase (ERK) activation in glial cells plays a crucial role in cancer-induced bone pain. From day 4 to day 21 after the intra-tibia inoculation with Walker 256 mammary gland carcinoma cells, significant mechanical allodynia was observed as indicated by the decrease of mechanical withdrawal thresholds in the von Frey hair test. Intra-tibia inoculation with carcinoma cells induced a vast and persistent (>21 D) activation of ERK in the bilateral L2–L3 and L4–L5 spinal dorsal horn. The increased pERK1/2-immunoreactivity was observed in both Iba-1-expressing microglia and GFAP-expressing astrocytes but not in NeuN-expressing neurons. A single intrathecal injection of the selective MEK (ERK kinase) inhibitors PD98059 (10 μg) on day 12 and U0126 (1.25 and 3 μg) on day 14, attenuated the bilateral mechanical allodynia in the von Frey hair test. Altogether, our results suggest that ERK activation in spinal microglia and astrocytes is correlated with the onset of allodynia and is important for allodynia maintenance in the cancer pain model. This study indicated that inhibition of the ERK pathway may provide a new therapy for cancer-induced bone pain.

Key words: extracellular signal-regulated kinase, spinal cord, allodynia, cancer-induced bone pain, astrocyte, microglia.

INTRODUCTION

Bone cancer pain is one of the most common symptoms in patients of late stage cancer. Breast carcinoma and prostate carcinoma are the most common causes of pain from osseous metastasis, and 70% of patients with advanced breast or prostate carcinoma have skeletal metastasis. Skeletal metastases are present in >90% of patients who die from breast or prostate carcinoma (Coleman, 1997; Peng et al., 2006). Thus, pain associated with tumor cells that have metastasized to the bone is a frequent and severe complication of cancer. Unfortunately, current therapies can be ineffective, and even when these therapies are effective, the duration of the patients' survival typically exceeds the duration of pain relief. Better understanding of the mechanisms underlying cancer-induced bone pain can lead to the development of effective therapies. Experimental animal models of pain have provided insight into the mechanisms underlying cancer-induced bone pain and may inform the development of therapies (Honoré et al., 2000; Mantyh et al., 2002; Urch et al., 2005; Niiyama et al., 2007; Colvin and Fallon, 2008; Yamamoto et al., 2008; Zhang et al., 2008a,b; Geis et al., 2010; Mantyh et al., 2010; Bloom et al., 2011; Otis et al., 2011).

The mitogen-activated protein kinases (MAPK) transmit extracellular stimuli into intracellular transcriptional and post-translational responses. The MAPK family includes intracellular signal transduction pathways (extracellular signal-regulated kinase (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK)). Activation of MAPK pathways in the spinal dorsal horn plays an important role in central sensitization (Boulton et al., 1991; Rosen et al., 1994; Ji and Suter, 2007; Hao et al., 2008). The extracellular signal-regulated kinases ERK1 and ERK2 also named as p44 and p42 MAPK are activated by membrane depolarization and calcium influx (Boulton et al., 1991), activated by dual phosphorylation of their regulatory tyrosine and threonine residues by an upstream kinase, mitogen-activated protein kinase (MEK). Several studies have demonstrated the involvement of the activation of ERK1/2 in the neuronal plasticity and activity of the central nervous system. For example, ERK1/2 has been implicated in learning, memory and pain hypersensitivity (Ji et al., 2002; Zhuang

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Abbreviations: CSF, cerebral spinal fluid; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; ERK, extracellular signal-regulated kinase; IR, immunoreactive; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; PBS, phosphate buffer saline; pNR1, phosphorylation of the NR1; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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et al., 2005; Gao et al., 2009; Kim et al., 2009; Gao and Ji, 2009). Previous studies have found that ERK1/2 is activated in the spinal dorsal horn after inflammation and nerve injury, and prolonged ERK1/2 phosphorylation was required for central sensitization during the development of hyperalgesia and allodynia. Intrathecal administration of U0126 or PD98059, inhibitors of MEK (ERK kinase), have been shown to reverse the pain hypersensitivity induced by peripheral tissue and nerve injury (Zhuang et al., 2005; Crown et al., 2006; Cruz, et al., 2005; Gao et al., 2009; Kim et al., 2009).However, little is known about the possible involvement of the activation of ERK in the spinal cord in cancer-induced bone pain. Our previous behavioral studies have demonstrated that injection of Walker 256 rat mammary gland carcinoma cells into the tibia of rats could produce mechanical allodynia (Mao-Ying et al., 2006).

In the present study, we found that the phosphorylation of ERK1/2 was increased in both microglia and astrocytes of the spinal dorsal horn in a model of cancer-induced bone pain. The activation of pERK1/2 contributed to mechanical allodynia, because a single intrathecal injection of MEK (ERK kinase) inhibitors PD98059 and U0126 by lumbar puncture attenuated mechanical allodynia. These results demonstrate that activated ERK1/2 in microglia and astrocytes of the spinal dorsal horn contribute to cancer-induced bone pain.

EXPERIMENTAL PROCEDURES

Animals

Adult female Wistar rats weighing 160–200 g were used in all experiments. All animals were kept under controlled conditions (a temperature-controlled room (24 ± 0.5 °C), a 12:12 h light cycle (07:00–19:00 light), with free access to food and water). All animal experiments were conducted in accordance with the IASP's guidelines for pain research (Zimmermann, 1983).

Surgical procedures

Walker 256 rat mammary gland carcinoma cells were used in the experiment. Suspensions of 1×10^8/ml tumor cells in phosphate buffer saline (PBS) were prepared as previously described (Mao-Ying et al., 2006). Five ml of Walker 256 carcinoma cells was obtained from an ascetic tumor-bearing rat, and 5 ml of PBS was added. The solution was centrifuged at 1200*g* for 3 min to collect the carcinoma cells. The precipitation was washed twice with PBS and diluted to the concentration used in the experiment. The surgical procedure was modified from previously reported method (Medhurst et al., 2002; Mao-Ying et al., 2006; Zhang et al., 2008a,b; Zhao et al., 2010; Tong et al., 2010a,b; Liu et al., 2011). After rats were anesthetized with sodium pentobarbital (i.p. 50 mg/kg), 4 μl of Walker 256 carcinoma cells was slowly injected into the right tibia cavity of each rat using a 10-μl microinjection syringe without any incision. Briefly, after the rats were anesthetized, the right leg was shaved and the skin was disinfected with 70% (v/v) ethanol. A 23-gauge needle drilled a hole at the site of intercondylar eminence of the tibia and then was replaced with a 10-μl microinjection syringe containing 4 μl of carcinoma cells and 4 μl of absorbable gelatin sponge dissolved in saline to seal the drilled hole. After 1 min of delay to allow cells to fill the bone cavity, the syringe was removed, and the limb was dusted with penicillin powder. For the sham group (control), 4 μl of PBS was injected instead of carcinoma cells into the tibia.

Drug administration

Two specific MEK inhibitors, PD98059 and U0126, were used in the experiment. MEK acts upstream of ERK1/2, and the MEK inhibitors successfully attenuate ERK1/2 phosphorylation (Alessi et al., 1995). PD98059 was purchased from Calbiochem (San Diego, California, USA), and U0126 was purchased from Sigma–Aldrich (St. Louis, MO, USA). PD98059 was dissolved in 20% dimethyl sulfoxide (DMSO) and U0126 was dissolved in 10% DMSO. PD98059 (10 μg) and U0126 (1.25 μg, 3 μg) were used respectively in the experiment and controls were treated with the same amount of DMSO. Rats were anesthetized with 2% isoflurane. After shaving the lumbar region and sterilizing it with 70% ethanol, animals were given a lumbar puncture at the L5–L6 interspace using a 0.5-inch 30-gauge needle. Drugs were administered into the cerebral spinal fluid (CSF) space through the needle (Xu et al., 2006).

Western blots

After inoculation with carcinoma cells, rats ($n = 4$) were killed on day 3, 7, 14, and 21. The L4–L5 spinal cord was dissected, the segment was cut into a left and right half from the ventral midline. The right half was used in the experiment. Protein lysate, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and Western blotting were performed as previously described (Rosen et al., 1994; Mao-Ying et al., 2006). The protein was extracted by homogenization in a SDS sample buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% SDS, 1 mM each, PMSF, NaF, $NaVO_3$, 1 μg/ml each, leupeptin, pepstatin, aprotinin) and followed by centrifugation at 12000*g* for 20 min. The protein concentration of the supernatant was determined by BCA Protein Assay Kit (Pierce, Rockford, USA), and 30 μg of protein was loaded on each lane of 10% SDS–PAGE. The membrane was blocked by 5% bovine serum albumin in TBS-T (50 mM Tris–HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20) overnight. The blot was probed with rabbit anti-phosphorylated ERK1/2 antibody (1:1000, Cell signaling Technology, MA, USA) or rabbit anti-ERK1/2 antibody (total ERK1/2, 1:3000, Cell signaling Technology, MA, USA) for 3 h at room temperature, then incubated with HRP-anti-rabbit (1:1000, Santa Cruz, CA, USA) antibody for 1 h at room temperature. Developed in ECL (Pierce, Rockford, USA) solution for 3 min, and exposed onto Kodak X-OMAT AR Film (Eastman Kodak, Rochester, USA) for 1.5 min. Densitometric analysis of pERK1/2 bands and total ERK1/2 bands were performed by using Syngene software (GeneGnome, Syngene, MD, USA). The same size square was drawn around each band to measure the density and subtract the background near that band. pERK1/2 levels were normalized against total ERK1/2 levels and expressed as % of total ERK1/2.

Immunohistochemistry

Rats were anesthetized with sodium pentobarbital, perfused through ascending aorta with 500 ml of 4% paraformaldehyde (4 °C) followed by 250 ml saline (37 °C) on day 7, day 14, and day 21 after carcinoma cells inoculation. The L2–L5 spinal cords were removed and post fixed in the same fixative solution for 4 h, and then cryoprotected over night in 20% sucrose. For immunohistochemistry, the spinal cord was cut at 30 μm on a freezing microtome (Leica 2000, Germany). The sections were stored at −20 °C in a cryoprotective solution until further usage. Sections were washed in PBS, treated with 0.75% Triton X-100 and 1% H_2O_2 in 0.01 M PBS for 1 h, and blocked with 4% donkey serum in 0.3% Triton X-100 for 1 h at 37 °C. Then the sections were incubated with rabbit anti-phosphorylation ERK1/2 antibody (1:200, Cell signaling Technology, MA, USA) at 4 °C over night. Secondary reactions were with biotinylated donkey anti-rabbit immunoglobulin (1:400, Vector Laboratories, CA, USA) for 1 h,

followed by avidin–biotin–peroxidase complexes (1:200, Vector Laboratories, CA, USA) for 1 h. The sections were washed with 0.01 M PBS for 15 min three times and then mounted on gelatinized slides, dehydrated through a series of ethanol solutions, cleared in xylene, and covered with glass coverslips. Four rats were used in quantitative measurement of pERK1/2-immunoreactive (IR) cell profiles in L2–L3 and L4–L5 spinal dorsal horn. The number of pERK1/2-IR cells was counted in lamina I–II and lamina III–IV of the ipsilateral spinal dorsal horn that was captured by using a computerized image analysis system (Leica Qwin 500). The area sampled was in the lamina I–II and half of the lamina III–IV. For immunofluorescence, the sections were washed three times and blocked with 4% donkey serum in 0.3% Triton X-100 for 1 h at 37 °C, and incubated with rabbit anti-phosphorylation ERK1/2 antibody (1:200, Cell signaling Technology, MA, USA) at 4 °C over night, then incubated with donkey fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson immunolab, PA, USA) for 1 h at 37 °C. For double immunofluorescence, sections were incubated with a mixture of rabbit anti-pERK and mouse anti-NeuN (neuronal marker, 1:500, Chemicon, CA, USA), mouse anti-GFAP (astrocyte marker, 1:500, Sigma–Aldrich, MO, USA), mouse anti-Iba-1 (microglia marker, 1:200, Chemicon, CA, USA) antibodies at 37 °C for 1 h, 4 °C overnight. Then the sections were incubated with a mixture of donkey FITC-conjugated (green) and donkey Alexa Fluor 594-conjugated (red, 1:500, Chemicon, CA, USA) secondary antibodies for 1 h at 37 °C. The stained sections were examined with Olympus FV1000 fluorescence microscope. At least four rats from each group and time point were analyzed. A minimum of 10 sections randomly selected from L2–L3 and L4–L5 spinal cord of each rat were counted to get average data per rat. Four rats of each group were used for statistical analysis. For immunofluorescence, at least one section from L4–L5 spinal cord of each rat and four rats of each group were used for statistical analysis.

Behavioral tests

Eight rats in each group were used in behavior tests, the day of operation was referred to as day 0. Mechanical allodynia was assessed using a von Frey hair (Stoelting, Wood Dale, IL, USA) filament as previously described (Dixon, 1980; Mao-Ying et al., 2006). An ascending series of von Frey hairs with logarithmically incremental stiffness (0.40, 0.60, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g) were used in the experiment. The test began with the application of the 2.0 g von Frey hair, and the plantar surface of right hand paw was stimulated in the test. Animals were habituated to the environment daily for at least 2 days before baseline testing. For testing the paw withdrawal thresholds, animals were put into the experimental environment for 30 min before stimulation. The pre-drug baseline was assessed 1 h before intrathecal injection on day 12 and day 14. All the tests were performed blinded with respect to drugs injected.

Statistical analysis

All data were analyzed with one-way ANOVA following Bonferroni post-test, and were presented as mean ± standard error of the mean (SEM). For behavioral data, the drug-administration groups were compared to vehicle control group and the different time points after drug-administration in model groups (carcinoma cells inoculation) were compared to before drug-administration. Eight rats were used in the behavioral test. Four rats were included in each group for quantification of Western blots results and immunohistochemistry results. The sham-operated rats were used as control and all groups were compared to tissues originating from control rats. $P < 0.05$ was set as the threshold of significance. The statistical analysis for behavior test was performed blinded. The statistical analysis for Western blots results and immunohistochemistry results were performed by the experimenters (not blinded).

RESULTS

Time course of the development of bilateral allodynia after intra-tibia inoculation with Walker 256 mammary gland carcinoma cells

The paw withdrawal thresholds to von Frey hair stimulation in the bilateral side were measured. A significant decrease was found in the bilateral paw on day four after inoculation with carcinoma cells compared to the control rats (Fig. 1A, B). Radiographs on alive rats with bone cancer had been taken and obvious bone destruction in the ipsilateral tibia was observed (Fig. 1C–F). The postmortem verifications after the end of the experiment were carried out, no obvious bone destruction in the contralateral tibia or other bones were observed, indicating that, no metastasis was found in the contralateral tibia or other bones at least during the observation window (within around 30 days) after the inoculation with cancer cells (data not shown).

Sustained activation of ERK in the spinal dorsal horn after intra-tibia inoculation with Walker 256 mammary gland carcinoma cells

We then examined whether ERK1/2 was activated in the cancer-induced pain model using western blots. pERK1/2 (44 and 42 kD) and total ERK1/2 (42 and 44 kD) were detected on the same membrane. The representative original immunoblotting bands detected in the spinal dorsal horn were obtained from five groups of rats ($n = 4$) including control, day 3, day 7, day 14 and day 21 after injection of carcinoma cells. We found that pERK1 and pERK2 were significantly increased in the spinal cord on day 3, day 7, day 14 and day 21 after injection of carcinoma cells (Fig. 2).

The number of pERK1/2-IR cells was increased in dorsal horn of the spinal cord after intra-tibia inoculation with Walker 256 mammary gland carcinoma cells

The immunohistochemistry results showed that pERK1/2 expression was very low in control spinal cord (Fig. 3A, B). An increased activation of ERK1/2 was obviously found in the bilateral L2–L3 spinal dorsal horn on day 7 and lasted until day 21 after carcinoma cell injection, and also pERK1/2 was increased in L4–L5 spinal dorsal horn on day 7 and lasted until day 21 on the ipsilateral side (Fig. 3C, D). The most obvious increase was found in the superficial dorsal horn, and pERK activation was also found in the deep dorsal horn.

ERK phosphorylation in neurons, astrocytes, and microglia after intra-tibia inoculation with Walker 256 mammary gland carcinoma cells

To identify the cell types that expressed increased pERK1/2, we used double immunofluorescence of pERK1/2 with frequently used cell-specific markers: NeuN (neurons), GFAP (astrocytes) and Iba-1 (microglia). In the spinal cord of control rats, pERK1/2 was co-expressed with neurons and microglia, very little co-expression with astrocytes (Fig. 4A, D, G). After inoculation with

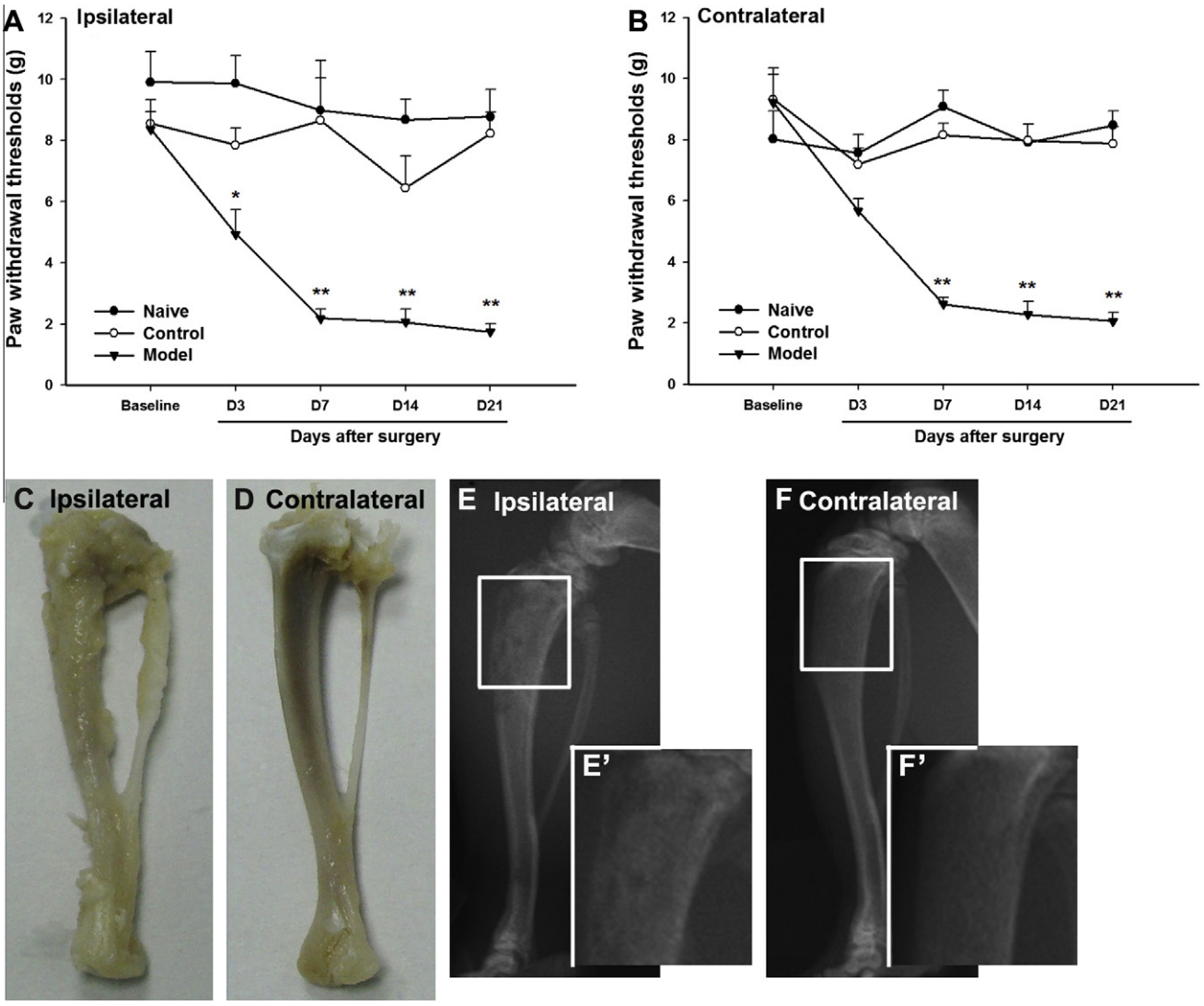

Fig. 1. Bone destruction and changes of mechanical response thresholds to von Frey hair of the bilateral hind paws in rats inoculated with intra-tibia Walker 256 cells (4×10^5). (A–B) BCIP animals have bilaterally mechanical allodynia evidenced by a decrease in paw withdrawal thresholds to mechanical stimuli from day 4 until day 21. (C–D) Typical photograph of the rat tibias 21 days after the intra-tibia inoculation with Walker 256 cells. See the obvious bone destruction of the ipsilateral tibia (D). (E–F) Typical radiographs of the rats 21 days after the intra-tibia inoculation with Walker 256 cells. Data are expressed as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control rats.

carcinoma cells, we found that pERK1/2 was co-expressed with neurons (Fig. 4B, C), microglia (Fig. 4E, F) and astrocytes (Fig. 4H, I) on day 14 and day 21. Double staining indicated that pERK1/2 was increased in Iba-1-IR microglia and GFAP-IR astrocytes (Fig. 4M) after inoculation with carcinoma cells. These results suggest that the increased pERK1/2 in microglia and astrocytes participated in the development of bone cancer-induced mechanical allodynia.

Intrathecal MEK inhibitors attenuated the mechanical allodynia induced by intra-tibia inoculation with Walker 256 mammary gland carcinoma cells

To determine the role of pERK1/2 in cancer-induced bone pain, the effects of MEK inhibitor PD98059 (10 μg) and U0126 (1.25 and 3 μg) delivered into the L5–L6 CSF space by a single lumbar puncture were assessed. Control injections were 20% DMSO (vehicle for PD98059) and 10% DMSO (vehicle for U0126). Paw withdrawal thresholds were tested at 1 h before intrathecal injection and at different time points after injection. The paw withdrawal thresholds decreased on day 12 after inoculation with carcinoma cells, and both PD98059 and U0126 increased paw withdrawal thresholds after intrathecal administration. PD98059 attenuated the mechanical allodynia on the bilateral sides at 3 h after intrathecal injection on day 12. DMSO had no effect on mechanical allodynia (Fig. 5A, B). Two doses of U0126 reduced the bilateral mechanical allodynia on day 14 after inoculation with carcinoma cells, as both the high dose (3 μg) and the low dose (1.25 μg) of U0126 reduced mechanical allodynia at 1 h after intrathecal injection and lasted for more than 5 h in the ipsilateral side (Fig. 5C).

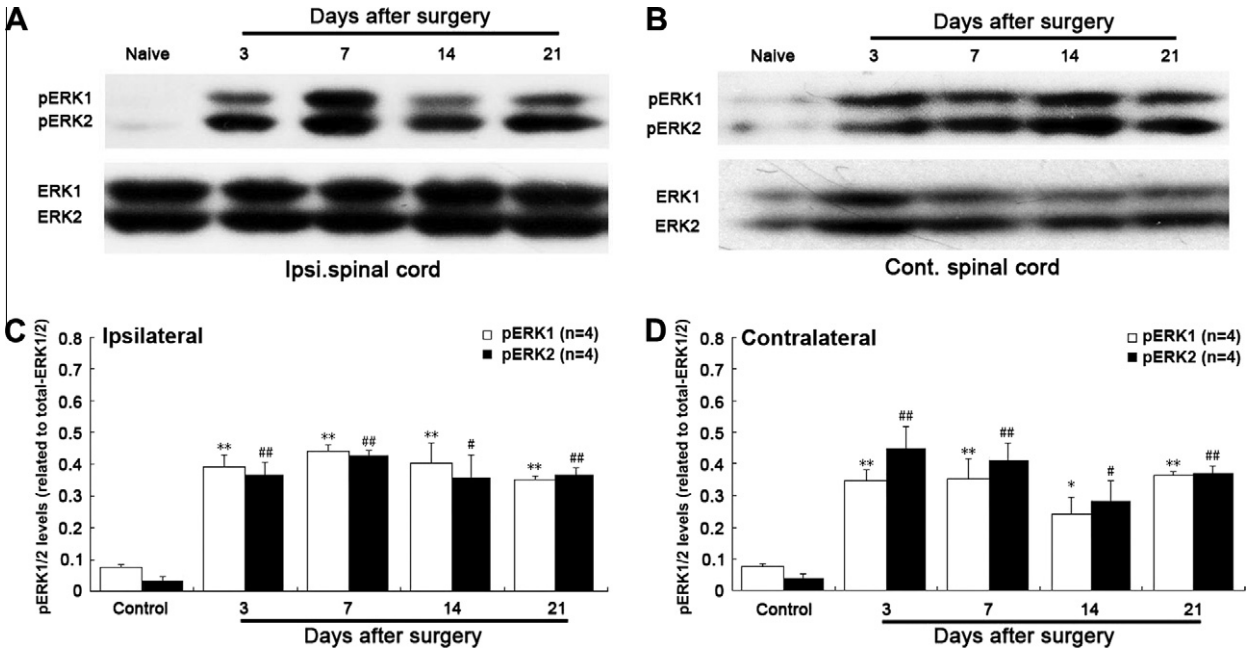

Fig. 2. Time course of pERK1/2 upregulation in the L4–L5 spinal cord after intra-tibia inoculation with Walker 256 carcinoma cells. (A,B) Representative Western blots for pERK1/2 with total ERK1/2 as the loading control, on the ipsilateral and contralateral side of control (PBS-injected) and model (carcinoma cells inoculated) groups. (C,D) Densitometric analysis showing a similar increase in the levels of pERK1 and pERK2 in the bilateral L4–L5 spinal cord. pERK1/2 levels were normalized against total ERK1/2 levels. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with control pERK1; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with control pERK2. $n = 4$. Values are expressed as the mean ± SEM.

DISCUSSION

In addition to the roles in inflammatory and neuropathic pain (Huang et al., 2004; Zhuang et al., 2005; Guo et al., 2007; Zhang et al., 2007), our data shows that ERK activation in the spinal cord plays a role in cancer-induced bone pain. In the present report, we have demonstrated that ERK1/2 activation in the spinal cord has an important role in pain hypersensitivity in a model of bone cancer pain induced by intra-tibia inoculation with Walker 256 mammary gland carcinoma cells. Although it is well known that ERK phosphorylation is increased in the spinal cord and dorsal root ganglion (DRG) under inflammatory and neuropathic pain conditions (Huang et al., 2004; Zhuang et al., 2005; Guo et al., 2007; Zhang et al., 2007), the time course and cellular localization as well as the possible role of ERK activation in the spinal cord have not been investigated in the cancer-induced bone pain. The present study detected a unique sustained activation of ERK in both microglia cells and astrocytes in spinal cord. We also found that the MEK inhibitors PD98059 and U0126 both reduced mechanical allodynia in cancer-induced bone pain model. These results indicate that the activation of ERK in the spinal cord contributes to bone cancer pain.

Implantation of carcinoma cells into the tibia or calcaneus bones of animals produces mechanical allodynia and heat hyperalgesia (Asai et al., 2005; Khasabov et al., 2007; Miao et al., 2010). In the present study, the paw withdrawal thresholds in response to von Frey hair stimulation on the plantar surface of the hand paw stable decreased from day 4 after carcinoma cells inoculation and lasted until the final day in which we tested responses (day 21). These results suggest that developed mechanical allodynia was present in our model, and is consistent with the results of our previous study (Mao-Ying et al., 2006). Peripheral sensitization (increased sensitivity in peripheral sensory neurons) and central sensitization (increased sensitivity in spinal dorsal horn neurons and brain neurons) are the main mechanisms of chronic pain (Millan, 1999; Woolf, 2011). Previous studies have demonstrated that the activation of MAPK pathways contributes to peripheral and central sensitization (Obata and Noguchi, 2004; Ji et al., 2009), and pERK1/2 expression has been regarded as a marker for central sensitization (Gao et al., 2009). However, the role of pERK1/2 activation in cancer-induced bone pain has not been clear to date. In our present study, pERK1/2 was activated in the L4–L5 spinal dorsal horn bilaterally from day 3 to day 21 following inoculation with carcinoma cells, and interestingly, pERK1/2 activation was found not only in the L4–L5 spinal dorsal horn but also in the L2–L3 spinal dorsal horn. After inoculation with MRMT-1 carcinoma cells, a fast blue retrograde neuronal tracer found that the sensory neurons innervating the tibia were mainly observed in primary afferent fibers of the L3 DRG and, to a lesser extent, in L1–L2 and L4–L5 of the DRG (Kaan et al., 2010). Also, in a cancer-induced bone pain model of mice, spinal synaptic transmission was shown to be enhanced in the substantia gelatinosa (SG, lamina I0049) of L2–L3 and L4–L5 lumbar levels which provides evidence of widespread spinal sensitization (Yanagisawa et al., 2010). Our results suggest that the inoculation with carcinoma cells into the tibia induced central sensitization through pERK1/2 activation in a wide area of spinal cord through an unknown mechanism.

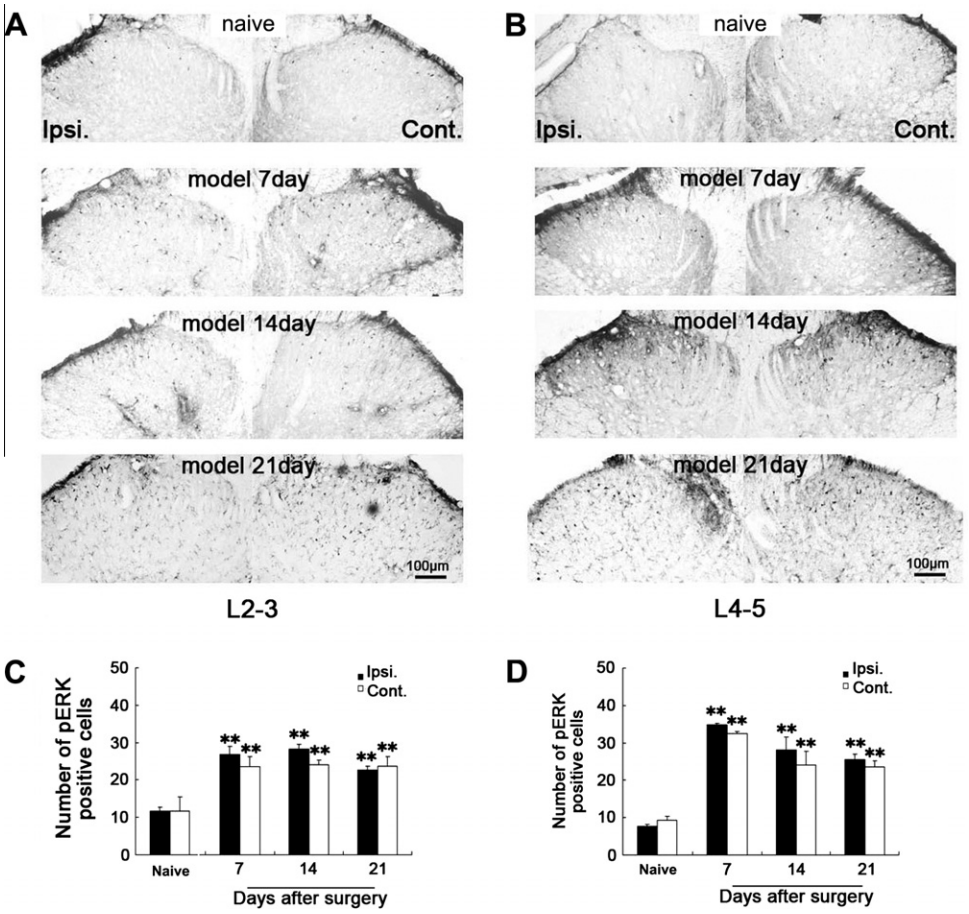

Fig. 3. Time course of pERK1/2 expression in the spinal dorsal horn at the L2–L3 and L4–L5 lumbar levels. (A) L2–L3 sections (30 μm) immunostained for pERK1/2 in control and model groups on day 7, day 14 and day 21. (B) L4–L5 sections (30 μm) immunostained for pERK1/2 in control and model groups on day 7, day 14 and day 21. (C) Quantification of the number of pERK1/2-IR cells in the L2–L3 spinal dorsal horn. (D) Quantification of the number of pERK1/2-IR cells in the L4–L5 spinal dorsal horn. All data are represented as the mean ± SEM, $n = 4$, scale bar = 100 μm. $^{**}p < 0.01$, compared to control by one-way ANOVA.

In previous studies, the activation of MAPK pathways was shown to contribute to neuropathic pain and inflammatory pain in different cell types at different times. For example, p38 and ERK phosphorylation were first increased in spinal neurons and microglia after inflammation and nerve injury, then decreased in neurons and microglia but increased in astrocytes at the late stage of neuropathic pain. JNK phosphorylation has been shown to primarily increase in astrocytes after nerve injury (Jin et al., 2003; Mizushima et al., 2005; Hao et al., 2008; Gao et al., 2009; Gao and Ji, 2010). Activation of ERK in spinal cord neurons is associated with the activation of nociceptive-specific sensory fibers (Hao et al., 2008) that contribute to both behavioral and cellular central sensitization. Interestingly, the significant increase of pERK was observed in glial cells but not in neurons in our model. Thus, the mechanism of cancer-induced bone pain seems quite different from that of neuropathic pain and inflammatory pain. In the present study, ERK phosphorylation significantly increased in microglia and astrocytes on day 14 after inoculation and lasted until day 21. Thus, it appears that the activation of ERK1/2 in microglia and astrocytes is more important than in neurons for the maintenance of cancer-induced bone pain. Based on the behavioral results, we did not detect which types of the increased pERK1/2-IR cells were in the early stage (< 4 day) of bone cancer pain. The pERK1/2-IR neurons peaked at 10 min after injecting complete freunds adjuvant (CFA) into the plantar surface of the hind paws in rats (Peng et al., 2009), and pERK1/2 activation in neurons and microglia was detected within 2 days after CCI (Gao et al., 2009). Considering the findings above, we hypothesize that the increasing of pERK1/2-IR cells may be found in neurons or both neurons and microglia before day 4.

The MEK inhibitors U0126 and PD98059 were used to identify the function of ERK activation. Intrathecal administration of PD98059 and U0126 reduces neuropathic pain and inflammatory pain through various downstream mechanisms (Daulhac et al., 2010; Kaan et al., 2010). Substantial evidence has established that the phosphorylation of *N*-methyl-D-aspartate receptor (NMDAR) contributes to central sensitization in the spinal cord after nerve injury or inflammation. U0126 inhibits the phosphorylation of the NR1 subunit (pNR1) and decreases in mechanical hyperalgesia in diabetes-induced painful neuropathy suggest that pNR1 is regulated by ERK1/2 (Zhao et al., 2007). PD98059 inhibits the induction of cyclooxygen-

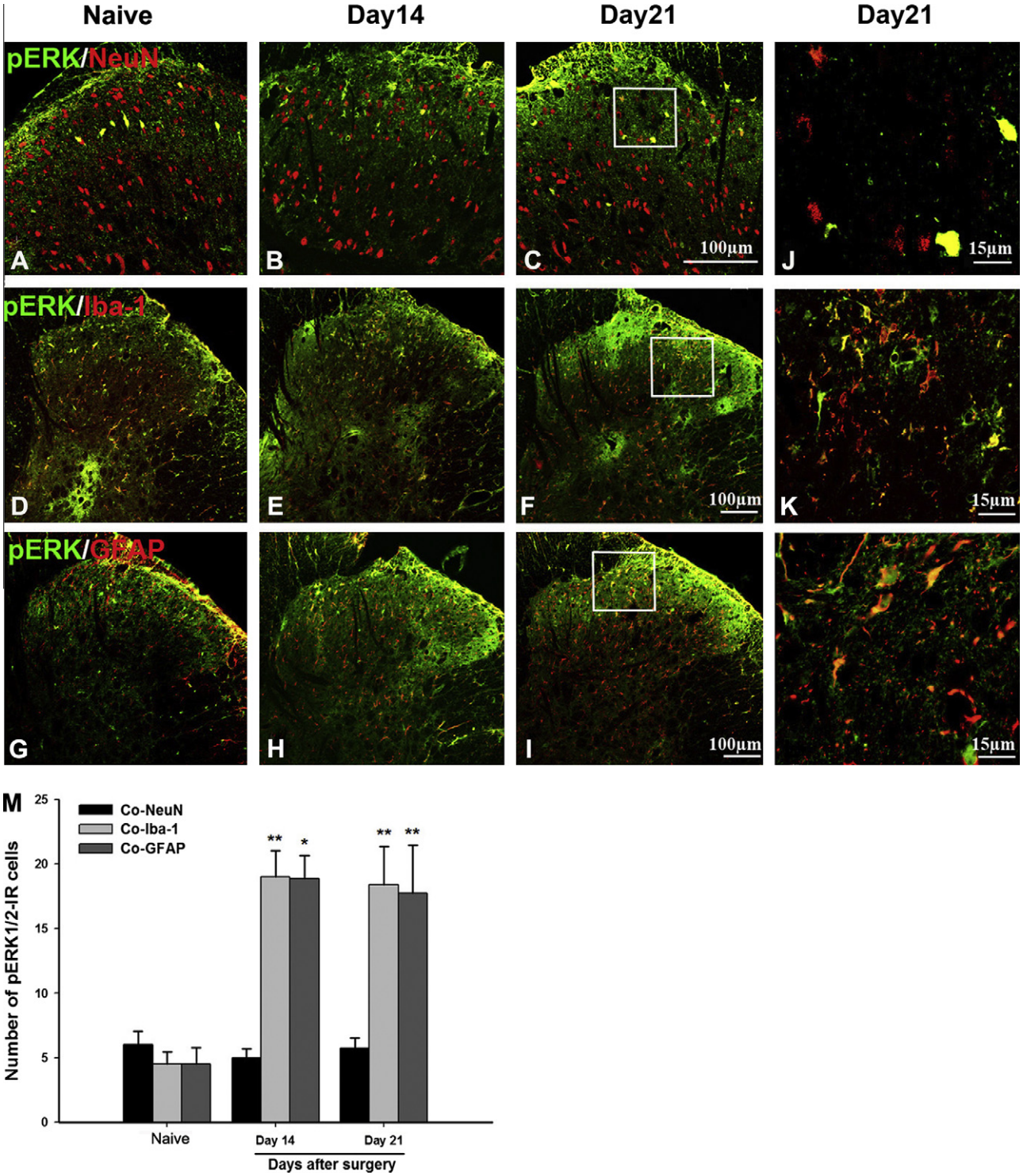

Fig. 4. Double immunofluorescence of pERK1/2 (A–L) and NeuN (A–C, J) or Iba-1 (D–F, K) or GFAP (G–I, L) in the ipsilateral L4–L5 spinal dorsal horn (lamina I–III) of the control group and the model groups of day 14 and day 21 after carcinoma cells inoculation. (A–C) pERK1/2 expression in neurons. Double staining appeared yellow. (D–F) pERK1/2 expression in microglia. Double staining appeared yellow. (G–I) pERK1/2 expression in astrocytes. Double staining appeared yellow. (J–L) The higher magnification images of C, F, I, indicated in the white boxes. (M) Statistic analysis of pERK1/2-IR cells co-expressed with NeuN, Iba-1 and GFAP. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared to control by one-way ANOVA. Scale bar = 100 μm in A–I, 15 μm in J–L. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ase-2 in spinal microglia and the spinal release of prostaglandin E2 (Zhao et al., 2007), which was an important factor in central sensitization. In the present study, intrathecal PD98059 on day 12 and U0126 on day 14 after inoculation with carcinoma cells attenuate the bilateral mechanical allodynia. From these results, it appears that,

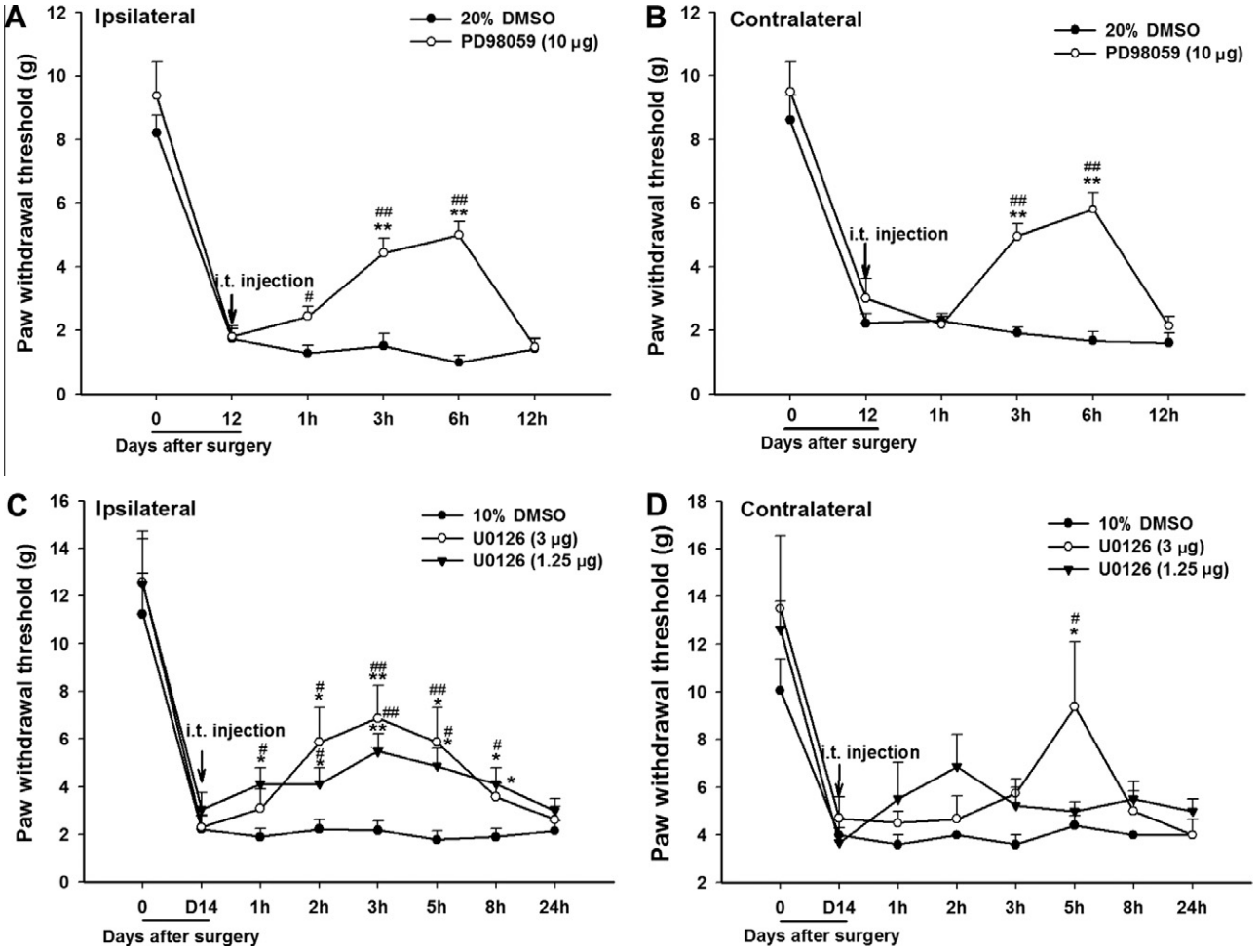

Fig. 5. Inhibition of ERK1/2 activation partially reversed the bilateral mechanical allodynia of the hind paw of rats after intra-tibia inoculation with Walker 256 carcinoma cells. (A, B) Intrathecal injection of MEK (ERK kinase) inhibitor PD98059 (10 μg) attenuated the bilateral mechanical allodynia on day 12 after carcinoma cells inoculation. (C, D) Two doses of the MEK (ERK kinase) inhibitor U0126 (1.25 and 3 μg) attenuated the bilateral allodynia of the hind paws on day 14 after carcinoma cells inoculation. DMSO had no effect on cancer-induced bone pain. The results are represented as the mean ± SEM. $^{\#,\#\#}p < 0.05$ and $p < 0.01$ compared with DMSO, $^{*,**}p < 0.05$ and $p < 0.01$ against the pre-administration by one-way ANOVA. The numbers of tested rats were 8 for each group.

after inoculation with carcinoma cells, ERK1/2 is essential for intracellular signaling in glial cells, which possibly leads to the production of pro-nociceptive factors. However, the possible involvement of JNK and p38 in cancer pain can not be ignored and remains to be studied. In addition, Gao et al. reported an interesting finding that ERK activation in spinal cord after intraplantar injection of CFA was inhibited by JNK inhibitor suggesting a possible interaction between JNK activation and ERK activation (Peng et al., 2009). Hence, the possible modulating effect of spinal JNK on ERK activation in cancer pain remains to be further investigated.

CONCLUSION

In summary, the inoculation with Walker 256 rat mammary gland carcinoma cells into the tibia of rats induced bone cancer pain, and rats developed mechanical allodynia. The resulting hypersensitivity was attenuated by the MEK inhibitors PD98059 and U0126. Increased ERK phosphorylation in spinal glial cells play an important role in cancer-induced bone pain and ERK activation in glial cells should be investigated in future studies. Inhibition of the ERK pathway may provide a new therapy for cancer-induced bone pain.

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