Changes in response properties of nociceptive dorsal horn neurons in a murine model of cancer pain

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Abstract: Pain associated with cancer that metastasizes to bone is often severe and debilitating. A better understanding of the neural mechanisms that mediate cancer pain is needed for the development of more effective treatments. In this study, we used an established model of cancer pain to characterize changes in response properties of dorsal horn neurons. Fibrosarcoma cells were implanted into and around the calcaneus bone in mice and extracellular electrophysiological recordings were made from wide dynamic range (WDR) and high threshold (HT) dorsal horn neurons. Responses of WDR and HT neurons evoked by mechanical, heat, and cold stimuli applied to the plantar surface of the hind paw were compared between tumor bearing mice and control mice. Mice exhibited hyperalgesia to mechanical and heat stimuli applied to their tumor-bearing hind paw. WDR neurons in tumor-bearing mice exhibited an increase in spontaneous activity, and enhanced responses to mechanical, heat, and cold stimuli as compared to controls. Our findings show that sensitization of WDR neurons, but not HT neurons, contributes to tumor-evoked hyperalgesia.

Key words: cancer pain; dorsal horn; electrophysiology; hyperalgesia; sensitization

1 Introduction

Pain is a common symptom reported by patients with cancer[1]. Approximately 90% of patients with end-stage cancer report pain[1-3]. Metastasis of tumor cells to bone often occurs in patients with breast, lung, and prostate cancer[4] and patients with bone metastasis may experience severe pain[5-9]. Once bone metastasis occurs, the frequency of pain is greater than 60% for patients with sarcomas, breast cancer, multiple myeloma, or lung cancer[10]. Thus, pain associated with tumor cells that have metastasized to bone is a frequent and debilitating complication of cancer.

Understanding the neurobiology underlying cancer pain is critical for improving pain management. Animal models of cancer pain using mice[11-20] and rats[21] have been developed and are providing new information on the mechanisms that contribute to cancer-related pain. Implantation of tumor cells into bone produces ongoing nocifensive behaviors[14,17,19], as well as increased withdrawal responses to mechanical[17,19,21,22], heat[14], and cold stimuli[19,23] applied to the tumor-bearing paw.

Studies using these models of cancer pain have already identified a number of mediators, such as endothelin-1 (ET-1), bradykinin, tumor necrosis factor alpha (TNFα), and nerve growth factor (NGF), that contribute to allodynia and hyperalgesia associated with tumor growth[19,24-26]. For example, ET-1 and TNFα are released at the tumor site and blockade of their receptors attenuates tumor-evoked hyperalgesia[19,27]. In naive animals, subcutaneous administration of ET-1 and TNFα sensitizes C nociceptors to mechanical and heat stimuli[28-30].

Using a model in which fibrosarcoma cells are implanted into and around the calcaneus bone[19], we have shown that about 35% of C nociceptors exhibited ongoing, spontaneous activity and were sensitized to heat stimuli[31]. Furthermore, peripheral neuropathy coincided with tumor growth as evidenced by a decrease in the number of epidermal nerve fibers (ENFs) in the plantar skin overlying the tumor site[31] with sparing of the ENFs that contained calcitonin gene-related peptide, (CGRP)[32,33], a neuropeptide associated with nociceptive signaling[34,35].

Although C nociceptors exhibited ongoing activity and sensitization to heat stimuli, several studies suggest that central sensitization[37,38] also contributes to cancer pain. For example, neurochemical changes in the dorsal horn...
that occur during tumor growth are consistent with central sensitization\[17, 21, 39\]. In one model of cancer pain in rats, direct evidence for central sensitization has been shown in electrophysiological studies\[23, 40\].

In the present study, we characterized changes in response properties of nociceptive dorsal horn neurons following implantation of fibrosarcoma cells into and around the calcaneus bone. Adult male C3H/HeNCr mice (National Cancer Institute, Frederick, MD) weighing 20-30 g were used in these experiments. NCTC 2472 fibrosarcoma cells (ATCC, Manassas, VA) were maintained as described previously\[41\]. Briefly, fibrosarcoma cells were grown to confluency in 75 cm\(^2\) flasks in NCTC 135 medium (pH 7.4) containing 10% horse serum and prepared for implantation by creating a cell suspension with trypsin. Cells were counted using a hemocytometer, pelleted and resuspended in phosphate buffered saline for implantation. Mice were briefly anesthetized with halothane (2%-3%) and fibrosarcoma cells (2×10\(^5\) cells/10 µL) were injected into and around the calcaneus bone of the left hind paw using a 0.3 mL insulin syringe with a 29.5 gauge needle, as described previously\[19\].

### 2 Results

#### 2.1 Hyperalgesia following implantation of fibrosarcoma cells

Mechanical hyperalgesia was determined using a von Frey (Semmes-Weinstein) monofilament (Stoelting Co, Wood Dale, IL) with a bending force of 3.4 mN applied to the plantar surface of the hind paw. Mice were placed on a mesh platform and the monofilament was applied 10 times, each for a duration of 1-2 s with an interstimulus interval of approximately 5 s. Tumor-evoked mechanical hyperalgesia was defined as an increase in the frequency of paw withdrawals from baseline values. Implantation of fibrosarcoma cells into and around the calcaneus bone in mice produced hyperalgesia to mechanical and heat stimuli (Fig.1), consistent with our earlier reports\[31, 42, 67\]. The mean frequency of paw withdrawals to the von Frey monofilament for the tumor-bearing hind paw increased from baseline levels of (15 ± 2.6)% to (49.0 ± 3.8)% by day 3 after implantation. The mean frequency of withdrawal further increased until day 10 [(70.7 ± 4.0)%] and remained elevated throughout the testing session. Paw withdrawal frequency for the contralateral control hind paw was unchanged during the time course.

Hyperalgesia to heat was also produced following tumor growth. Heat hyperalgesia was determined by measuring paw withdrawal latency to a radiant heat source\[43\]. Mice were placed on a glass platform, and radiant heat was applied to the plantar surface of the hind paw. Latency (s) to paw withdrawal was determined. Mean paw withdrawal latency for each paw was calculated from 3 trials each separated by 1 min. Heat hyperalgesia was defined as a decrease in paw withdrawal latency from baseline values. Mean paw withdrawal latency decreased from (9.2 ± 0.5) s to (7.9 ± 0.5) s by 3 d following implantation of fibrosarcoma cells, and decreased further to (5.3 ± 0.5) s by day 18. Paw withdrawal latencies for the contralateral control
paw remained unchanged. Thus, implantation of fibrosarcoma cells into and around the calcaneus bone in mice produced mechanical and heat hyperalgesia.

2.2 Response properties of mouse dorsal horn neurons

2.2.1 General characteristics

Extracellular recordings were made from 45 nociceptive dorsal horn neurons in control mice (n=25) and 42 nociceptive neurons in tumor-bearing mice (n=29) that exhibited hyperalgesia. For these experiments, mice were anesthetized with acepromazine maleate (20 mg/kg, i.p.) and sodium pentobarbital (Nembutal, 48 mg/kg, i.p.). Supplemental doses of sodium pentobarbital (15 mg/kg) were added as needed to maintain areflexia. The lumbar enlargement was exposed by a laminectomy, and mice were secured in a spinal frame. Extracellular recordings from dorsal neurons at the L3 - L5 spinal levels with receptive fields (RFs) located on the plantar surface of the hind paw were obtained using stainless steel microelectrodes (10 MΩ; Frederick Haer and Co., Brunswick, ME), lowered into the spinal cord using an electronic micromanipulator (Burleigh, Fisher, NY) in 3 µm steps. Dorsal horn neurons were identified using mechanical stimulation (stroking the skin and mild pinching with the experimenter’s fingers) of the plantar surface of the hind paw. Each spinal neuron was characterized based upon its responses to graded intensities of mechanical stimuli applied to the RF. Innocuous stimuli consisted of stroking the skin with a cotton swab or the experimenter’s fingers. Noxious stimuli consisted of mild pinching with the experimenter’s fingers. Neurons were classed according to responses evoked by mechanical stimuli as (1) low threshold - excited maximally by innocuous stimulation; (2) wide dynamic range (WDR) - responded in a graded fashion to increasing intensity of stimulation beginning with innocuous stimuli; and (3) high threshold (HT) - response evoked by noxious stimulation only. Only nociceptive dorsal horn neurons (WDR and HT) were studied further.

Figure 2 shows the locations of recording sites for 24 of 45 neurons determined histologically in control mice and for 30 of 42 neurons in tumor-bearing mice. The recording sites were obtained from electrolytic lesions made at the end of each experiment. There were no differences in the proportion of WDR or HT neurons located in the superficial or deep dorsal horn of tumor-bearing compared to control mice. Moreover, there were no differences in the response properties of WDR or HT neurons located in the superficial or deep dorsal horn for either control mice or tumor-bearing mice.

A greater proportion (76%) of WDR neurons exhibited ongoing activity in tumor-bearing mice than in control mice (54%, P < 0.05) and the discharge frequency of the ongoing activity for WDR neurons in tumor-bearing mice was approximately 3 times greater than that of WDR neurons in control mice (P < 0.01). In contrast, there was no difference in the proportion of HT neurons that exhibited ongoing activity between control and tumor-bearing mice.

2.2.2 Responses to mechanical and thermal stimuli

Responses to mechanical stimuli (brush, von Frey monofilaments, and pinching with serrated forceps), applied to the RF for 2 s, were determined. Thermal stimuli were applied using a Peltier-type thermode (contact area of 1 cm²) starting from a base temperature of 32°C. Heat stimuli (35 °C to 51 °C, 5-s duration) were delivered in ascending order of 2 °C increments at a ramp rate of 18 °C/s with an interstimulus interval of 60 s. Cold stimuli (28 °C to 0 °C, 10-s duration) were applied in descending order of 4 °C decrements with a ramp rate of 8 °C/s and interstimulus interval of 180 s.

Implantation of fibrosarcoma increased responses of WDR neurons to mechanical stimuli applied to the hind paw. Examples of responses of WDR neurons to mechanical stimuli in a control and a tumor-bearing mouse are shown in Fig. 3A. Figure 3B shows mean number of impulses of all WDR neurons in tumor-bearing mice was greater than those in control mice. Importantly, WDR neurons in tumor-bearing mice exhibited greater responses (9.5 ± 1.0 impulses) to the mechanical stimulus used in the behavioral studies (3.4 mN) than did WDR neurons in control mice.
Fig. 3. Responses of nociceptive dorsal horn neurons to mechanical stimuli. 

A: Examples of the responses of WDR neurons from a control and a tumor-bearing (cancer) mouse to mechanical stimuli (brush, von Frey monofilaments, and pinch). 

B: Mechanical stimuli evoked greater mean (± SEM) number of impulses from WDR neurons in tumor-bearing compared to control mice. 

C: Examples of the responses of HT neurons from a control and a tumor-bearing mouse to mechanical stimuli. 

D: There were no differences in the mean (± SEM) number of impulses from HT neurons evoked by the mechanical stimuli between tumor-bearing and control mice. *$P < 0.05$, ****$P < 0.0001$ for number of impulses between tumor-bearing and control mice for each mechanical stimulus. Reprinted from reference 67, with permission from Elsevier.
mice. Whereas responses of WDR neurons to mechanical stimuli were increased in tumor-bearing mice, responses of HT neurons were unchanged. This is illustrated in Fig. 3C which shows that there were no differences in the mean number of impulses evoked by the mechanical stimuli between HT neurons in tumor-bearing and control mice.

WDR neurons, but not HT neurons, in tumor-bearing mice exhibited greater responses to heat stimuli applied to the hind paw. Examples of responses of individual WDR neurons to heat in a control and a tumor-bearing mouse are shown in Fig. 4A. Figure 4B shows that WDR neurons in tumor-bearing mice exhibited greater responses to temperatures at 43 °C and above than did the WDR neurons in control mice.

HT neurons in both the control and tumor-bearing mice responded to noxious but not innocuous (i.e., 37 °C) heat. Figure 4C shows that HT neurons in tumor-bearing mice exhibited greater responses only to the 49 °C and 51 °C stimuli than did the HT neurons in control mice. However, there was no difference in heat threshold for HT neurons in tumor-bearing (39.4 ± 0.8 °C) or control (38.9 ± 0.4 °C) mice. Thus, HT neurons in cancer mice exhibited increased responses only to temperatures above those that induced paw withdrawals in our behavioral studies (33 °C-39 °C) and most likely do not contribute to the hyperalgesia produced by tumor growth.

WDR neurons in tumor-bearing mice also exhibited sensitization to cold applied to the hind paw. Examples of responses of WDR neurons to cold in a control and a tumor-bearing mouse are shown in Fig. 5A. The WDR neurons in both the control and tumor-bearing mice responded to noxious (i.e., 8 °C and 0°C) cold in a graded fashion. However, responses of the WDR neuron in the tumor-bearing mouse were greater than those of the WDR neuron in the control mouse. Figure 5B shows that WDR neurons in tumor-bearing mice exhibited greater mean responses to temperatures of 12 °C and below than did the WDR neurons in control mice. Response thresholds of WDR neurons to cold stimuli in tumor-bearing mice (23.1 ± 1.7 °C) were not different from those of WDR neurons in control mice (19.3 ± 2.3 °C).

There were no differences in responses of HT neurons to cold stimuli between tumor-bearing and control mice. HT neurons in both tumor-bearing and control mice exhibited increased responses to increasingly colder temperatures applied to the hind paw (Fig. 5C). However, there were no differences in the number of impulses evoked at each temperature or in the cold threshold temperature for HT neurons between control and tumor-bearing mice. Thus, HT neurons likely did not contribute to the cold hy-
peralgesia exhibited by tumor-bearing mice shown previously[19].

3 Discussion

In the model of cancer pain used in these studies, hyperalgesia to mechanical, heat, cold stimuli are, at least in part, due to sensitization of WDR neurons. The proportion of WDR neurons that exhibited ongoing activity was greater and the discharge frequency of that ongoing activity was higher in tumor-bearing than in control mice. This ongoing may contribute to ongoing nociceptive behaviors exhibited in this model[19]. Also, WDR neurons exhibited greater responses to mechanical, heat and cold stimuli in tumorbearing compared to control mice. In contrast, responses of HT neurons in tumor-bearing mice were similar to those in control mice. Thus, sensitization of WDR, but not HT, neurons likely contributed to tumor-evoked hyperalgesia in this murine model of cancer pain. Our results are consistent with earlier reports by Dickenson and colleagues[23] who showed that neurons classed as WDR were sensitized following implantation of mammary carcinoma cells into the tibia of rats.

Differential sensitization of WDR but not HT neurons observed in our study and by Dickenson and colleagues[23] may be unique to cancer pain in that both WDR and HT neurons exhibit increased levels of ongoing activity in other models of pain, including incisional pain[44], arthritis[45, 46], paw inflammation[47], skin freeze injury[48], activation of C nociceptors by mustard oil[49], capsaicin evoked pain[50, 51] and neuropathic pain[52]. It has been demonstrated that neurochemical changes in the spinal cord of mice with tumor-evoked hyperalgesia differ from those in mice with hyperalgesia produced by inflammation or nerve injury[53]. Thus, changes in neurochemical organization of the dorsal horn, and the ensuing changes in response properties of dorsal horn neurons, may differ depending upon the nature of the injury.

3.1 Mechanical hyperalgesia and sensitization of nociceptive dorsal horn neurons to mechanical stimuli

Injury to peripheral tissues sensitizes nociceptors and nociceptive dorsal horn neurons, and produces hyperalgesia[54-56]. In our model of cancer pain, we found that a proportion of C nociceptors were sensitized to heat stimuli in mice with tumor-evoked hyperalgesia[51] and this sensitization likely contributed to the tumor-evoked hyperalgesia to heat re-
ported in the present study. Thresholds to mechanical stimuli in sensitized C nociceptors were not decreased suggesting that these C nociceptors may not contribute to tumor-evoked mechanical hyperalgesia. Responses to suprathermal mechanical stimuli were not examined. Similarly, mechanical thresholds did not change following inflammation, but nociceptors exhibited increased responses to suprathermal stimuli[57]. It is therefore possible that C nociceptors in tumor-bearing mice may have been sensitized although their thresholds to mechanical stimuli may not have decreased. Regardless, sensitization of nociceptive dorsal horn neurons may also contribute to tumor-evoked mechanical hyperalgesia. Sensitization of nociceptive dorsal horn neurons contributes to mechanical hyperalgesia in models of inflammatory and neuropathic pain[58-60].

In the present study, responses of WDR neurons to a variety of mechanical stimuli were higher in tumor-bearing compared to control mice. Importantly, responses of WDR neurons to the von Frey monofilament (3.4 mN bending force) used to assess mechanical hyperalgesia were almost 7 times greater than the responses of WDR neurons in control mice. These findings are consistent with our recent report that threshold force for evoking a paw withdrawal decreases in tumor-bearing mice[42]. These changes were not observed in HT neurons. Our observation that WDR neurons likely contribute to tumor-evoked mechanical hyperalgesia in this model of cancer pain is consistent with the findings of Dickenson and colleagues in that central sensitization of superficial WDR, but not HT, neurons contributed to tumor-evoked mechanical hyperalgesia in rats[23]. Sensitization of WDR but not HT neurons to mechanical stimuli may be unique to cancer pain since both types of nociceptive neurons are sensitized to mechanical stimuli in models of diabetic neuropathy[61], peripheral nerve injury[62], inflammatory[45, 46, 63], and capsaicin-evoked[64] pain.

3.2 Thermal hyperalgesia and sensitization of nociceptive dorsal horn neurons to thermal stimuli

In their initial description of the model of cancer pain used in the present study, Wacnik and colleagues reported that implantation of fibrosarcoma cells into and around the calcaneus produced hyperalgesia to mechanical and cold stimuli applied to the plantar surface of the hind paw[19]. Whether tumor-bearing mice exhibited hyperalgesia to heat was not reported. We now demonstrate that this model of cancer pain also produces hyperalgesia to heat. In a previous study, we found that C nociceptors located in the plantar skin of the hind paw were sensitized to heat, as evidenced by decreased threshold temperatures and increased responses to suprathermal stimuli[31]. Thus, tumor-evoked sensitization of C nociceptors is associated with tumor-evoked heat hyperalgesia.

The findings of the present study are consistent with the findings of Dickenson and colleagues that nociceptive dorsal horn neurons are sensitized to heat in tumor-bearing rats. In comparison, WDR neurons in models of neuropathic pain do not exhibit sensitization to heat[58, 59, 64, 65]. Following zymosan-induced inflammation, intradermal injection of capsaicin, and freeze injury of the skin, both WDR and HT neurons exhibited sensitization to heat with decreased response thresholds and increased responses to suprathermal stimuli[47, 48, 51].

Few studies have compared the responses of WDR and HT neurons to cold stimuli in animals exhibiting hyperalgesia to cold. Following spinal cord injury, rats exhibited nocifensive behaviors to innocuous cold stimuli[66]. In these rats, the percentage of both WDR and HT neurons that responded to cold stimuli increased. Similarly, we reported that a mild freeze injury to the plantar skin lowered response thresholds and increased responses of both WDR and HT neurons to suprathermal cold (and heat) stimuli[49]. In the present study, WDR (but not HT neurons) in tumor-bearing mice exhibited sensitization to cold stimuli as responses of these neurons to noxious cold stimuli (≤ 12 °C) were greater than responses of WDR neurons in control mice. Sensitization of WDR neurons is consistent with the hyperalgesia to cold reported previously for this model of cancer pain[19]. In rats, implantation of mammary carcinoma cells into the tibia also produced cold hyperalgesia[21]. Thus, tumor-evoked sensitization of WDR neurons to thermal stimuli contributes to tumor-evoked hyperalgesia to heat and cold stimuli.

4 Conclusions

Hyperalgesia produced by implantation of fibrosarcoma cells into and around the calcaneus appears to involve sensitization primarily of WDR, but not HT, nociceptive dorsal horn neurons. Our findings support the notion that the mechanisms underlying cancer pain differ from those that contribute to inflammatory and neuropathic pain in that WDR neurons became sensitized in mice with tumor-evoked hyperalgesia compared to sensitization of both WDR and HT neurons in other models of chronic pain. Understanding the unique changes in the physiology and neurochemistry of the dorsal horn produced by tumor growth
may provide new opportunities for improved management of cancer pain.

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