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DOWNREGULATION OF SPINAL ENDOMORPHIN-2 CORRELATES WITH MECHANICAL ALLODYNIA IN A RAT MODEL OF TIBIA CANCER

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Abstract—The endogenous tetrapeptide endomorphin-2 (EM2) participates in pain modulation by binding to pre- and/or post-synaptic μ opioid receptor (MOR). In the present study, pathological expression and antinociceptive effects of EM2 at the spinal level were investigated in a rat model of bone cancer pain. The model was established by introducing Walker 256 mammary gland carcinoma cells into the tibia medullary cavity. Immunohistochemical staining for EM2 showed a markedly reduced EM2-immunoreactivity in the ipsilateral spinal dorsal horn on days 6, 12 and 18 post Walker 256 inoculation ($p < 0.05$). Intrathecal injection (i.t.) of EM2 significantly attenuated cancer-induced mechanical allodynia ($p < 0.05$) which could be blocked by β-funaltrexamine (β-FNA), the μ receptor antagonist ($p < 0.05$). Furthermore, topical application of EM2 dose-dependently inhibited the electrically evoked C-fiber responses and postdischarge of wide dynamic range (WDR) neurons within the spinal cord ($p < 0.05$), and pretreatment with β-FNA abolished the hyperactivity of these neurons. Compared with the antinociception of morphine which took effect from 40 min to 100 min post application, the analgesic action of EM2 was characterized by quick onset and short-lived efficacy ($p < 0.05$), being most potent at 10 min and lasting about 20 min. These findings indicate that the down-regulated spinal EM2 is an important contributor to the neuropathological process of bone cancer pain and enhancing activation of EM2/μ receptor signaling might provide a therapeutic alternative to optimizing the treatment of cancer-induced bone pain.

Key words: cancer-induced bone pain, endomorphin-2, morphine, allodynia, nociceptive information transmission.

INTRODUCTION

Cancer pain is one of the most common symptoms of patients with malignant tumor and is hard to control (Foley, 1999; Portenoy and Lesage, 1999). Cancer-induced bone pain (CIBP), the debilitating suffering associated with bone cancer, arises mainly from carcinoma bone metastasis (Mercadante, 1997; Mundy, 1997). The major clinical symptoms of CIBP include continuous ongoing pain, referring to constant background pain accompanying and exacerbating with progression of cancer, and breakthrough pain that spontaneously occurs as an intermittent episode of extreme pain, more frequently provoked through weight bearing or movement, at the deteriorating state of cancer (Goblirsch et al., 2005; Schmidt et al., 2010). Breakthrough pain is more difficult to relieve by common analgesics as compared with ongoing pain (Zeppetella, 2008). Clinically, treatment of intense CIBP often resorts to systemic morphine with high dosages (Honore et al., 2000b) which functions mainly by activating μ-opioid receptors (MORs) pre- and/or post-synaptically (Wang et al., 2005; Macey et al., 2014; Mizoguchi et al., 2014; Zhang et al., 2014). However, morphine therapy is compromised by its paralleling adverse side effects and tolerance (Cleeland et al., 1994). Furthermore, morphine shows a delayed action of maximal potency for antiallodynic effect at lower doses (~30 min post application) (Przewlocka et al., 1999; Zeppetella, 2008), not coincident with the "time window" of breakthrough pain characterized by sudden onset and a duration of −35 min (Zeppetella, 2008); hence ineffective in breakthrough pain relief.

Endomorphin-2 (EM2), an opioid tetrapeptide, together with its analog EM1 comprises endogenous endomorphins (Zadina et al., 1997) which, like morphine, engage in pain suppression by activating pre- and post-synaptic MORs associated with nociceptive circuits (Sakurada et al., 1999; Zadina et al., 1999; Smith et al., 2001; Wu et al., 2003; Fichna et al., 2007). EM2 was immunohistochemically demonstrated to be much more abundantly expressed than EM1 within the spinal cord,

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Abbreviations: β-FNA, β-funaltrexamine; ANOVA, analysis of variance; CIBP, Cancer-induced bone pain; DRG, dorsal root ganglion; EM2, endomorphin-2; EM2-IP, EM2-immunopositive; MOR, μ opioid receptor; PB, phosphate buffer; PBS, phosphate-buffered saline; POD, post-operative day; PWT, paw withdrawal threshold; RPM, rotations per minute; WDR, wide dynamic range.

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suggesting it plays a more crucial role in antinociception at the spinal level (Martin-Schild et al., 1998, 1999; Schreff et al., 1998; Pierce and Wessendorf, 2000; Smith et al., 2001). It has been reported that spinal EM2 was downregulated following sciatic nerve ligation (Smith et al., 2001). Involvement of endogenous spinal opioid mechanisms in antinociception was also suggested by using a CIBP model (Menendez et al., 2003). In CIBP, unique neurochemical changes (i.e. neurochemical changes different from those with inflammatory and neuropathic pain), may occur (Schmidt et al., 2010). Whether and how EM2 content changes within the spinal cord in the CIBP model has not yet been characterized. For better understanding of the mechanisms underlying CIBP, the present study aims to investigate the changing pattern of EM2 and its antinociceptive effect at the spinal level by using a combination of immunohistochemistry, electrophysiological and pharmacological techniques on a tibia cancer-bearing rat model of CIBP.

EXPERIMENTAL PROCEDURES

All surgical procedures and behavior tests of the present experiment were approved by the Committee of Animal Use for Research and Education of the Fourth Military Medical University (Xi'an, P.R. China) and all the surgeries were performed under general anesthesia with sodium pentobarbital (40–60 mg/kg i.p., Fluka, Buchs, Switzerland) to alleviate the animals' suffering and efforts were made to minimize the number of animals used.

Animals

Adult female *Sprague–Dawley* rats weighing 180–220 g at the beginning of the experiment were used. Three rats were housed in each individual cage with food and water *ad libitum* under a 12:12-h light–dark cycle (lights on at 06:00 AM, off at 18:00 PM).

Cell preparation

The procedures of cell preparation were in accordance with the previous study (Tong et al., 2010; Chen et al., 2013). Briefly, Walker 256 rat mammary gland carcinoma cells (The Fourth Military Medical University, Xi'an, China) (0.5 ml, 2×10^7 cells/ml) were injected into the abdominal cavity of the SD rats. After 7–10 days, ascitic fluid was extracted and centrifuged for 3 min at 1500 rpm. Cells were diluted to achieve final concentrations of 5×10^5 cells/10 μl 0.01 M phosphate-buffered saline (PBS, pH 7.4) for injection and kept on ice until injected into rats. The cells in the same final concentrations were boiled for 20 min for the sham group.

Surgery

As described in our previous studies (Tong et al., 2010; Chen et al., 2013), following complete anesthesia with sodium pentobarbital (i.p. 50 mg/kg), the skin of the right leg of the rat was cut and a 10 μl volume of Walker 256 carcinoma cells (5×10^5 cells) was slowly injected into the intramedullary cavity of the tibia. The syringe was then removed and the injection site was closed using bone wax. Then penicillin was applied to the wound. In the sham group, all the procedures were the same as those of the model group, except that the equal volume of heat-treated carcinoma cells was administered instead.

Intrathecal implantation

The intrathecal implantation was made as previously reported (Mei et al., 2009). Briefly, a midline incision (3 cm) was made at the level of the thoracic vertebrae 3–4 (T3–4) at the back of the rats, under pentobarbital sodium anesthesia (45 mg/kg, i.p.). An appropriate length of PE-10 tube (I.D. 0.28 mm and O.D. 0.61 mm) (Clay Adams, Parsippany, NJ, USA) was inserted from the T8 to the lumbar 3 (L3) level of the spinal cord, and 2 cm of free ending was left in the upper thoracic region. Rats were allowed to recover for 3–5 days. Only the animals that were neurologically normal and showed complete paralysis of the tail and bilateral hind legs after administration of 2% lidocaine (10 μl) through the intrathecal catheter were used for the following experiments.

Drug administration

EM2 (Sigma, St. Louis, MO, USA), morphine (Sigma), β-funaltrexamine (β-FNA, Sigma) or saline was intrathecally (i.t.) administered. In order to observe the effect of intrathecal EM2, a total of 24 CIBP rats were used on day 14 post-cancer cell injection: six of these were injected of 10 μl saline, six received injection of 1 μg EM2, six were injected with 3 μg EM2, and six were injected with 10 μg EM2. To study the influence of β-FNA (antagonist of MOR) on the antinociceptive effect of EM2, 24 CIBP rats were divided into four groups randomly, and were individually pretreated with i.t. injection of 10 μg β-FNA or 10 μl saline. Because β-FNA has transient kappa agonist activity and long-lasting MOR antagonist actions (Ward et al., 1982), it was injected 24 h prior to EM2 or saline administration, as in previous studies (Frankel et al., 1999; Martin et al., 2002; Ward et al., 2006). Then we compared the analgesic effect of EM2 and morphine on the CIBP rats 14 days after cancer cell injection. Eighteen CIBP rats were randomly divided into three groups: saline ($n = 6$), 10 μg EM2 ($n = 6$), or 30 μg morphine ($n = 6$). Drugs were dissolved in saline and injected in a volume of 10 μl, followed by a 5 μl saline flush.

Behavioral testing

In our previous study, we observed that mechanical allodynia was stable from 12 days to 18 days (Chen et al., 2013). So we chose day 14 and day 15, when the mechanical allodynia reached the highest level and was stable, to perform behavioral and electrophysiological experiments. As described previously (Mei et al., 2009; Tong et al., 2010), rats were placed in inverted plastic boxes ($30 \times 30 \times 50$ cm^3) on an elevated mesh floor and the ipsilateral hindpaws were pressed with a logarithmic series of eight calibrated Semmes–Weinstein monofilaments (von-Frey hairs; Stoelting, Kiel, WI, USA). Log