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Involvement of EphB1 receptor/ephrinB1 ligand in bone cancer pain

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ABSTRACT

In prior studies, Eph/ephrin system was demonstrated to be involved in inflammatory and neuropathic pain modulation. The present study was to investigate whether the spinal Eph/ephrin signaling was involved in modulation of spinal inflammatory cytokines in bone cancer pain (BCP) of rats. BCP was induced by intra-tibial inoculation of Walker 256 mammary gland carcinoma cells. The expressions of EphB1/ephrinB1 in spinal cord (SC) and dorsal root ganglia (DRG) were determined. At 16 days post inoculation, the pain relieving effect and the mRNA levels of inflammatory cytokines were detected after intrathecal administration of EphB1-Fc (blocker of EphB1 receptor, 10 μg). The results showed that the EphB1/ephrinB1 expression was significantly increased in SC, but ephrinB1 was decreased in DRG after Walker 256 inoculation. The mechanical allodynia induced by bone cancer was significantly alleviated by intrathecal administration of EphB1-Fc. Furthermore, the RT-PCR analysis showed that the mRNA levels of IL-1β, IL-6 and TNF-α were significantly increased at 16 days post Walker 256 inoculation and were significantly suppressed by intrathecal administration of EphB1-Fc in SC. We concluded that Eph/ephrin might be involved in the maintenance of mechanical allodynia, via modulating the expression of spinal inflammatory cytokines, in the present rat model of BCP. This study suggested that Eph/ephrin signaling would be a potential target for the treatment of BCP.

Cancer remains a major cause of death. With improvement of medical treatment, patient's life expectancy has been significantly extended, but the cancer-induced pain is extremely disruptive to patient's life quality [7]. Recently, several successful cancer pain models in rats were established. These models have promoted the understanding of mechanisms underlying cancer pain and could help to deal with cancer pain.

The erythropoietin-producing human hepatocellular carcinoma receptors (Ephs) are the largest subfamily of receptor tyrosine kinases (RTKs), which include A (A1–A8) and B (B1–B4, B6) types. The ligands of Ephs are named Eph-receptor-interacting proteins (ephrins), which are divided into two types: glycosylphosphatidylinositol (GPI)-anchored A-type (A1–A5) and transmembrane B-type (B1–B3) [6]. Studies have shown that EphB/ephrinB system was involved in modulation of inflammatory and neuropathic pain [1,5]. However, there is no report about the role of EphB/ephrinB in bone cancer pain (BCP) till now. It has been documented that EphB/ephrinB modulated spinal nociceptive processing through a mitogen-activated protein kinases (MAPKs)-dependent mechanism [12]. And activating MAPKs-dependent cell signaling cascades could result in a rapid pro-inflammatory response which produces inflammatory cytokines such as IL-1β, IL-6 and TNF-α [18]. And reports have demonstrated that these inflammatory cytokines were important for the induction and maintenance of pain [11,20].

Thus, the present study was to investigate whether the spinal Eph/ephrin signaling was involved in modulation of spinal inflammatory cytokines in BCP of rats.

Female Wistar rats (150–170 g, from Shanghai Laboratory Animal Center, China Academy Sciences, Shanghai) were kept under controlled conditions (23 ± 0.5 °C, 12 h alternating light–dark cycle, free food and water ad libitum). All tests were carried out in a temperature-controlled room (23 ± 0.5 °C) from 8 a.m. to 12 a.m. to avoid behavioral variation by circadian rhythm. The experimental procedures were approved by Animal Care and Use Committee of Fudan University, and were consistent with the NIH's Guide for the Care and Use of Laboratory Animals and the Ethical Issues of the IASP [23].

According to the previous method [8], the rats were anesthetized with chloral hydrate (i.p. 400 mg/kg). After hair shaved, skin disinfected, a 23-gauge needle was punctured through the skin, aimed at the inner side of intercondylar eminence, pierced 1 cm below the knee joint into the medullary cavity of tibia, and

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then removed and replaced with a 10 μl microinjection syringe. Walker 256 rat mammary gland carcinoma cells from ascitic fluid (4×10^4) in 4 μl phosphate buffered saline (PBS) or 4 μl PBS alone (sham group) were slowly injected into the tibial cavity. The syringe was removed 1 min later to prevent the cells from leaking out along the injection track.

Mechanical allodynia was measured by the hind paw withdrawal response to von Frey hair stimulation as previously described [17]. A series of nine calibrated von Frey hairs were applied to the central region of the plantar surface of one hind paw in ascending order (1 g, 1.4 g, 2 g, 4 g, 6 g, 8 g, 10 g, 15 g, 26 g). The hair was applied only when the rat was stationary and standing on all four paws. And the stimulation was sustained for 2 s. A withdrawal response was considered valid only if the hind paw was completely removed from the customized platform. Each hair was applied five times at 5 s intervals. If withdrawal response was not induced more than twice during five applications of a hair, the next ascending hair in the series was applied in a similar manner. Once the hind paw was withdrawn from a particular hair three out of the five consecutive applications, the rat was considered responsive to that hair. Then the next descending hair was applied until the hind paw was withdrawn less than three out of the five times. The paw withdrawal threshold (PWT) was defined as the lowest hair force in grams that produced at least three withdrawal responses in five tests.

At 16 days post Walker 256 inoculation, EphB1-Fc chimera (10 μg, Sigma, E9277), an EphB1 receptor blocking reagent, was administrated intrathecally as previously published [16]. In brief, EphB1-Fc, the human immunoglobulin G (IgG) Fc fragment (2 μg, Jackson, Fc control) or PBS was administrated intrathecally via lumbar puncture under inhalational anesthesia with isoflurane. For intrathecal injection (I.T.), a modified lumbar puncture technique was used as described by Kim et al. [4]. A volume of 20 μl was injected slowly. The syringe was held for 10 s before removal to prevent outflow of the solution.

After being sacrificed, rats were perfused with fresh 4% paraformaldehyde. The lumbar spinal cord (L4–L6) was removed, postfixed overnight and subsequently immersed in 30% sucrose. 20 μm-thick sections were prepared and rinsed in 0.01 M PBS followed by blocked in 0.3% H_2O_2 for 10 min. The sections were rinsed in 0.01 M PBS and preincubated for 30 min at room temperature in 3% normal goat serum in 0.01 M PBS with 0.3% Triton-X 100 (NGST). Then the sections were incubated in primary rabbit polyclonal anti-ephrinB1 antibody (1:50, Santa Cruz) or rabbit polyclonal anti-EphB1 antibody (1:50, Santa Cruz) diluted in 1% NGST at 4 °C for 24 h. After washing three times in 0.01 M PBS, the sections were incubated in biotinylated goat anti-rabbit IgG (1:200, Jackson) for 1 h at 37 °C, washed three times in 0.01 M PBS and incubated for 1 h in avidin–biotin–peroxidase complex (1:200, Vector) at 37 °C. Finally, the sections were washed three times in 0.01 M PBS and the immunoreactive products were visualized by catalysis of 3,3-diaminobenzidine. Then the sections were mounted, air-dried, dehydrated with alcohol, cleared with xylene and cover slipped. The sections were observed using Leica Q500 IW image analysis system.

Total RNA from lumbar spinal cord (L4–L6) was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's recommendations. The amount of RNA was measured spectrophotometrically. 1 μg of total RNA was reversely transcribed into cDNA using the M-MLV reverse transcriptase (LifeFeng Biotech) with oligo (dT). PCR reactions were performed in the presence of the oligonucleotide primers as shown in Table 1. β-Actin was used as housekeeping gene for an internal control. Each PCR production (10 μl) was electrophoresed in 2% agarose gel containing ethidium bromide staining and scanned with ultraviolet transilluminator (GDS 8000, Gene Tools from Syngene Software, UK).

The lumbar spinal cord (L4–L6) and DRG (L4, L5) were homogenized at 4 °C in RIPA (Beyotime) supplemented with 1 mM phenylmethyl sulfonylfluoride (Beyotime) and the protein concentration was measured by the bicinchoninic acid (Beyotime) method. The equivalent amounts of proteins (30 μg) were separated by 8% or 10% SDS-PAGE and transferred (300 mA for 1.5 h) onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% nonfat milk at room temperature for 2 h and then incubated overnight at 4 °C with a primary antibody (anti-EphB1 1:200, anti-ephrinB1 1:200) or horseradish peroxidase (HRP)-conjugated anti-GAPDH (Kangchen). HRP-conjugated goat anti-rabbit IgG (Jackson) was used as secondary antibody at a dilution of 1:10,000. The chemiluminescence was detected with the ECL system (Millipore).

Data were presented as mean ± SEM and were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test, using SPSS 13.0 statistical software. $P < 0.05$ was considered statistically significant.

Immunohistochemistry staining was used to examine the expression of ephrinB1 and EphB1 in spinal cord (SC). As displayed in Fig. 1, the expression of EphB1 was moderate and the level of ephrinB1 was quite low on dorsal horn (DH) in normal and sham groups (16 days after intra-tibial inoculation of PBS). In tumor-bearing rats, the number of EphB1 positive cells was strongly increased and ephrinB1 was significantly increased in not only superficial layer but also deep layer.

Further Western blot analysis showed that in SC and DRG, the protein expression of ephrinB1 and EphB1 was comparable between normal and sham rats. At 8 and 16 days post Walker 256 inoculation, the protein levels of ephrinB1 and EphB1 in SC were significantly increased compared with those of normal and sham rats (Fig. 2A and C). However, the level of ephrinB1 in DRG was markedly decreased after inoculation of Walker 256 cells, while the EphB1 level was not significantly changed compared with that of normal and sham rats (Fig. 2B and D).

Compared with normal and sham rats, tumor-bearing rats displayed a profound decrease in ipsilateral PWT in a time-dependent manner, reaching a lowest level at 16th day post inoculation. Compared with that of normal rats, PWT of sham group was decreased at 2nd and 4th day but there was no significant difference between sham and normal group after 6 days of inoculation (Fig. 3A). Administration of EphB1-Fc (10 μg, I.T.) significantly alleviated mechanical allodynia induced by bone cancer. This effect appeared at 2 h, peaked at 6 h and disappeared at 12 h, 24 h after injection (Fig. 3B). However, Human-Fc or PBS (I.T.) had no pain-alleviating effect.

Our data demonstrated that low levels of IL-1β, IL-6 and TNF-α mRNA were present in SC of sham rats. The mRNA levels of IL-1β, IL-6 and TNF-α in SC were markedly augmented at 8 and 16 days post inoculation of Walker 256 (Fig. 4A and C). At 2 h after intrathecal administration of EphB1-Fc, the mRNA levels of IL-1β, IL-6 and TNF-α were significantly decreased compared with those of PBS-treated rats, while injection of Human-Fc failed to alter the mRNA levels of these inflammatory cytokines (Fig. 4B and D).

The present study was carried out in a modified rat model of BCP induced by intra-tibial inoculation of Walker 256 mammary gland carcinoma cells [8,9]. Here we detected the effects of EphB1/ephrinB1 in this model. The results showed that intra-tibial inoculation of Walker 256 cells induced significant mechanical allodynia and significantly increased spinal expression of EphB1/ephrinB1 protein. EphB1-Fc significantly alleviated the mechanical allodynia induced by bone cancer. Our data indicated that the Eph/ephrin might be involved in the maintenance of BCP in rats. Further, RT-PCR analysis showed that the mRNA levels of spinal inflammatory cytokines were significantly increased after Walker 256 inoculation and markedly decreased after intrathecal