

Original Article

Effects of EphB1 inhibitor on tibia cancer pain in rats and its related mechanism

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Abstract: Objective: To investigate the effects of EphB1 inhibitor (EphB1-Fc) on tibial cancer pain in rats by intrathecal injection and its related mechanism. Methods: Thirty two male SD rats successfully underwent sheath built-in tube were assigned into 4 groups (n=8) according to random number table: sham operation group (C group, as normal control group), model group (BCP group), model + solvent group (BCP + DMSO group), model + EphB1-Fc group (BCP + E group). A model of bone cancer pain (BCP) was established by injecting Walker256 rat breast cancer cells into the medullary cavity of left tibia. Intramuscular administration was performed 1 day after BCP modeling, once a day for 14 days. The rats in BCP + DMSO and BCP + E groups were respectively injected intrathecally with 20 μ l 10% DMSO and 10 μ g EphB1-Fc for 20 μ l. The mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) of rats' left hindlimb were measured at 1 day before operation and 1, 3, 5, 7 and 14 days after operation. On the 14th day after operation, Western blot was adopted to detect phosphorylation of JNK and activation of NF- κ B p65 in the rat's spinal dorsal horn of each group. Results: Praxiology: compared with C group, the MWT and TWL of BCP group decreased significantly from 5th to 14th day after operation. Compared with BCP group 1 day before operation, the MWT and TWL of that showed a progressive reduction from 5th to 14th day after operation, and it reached the maximum level on the 14th day after the inoculation of tumor cells. Thermal and mechanical hyperalgesia caused by tumor cell implantation were significantly inhibited from 5th to 14th day after operation through successive intrathecal injection of EphB1-Fc, an inhibitor of EphB1 receptor signaling pathway; meanwhile, repeated use of EphB1-Fc significantly inhibited the activation of NF- κ B p65 protein ($P=0.02$) and the phosphorylation of JNK protein ($P=0.01$) induced by BCP. Conclusion: EphB1-Fc can block the activation of JNK and NF- κ B p65 pathway by inhibiting the binding of EphB1 and its receptor, thus alleviating the tibia cancer pain in rats.

Keywords: EphB1, EphB1-Fc, bone cancer pain (BCP), spinal cord, JNK, NF- κ B

Introduction

Bone is one of the common metastatic sites of cancer, and bone cancer pain (BCP) is a common clinical chronic pathological pain, mainly manifested as hyperalgesia, and abnormal hyperalgesia. On the grounds of its unclear pathogenesis and poor treatment effects, BCP has serious effects on patients' life, therefore, it is very urgent to further explore the mechanism of BCP, and find new drugs and gene targets to prevent and treat BCP [1, 2]. Tumor tissues contain many different types of cells, such as macrophage, neutrophile, lymphocyte, etc. [3]. These cells can secrete a variety of sub-

stances which can excite the primary afferent neurons, including prostaglandin, endothelin, cytokine, etc. Binding with their corresponding receptors in the primary afferent neurons, these substances give rise to the excitement of neurons, transmit nociceptive information into the central nerve, and thus cause pain.

Eph receptor is currently known as the largest receptor family tyrosine kinase. Recently, researchers have found increasing significances of Eph receptor. In addition to regulating a variety of cells' transmembrane signal transduction apoptosis, Eph receptor also plays an important role during the process of organic deve-

lopment, especially in the development of central nervous system. Recent studies have found that Eph receptor signaling system also has a strong effect on the regulation of neuralgia and inflammatory pain [4, 5]. Therefore, it is of great theoretical and practical significance to further study the mechanism of BCP and to seek for a new and more effective cancer pain target. This study aims to investigate the effect of EphB1 inhibitor (EphB1-Fc) by intrathecal injection, in order to further explore the mechanism of EphB1 signaling pathway on tibial cancer pain in rats.

Materials and methods

Experimental animals

Thirty-two healthy adult male SD rats, (6-8 weeks old, weight 180-220 g) were provided by the Southern Medical University Experimental Animal Center. The rats were given a free diet in a temperature-controlled room at 20-24°C, and were performed the experiment after 5 days' adaptation of this environment.

Experimental grouping

Thirty-two rats were divided into 4 groups (n=8) in accordance with random number table: normal control group (C group), model group (BCP group), model + solvent group (BCP + DMSO group), model + EphB1-Fc group (BCP + E group). A model of BCP was established by injecting Walker256 rat breast cancer cells into the medullary cavity of left tibia. Intramuscular administration was performed 1 day after BCP modeling, once a day for 14 days. The rats in BCP + DMSO and BCP + E groups were respectively injected intrathecally with 20 µl 10% DMSO and 10 µg EphB1-Fc for 20 µl. This study followed the guidelines for the protection and usage of experimental animals and was approved by the Experimental Animal Ethics Committee.

Experimental methods and observation indexes

Experimental instruments and materials: EphB1-Fc (Sigma Company, the USA), DMSO (Beijing Super Chemical Instrument Co., Ltd, China), Anti-p-JNK (Celling Signaling Company, the USA), Anti-NF-κB p65 (Abcam Company, the USA), Anti-GAPDH (Beijing Zhongshan Jinqiao

Biotechnology Co., Ltd, China), Von Frey filaments (North Coast Medical Company, the USA), thermal radiation stimulus machine (Institute of Biotechnology of Chinese Academy of Medical Sciences).

Determination of pain praxiology in rats

Determination of mechanical withdrawal threshold (MWT): The rats were placed in a transparent organic glass box (22 cm*12 cm*22 cm) with a metallic sieve (1 cm*1 cm) at the bottom. Adapting for 30 minutes, the middle part of the pelma of rat's left hind limb was stimulated in a vertical direction by Von Frey cellosilk with different folding force for 6-8 seconds. Rats appearing actions like paw withdrawal or paw licking was seen as a positive action, otherwise it was a negative one. The interval between each stimulation was 60 seconds. 50% MWT was calculated by up-down method [3].

Determination of thermal withdrawal latency (TWL): The rats were placed in a transparent organic glass box with a 3 mm thick glass plate at the bottom. According to Hargreaves method, thermal radiation stimulus machine (10 V, 50 W, the facula diameter is 0.8 cm) was used to irradiate rat's pelma. TWL was the duration from the beginning of irradiation to rats beginning to lift legs. The automatic cut-off time was 25 seconds in order to prevent the occurrence of tissue damage. Each animal was measured 5 times with interval time of 5 minutes. The mean of three middle stable data was as TWL value.

Western blot

Rats received intraperitoneal injection of 10% chloral hydrate (300-350 mg/kg) for deep anesthesia, and then sacrificed immediately. Rats' L4-L6 sections of spinal cord were weighed and performed ultrasonic homogenated in a low temperature. The nucleoprotein extraction was performed according to the instruction on the kit followed by centrifugation (14000 g) for 15 minutes at 4°C, then the supernatant was collected. After quantifying the concentration of the collected protein by BCA method, they were denatured with buffer, of which the volume was four times of the protein, for 5 minutes at 95°C. Then, the equivalent amount of nucleoproteins was performed

Effect and related mechanism of EphB1 inhibitor

Table 1. Effects of EphB1-Fc on MWT of BCP rats

	-1 d	1 d	3 d	5 d	7 d	14 d
C	14.12±4.19	13.83±2.55	14.66±4.96	13.42±4.28	15.42±3.39	14.33±4.74
BCP	14.75±4.49	12.33±2.25	11.25±1.69	8.25±1.99**	6.50±0.78**	4.21±1.11**
BCP + DMSO	14.58±4.84	12.42±1.86	12.00±1.09	8.32±1.13	6.92±1.72	4.67±1.48
BCP + E	15.48±3.73	13.29±1.23	12.75±1.92	11.00±1.23#	9.49±1.9##	6.58±1.15##

Note: **P<0.01, compared with C group. #P<0.05, ##P<0.01, compared with BCP group.

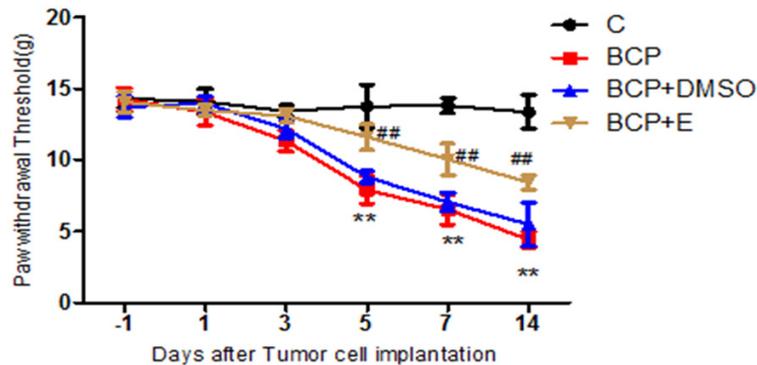


Figure 1. Effects of EphB1-Fc on MWT of BCP rats. Data were expressed as mean \pm SD (n=8). C: Control group (C group); BCP: BCP group; BCP + DMSO: BCP + DMSO group; BCP + E: BCP + EphB1-Fc group. **P<0.01, compared with C group; ##P<0.01, compared with BCP group.

electrophoresis on 10% SDS polyacrylamide gel with constant voltage. Then the separated proteins were transferred to nitrocellulose membrane, blocked by 3% BSA for 2 hours at room temperature, and washed three times by washing buffer, each time for 5 minutes. Anti-p-JNK rabbit, NF- κ B p65 polyclonal antibody and anti-GAPDH mouse polyclonal antibody and the membranes were incubated at 4°C overnight, which were re-warmed for 30 minutes, washed three times by washing buffer, each time for 5 minutes, in next day morning. The secondary antibody labeled with alkaline phosphatase was added and they were oscillated and incubated for 2 hours at room temperature and then washed three times by washing buffer, each time for 5 minutes. NBT/BCIP was used to detect reaction bands which were performed semiquantitative analysis by Image J Launcher software of Broken Symmetry Software Company.

Statistical method

SPSS 16.0 statistical software was used to analyze and process the data. The measurement data were expressed as mean \pm standard

deviation (mean \pm SD). Repeated measures analysis of variance or single factor variance analysis was used to analyze data, and post-hoc analysis to analyze the comparison between every two groups. The difference was statistically significant with P<0.05.

Results

Effects of EphB1-Fc on mechanical withdrawal threshold (MWT) of BCP rats

There was no significant difference in MWT among groups at 1 d before operation (P>0.05). Compared with C group, the MWT of BCP group decreased obviously on the fifth, seventh and fourteenth day after operation (P<0.01). Compared with BCP group, there was no significant difference in MWT of BCP + DMSO group at each time point, while the MWT of BCP + E group increased obviously on the fifth, seventh and fourteenth day after operation (P<0.05, P<0.01). See **Table 1** and **Figure 1**.

Effects of EphB1-Fc on thermal withdrawal latency (TWL) of BCP rats

Compared with C group, the TWL of BCP group decreased significantly on the fifth, seventh and fourteenth day after operation (P<0.01). Compared with BCP group, there was no significant difference in TWL of BCP + DMSO group at each time point, while the TWL of BCP + E group increased obviously on the fifth, seventh and fourteenth day after operation (P<0.01). See **Table 2** and **Figure 2**.

Western blot

The result showed that p-JNK protein was expressed in each group on the 14th day after

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Table 2. Effects of EphB1-Fc on TWL of BCP rats

Group	-1 d	1 d	3 d	5 d	7 d	14 d
C	15.18±1.34	15.33±1.39	14.78±1.15	13.94±1.64	13.69±1.41	15.54±0.55
BCP	14.87±0.78	13.32±0.72	12.38±1.55	8.66±0.98**	5.91±0.84**	3.98±0.64**
BCPI + DMSO	15.99±0.58	13.93±0.96	12.58±1.18	8.90±0.73	6.47±0.76	4.61±0.75
BCP + E	14.65±2.2	13.50±1.11	13.17±0.55	10.49±0.79##	7.57±0.55##	8.71±0.76##

Note: **P<0.01, compared with C group. ##P<0.01, compared with BCP group.

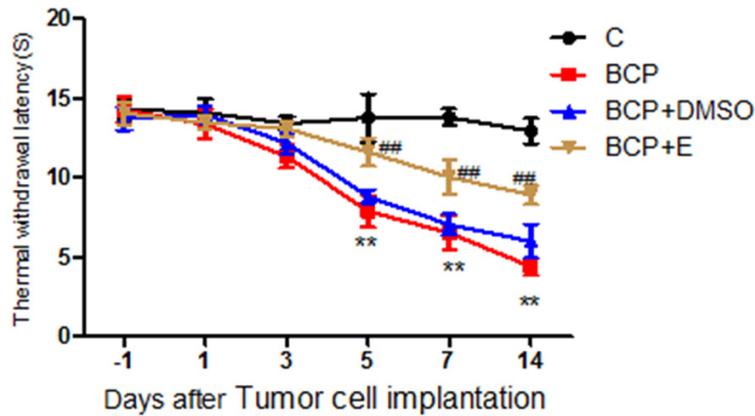


Figure 2. Effects of EphB1-Fc on TWL of BCP rats. Data were expressed as mean ± SD (n=8). C: Control group (C group); BCP: BCP group; BCP + DMSO: BCP + DMSO group; BCP + E: BCP + EphB1-Fc group. **P<0.01, compared with C group; ##P<0.01, compared with BCP group.

Table 3. Western blot detecting the effects of EphB1-Fc to the phosphorylation of JNK protein and the activation of NF-κB p65 protein in spinal dorsal horn of BCP rats on the 14th day after operation

Group	NF-κB p65 (fold change)	p-JNK (fold change)
C	1.000±0.000	1.000±0.000
BCP	2.519±0.617**	2.885±0.698**
BCP + DMSO	2.472±15.91	2.521±0.513
BCP + E	1.787±0.144##	1.767±0.263##

Note: **P<0.01, compared with C group. ##P<0.01, compared with BCP group.

operation and, compared with C group, the expression of p-JNK protein in BCP group was significantly increased (P<0.01). Besides, compared with BCP group, the expression of p-JNK protein in BCP + E group was significantly decreased (P<0.01). Meanwhile, NF-κB p65 protein was also expressed in the nucleus of each group. Compared with C group, the expression of NF-κB p65 protein in the nucleus was significantly increased in BCP group (P<0.01).

Compared with BCP group, the expression of NF-κB p65 protein in the nucleus of BCP + E group was significantly decreased (P<0.01). See Table 3 and Figure 3.

Discussion

Bone is a common metastasis site of cancer, the bone metastasis rate of patients with lung cancer, breast cancer and prostatic cancer can reach 85% [6, 7]. Numerous researches have indicated that BCP is a kind of chronic pain which has distinctive and complex mechanism. Therefore, to understand the

BCP, and to seek the optimum treatment method, the mechanism of the occurrence and development of BCP should be initially found out. Studies have reported the role of EphB1/ephrinB1 signaling pathway in tibia cancer pain rats, but the effect of using the Eph signal system inhibitor to control BCP and its related mechanism has remained to be studied. This study established a rat model of BCP in the light of the literature method [3, 20]. Testing the MWT and TWL of rats, the study has found that there was no significant difference in the base value among the groups 1 d before operation. Compared with sham operation group (C group), the MWT and TWL of rats in BCP group were all significantly decreased on the fifth, seventh, fourteenth day after operation, indicating the successful establishment of BCP model. Praxiology study also suggested that, compared with the BCP group, EphB1-Fc could significantly increase MWT and TWL (P<0.01) in BCP rats at each time point after operation and ameliorate the thermal hyperalgesia and mechanical hyperalgesia of BCP rats. Afterwards, this study investigated the molecu-

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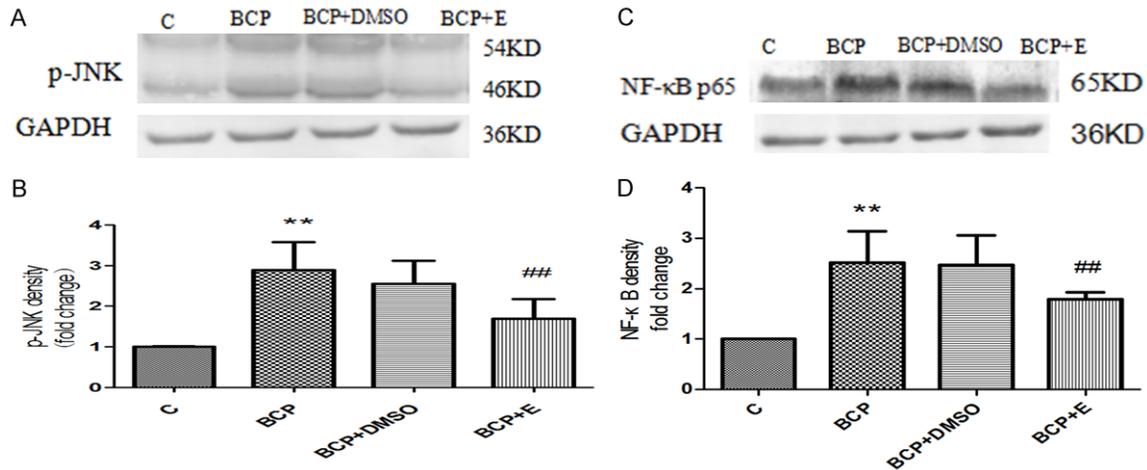


Figure 3. Western blot detecting the effects of EphB1-Fc to the phosphorylation of JNK protein and the activation of NF-κB p65 protein in spinal dorsal horn of BCP rats. GAPDH was used as an internal standard. The expression levels of p-JNK and NF-κB p65 on the spinal cord were quantized. Data were expressed as mean ± SD (n=8). **P<0.01, compared with C group; ##P<0.01, compared with BCP group.

lar mechanism of EphB1-Fc, which could improve BCP praxiology.

JNK is a kind of serine or threonine kinase, also known as stress activated protein kinase (SAPK). It can be activated by a variety of factors, such as inflammatory factor, bacterial endotoxin, ischemia, hypoxia, etc. The phosphorylated JNK can regulate and control the functional activity of various substrates, resulting in changes of cell function [8]. In recent years, more and more studies have showed that SAPK/JNK pathway plays a vital role in the regulation of pain sensitization [9, 10]. Guo et al. found that the expression of phosphorylated JNK protein was increased in spinal dorsal horn of BCP rats and intrathecal injection of JNK inhibitor SP600125 could effectively reduce BCP [11, 12]. The study of Tang et al. confirmed that the spinal JNK/MCP-1 signal pathway may be involved in the maintenance of rat's BCP [13]. The results of this study showed that the expression of p-JNK protein in the spinal dorsal horn of BCP rats was significantly increased and positively correlated with the pain praxiology, which indicated that activation of JNK in the astrocytes might be involved in the induction and maintenance of BCP. This study also found that intrathecal injection of EphB1's receptor antagonist (EphB1-Fc) not only significantly improve the hyperalgesia of BCP rats, but also remarkably reduce the expression of p-JNK in spinal dorsal horn of BCP rats. This showed the

reason why EphB1-Fc could improve the hyperalgesia of BCP rats might result from that it could inhibit the activation of JNK in spinal dorsal horn of BCP rats. Thus, it gives a further inference that EphB1 signal may be involved in the occurrence and maintenance of rats' BCP by activating the JNK signaling pathway.

Nuclear transcription factor-κB is a nuclear protein factor, which can specifically bind with κB enhancer sequence (GGGACTTCC) of immune globulin K chain gene, and is expressed in almost all neurons and glial cells in the peripheral and central nervous system, and is an important signal transduction molecule in the regulation of immune and inflammatory gene expression in the central nervous system [13]. The activated NF-κB entering nucleus can promote the transcription of a variety of inflammatory factors and release a large number of pro-inflammatory factors [14]. These proinflammatory factors play a role in promoting pains [15, 16]. So it is possible to reduce chronic pain by blocking NF-κB pathway with inhibitors or down-regulating its expression [17]. These studies have suggested that NF-κB is involved in the development and maintenance of pain. Zhao found that NF-κB signaling pathway worked in the degree of damage of bone tissue, especially in the process of progressive osteolysis, which was related to the degree of severity and seizure frequency of pain, besides, it was involved in the inflammatory reaction of BCP

[18]. In addition, Hartung et al. found that the expression of phosphorylation of nuclear transcription factor- κ B increased in spinal dorsal horn of BCP rats [19-21]. And Xu et al. confirmed that intrathecal injection of minocycline could cause antinociception for the rats with BCP by restraining NF- κ B signal pathway [22, 23]. These results suggested that NF- κ B might be involved in mediating the mechanical hyperalgesia in BCP rats. NF- κ B is a transcription factor protein family, including 5 subunits: Rel, p65, RelB, p50, and p52. The activation of NF- κ B generally refers to the phosphorylated p65 transporting from the cytoplasm into the nucleus, regulating the expression of the corresponding genes. In this study, Western blot method was used to detect the nuclear translocation of NF- κ B p65 in spinal dorsal horn of rats. The results suggested that the expression NF- κ B p65 in the nucleus of rat's spinal cord in BCP group increased and was positively correlated with pain praxiology, and EphB1 receptor antagonist (EphB1-Fc) could significantly reduce the expression NF- κ B p65 in the nucleus of rat's dorsal spinal cord in BCP group, which not only further indicated that the NF- κ B of spinal dorsal horn might be involved in the formation and maintenance of BCP, but also illustrated that the inhibition of BCP's hyperalgesia through intrathecal injection of EphB1-Fc might be related to the attenuation of nuclear translocation of NF- κ B in rat's spinal dorsal horn with BCP. The above results suggest that EphB1 might participate in the formation and maintenance of BCP in rats by activating transcription factor NF- κ B.

BCP is a chronic pain with complex and distinctive mechanism, which includes inflammatory pain and pathological neuralgia. It is produced mainly because of the release of sensitizing factors from cancer cells and their associated stromal cells, which promotes peripheral and central sensitization, leading to the BCP of continuance and development. Studies of Zhou et al. have shown that glial cell is a new target for BCP, so it gives a speculation that the EphB1 signal system may promote the release of pro-inflammatory factor from spinal cord glial cells after the activation of JNK and NF- κ B p65 pathway in spinal cord, and further promote the formation and maintenance of BCP, which has required more experimental researches to confirm [24].

This research found that intrathecal administration of exogenous EphB1 antagonist (EphB1-Fc) significantly alleviated thermal hypersensitivity and mechanical hyperalgesia of BCP rats, suggesting that EphB1 may be involved in the occurrence of BCP. Besides, EphB1-Fc, could restrain the activation of JNK and NF- κ B p65 in spinal dorsal horn, indicating that EphB1 signal system may promote the formation and maintenance of BCP by activating JNK and NF- κ B p65 in spinal cord and this pathway may become a new target for the treatment of BCP.

Disclosure of conflict of interest

None.

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