Neuroscience 217 (2012) 172–181

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

X.-W. WANG, [a,c,1] T.-T. LI, [b,1] J. ZHAO, [a,c] Q.-L. MAO-YING, [a,c] H. ZHANG, [a,c] S. HU, [a,c] Q. LI, [a,c] W.-L. MI, [a,c] G.-C. WU, [a,c] Y.-Q. ZHANG [b*] AND Y.-Q. WANG [a,c*]

[a] *Department of Integrative Medicine and Neurobiology, State Key Laboratory of Medical Neurobiology, Shanghai Medical College, Fudan University, Shanghai, China*

[b] *Institute of Neurobiology, Institutes of Brain Science, Fudan University, Shanghai, China*

[c] *Institute of Acupuncture Research (WHO Collaborating Center for Traditional Medicine), Fudan University, Shanghai, China*

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

*Corresponding authors. Addresses: Institute of Neurobiology, Institutes of Brain Science, Fudan University, 138-Yi-Xue-Yuan Road, Shanghai 200032, China. Tel: +86-21-54237635; fax: +86-21-54237635 (Y.-Q. Zhang), Department of Integrative Medicine and Neurobiology, Shanghai Medical College, Fudan University, P.O. 291, 138 Yi-Xue-Yuan Road, Shanghai 200032, China. Tel: +86-21-54237496; fax: +86-21-54237526 (Y.-Q. Wang).

E-mail addresses: yuqiuzhang@fudan. edu. cn (Y.-Q. Zhang), wangyanqing@shmu. edu. cn (Y.-Q. Wang).

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.

[1] These authors contributed equally to this work.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.04.065

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,