

Impacts of anti-nerve growth factor antibody on pain-related behaviors and expressions of opioid receptor in spinal dorsal horn and dorsal root ganglia of rats with cancer-induced bone pain

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Abstract

Objective: To investigate the impacts of anti-nerve growth factor antibody on pain-related behaviors and expressions of μ -opioid receptor in spinal dorsal horn and dorsal root ganglia of rats with cancer-induced bone pain.

Methods: The rats were randomly grouped and then injected with 10 μ l of phosphate buffer saline or Walker256 tumor cells into the upper segment of left tibia. Thirteen days after the injection, the intrathecal catheterization was performed, followed by the injection of saline, anti-nerve growth factor, nerve growth factor, and naloxone twice a day. The pain ethological changes were measured at the set time points; the expression changes of μ -opioid receptor protein and mRNA in spinal dorsal horn and dorsal root ganglia were detected on the 18th day.

Results: After the tumor cells were injected into the tibia, hyperalgesia appeared and the expression of μ -opioid receptor protein and mRNA in spinal dorsal horn and dorsal root ganglia was increased, compared with the sham group; after intrathecally injected anti-nerve growth factor, the significant antinociceptive effects appeared, and the μ -opioid receptor expression was increased, compared with the cancer pain group; the μ -opioid receptor expressions in the other groups showed no statistical significance. The naloxone pretreatment could mostly inverse the antinociception effects of anti-nerve growth factor.

Conclusions: Anti-nerve growth factor could reduce hyperalgesia in the cancer-induced bone pain rats, and the antinociceptive effects were related with the upregulation of μ -opioid receptor.

Keywords

Cancer-induced bone pain, nerve growth factor, pain-related behaviors, opioid receptor, intrathecal injection

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Introduction

Cancer-induced bone pain (CIBP) is a complex pain syndrome, which might seriously impact patients' life qualities. Clinically, it was treated by the radiation therapy, bisphosphonates, radiofrequency ablation, and other methods, but large doses of morphine would be needed for analgesia, while the analgesic effects were not ideal even accompanied by serious side effects. With the successful establishment of CIBP animal model in recent years, the performance of which was similar to that of CIBP in human, it was found that the signaling transduction of CIBP was different from

inflammatory pain¹ and neuropathic pain.² The roles of endogenous opioid system inside the spinal cord and upper nerve center toward the pathophysiological

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processes of pain had received more and more attention. The opioid receptors were not only the action targets of exogenous opioids but also the action site of endogenous opioids. Therefore, the opioid receptors would directly impact the modulation of pain as well as the intervention effects. A recent study has shown³ that in the spinal ganglia of CIBP rat model, the expressions of μ -opioid receptor (MOR) in the primary afferent neurons of calcitonin gene-related peptide (CGRP) and transient receptor potential vanilloid type-1 were significantly downregulated, whereas in the spinal ganglionic neurons of mouse model with inflammatory pain, the MOR expression was not downregulated, suggesting that the downregulation of the MOR expression might be one of the main reasons that the CIBP treatment required a larger dose of morphine than the inflammatory pain, while the analgesic effects were still poor.^{4,5} However, it was still unclear about the causes that reduced the expression of MOR in the spinal ganglionic neurons of CIBP.

Our previous studies showed that the nerve growth factor (NGF) could exacerbate the harm feelings in CIBP rats; the expressions of NGF protein and mRNA, as well as those of NGF receptors, in the dorsal root ganglia (DRG) and spinal dorsal horn were upregulated,^{6,7} which is consistent with the previous study results.^{8–10} NGF played an important role in inflammatory pain¹¹ and neuropathic pain.¹² A recent study has reported that¹³ in the inflammatory pain model, NGF could upregulate the number and efficacy of sensory neuron MOR. But it has not yet been reported whether NGF would have the modulatory effects toward MOR in CIBP model.

This study established the CIBP rat model and then intrathecally applied anti-NGF, aiming to observe the changes of pain-related behaviors, expressions of MOR protein and mRNA, and further to observe whether the naloxone pretreatment could reverse the antinociceptive effects of anti-NGF, and to discuss relationships of NGF and MOR.

Materials and methods

Experimental design

Female Sprague-Dawley rats, with an initial body weight of 200–220 g, were provided by Animal Experimental Center of Shengjing Hospital of China Medical University, and this study was approved by the Ethics Committee of China Medical University.

The rats were randomly grouped into the sham group, the sham + anti-NGF group, the cancer pain group, the cancer pain + NGF group, the cancer pain + anti-NGF group, and the cancer pain + NLX + anti-NGF group ($n = 15$). The rats in

the sham group and the sham + anti-NGF group were injected with 10 μ l of phosphate buffer saline (PBS) into the left tibia; the cancer pain groups were injected with 10 μ l of Walker256 tumor cells (provided by the Cancer Institute of Chinese Academy of Medical Sciences).

The intrathecal catheterization on the rats was performed on the 13th day, and before the catheterization, the pain behavioral tests were conducted; the injection was not performed on the day of catheterization and it started only from the second day of catheterization. The medication was injected through the catheter twice per day: the sham group and the cancer pain group were injected with only 10 μ l of saline; the sham + anti-NGF group and the cancer pain + anti-NGF group were injected with 10 μ l of anti-NGF (diluted with normal saline, 1 μ g/ μ l Santa Cruz); the cancer pain + NGF group was injected with 10 μ l of NGF (diluted with normal saline, 0.1 μ g/ μ l; Sigma St. Louis, MO, N2513); and the cancer pain + anti-NGF + NLX group was intrathecally injected with naloxone (Naloxone, NLX) 10 μ g/25 μ l/rat (diluted with normal saline, 0.4 μ g/ μ l 0.5 h) and then injected with 10 μ l of anti-NGF through the catheter 30 min later. Each group was injected the medication twice a day for five consecutive days, and 10 μ l of saline was injected each time after the medication injection and the tube was then sealed by heating.

On the 18th day of modeling, after the drug injection, the pain tests were completed within 2 h, and the rats were anesthetized with chloral hydrate, decapitated, and the L4-5 spinal cord was carefully removed; the left spinal dorsal horn was then rapidly removed, the DRG along the L4-5 spinal nerve was isolated and immediately frozen in liquid nitrogen at -80°C for later use.

The pain-related behavior analysis. The pain-related behavior observation was performed when the tumor cells were injected for 7, 13, 15 (two days after the intrathecal catheterization), 17 (four days after the intrathecal catheterization), and 18 (five days after the intrathecal catheterization) days.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University.

Establishment of tibial CIBP model and intrathecal catheterization

The rat tibial CIBP model was established according to the method of Medhurst et al.¹⁴ The rats were anesthetized with chloral hydrate (10% concentration, 0.35 ml/

100 g), the lateral fur of the left hind limb was sheared, the skin was disinfected, the skin above the upper tibial segment was incised for about 1 cm, the tibia was exposed and a 5-ml syringe needle was used to drill the tibia and the needle broke the tibia. Then a 1-ml curved syringe needle was used to gently probe the marrow cavity, and a 25- μ l micro syringe was inserted into the bone marrow cavity along with pinhole, and 10 μ l of Walker256-containing PBS cell suspension was slowly injected, which contained a total of 10^4 tumor cells; the injection time was 2 min, and after the injection, the amalgam mixture was quickly used to seal the pinhole, then 75% ethanol and normal sterile saline were used to rinse the incision, and the skin was then sutured layer by layer. The sham group was injected with an equal volume of PBS solution on the left upper tibia, and the rest operations were the same as the cancer pain group.

The intrathecal catheterization referred by Huang and Zhang¹⁵ was performed. In brief, the PE-10 catheter tip was placed close to L5 DRG, and was confirmed as the correct position at the end of the experiment.¹⁶ The animals, without any sensory and motor damage, were used as research subjects. All rats were individually raised after the catheterization.

Observation of general behaviors

All rats were individually raised after the catheterization to avoid them bite each other and behaviors such as posture, walking posture, with or without autophagy, and clumsiness in hind limb were observed.

Observation of spontaneous foot-constriction frequency

The rats were placed in transparent plexiglass boxes, while they could walk freely. The spontaneous foot-constriction frequency of left hind paw within 5 min was then observed.

Determination of paw withdrawal thermal latency¹⁷

The thermalgia threshold was determined by using the BME-410A Thermalgia Instrument (Institute of Bioengineering, Chinese Academy of Medical Sciences). The rats were placed in a transparent organic glass cage for observation, and when they kept quiet for 30 min, the thermal radiation source was focused on the middle bottom of left toe, the latency from the beginning of irradiation to till the rat lifted its foot or hind away was set as the thermalgia threshold; the measurement was repeated three times, with the interval of 10 min, the mean value was then used as the final value. To prevent the burns, the maximal irradiation time was 20 s each time.

Determination of paw withdrawal mechanical threshold

The rat was placed in a quiet environment, and the von Frey wire (Stoelting) was used for the determination of paw withdrawal mechanical threshold (PWMT) by the “up and down” method as reported by Chaplan et al.¹⁸ The rats were placed on a metal net and covered with one transparent plexiglass box. They were let to adapt to the environment for 30 min and then a series of standard von Frey wires was used to stimulate the middle skin of rat's left toe in a certain order, until the wire slightly bent into S-shape; this stimulus was continued for 6–8 s, and it was observed whether the foot-constriction reaction occurred. If the quick foot-constriction reaction of the rat immediately appeared in the stimulation time or at the time when the von Frey wire was removed, this phenomenon was recorded as positive reaction. However, the body movement-caused foot constriction was not recorded as a positive reaction, and the test was performed for a total of 10 times, with the stimulus interval of 10 min.

Western blot

The membrane protein was extracted and quantificated according to the instructions of kits (P0033 and P0009, Beyotime). Samples (each 20 ng) were then separated on 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). The PVDF membranes were blocked with the blocking buffer (5% nonfat milk in Tris-buffer saline containing 0.05% Tween-20, TTBS) and incubated with MOR antibody (abcam, ab51140) or β -actin antibody (sc-130656, Santa Cruz), diluted 1:1000 in blocking buffer, at 4°C overnight. After washing with TTBS, the membrane were then probed with horseradish peroxidase (HRP)-conjugated second (ZB-2307, ZSGB-BIO) antibody diluted 1:2000 in blocking buffer, for 2h at room temperature. Secondary antibody binding was detected by reaction with ECL plus reagent (GE Healthcare, Piscataway, NJ, USA). Gel-Pro analyzer software was applied to analyze the optical density of the bands. The relative expression of MOR protein = optical density value of each group/density value of the sham group \times 100%.

Determination of MOR mRNA expression

The total RNA of the left spinal dorsal horn and L4-5 DRG was extracted by the Trizol Reagent (Invitrogen Co.), and the UV spectrophotometer was used to detect the RNA concentration and to calculate the content; the same amount of RNA was added into the reverse transcription system to synthesize cDNA. The primer sequences of MOR were as follows: upstream primer 5'-CAG CCC TTC CAT GGT CAC AG-3'; downstream

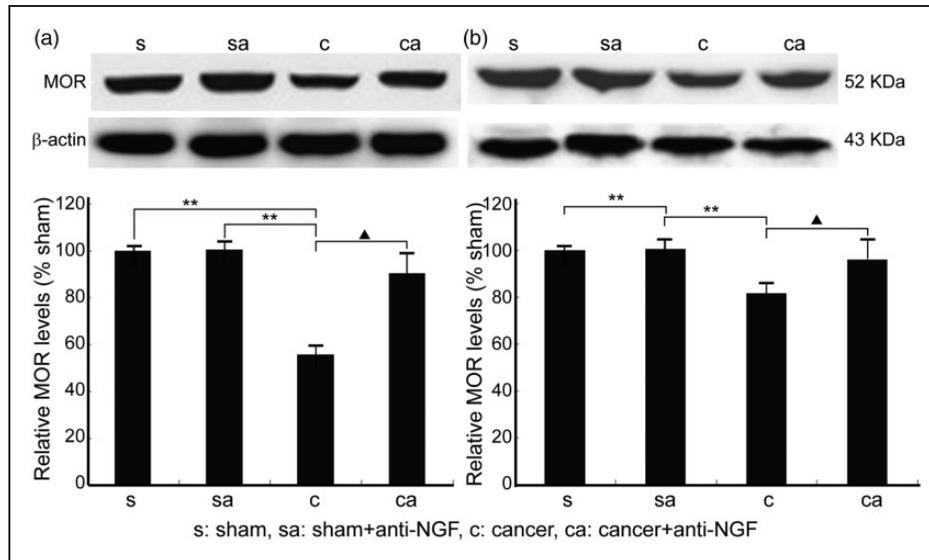


Figure 1. Western blot analysis of MOR in DRG (a) and the dorsal horn spinal cord (b). The upper panel displays the bands of MOR and β -actin, and the lower panel indicates the statistical results of Western blot analysis. MOR in both DRG and the dorsal horn spinal cord was downregulated in the cancer pain group, $n = 13$ rats for each group (two DRG in each rat), while the intrathecal injection of anti-NGF antibody (from Day 14 to 18, twice daily) significantly increases MOR expression in cancer anti-NGF group, $n = 13$ rats for each group (two DRG in each rat). Values of histograms are expressed as means \pm S.E.M (standard error of the mean). * and Δ indicate $p < 0.05$, and ** indicates $p < 0.01$. MOR: μ -opioid receptor; DRG: dorsal root ganglia; NGF: nerve growth factor.

primer 5'-TAC TGG TCG CTA AGG CGT CTG-3'. The primer sequences of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: upstream primer 5'-CCG AGG GCC CAC TAA AGG-3'; downstream primer 5'-TGC TGT TGA AGT CAC AGG AGA CA-3'. The Light Cycler Real-Time PCR amplifier (Roche Co.) was used to amplify MOR and GAPDH, and the amplification conditions were as follows: denaturation at 95°C for 30 s; denaturation at 95°C for 10 s, annealing at 58°C for 15 s, extension at 72°C for 10 s, with a total of 40 cycles, and then extension at 72°C for 10 min. The relative expression of MOR mRNA was calculated using $2^{-\Delta Ct}$, where $\Delta Ct = Ct \text{ MOR} - Ct \text{ GAPDH}$ and then based on $\Delta\Delta Ct = \Delta Ct \text{ MOR} - \Delta Ct \text{ GAPDH}$, we calculate $2^{-\Delta\Delta Ct}$. The melting curve was then produced to determine the specificities of amplified products.

Statistical analysis

Data were expressed as mean \pm standard deviation. Statistical analysis was performed with SPSS13.0 software. The behavioral tests were analyzed using repeated measures analysis of variance (ANOVA) followed by post hoc Scheffé's multiple comparisons. Western blot and reverse transcription polymerase chain reaction (RT-PCR) data were analyzed with one-way ANOVA followed by the Scheffé's multiple comparison procedure. $p < 0.05$ was set as the level of statistical significance.

Results

General information

The number of rats that died during the experimental observation period was two, two, one, and one, respectively, in the cancer pain group, the cancer pain + anti-NGF group, the cancer pain + NGF group, and the cancer pain + anti-NGF + NLX group; as the difference was not statistically significant, these were excluded in this study. The cancer pain + NGF group and the cancer pain + anti-NGF + NLX group had one case which exhibited mass inside the left tibia, the left lower limb paralyzed and exhibited the biting phenomenon, and therefore, they were excluded in this study.

Expressions of MOR protein

Relative expression of MOR in DRG and spinal dorsal horn is shown in Figure 1. Eighteen days after the inoculation of Walker256 tumor cells, the MOR expressions in the spinal dorsal horn and the DRG neurons of the cancer pain group were decreased ($n = 13$ for each group), compared with the sham group, $p < 0.05$. After intrathecally injected anti-NGF, the MOR expressions were increased compared with the cancer pain group, and the differences were significant. The comparison among the cancer pain + anti-NGF group, the sham group and the sham + anti-NGF group, the MOR expressions showed no significant difference, suggesting that anti-NGF could

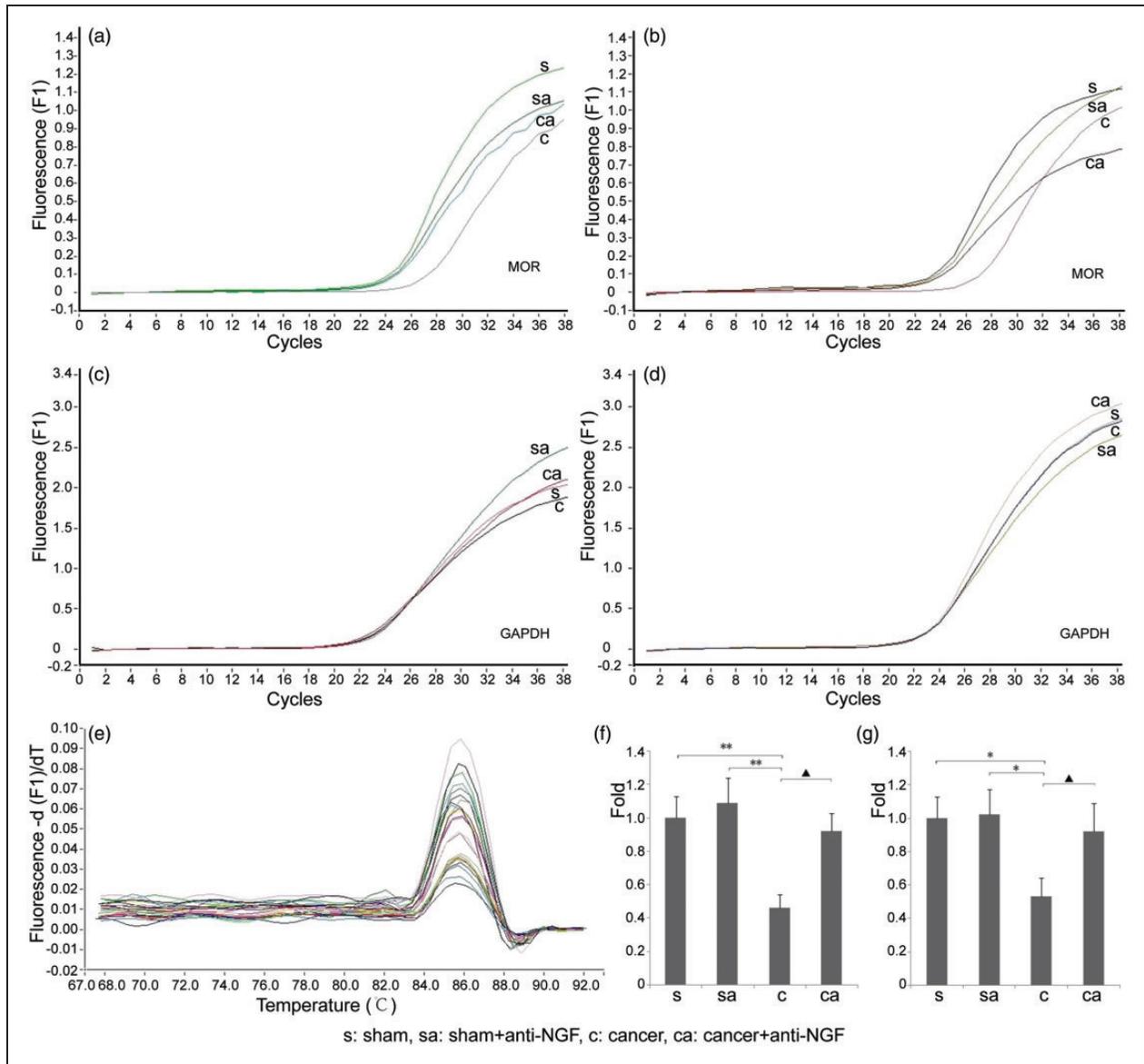


Figure 2. Real-time RT-PCR analysis of MOR mRNA in DRG (a) and spinal dorsal horn (b). GAPDH in DRG (c) and the dorsal horn spinal cord (d). (e) The dissociation curves. Statistical results of RT-PCR in DRG (f) and the dorsal horn spinal cord (g). $n = 13$ rats for each group (two DRG in each rat). Values of histograms are expressed as means \pm S.E.M (standard error of the mean). * and Δ indicate $p < 0.05$, and ** indicates $p < 0.01$. RT-PCR: reverse-transcription polymerase chain reaction; MOR: μ -opioid receptor; DRG: dorsal root ganglia; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

increase the MOR protein expressions in DRG and spinal dorsal horn of cancer rats, while the sham + anti-NGF group did not show this change.

Expressions of MOR mRNA

The real-time quantitative RT-PCR detection results were shown in Figure 2. Eighteen days after the inoculation of tumor cells, as for the DRG neurons, compared with the sham group (1.00 ± 0.13), the MOR mRNA expression of

the cancer pain group was decreased ($2^{-\Delta\Delta C_t}$: 0.45 ± 0.09 ; $p < 0.01$, $n = 13$ rats for each group). After intrathecally injected anti-NGF, the MOR mRNA expression of the cancer pain + anti-NGF group was increased (0.91 ± 0.10) and compared with the cancer pain group, the difference was significant ($p < 0.05$, $n = 13$ rats for each group); the comparison among the cancer pain + anti-NGF group, the sham group, and the sham + anti-NGF group showed no significant difference. In the spinal dorsal horn, the MOR mRNA

Table 1. The frequency of paw withdrawal in each CIBP group within 5 min (times, $\bar{x} \pm s$).

Group	Base value	Post-inoculation 7 days	Post-inoculation 13 days	Post-inoculation 15 days	Post-inoculation 17 days	Post-inoculation 18 days
Sham	5.1 ± 1.2	5.2 ± 1.1	5.2 ± 0.9	4.9 ± 1.1	5.3 ± 1.0	5.4 ± 1.2
Sham + anti-NGF	5.2 ± 1.3	5.1 ± 1.0	5.0 ± 1.2	5.4 ± 1.0	5.2 ± 1.1	5.3 ± 1.2
Cancer	5.1 ± 1.2	8.2 ± 1.4	25.5 ± 3.3**	28.4 ± 4.4**	29.4 ± 4.1**	29.2 ± 4.8**
Cancer + NGF	5.0 ± 1.2	8.1 ± 1.4	25.8 ± 3.9**	39.3 ± 5.4**#	32.4 ± 3.9**	27.1 ± 3.4**
Cancer + anti-NGF	4.9 ± 1.2	8.3 ± 1.3	24.7 ± 3.6**	9.7 ± 1.2###	8.1 ± 1.3###	6.5 ± 1.5###
Cance + NLX + anti-NGF	5.2 ± 1.1	8.1 ± 1.1	26.7 ± 3.8**	27.2 ± 3.4** $\Delta\Delta$	26.6 ± 3.9** $\Delta\Delta$	27.1 ± 3.4** $\Delta\Delta$

Note: NLX: naloxone; NGF: nerve growth factor; CIBP: cancer-induced bone pain. * or ** indicates $p < 0.05$ or $p < 0.01$, respectively, compared with sham group; # or ### indicates $p < 0.05$ or $p < 0.01$, compared with cancer group; $\Delta\Delta$ indicates $p < 0.01$ between Cancer + NLX + anti-NGF and cancer + anti-NGF groups, $n = 13$ for each group.

Table 2. Paw withdrawal thermal latency in each CIBP group (s, $\bar{x} \pm s$).

Group	Base value	Post-inoculation 7 days	Post-inoculation 13 days	Post-inoculation 15 days	Post-inoculation 17 days	Post-inoculation 18 days
Sham	12.3 ± 1.3	11.3 ± 1.4	12.6 ± 1.3	11.2 ± 1.5	11.5 ± 1.3	12.9 ± 1.3
Sham + anti-NGF	12.4 ± 1.4	12.1 ± 1.5	12.3 ± 1.2	12.4 ± 0.8	12.2 ± 1.1	11.8 ± 1.3
Cancer	12.4 ± 1.2	9.2 ± 1.2	8.8 ± 0.9*	7.4 ± 1.2**	6.1 ± 0.5**	3.8 ± 0.5**
Cancer + NGF	12.0 ± 1.3	9.1 ± 1.4	8.6 ± 0.9*	8.9 ± 0.9*	5.9 ± 0.5**	3.3 ± 0.4**
Cancer + anti-NGF	12.9 ± 1.2	9.3 ± 1.0	8.5 ± 1.1*	10.6 ± 1.2###	10.8 ± 1.6###	11.3 ± 1.2###
Cance + NLX + anti-NGF	12.2 ± 1.5	8.3 ± 1.1	8.7 ± 0.9*	8.6 ± 0.9** $\Delta\Delta$	6.9 ± 0.8** $\Delta\Delta$	4.5 ± 0.7** $\Delta\Delta$

Note: CIBP: cancer-induced bone pain; NLX: naloxone; NGF: nerve growth factor. * or ** indicates $p < 0.05$ or $p < 0.01$, respectively, compared with sham group; # or ### indicates $p < 0.05$ or $p < 0.01$, compared with cancer group; $\Delta\Delta$ indicates $p < 0.01$ between Cancer + NLX + anti-NGF and cancer + anti-NGF groups, $n = 13$ for each group.

Table 3. Paw withdrawal mechanical threshold in each CIBP group (g, $\bar{x} \pm s$).

Group	Base value	Post-inoculation 7 days	Post-inoculation 13 days	Post-inoculation 15 days	Post-inoculation 17 days	Post-inoculation 18 days
Sham	12.1 ± 2.4	11.9 ± 2.4	11.2 ± 2.3	11.4 ± 2.2	11.1 ± 1.9	11.3 ± 1.3
Sham + anti-NGF	12.3 ± 2.5	11.4 ± 2.2	12.6 ± 1.9	13.3 ± 1.8	11.4 ± 1.7	11.2 ± 1.3
Cancer	12.4 ± 1.2	9.2 ± 1.2	5.6 ± 1.2**	4.3 ± 0.8**	2.7 ± 0.9**	2.2 ± 1.1**
Cancer + NGF	12.0 ± 1.3	9.1 ± 1.4	5.9 ± 0.9**	5.1 ± 0.9**	2.1 ± 0.5**	1.8 ± 0.3**
Cancer + anti-NGF	12.9 ± 1.2	9.3 ± 1.0	5.8 ± 1.1**	8.9 ± 0.7*###	9.5 ± 1.3###	9.2 ± 1.5###
Cance + NLX + anti-NGF	12.2 ± 1.5	8.3 ± 1.1	5.9 ± 0.9**	4.7 ± 0.8** $\Delta\Delta$	2.9 ± 0.6** $\Delta\Delta$	2.8 ± 0.5** $\Delta\Delta$

Note: CIBP: cancer-induced bone pain; NLX: naloxone; NGF: nerve growth factor. * or ** indicates $p < 0.05$ or $p < 0.01$, respectively, compared with sham group; ### indicates $p < 0.01$, compared with cancer group; $\Delta\Delta$ indicates $p < 0.01$ between cancer + NLX + anti-NGF and cancer + anti-NGF groups, $n = 13$ for each group.

showed the same trend as that in the DRG neurons, and the MOR mRNA expressions ($2^{-\Delta\Delta Ct}$) were 1.00 ± 0.12 , 1.01 ± 0.14 , 0.52 ± 0.10 , and 0.91 ± 0.16 in the sham group, the sham + anti-NGF group, the cancer pain group, and the cancer pain + anti-NGF group, respectively. When compared with the sham group, the MOR mRNA expression in the cancer pain group was decreased ($p < 0.05$, $n = 13$ rats for each group); after intrathecally

injected anti-NGF, the MOR mRNA expression in the cancer pain + anti-NGF group was increased than in the cancer pain group ($p < 0.05$, $n = 13$ rats for each group).

Pain-related behavior

The pain-related behavioral tests were shown in Tables 1 to 3. The intrathecal application of NGF increased the

number of spontaneous foot constriction in CIBP rats, shortened the heat radiation latency, and decreased PWMT, suggesting that NGF could increase the hyperalgesia of CIBP rats. The intrathecal application of anti-NGF increased the number of spontaneous foot constriction in CIBP rats, shortened the heat radiation latency, and decreased PWMT, suggesting that anti-NGF had significant antinociceptive effects; the naloxone pretreatment could mostly reverse the antinociceptive effects of anti-NGF, suggesting that anti-NGF's antinociceptive effects might be related to MOR.

Discussion

The results of this study showed that after injecting the Walker256 tumor cells into the upper segment of rat tibial cavity, the number of spontaneous foot constriction was increased, the heat radiation latency was shortened, and PWMT was decreased, indicating that the tibial CIBP rat model was successfully established.

CIBP was a chronic pain with extremely complex mechanism, which involved nerve damage-caused pathogenic rational pains, as well as the inflammatory pains caused by the tumor cells' oppression, ischemia, and the release of cytokines and other inflammatory mediators, and the chronic pain syndrome of CIBP was thus constituted. Previous findings⁶⁻¹⁰ showed that NGF played important roles in the development of CIBP, and blocking NGF or its receptor could significantly relieve the pain, which is consistent with the findings of Kumar and Mahal¹⁹ and Hu et al.²⁰ NGF could mediate inflammation and immune response, causing pain-related hypersensitivity. When the tissues were under noxious stimuli, NGF could bind with the receptors, activated neuronal terminal TrkA and P75 receptors,^{21,22} and regulated the internal flow of calcium ions, thereby activating the intracellular signaling cascade reaction, modulating and activating different ion channels, causing central sensitization, and resulting in pain-related hypersensitivity or allodynia.^{23,24}

In this study, the doses of intrathecally injected anti-NGF and NGF were 10 µg, which is consistent with that of Obata et al.²⁵ and Xanthos et al.²⁶ in studying inflammatory pain and neuropathic pain. The pain-related behavioral tests showed that after intrathecally injected anti-NGF, the number of spontaneous foot constriction was decreased, the heat radiation latency was prolonged, and PWMT was increased, suggesting that anti-NGF could significantly alleviate the effects of CIBP in rats; while the intrathecal injection of anti-NGF into the sham group showed no pain-related behavioral changes, suggesting that NGF did not participate the pain signal transduction in normal rats or could not play a major role, meanwhile, it is also suggested that NGF played an important role in the occurrence and development of

CIBP. In this study, after intrathecally injected NGF into the CIBP rats, compared with the cancer pain group, the nociceptive feeling showed the trend of exacerbation, but the difference was not statistically significant, considering NGF was highly expressed in spinal dorsal horn and DRG of CIBP rats, and the rats were in the hyperalgesia state, the TrkA and P75 receptors were in the saturated state, the exogenous NGF did not further exacerbation of nociceptive effects significantly, but the specific reasons still needed further studies.

The results of Western blot and real-time PCR showed that the expressions of MOR protein and mRNA in the spinal dorsal horn and DRG of CIBP rats were significantly reduced, which is consistent with the findings of Yamamoto et al.³ After intrathecally injected anti-NGF, the MOR protein and mRNA were upregulated, suggesting that the relief of CIBP by anti-NGF was related with the upregulation of MORs. To verify this point, we performed the naloxone pretreatment, opioid receptor antagonist; before the intrathecal injection of anti-NGF, the naloxone dose was selected as 10 µg/25 µl (0.4 µg/µl), referring to the conventional dosage selected previously,²⁷ this dose could completely block MOR. The results of this study showed that the naloxone pretreatment mostly overturned the antinociceptive effects of anti-NGF, suggesting that the antinociceptive effects of anti-NGF were associated with the upregulation of MORs.

When in CIBP, NGF was highly expressed in spinal dorsal horn and DRG, anti-NGF could relieve CIBP and inhibit the development of tumors.²⁸ Previous study¹¹ showed that in the DRG of rats with inflammatory pain, all MOR-positive neurons and NGF receptors TrkA and P75^{NTR}-marked positive neurons were co-expressed on the CGRP-labeled sensory neurons, suggesting that NGF could regulate the expression of MOR through activating the expressions of TrkA and P75^{NTR} receptors. In this study, MOR was downregulated when CIBP occurred, which then significantly increased after using anti-NGF, accompanied with the increase of thermal hyperalgesia and PWMT, which fully demonstrated that in CIBP, NGF was also the MOR regulator.

However, due to the complexity of CIBP and the pleiotropia of NGF, the blocking NGF pathway might produce complex reactions.²⁹ The roles of NGF and its receptors in neuronal sensitization and activation of downstream signaling pathways were unclear. Molliver et al.³⁰ and Cahill et al.³¹ reported that in inflammatory pain, the highly expressed NGF could upregulate MOR, but MOR was reduced in CIBP, so we could not rule out other mechanisms that were involved in modulating MOR, such as the roles of NGF in activating G protein-coupled receptor kinases (GRKs) in CIBP, as well

as the roles of β -arrestin pathway in modulating MOR. A previous study also showed that³² GRKs and β -arrestin played important roles in downregulating MOR and the desensitization process.

But we could not rule out the roles of endogenous opioid system in the modulation process of MOR by NGF,^{33,34} meanwhile, the participation of endogenous opiates could not be ruled out during anti-NGF-relieved CIBP, naloxone blocked MOR, thereby blocking the action sites of endogenous opiates, leading to the disappearance of anti-NGF's analgesic effects. However, some studies toward the CIBP model also used naloxone,^{1,35} while found no pain-related behavior changes, and excluded the regulations of endogenous opioid peptides on CIBP. Our research group would further study the direct and indirect factors that caused the downregulation of MOR in CIBP from aspects such as the changes of endogenous opiates, GRKs and β -arrestin, as well as the co-expression of NGF receptor TrkA, P75, and MOR.

In summary, the CIBP rats would exhibit hyperalgesia, as well as the downregulation of MOR in spinal dorsal horn and DRG; anti-NGF could reduce hyperalgesia in CIBP rats, and this kind of antinociceptive effects was related with the upregulation of MOR.

Declaration of Conflicting Interests

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