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A rat model of bone cancer pain induced by intra-tibia inoculation of Walker 256 mammary gland carcinoma cells

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Abstract

This study described a modified rat model of bone cancer pain. Syngeneic Walker 256 mammary gland carcinoma cells were injected into the tibia medullary cavity via intercondylar eminence. Series of tests were carried out including bone radiology, bone histology, ambulatory pain, thermal hyperalgesia, mechanical allodynia, weight bearing ability, and electrophysiological recording from primary afferent fibers. The rats inoculated with carcinoma cells showed significant ambulatory pain, mechanical allodynia, and reduction in weight bearing, as well as increased incidence of spontaneous activity in Aβ fibers in affected limb, whereas PBS (vehicle) or heat-killed cells (sham) injected rats showed no significant difference in comparison to normal rats. The pain hypersensitive behaviors were aggravated with time and destruction of bone. Interestingly, mechanical allodynia was also observed in the contralateral limb, indicating the involvement of 'mirror image' pain in bone cancer pain. In summary, the present study provided a useful and easily established rat model of bone cancer pain which will contribute to further study of the mechanisms underlying cancer pain.

Keywords: Bone cancer pain; Walker 256 mammary gland carcinoma cells; Ambulatory pain; Mechanical allodynia; Weight bearing difference

As improvements in cancer detection and treatment have extended the life expectancy of cancer patients, more attention has been focused on improving the patients' quality of life. Approximately 30–50% of all cancer patients will experience moderate to severe pain, and 75–95% of patients with advanced-stage or metastatic cancer will experience substantial, life-altering cancer-induced pain [1,2]. According to the guidelines of the World Health Organization's 'analgesic ladder', treatment with non-steroidal anti-inflammatory drugs and/or opioids, great progress has been made in cancer pain relieving. However, it has been reported that 45% of cancer patients have inadequate and undermanaged pain control because of the relative ineffectiveness and the side effects of current treatments [3–5].

The common type of cancer pain was bone cancer pain that was difficult to treat. It was a serious clinical pain syndrome, which mostly occurred in patients with primary bone cancer or secondary bone metastasis from distant sites such as breast, prostate, and lung cancer [5]. This type of pain was dull and constant, increased with time, and was exacerbated by the use of involved bone. As bone cancer progressed, breakthrough pain, which was an intermittent episode of extreme pain, occurred spontaneously or more commonly by weight bearing or movement of the affected bone [6,7].

The recently published animal models of localized but progressive bone destruction have allowed greater insight into the plasticity of peripheral and central nervous system,

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and the further future studies on these models are expected to lead to new approaches for cancer pain management [8–11]. In these models, inoculation of cancer cells into the intramedullary cavity of femur or tibia of syngeneic animals produced a series of behavioral, cellular, and neurochemical changes correlated with cancer growth and bone destruction, including the development of mechanical hyperalgesia and mechanical allodynia, the changes of weight bearing, and astrocyte hypertrophy.

In this article, we described a modified model of bone cancer pain with unique bilateral mechanical allodynia induced by inoculating another mammary gland carcinoma cells derived from Wistar rats—Walker 256 cells into the tibia cavity of rats. Bone destruction and the time course of pain-related behavioral changes were evaluated. This is the first description of bilateral mechanical allodynia in an animal model of bone cancer pain.

Materials and methods

Animals. Female Wistar rats weighing 150–170 g were kept under controlled conditions (24 ± 0.5 °C, 12 h alternating light-dark cycle, food and water ad libitum). All experiments were conducted in accordance with the NIH guide for the care and use of laboratory animals and the Ethical Issues of the IASP [12].

Preparation of cells. Walker 256 rat mammary gland carcinoma cells (kindly provided by Institute of Radiation Medicine, Fudan University) were derived from Wistar rat. Ascitic cancer cells 0.5 ml (2×10^7 cells/ml) were injected into the abdominal cavity of the Wistar rats. After 6–7 days, ascitic fluid was extracted from above rats. Then cells were collected by centrifugation of 2 ml ascitic fluid for 3 min at 1200 rpm. The pellet was washed with 10 ml PBS and re-centrifuged for 3 min at 1200 rpm. Before the final pellet was re-suspended in an appropriate volume to achieve final concentrations for injection, the pellet was suspended in 10 ml PBS and cells were counted using a haemocytometer. The cell suspension was kept on ice until injected into rats. For the sham group, Walker 256 cells were prepared in the same final concentrations for injection and boiled for 20 min.

Surgery. All animals (except the normal group) were anesthetized with sodium pentobarbital (i.p. 50 mg/kg). Bilateral superficial incisions were made in the skin overlying the patella after disinfected with 70% v/v ethanol. Then more incisions were cut along the patellar ligament in order to expose the tibia head with minimal damage. A 23-gauge needle was inserted at the site of intercondylar eminence and pierced 7 mm below the knee joint into the medullary cavity of tibia. The needle was then removed and replaced with a 29-gauge needle (long thin blunt needle) attached to a 10 μl microinjection syringe. Then, carcinoma cells (4×10^5 or 4×10^3), heat-killed carcinoma cells (sham group) in 6 μl PBS, or 6 μl PBS only (vehicle group) were slowly injected into the right tibia cavity. Simultaneously 6 μl PBS was injected into the left tibia cavity in all these animals. The syringe was left in place for an additional 2 min to prevent the carcinoma cells from leaking out along the injection track. The injection site was closed using bone wax while the syringe was removed. The wound was closed and dusted with penicillin powder after the injection site was closed using gelatin sponge. All animals were allowed to recover from the inoculation surgery for 3 days prior to any experimentation.

Bone radiological detection. To assess the tibia bone destruction by tumor, tibia bone radiographs and histological staining were performed in this study. Rats were placed on a clear plane plexiglass and exposed to an X-ray source under sodium pentobarbital anesthesia on day 12 and 20 after cancer cell inoculation. By using E-COM Digital Radiographer System (E-COM Technology Co. Ltd., Guangdong, China), tibia radiographs were taken from both hind limbs of normal rats, Walker 256 mammary cells, heat-killed Walker 256 cells, and PBS treated rats ($n = 4$ for each group).

Bone histology. On day 20 after cancer cell inoculation, rats were anesthetized with overdose of sodium pentobarbital and transcardially perfused with 300 ml of 0.9% normal saline followed with 300 ml 4% paraformaldehyde. Bilateral tibia bones were removed and decalcified in decalcifying solution for 24 h. The bones were rinsed, dehydrated, and then embedded in paraffin, cut into 7 μm cross-sections using a rotary microtome (Reichert-Jung 820, Cambridge Instruments GmbH, Germany), and stained with hematoxylin and eosin to visualize the extent of tumor infiltration and bone destruction.

Behavioral assays for ambulatory pain. Rats were placed in a large plastic observation box with smooth floor. According to the extent of limb use during spontaneous ambulation, scores were given as following: (0) normal use, (1) slight limp, (2) extent between (1) and (3), (3) severe limp, and (4) complete lack of limb use. Testing was blind with respect to group.

Mechanical allodynia test. Animals were placed in a plastic cage ($26 \times 20 \times 14$ cm^3) with a plexiglass floor, containing 1.5 mm diameter holes in a 5 mm grid of perpendicular rows throughout the entire area of the platform. After 15 min accommodation, mechanical allodynia was measured as the hind paw withdrawal response to von Frey hair stimulation according to the up-down method described by Dixon [13]. Testing was blind with respect to group. An ascending series of von Frey hairs with logarithmically incremental stiffness (0.40, 0.60, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g) (Stoelting, Wood Dale, Illinois, USA) were applied perpendicular to the mid-plantar surface (avoiding the less sensitive tori) of each hind paw. Each von Frey hair was held about 1–2 s, with a 10-min interval between each application. A trial began with the application of the 2.0 g von Frey hair. The positive response was defined as a withdrawal of hind paw upon the stimulus. Whenever a positive response to a stimulus occurred, the next lower von Frey hair was applied, and whenever a negative response occurred, the next higher hair was applied. The testing consisted of five more stimuli after the first change in response occurred, and the pattern of response was converted to a 50% von Frey threshold using the method described by Dixon [13].

Hargreaves test. Using the Model 390 Paw Stimulator Analgesia Meter for paw stimulation (IITC/Life Science Instruments, USA), the paw withdrawal latency (PWL) to radiant heat was examined for evidence of heat hyperalgesia in animals according to Hargreaves test [14]. The rats were placed beneath an inverted, clear plastic cage upon an elevated floor of window glass. After an adaptation period of 30 min, radiant heat was applied to the plantar surface of each paw until the animal lifted its paw from the glass. The intensity of radiant heat was adjusted to elicit the response around 10–12 s in normal rats and the heat was maintained at a constant intensity. A cut-off time of 20 s was imposed on the stimulus duration to prevent tissue damage. The time from onset of radiant heat application to withdrawal of the rat's hind paw was defined as the PWL. Both hind paws were tested independently with a 10-min interval between trials. Testing was blind with respect to group.

Weight bearing experiment. Hind limb weight bearing was measured using an Incapacitance Analgesia Tester (Institute of Biomedical Engineering, Chinese Academy of Medical Science, Tianjin, China). Testing was blind with respect to group. The rats were placed in a Perspex chamber and each hind paw was stably contacted to a separate force transducer pad. The average was set to record the load on the transducer over 10 s and the results were presented as weight bearing difference (body weight on left limb—on right limb) between two hind limbs.

Electrophysiological study. Single fiber recording from the tibial nerve was performed in normal and Walker 256 mammary carcinoma cells (4×10^5) treated rats. Of rats with Walker 256 mammary carcinoma cells, only those determined to be mechanical allodynia were used. Animal was anesthetized with i.p. injection of urethane (1.5 g/kg, supplemented about 0.5 g/kg as necessary during experiment). Core temperature, respiration, heart rate, electrocardiogram, and arterial blood pressure were continuously monitored and maintained under the physiological criteria.

The right (ipsilateral to Walker 256 cells inoculated limb) sciatic nerve was exposed and a mineral oil poll was made by the cut edge of skin. The tibial nerve was detached from the sural and peroneal nerves. Microfilament containing one or two unit activity was teased apart using sharpened forceps and cut centrally, and then placed on a single platinum recording

electrode. A reference electrode was inserted in the surrounding tissues. The action potential was amplified with an AC-coupled amplifier, filtered, and input into an oscilloscope, then recorded and stored on computer. An A/D converter card (SMUP-PC, Shanghai Medical College, Fudan University, China) was used to digitize and store data.

The conduction velocity of each unit was determined by electrical stimulation using two fine needle electrodes inserted into the skin just proximal to the receptive field.

Statistical analysis. The mean values and the standard errors were calculated for behavioral assay. Data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Newman–Keuls test or χ^2 test based on necessary, using SPSS 10.0 statistical software. Criteria for significance in all analyses were considered as $P < 0.05$.

Results

Radiological and histological evaluation of bone destruction

Bone destruction was monitored using radiological and histological methods. No radiological change was found in rats treated with PBS or heat-killed cells as well as in normal rats. However, 8 days after injection with 4×10^5 Walker 256 cells, the tibia bone showed signs of radiolucent lesion in the proximal epiphysis, close to the injection site. By day 20 after inoculation, further deterioration was detected with medullary bone loss and full thickness uni- and/or bicortical bone loss. No radiological changes were observed on contralateral tibia bone up to 20 days after inoculation. Representative examples are presented in Fig. 1.

Sections obtained from the proximal end of tibia 20 days after the intra-tibia injection were stained with hematoxylin and eosin. Twenty days after injection, bone destruction was not observed in either vehicle or sham group animals. In contrast, tumor growth and various degrees of bone destruction were observed in the animals received live Walker 256 carcinoma cells. Remodeling of the bone was also observed in the affected bone (Fig. 2). In cases of severe bone destruction, the tumor destroyed bone matrix and periosteum and grew outside of the bone.

Time course of ambulatory pain

In the vehicle and sham group, rats showed no significant difference of hind limb use in comparison to normal rats. In contrast, all rats injected with live cells showed apparent limp on the injected hind limb over days following injection. Rats injected with 4×10^5 cells showed significant limp from day 7 ($P < 0.01$), whereas rats injected with 4×10^3 showed slight, but significant, limp from day 10 ($P < 0.05$). Moreover, rats received inoculation of higher number of cells showed more severe limp than that received lower number of cells (Fig. 3).

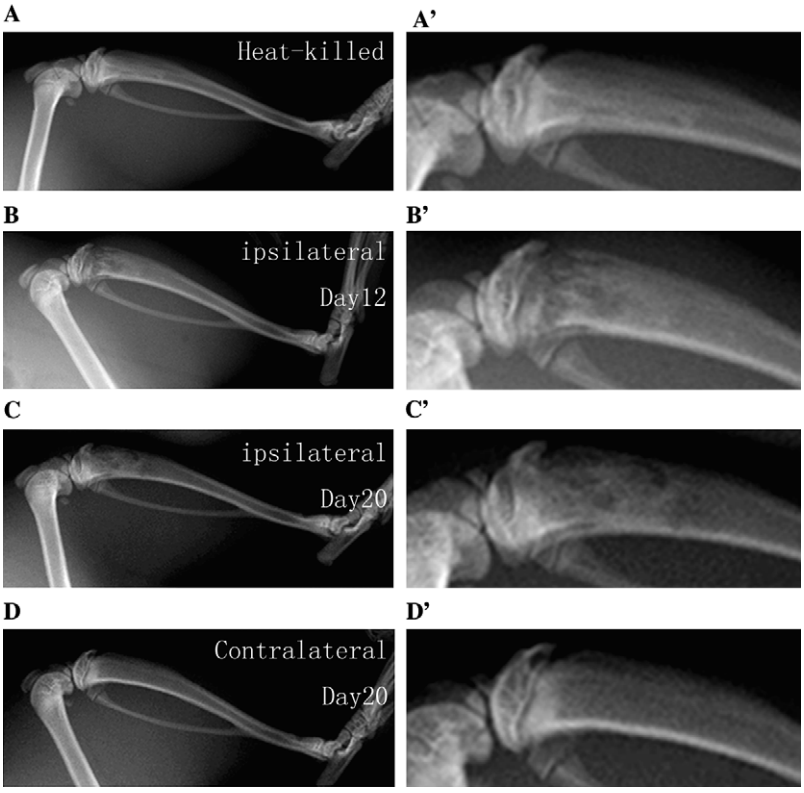

Fig. 1. Radiographs of the tibia bone inoculated with heat-killed Walker 256 cells (A), and live Walker 256 cells 12 and 20 days after inoculation in the ipsilateral (B and C) and contralateral (D) hind limbs. (A′–D′) showing the proximal end of the bones with a higher magnification. Radiograph of the ipsilateral Walker 256 injected and the contralateral tibia from the same animal, 20 days after inoculation. Note the lack of any effect on the contralateral side.

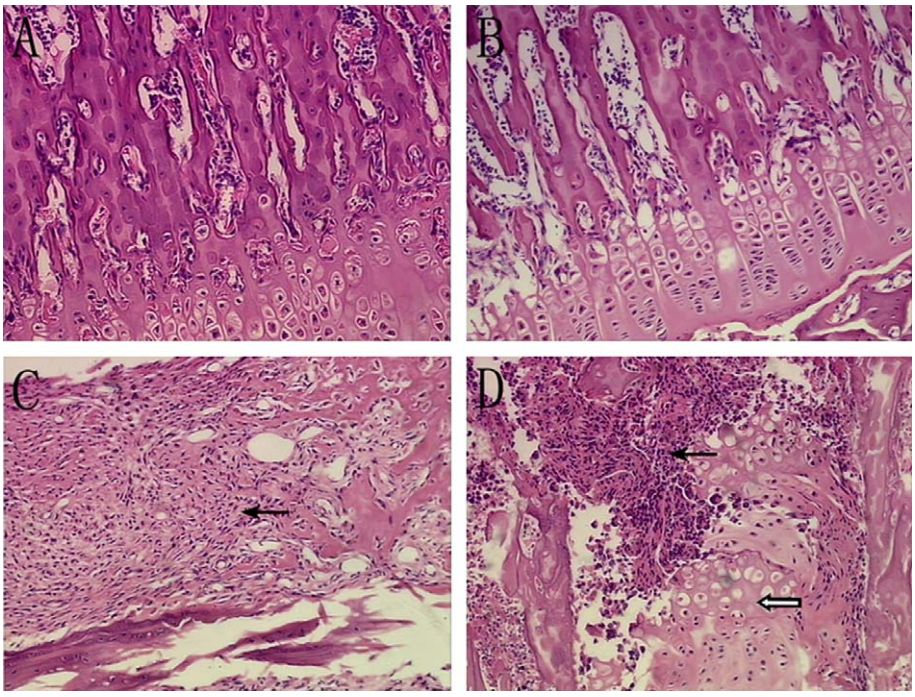

Fig. 2. Histology of tibial bone destruction (Hematoxylin-eosin stain). The sections (7 μm) were taken from tibial bone 20 days after surgery. (A) Bone injected with PBS; (B) bone injected with heat-killed cells; (C) showing tumor growth and bone destruction after injected with 4×10^5 Walker 256 mammary gland carcinoma cells (tumor cells are marked by black arrow); (D) showing newly formed bone after injected with 4×10^5 Walker 256 mammary gland carcinoma cells (tumor cells are marked by black arrow while newly formed bone is marked by hollow arrow).

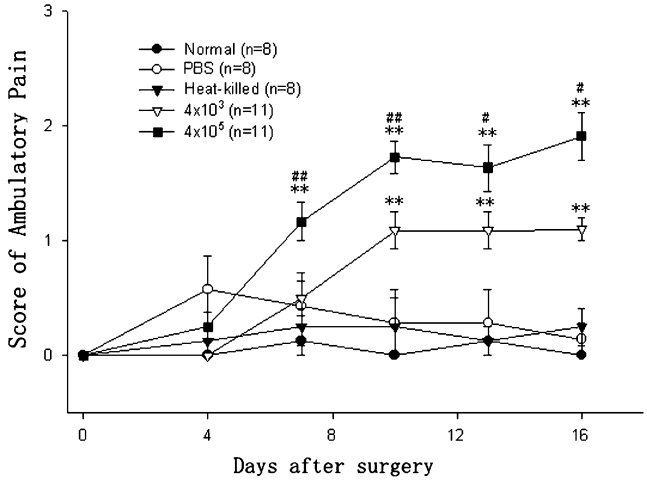

Fig. 3. Changes of ambulatory score in normal rats and rats received intra-tibial inoculations of syngeneic Walker 256 mammary gland cells (4×10^3 or 4×10^5 cells), heat-killed cells, and PBS. Data are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. 4×10^3 cells rats.

Time course of mechanical allodynia

Rats inoculated with live cells displayed a profound decrease in paw withdrawal threshold to von Frey hair stimulation, not only on the right hind limb received live cells but also on the left received PBS to the same extent (Fig. 4). Rats treated with 4×10^5 cells displayed a significant decrease in paw withdrawal threshold to von Frey hair stimulation from day 4 ($P < 0.05$), while rats treated with 4×10^3 cells displayed significant decease in paw withdrawal threshold from day 6 ($P < 0.01$). In contrast, no significant difference in paw withdrawal threshold was observed among vehicle, sham, and normal group rats.

Time course of thermal hyperalgesia

Rats injected with 4×10^3 and 4×10^5 cells showed no significant change in paw withdrawal latency to radiant heat stimulation on both hind paws during the whole experiment ($P > 0.05$, data not shown). No detectable difference was found among normal, vehicle, sham, and live cells tread rats.

Time course of weight bearing ability

Rats injected with 4×10^5 cells showed significant reduction in weight bearing on the ipsilateral hind limb from day 10 following intra-tibia injection ($P < 0.01$). On the contrary, vehicle, sham, and 4×10^3 cells treated rats showed no significant difference in hind limb weight bearing over 16 days following injection in comparison to normal rats (Fig. 5).

Single fiber recording

In 8 normal and 7 Walker 256 cells (4×10^5) treated rats, a total of 222 and 219 mechanosensitive afferents

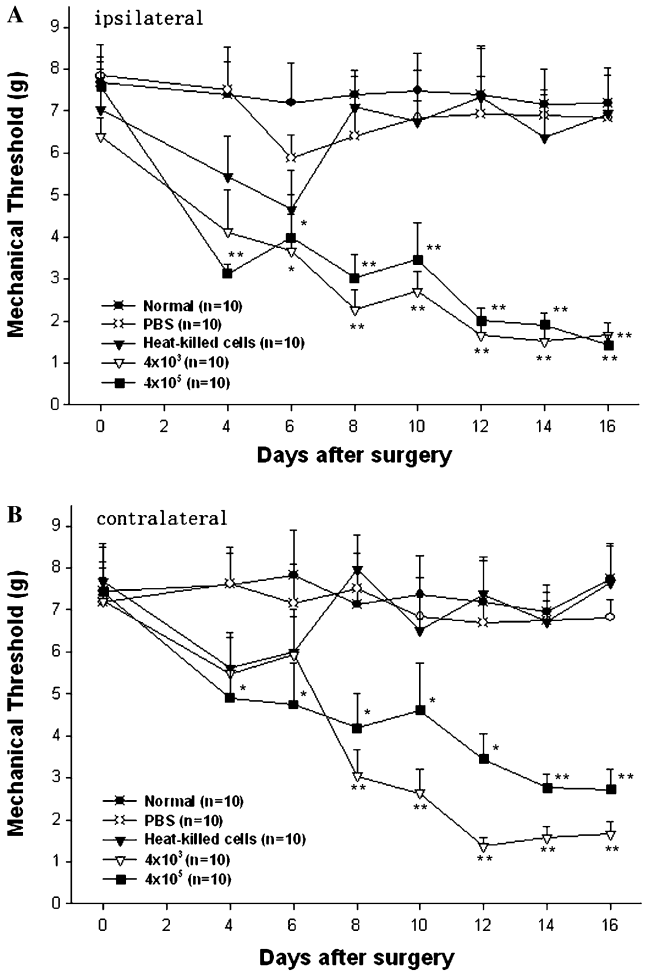

Fig. 4. Changes of mechanical response thresholds to von Frey hair of the ipsilateral (A) and contralateral (B) paw in normal rats and rats received intra-tibial inoculations of syngeneic Walker 256 mammary gland cells (4×10^3 or 4×10^5 cells), heat-killed cells, and PBS. Data are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal rats.

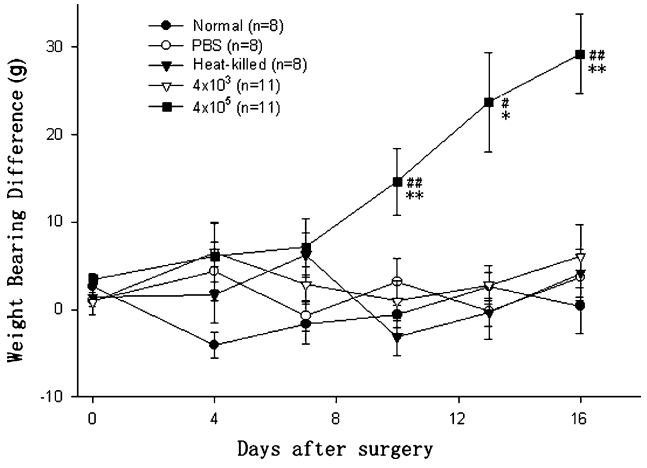

Fig. 5. Time course of hind limb weight bearing difference in normal rats and rats received intra-tibial inoculations of syngeneic Walker 256 mammary gland cells (4×10^3 or 4×10^5 cells), heat-killed cells, and PBS. Data are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.05$ vs. 4×10^3 cells rats.

in the tibial nerve were recorded, respectively. All of the fibers in rats with Walker 256 cells treatment were recorded at 10–19 days after inoculation. All the units were characterized by A fibers, most of them were Aβ fibers. The mean conduction velocity was 29.37 ± 10.05, and 25.92 ± 5.24 m/s, respectively, in normal and Walker 256 cells treated rats. Different from normal rats in which 14.1% of fibers were spontaneously active, 25.0% of fibers in rats with Walker 256 cells treatment exhibited spontaneous activity. The incidence of spontaneous activity in Aβ fibers had significant difference between normal and Walker 256 cells treated rats (χ^2 test, $\chi^2 = 8.035$, $P < 0.01$). More than half of the spontaneous discharges (64%) had a regular firing pattern, the remainder a slowly irregular or bursting firing pattern. Fig. 6 illustrates the three patterns of spontaneous activity in three independent fibers. No significant difference in the incidence of the firing patterns of fibers was found between normal and Walker 256 cells treated rats.

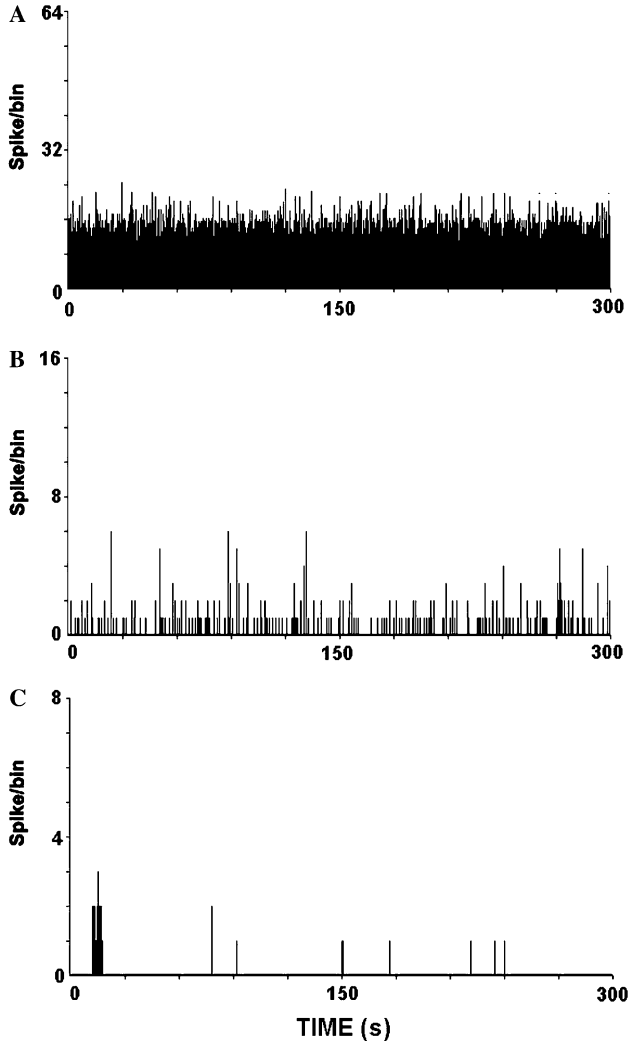

Fig. 6. Three examples of Aβ fibers showing regular (A), irregular (B), and bursting (C) patterns of spontaneous activity.

Discussion

Several models of bone cancer pain have recently been developed in mice [9,11] and rats [8,10]. Inoculation of different cancer cells into the intramedullary cavity of femur or tibia of syngeneic animals produces a series of behavioral, cellular, and neurochemical changes correlated with cancer growth and bone destruction [8–11]. These models, which could parallel the clinical bone cancer pain very well, promoted further study of the mechanisms underlying cancer pain.

According to the rat bone cancer pain model described by Medhurst et al. [10], here we established a modified bone cancer pain model by inoculating an alternative and available breast cancer cell line, Walker 256 mammary gland carcinoma cells, into tibia cavity of syngeneic Wistar rats via a new injection site. In the present study, incision was cut along the patellar ligament to expose the tibia head and carcinoma cells were injected into the medullary cavity of tibia via intercondylar eminence. The surgical procedure was taken carefully to minimize the damage to the knee joint. The results demonstrated that the surgical procedure per se did not affect the basal behavioral responses in von Frey, radiant heat and weight bearing test. No significant difference was found between normal rats and vehicle group rats even on the early days after inoculation, indicating that the function of knee joints kept intact.

Cancer pain is a complicated clinical syndrome and still remains a serious medical problem due to the lack of elucidation of its mechanisms. Bone cancer pain, one of the most serious cancer pain, was usually induced by primary bone cancer or secondary bone metastasis from breast, prostate, lung cancer, etc. [5]. The severity of the pain is closely correlated with the extent of bone destruction [15]. The pain progressively becomes heavy with cancer growth and bone destruction, and breakthrough pain occurs spontaneously or is more commonly evoked by weight bearing or movement of the affected bone as bone destruction progresses [6,7]. In the present study, bone cancer developed from intra-tibial Walker 256 cells induced ambulatory pain, mechanical allodynia, and reduction in weight bearing, as well as increased incidence of spontaneous Aβ fibers on ipsilateral hind limb indicating the hypersensitivity of rats. However, thermal hyperalgesia was not observed after Walker 256 cells inoculation in our study. Ambulatory pain, one of the most recognized types of bone cancer pain in clinic, can be divided into ambulatory and touch-evoked pain. It is resulted from the voluntarily use of the affected limb, which occurs during normal walking, standing or sitting [16]. In this study, we tested ambulatory pain by quantifying the extent of voluntary walking by applying a limb-use-scale as formerly described. Consistent to the previously reports [8,10], rats injected with live cells showed a significant limp on the injected hind limb over days following injection and the extent of the indicated ambulatory pain seemed dependent on the inoculated cell number. Additionally, the extent of hind limb weight bearing ability showed similar dependence on the inoculated cell number. These results were consistent to the previously reported bone cancer pain model [10] and suggested that this modified model could also mimic well the key features of human bone cancer pain.

Interestingly and surprisingly, in this model, we observed that the paw withdrawal threshold in von Frey test was reduced not only in the ipsilateral hind limb to inoculation, but also in the contralateral hind limb, demonstrating the existence of bilateral mechanical allodynia. Since the Walker 256 mammary carcinoma cells are known to avidly metastasize throughout the body [17], we may be observing a general sickness behavior due to these metastases. However, after the unilateral intra-tibia injection of Walker 256 cells, it seemed that tumor cells did not metastasize to the contralateral tibia since the radiographs showed no bone destruction in it (Fig. 1).

A similar intriguing phenomenon termed as mirror-image pain has already been well described clinically in herpes zoster, reflex sympathetic dystrophy (RSD, also named complex regional pain syndrome-type I, CRPS I), and causalgia (CRPS II) as well as experimentally in various animal models of neuropathic pain [18]. Notably, although not as frequently as traumatic precipitating factors such as bone fracture [19], malignant diseases are also associated with RSD and have been clinically identified as other precipitating factors of RSD [20]. Malignancy associated RSD was reported to be mainly induced by cancers which are the most easily to metastasize to bone, including breast cancer [21], prostate cancer [22], lung cancer [23,24], etc. Moreover, RSD and bone cancer pain shared common responsiveness to bisphosphonates, potent antiosteoclastic agents, indicating that RSD and bone cancer pain might share at least partially common mechanism. However, this is the first animal experimental report of bilateral allodynia induced by bone cancer and the possible relationship of this cancer pain model with RSD and the underlying mechanisms remains to be investigated.

On the basis of results from the different unilateral nerve injury models, Koltzenburg et al. provided potential explanations for contralateral change, in which peripheral circulating factors, transmedian sprouting, central terminals of afferents or dendrites of motoneurons, as well as commissural interneuron in the spinal cord and brainstem were considered to be involved in mediating unilateral nerve injury-induced contralateral mirror effects [25]. In addition, a recent report from Watkins group demonstrated that 'mirror image' pain in neuropathic pain was correlated with spinal glia activation, proinflammatory cytokines production, and morphological changes within local nerve, suggesting the involvement of glia in the 'mirror image' pain [18,26]. Spinal glia has been supposed to be linked to the induction and maintenance of chronic exaggerated pain [27]. Schwei et al. reported that bone cancer induced a profound neurochemical reorganization of the spinal cord that was directly correlated with the extent of cancer-induced bone destruction, among which they observed

a massive astrocyte hypertrophy without neuronal loss [9–11]. Thus whether spinal glia plays an essential role in the 'mirror image' pain in the present model of bone cancer pain is an interesting question to be answered.

In contrast to the present results, the previous observations on mice and rats cancer pain models showed unilateral rather than bilateral mechanical allodynia. This discrepancy might be regarded as being related to the different animal species and cancer cell lines used in these studies. Indeed, previous report has demonstrated that different bone tumors give rise to a distinct pattern of bone cancer-related behaviors and neurochemical changes in the central nervous system [28].

Acknowledgments

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