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Dissociation between the relief of skeletal pain behaviors and skin hypersensitivity in a model of bone cancer pain

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Abstract

Recent studies have suggested that in humans and animals with significant skeletal pain, changes in the mechanical hypersensitivity of the skin can be detected. However, whether measuring changes in skin hypersensitivity can be a reliable surrogate for measuring skeletal pain itself remains unclear. To explore this question we generated skeletal pain by injecting and confining GFP-transfected NCTC 2472 osteosarcoma cells unilaterally to the femur of C3H male mice. Beginning at day 7 post-tumor injection, animals were administered vehicle, an antibody to the P2X3 receptor (anti-P2X3) or anti-NGF antibody. Pain and analgesic efficacy was then measured on days 21, 28 and 35 post-tumor injection using a battery of skeletal pain-related behaviors and von Frey assessment of mechanical hypersensitivity on the plantar surface of the hindpaw. Animals with bone cancer pain treated with anti-P2X3 showed a reduction in skin hypersensitivity but no attenuation of skeletal pain behaviors. Whereas animals with bone cancer pain treated with anti-NGF showed a reduction in both skin hypersensitivity and skeletal pain behaviors. These results suggest that while bone cancer can induce significant skeletal pain-related behaviors and hypersensitivity of the skin, relief of hypersensitivity of the skin is not always accompanied by attenuation of skeletal pain. Understanding the relationship between skeletal and skin pain may provide insight into how pain is processed and integrated and help define the preclinical measures of skeletal pain that are predictive endpoints for clinical trials.

INTRODUCTION

Skeletal pain is one of the leading causes of chronic pain and long-term disability. Thus, skeletal pain arising from osteoarthritis, osteoporosis, age-related bone fracture, and bone cancer frequently result in a decline in functional status with an increase in both morbidity and mortality [37; 44; 51]. Unfortunately, the incidence of skeletal pain frequently increases with age. Without new treatments that more effectively relieve skeletal pain, this burden is projected to rapidly grow with the increasing global life expectancy [10; 13; 15; 45].

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There are several reasons why skeletal pain remains a significant and largely untreated worldwide health issue. First, there are relatively few analgesic therapies that can effectively relieve skeletal pain without significant unwanted side effects. For example, non-steroidal anti-inflammatory drugs and opiates, the most commonly used therapies to treat skeletal pain, are accompanied by significant renal, hepatic, respiratory, dependency and/or functional status issues when these therapies are used long term [2; 61; 66]. Secondly there is a limited understanding of the underlying mechanisms that drive skeletal pain. This is, in large part, due to the paucity of preclinical skeletal pain models that closely mirror the severity and chronicity of human skeletal pain. Lastly, the available surrogates for skeletal pain in preclinical models (i.e. limb use, weight bearing, nocifensive behaviors, or day/night activity) are time and labor intensive compared to measuring skin hypersensitivity. These factors make it difficult to rapidly screen promising new pharmacological therapies to attenuate skeletal pain.

Recently, hypersensitivity of the skin has been detected in human patients and preclinical animal models of osteoarthritis, low back pain, and bone cancer pain [4; 33; 43; 56; 58; 59; 72]. Given that skin hypersensitivity can be measured much more quickly and easily than skeletal pain-related behaviors, a major question is whether skeletal pain-induced hypersensitivity of the skin is an appropriate and reliable surrogate for assessing skeletal pain.

In the present study we explore this question by measuring hypersensitivity of the skin and skeletal pain-related behaviors in a murine model of cancer-induced bone pain (CIBP) in combination with therapeutic administration of either anti-P2X3 or anti-NGF monoclonal antibodies. Using this approach we found that anti-P2X3 significantly reduced hypersensitivity of skin, but had no significant effect on attenuating skeletal pain-related behaviors. In contrast, anti-NGF, which has been shown to reduce skeletal pain in both humans and animals models [16; 21; 28; 30; 35; 36; 38; 42; 46; 54; 57], attenuated both mechanical hypersensitivity of the skin and skeletal pain-related behaviors. These results suggest that therapies that attenuate skeletal pain-induced skin hypersensitivity may not always predict therapies that also attenuate the underlying skeletal pain.

METHODS

Experiments were performed on adult, male C3H/HeJ mice (n= 49, numbers represent two independent experiments) (Jackson Laboratories, Bar Harbor, ME, USA), approximately 8–9 weeks old, weighing 25–30 g at the time of tumor cell injection. This strain was chosen for its histocompatibility with the NCTC clone 2472 sarcoma tumor line (American Type Culture Collection, Manassas, VA, USA), which has been previously shown to form osteolytic lesions in bone following intramedullary injection [11; 12]. The mice were housed in accordance with National Institutes of Health Guidelines and kept in a vivarium maintained at 22°C with a 12h alternating light–dark cycle and provided food and water *ad libitum*. All procedures adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain [74] and were approved by the Institutional Animal Care and Use Committee at the University of Arizona (Tucson, AZ, USA).

Surgery and implantation of cancer cells

Surgery was performed as previously described in [46]. In brief, an arthrotomy was performed following induction of general anesthesia with ketamine/xylazine (100 mg/kg ketamine and 10 mg/kg xylazine; s.c.). A hole was drilled, using a pneumatic dental high-speed hand piece, in the patellar groove of the femur. A needle was inserted into the intramedullary canal to core a pathway for the sarcoma cells. Mice were injected with Hanks buffered saline (HBSS) (5 μL, Sigma, St. Louis, MO, USA) or 5×10^4 osteolytic murine sarcoma cells (NCTC 2472, American Type Culture Collection, Manassas, VA, USA) transfected with green fluorescent protein (GFP) and suspended in HBSS. The injection site was sealed with a dental amalgam plug (Dentsply, Milford, DE), to confine the cells within the intramedullary canal, followed by irrigation with sterile saline. To prevent the patella from becoming displaced post-arthrotomy, muscles were secured back in position using a horizontal mattress suture. In addition, an incision closure was achieved using wound clips. After surgery, animals were individually housed and allowed to recover for one week before being handled for behavioral and radiological assessment.

Anti-NGF Treatment

The anti-NGF antibody (mAb911) was kindly provided by Drs. Kris Poulsen and David Shelton (Rinat/Pfizer, San Francisco, CA, USA). How this antibody was made and its physical properties have been previously described [23]. This antibody does not effectively cross the blood brain barrier. We have shown that anti-NGF effectively reduces the skeletal pain associated with malignant bone cancer [46]. A dose of 10 mg/kg delivered i.p. was used and was administered on the same schedule as the anti-P2X3 (12D4) antibody.

Anti-P2X3 Treatment

The anti-P2X3 sequestering antibody (12D4) was kindly provided by Drs. Kris Poulsen and David Shelton (Rinat/Pfizer, San Francisco, CA, USA). This antibody was made similarly to the anti-NGF, has similar pharmacokinetics and pharmacodynamics, and does not effectively cross the blood brain barrier. A dose of 30 mg/kg was delivered by intra-peritoneal injection (i.p.) starting at day 7 post-cancer cell injection, and every five days thereafter.

Exclusion Criteria

Animals were observed daily, and criteria for exclusion from the study (and euthanasia) included: rapid weight loss (>20% in one week), patella displacement, lack of intra-femoral cancer cell growth by the end of the study, prolonged digestive abnormalities (e.g., diarrhea or vomiting for over 3 days), severe ulcerative dermatitis or infected tumors, and paralysis. A total of 6 animals were excluded in accordance with the above criteria.

Behavioral Measures of Cancer Induced Bone Pain (CIBP)

Mice were acclimatized to the testing environment for 30 minutes on four consecutive days one week prior to naïve baseline behavioral testing. All behavioral testing was performed in the morning and completed before noon. Testing was performed at baseline (pre-sarcoma inoculation) and 21, 28, and 35 days post-sarcoma inoculation. Mice were assessed for cutaneous stimulus evoked-pain, spontaneous nocifensive behavioral indicators of pain,

weight born by the ipsilateral hindlimb, and rearing activity. Each individual behavioral measure was performed by the same experimenter for the duration of the experiment. These individuals were blinded to the treatments animals received.

Cutaneous stimulus evoked-pain (von Frey test)

Mechanical withdrawal thresholds were measured using calibrated von Frey monofilaments (Stoelting, Wood Dale, IL, USA). Animals were placed in plastic cages with a wire-mesh floor. The von Frey filaments were applied in ascending order, beginning with the filament of 0.6 g, to the mid-plantar surface of the ipsilateral hindpaw through the mesh floor. Each probe was applied to the foot until it bent. The time between filament applications was at least 5 seconds. An algorithm was used to compute the 50% withdrawal threshold based on sequentially increasing and decreasing the strength of the filament stimulus applied incrementally [9]. Both ipsilateral and contralateral responses were recorded.

Tail Flick Assay

Animals had approximately two thirds of their tails immersed in a 48°C water bath. The time it took for the mice to remove their tail completely out of the water was recorded three times per mouse. All investigators were blinded to the groups. The average tail flick latency was plotted.

Spontaneous nocifensive behaviors

Briefly, mice were placed in a large Plexiglass box raised 20 inches above the surface of the bench and allowed to acclimate for approximately 15 minutes. Each animal's movements were recorded from below using Sony Handycam DCR-SR68 cameras. Time spent in nocifensive behavior was assessed by a blinded observer over a 300 second observation period after a 15 minute habituation period. Nocifensive behaviors were defined as any of the following: (a) full guarding (lifting the affected limb and holding it against its body), (b) reduced weight-bearing (affected limb is held in such a way that the foot, or the side of the foot, is merely resting on the floor), (c) tending to the affected limb (abnormal grooming behavior directed solely to affected limb, specifically licking lower limb and foot), (d) flinching the affected limb and (e) sporadic hopping (intermittent jumps without utilizing affected limb). The cumulative duration of pain-related behaviors during the 300-second testing period were plotted. This method is the same as that reported previously [46].

Dynamic weight bearing (DWB)

The percentage of weight borne by each limb of a freely moving animal was measured using a floor-instrumented dynamic weight bearing system (DWB, BioSeb EB Instruments, Pinellas Park, FL). Mice were placed in a small Plexiglass cage (11 × 11 × 22 cm) and a camera was placed on top of the enclosure. Weight of all tested mice was recorded, as it is a required entry for the data acquisition. The animal was allowed to move freely within the apparatus for 5 minutes while the pressure data and live recording were transmitted to a laptop computer via a USB interface. Raw pressure data was automatically synchronized with images from the video camera and the averaged values were recorded on a computer. Using the BioSeb software v1.3, the operator manually "validated" each test period,

ensuring each print corresponded to the appropriate paw using the synchronized video feed as a reference. A zone is considered valid when the following parameters were detected: ≥4 g on one captor with a minimum of 2 adjacent captors recording ≥1 g. For each time segment where the weight distribution is stable for more than 0.5 sec, zones that met the minimal criteria are then validated and assigned as either right or left hind paw or front paw by the experimenter according to the video and the scaled map of activated captors. Data is presented as percent weight borne by ipsilateral hindlimb over the total weight borne by both hindlimbs. Dynamic weight bearing was performed as previously described [42; 53; 62].

Rearing Activity

The number of times an animal reared (simultaneously lifted both front paws up from the floor) over a 300 second period was recorded as a measure of voluntary activity. Rearing was assessed during the time the animal was freely moving in the DWB apparatus (see above).

Immunohistochemistry

At day 28 post-sarcoma inoculation, three sham mice and five CIBP mice inoculated vehicle-treated mice were deeply anesthetized with ketamine/xylazine (100 mg/kg ketamine, and 10 mg/kg xylazine, s.c.) and perfused intracardially with 20 mL of 0.1 M phosphate buffered saline (PBS, pH = 7.4 at 4°C) followed by 20 mL of 4% formaldehyde/12.5% picric acid solution in 0.1M PBS (pH 6.9 at 4°C). After perfusion, the ipsilateral and contralateral dorsal root ganglia (DRG) corresponding to lumbar regions L2 were removed and placed in 30% sucrose overnight at 4°C. Ganglia were frozen with a small amount of OCT (Optimal Cutting Temperature) embedding medium (Sakura Finetek USA Inc., Torrance, CA) and placed on the mounting block surface. The block was then inserted into the cryostat (Bright OTF5000, Hacker Industries Inc., Winnsboro, SC) and sections were cut 16 μm thick and stored at −20°C until use. Sections were removed from −20°C and allowed to reach room temperature and then washed in PBS for 30 min. Sections were blocked 1 h at room temperature in 3% normal donkey serum (NDS; Jackson ImmunoResearch, Cat# 017-11-121, West Grove, PA, USA) diluted in PBS with 0.3% Triton-X 100 (Sigma Chemical Co., Cat# X100). The sections were then incubated overnight at room temperature with primary antibodies made in 1% NDS and 0.1% Triton-X 100 in 0.1M PBS. The primary antibodies used were: anti-P2X3 (1:1,000 dilution; Alomone Labs APR-016; lot APR016AN0925, Jerusalem, Israel) and anti-TrkA (1:1,000; R&D Systems AF1056; lot VFA0111011, Minneapolis, MN, USA). To control for nonspecific staining, the primary antibodies were omitted which resulted in the abolishment of specific signal for all antibodies used (data not shown). After three washes in PBS, sections were incubated for 3 h at room temperature with a mixture of secondary fluorescent antibodies: Donkey anti-rabbit Cy3 (1:600; Jackson ImmunoResearch, lot 120534, West Grove, PA, USA) and Donkey anti-goat Alexa Fluor 488 (1:400; Jackson ImmunoResearch, lot 114772). Both mixtures were incubated in 1% NDS in 0.1% Triton-X 100 in PBS. Preparations were then washed three times for 10 min each in PBS and dehydrated through an alcohol gradient (2 min each, 70, 80, 90 and 100%), cleared in xylene (twice for 2 min), and coverslipped with Eukitt mounting media (Sigma Chemical Co., St. Louis, MO, USA). Preparations were allowed to dry at room temperature for at least 12 hours before imaging.

Image acquisition and analysis

Confocal images were acquired with an Olympus Fluoview FV1200 system (Olympus, Melville, NY, USA) equipped with LD (405, 440, 473, 559, 635 nm), Multiline Argon (457, 488, 515 nm), and HeNe(G) (534 nm) lasers. Sequential acquisition mode was used to reduce bleed-through from fluorophores. Images were obtained using 20x air and 60x oil objectives. The average dimensions of the images collected were 211.7 μm × 211.7 μm × 20 μm with each Z-axis slice being 1 μm/slice. All images displayed as a Z projection.

Number of neuronal cell bodies expressing P2X3 alone, TrkA alone, or co-expressing P2X3 and TrkA were counted in the image processing software, FIJI [55]. To quantify the overlap between TrkA+ and P2X3+ neuronal cell bodies in the DRG, digital images were acquired and analyzed on a minimum of three ipsilateral and contralateral DRG sections per animal. The percentage of total neurons that are TrkA+ and P2X3+ was calculated by dividing TrkA + or P2X3+ neuronal cell bodies by total number of neuronal cell bodies. Images used for illustrative purposes in this publication were globally brightened in FIJI. These images were not used for quantification of TrkA/P2X3 overlap.

Statistical analysis

All statistical analyses were calculated in GraphPad Prism software (GraphPad, La Jolla, CA, USA). One way-ANOVA was used to compare pain-related behavioral changes and TrkA and P2X3 changes between experimental groups, followed by a Tukeys' post-hoc test comparing each group to each other at each time point. Significance level was set at $P < 0.05$.

Results

Sustained blockade of P2X3 reduces skin mechanical hypersensitivity in CIBP mice

Our mouse model of sarcoma bone metastasis served as a platform to test whether or not skin pain serves as an effective surrogate for skeletal pain. In order to accomplish this we evaluated the effect of two antibody treatments that bind either P2X3 or NGF on a battery of skeletal pain-related behaviors and on cancer-induced mechanical hypersensitivity of the skin. Animals injected with sarcoma cells and treated with vehicle developed significant mechanical hypersensitivity of the hind paw skin by day 21-post sarcoma cell injection and paw withdrawal thresholds continued to decline for the duration of the study. This hypersensitivity did not extend to the tail, as tail flick latencies were not statistically different between any of the tested groups (Supplemental Figure 1). Mechanical hypersensitivity was relieved when sarcoma injected animals were treated with either anti-P2X3 (on days 28 and 35) or anti-NGF (on days 21, 28, and 35) (Figure 1). Throughout the study, sham mice and the contralateral limb of sarcoma injected mice exhibited paw withdrawal thresholds that did not significantly deviate from baseline values (2.34 g) (Figure 1B and data not shown).

Skeletal pain behaviors in CIBP mice were alleviated by anti-NGF but not anti-P2X3 treatment

The analgesic efficacy of anti-P2X3 and anti-NGF were tested in CIBP mice using a battery of skeletal pain-related behaviors. These behaviors include spontaneous nocifensive behaviors, weight born by the ipsilateral hind limb, and rearing activity of sarcoma-injected mice (Figs. 2A, B, and C). Sarcoma injected animals treated with vehicle exhibited increased spontaneous nocifensive behaviors on day 21 that continued for the duration of the experiment (Figure 2A, light gray bars). Anti-P2X3 administration in CIBP mice (Figure 2A, dark gray bars) resulted in no difference in spontaneous nocifensive behaviors when compared to tumor-bearing mice treated with vehicle (Figure 2A). In contrast, sarcoma bearing mice treated with anti-NGF (black bars) showed a significant decrease in spontaneous nocifensive behaviors that was significantly different from sarcoma + vehicle (Figure 2A).

The inability of anti-P2X3 treatment to reduce sarcoma-induced skeletal pain-related behaviors was also apparent in the weight bearing of the affected limb (Figure 2B). Sarcoma + vehicle (light gray bars) and sarcoma + anti-P2X3 treated (dark gray bars) mice showed a significant decrease in the amount of weight borne on the tumor-bearing limb over time and were not statistically different from each other at any time point. By day 35 post-sarcoma cell injection, the tumor-bearing hind limb of anti-P2X3 treated animals only bore approximately 20% of the weight borne by the hindlimbs (Figure 2B, dark gray bars). In contrast, sarcoma + anti-NGF treated animals (black bars) restored their ability to bear weight on the ipsilateral limb and was statistically different from sarcoma + vehicle (day 21 and 35, Figure 2B). Similarly, rearing activity in sarcoma + vehicle (light gray bars) and sarcoma + anti-P2X3 (dark gray bars) treated mice was not statistically different from each other (Figure 2C). However, sarcoma + anti-NGF treated animals (black bars) had significantly increased rearing activity compared to sarcoma + vehicle at day 21, 28 and 35 post sarcoma implantation (Figure 2C).

Minimal colocalization of P2X3 and TrkA in neurons of the dorsal root ganglia

To explore why there was an effect of anti-P2X3 treatment on skin pain but not skeletal pain, we analyzed the distribution of neurons in femur innervating ganglia that expressed TrkA, P2X3, and both TrkA and P2X3. We compared ipsilateral sham to ipsilateral sarcoma L2 dorsal root ganglia from day 28-post sarcoma implantation and quantified the number of neurons that were immunopositive for either receptor by confocal microscopy (Figure 3). Neurons that express TrkA (red bars, sham 26.6%±2.3 of total neurons, sarcoma 23.9%±4.0, mean±SEM) and P2X3 (green bars, sham 45.8%±3.9 of total neurons, sarcoma 46.3%±5.6, mean±SEM) were not statistically different between the two groups, suggesting there is no change in either receptor expression due to sarcoma growth and its associated pain. Others have shown that intrathecal NGF administration increases P2X3 expression in a subset of neurons [52], however here we show that peripheral expression of NGF (by cancer or stromal cells) does not result in an up-regulation of P2X3 expression in neurons in the dorsal root ganglia that innervate the tumor bearing bone. Consistent with previous reports [31], these two receptors label largely separate populations of neurons with limited overlap (yellow bars, sham 6.8±2.2, sarcoma 4.7%±1.5, mean±SEM).

DISCUSSION

Potential mechanisms that drive skeletal pain-induced hypersensitivity of the skin

The present study confirms previous results obtained in the mouse [19; 20; 25; 34], rat [31; 40; 67; 73], and human [58] that bone cancer pain can induce a hypersensitivity of the nearby skin. Currently, the mechanism(s) by which skeletal pain drives skin hypersensitivity are not well understood. However, there are at least three possible mechanisms that may be driving this skin hypersensitivity. The first and probably most well investigated mechanism is that skeletal pain is highly efficacious in driving central sensitization and in turn may affect signaling to and from higher centers of the brain resulting in changes to descending modulation and ascending facilitation of pain signaling [18; 26; 63–65; 68].

The second possible mechanism is spinal sensitization, which has been shown to occur in several chronic skeletal pain states such as osteoarthritis and bone cancer pain. In preclinical studies using these models, significant glia, neuronal, and inflammatory changes have been observed in the superficial lamina of the spinal cord [7; 17; 20; 24; 68; 71]. As many of these neurochemical changes evolve and increase with the escalating skeletal dysfunction and accompanying pain, these changes may also affect how even normally non-noxious sensory information from the uninjured skin is processed and perceived by the organism.

Third, most dorsal root ganglia (DRG) contain both the cell bodies of sensory neurons that innervate the skeleton and skin [29; 47]. For example, the DRGs that house the sensory nerve fibers that innervate the tumor-bearing femur (lumbar ganglia) also house the cell bodies of sensory neurons that innervate the plantar surface of the skin [32; 50]. Interestingly, previous studies have shown that in animal models of chronic skeletal pain, the DRGs that innervate the damaged or diseased skeleton show remarkable infiltration by macrophages and changes in supporting cells [50]. Importantly, these changes in the DRG in terms of inflammatory and supporting cells are not confined to the area around sensory nerve fibers that innervate the bone but rather appear to be disseminated throughout the entire ganglia and are equally likely to occur around DRG neurons that innervate the bone or the skin.

Analgesic therapies that attenuate skeletal pain-induced skin hypersensitivity do not always attenuate skeletal pain-related behaviors

To explore the linkage and potential use of skeletal pain-induced skin hypersensitivity as a surrogate for measuring skeletal pain itself, we focused on two therapies in the same mouse model of bone cancer pain. Both therapies, anti-P2X3 and anti-NGF, are mouse monoclonal antibodies that do not readily cross the blood brain barrier (BBB) and thus most likely exert their effects outside the BBB [49]. It should also be noted that previous studies [22; 31; 39; 67; 73] examining whether P2X3 antagonists can relieve bone cancer pain and/or bone cancer pain induced skin hypersensitivity all employed small molecule P2X3 antagonists which unlike, anti-P2X3 or anti-NGF, probably readily cross the BBB.

In the present study, sarcoma + vehicle animals displayed significant skeletal pain behaviors and mechanical hypersensitivity of the skin when compared to sham animals and baseline values. Sarcoma + anti-P2X3 treated animals showed a reduction in skin hypersensitivity but

no attenuation of skeletal pain behaviors when compared to the sarcoma + vehicle group. In marked contrast, sarcoma + anti-NGF treated animals showed a reduction in both skin hypersensitivity and skeletal pain behaviors when compared to the sarcoma + vehicle group. Interestingly, these results are in line with those observed by Hansen and colleagues who used the same mouse model of osteosarcoma [22] and a small molecule P2X3 receptor antagonist from which they concluded that while bone cancer can induce significant skeletal pain-related behaviors and hypersensitivity of the skin, relief of hypersensitivity of the skin is not always accompanied by attenuation of the underlying skeletal pain.

In light of the data presented above, a major question is what mechanisms might explain how anti-P2X3 could relieve bone cancer-induced hypersensitivity of the skin but not the underlying bone cancer pain itself? One possibility is that this difference may in part be explained by the unique populations of primary afferent sensory nerve fibers that innervate the skin vs. the skeleton. It has been shown that the number of P2X3 or TrkA expressing neurons in the DRG appears to be conserved between humans and rodents [70]. Previous studies have demonstrated that in the mouse, P2X3 receptors are expressed by sensory nerve fibers that also express Mas-related G protein-coupled receptor member D $(MrgprD)^+$ and IsolectinB 4 $(IB4)^+$ and that this population of P2X3 expressing sensory nerve fibers has been shown to densely innervate the skin but not the bone or joint [3; 14; 29; 41; 47; 69; 75]. In contrast, the great majority of sensory neurons that innervate the bone express TrkA [8; 60] (which is the cognate receptor for NGF) whereas only a minority of the sensory nerve fibers that innervate the skin express TrkA [5; 6; 27; 75]. One other possibility would be that NGF may induce an up-regulation of P2X3 receptors in TrkA+ DRG neurons. Indeed, Ramer et al [52] showed that intrathecal infusion of NGF induced an approximately 1.2 fold increase in the number of DRG neurons that express P2X3 receptor. In the present study we examined whether peripherally released NGF (whether it be by the tumor or stromal cells) also resulted in an up-regulation of P2X3 expression in neurons in the DRG that innervate the tumor bearing bone. However, in the present study there was no increase in the percentage of P2X3 or TrkA expressing neurons in the dorsal root ganglia in sarcoma vs. naïve animals. Together, the present results suggest that blockade of P2X3 only attenuates skin pain whereas sequestration of NGF attenuates both the skeletal pain itself and the skin pain induced by skeletal pain. While other mechanisms are probably involved in driving skeletal pain-induced hypersensitivity of the skin, the present data suggest that the neurochemistry and repertoire of nociceptors that transmit skin vs. skeletal pain may be different and that therapies that relieve skin pain may not always relieve skeletal pain and vice versa.

Is skeletal pain-induced hypersensitivity of the skin a major driver of skeletal pain-related behaviors?

A major question addressed in the present study was whether mechanical sensitivity of skin, that occurs with many skeletal pain states, could be a major player in driving “skeletal pain-related behaviors” such as dynamic weight bearing, limb use, and spontaneous guarding / flinching of the tumor bearing limb. Severe injury to skin (such as a severe burn on the plantar surface of the foot) would be expected to induce very significant changes in mechanical sensitivity of the foot and therefore affect the ability of one to place significant

weight on the affected foot. This has been shown in other pain models including inflammatory pain models of the hindpaw [53; 62]. Given that animals with bone cancer exhibit increased mechanical sensitivity in the hindpaw skin, could this skin sensitivity be a driving force behind the "skeletal pain behaviors" measured here? At least in the present model the answer to this question appears to be no, as relief of skin pain by anti-P2X3 treatment did not affect any of the skeletal pain measures in the present study. In contrast, anti-NGF relieved skeletal pain-related behaviors in this model and has been shown to relieve skeletal pain in humans, dogs, rats and mice [16; 21; 28; 30; 36; 38; 54]. Thus, while skin hyperalgesia as assessed by quantitative sensory testing may be common in humans with moderate to severe skeletal pain, these patients rarely self-report skin hypersensitivity as their attention maybe focused on obtaining relief from the underlying skeletal pain. Indeed, it has been noted by clinicians that cancer patients rarely report skin pain even when they are reporting severe cancer pain [56].

Conclusions

The present results suggest that the relief of skin hypersensitivity may not always be a predictive surrogate for measuring the relief of underlying skeletal pain itself. Measuring skin hypersensitivity is much more straightforward, easier and less time intensive than measuring skeletal pain-related behaviors. However, as the repertoire of sensory nerve fibers that innervate the skeleton is quite different from the repertoire of sensory nerve fibers that innervate the skin, it would not be surprising if the set of peripheral factors that drive skeletal pain are not in some ways different from the set of peripheral factors that drive skin pain. Developing a better understanding of how skeletal pain drives skin hypersensitivity should provide interesting and unique insights into how pain from different tissues and regions of the body is processed and integrated. However, using attenuation of skin hypersensitivity as the sole or a pivotal surrogate for measuring the relief of skeletal pain may lead to false positives (or negatives) in preclinical and clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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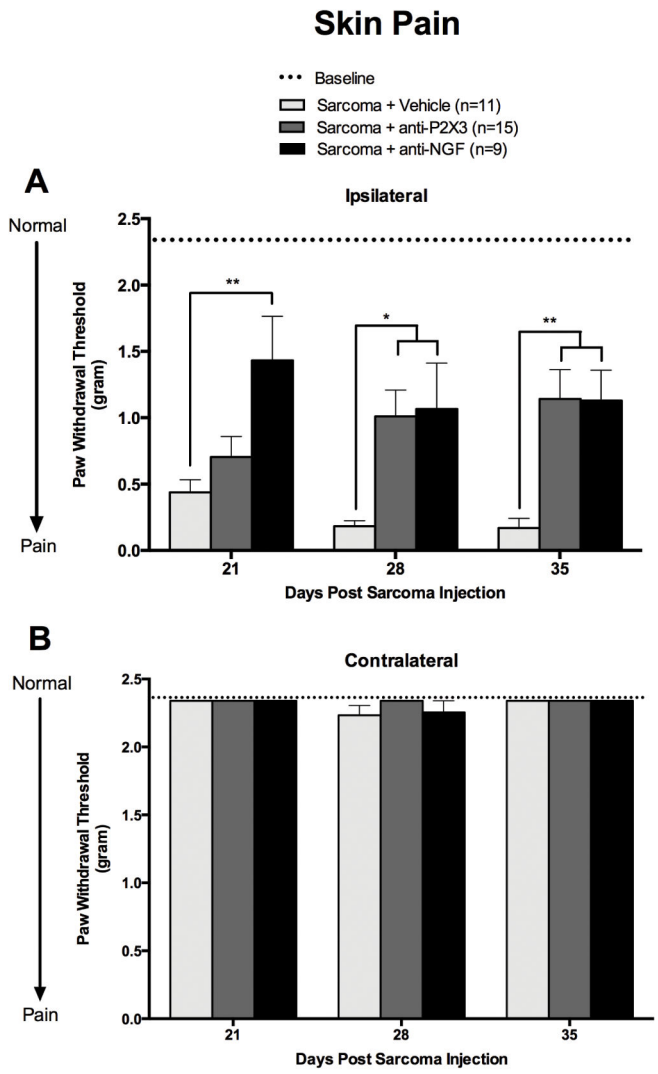

Figure 1. Mechanical hypersensitivity of the hindpaw occurs in mice with cancer-induced bone pain (CIBP) and is attenuated by treatment with anti-P2X3 or anti-NGF

Animals with CIBP, induced by injecting and confining tumor cells to one femur, treated with vehicle (PBS; light gray bars), developed mechanical hypersensitivity of the plantar surface of the hindpaw. This hypersensitivity of the skin increased with time and was present in the ipsilateral (A) but not contralateral plantar surface of the hindpaw (B). Sustained treatment with anti-P2X3 (30 mg/kg, i.p.; dark gray bars) significantly relieved mechanical hypersensitivity of the skin in sarcoma-injected mice at days 28, and 35 when compared to sarcoma + vehicle-treated animals. Anti-NGF treatment (10 mg/kg, i.p.; black bars) significantly reversed mechanical hypersensitivity of the skin at days 21, 28, and 35 when compared to vehicle-treated bone cancer bearing mice. Dashed line represents the baseline (naïve, pre-surgery) values. Error bars represent S.E.M. * $P \leq 0.05$ vs. vehicle, ** $P \leq 0.01$ vs vehicle.

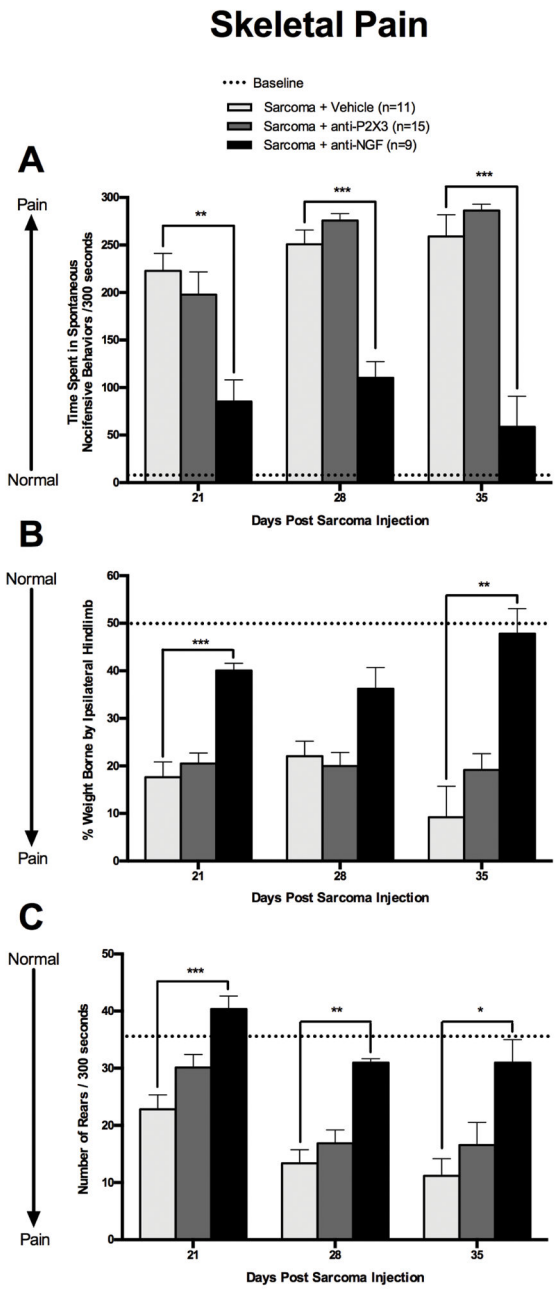

Figure 2. Skeletal pain related behaviors in animals with CIBP are reduced by treatment with anti-NGF but not anti-P2X3

Animals with CIBP treated with vehicle (PBS; light grey bars) exhibited several skeletal pain related behaviors including; increased spontaneous nocifensive behaviors (A), a decline in their ability to place weight on the affected limb (B), and a decline in their ability to rear on their hindlimbs for exploration purposes (C) was significant by day 21 post-tumor injection and these skeletal pain related behaviors increased with disease progression. Sustained anti-P2X3 treatment (30 mg/kg, i.p.; dark grey bars) was ineffective at reducing bone cancer-induced spontaneous nocifensive, changes in hindlimb weight bearing, and increasing rearing activity at all time points. However, animals treated with anti-NGF (10

mg/kg, i.p.; black bars) showed a significant reduction in time spent in spontaneous nocifensive behaviors (at all time points), an increased ability of animals to bear weight on the disease-burdened limb (at days 21 and 35), and an increase in rearing activity (at all time points) when compared to CIBP vehicle treated mice. Dashed line represents the baseline (naïve, pre-surgery) values. Error bars represent S.E.M. * $P \leq 0.05$, ** $P \leq 0.01$ vs. vehicle, *** $P \leq 0.001$ vs vehicle.

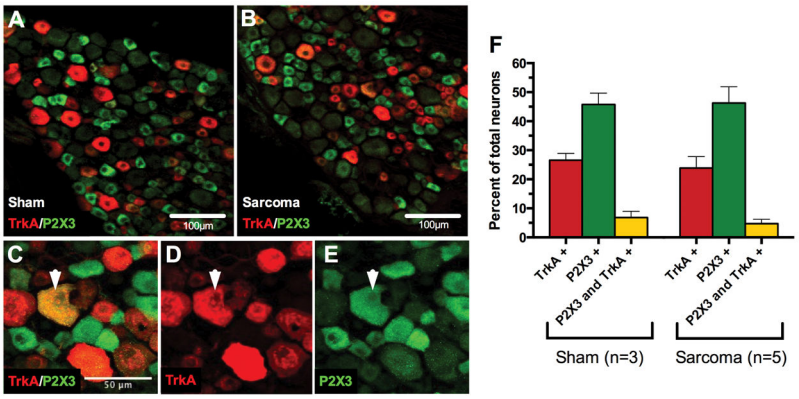

Figure 3. The expression and co-localization of TrkA and P2X3 in the cell bodies of sensory neurons in the L2 dorsal root ganglia that innervate the tumor bearing femur

L2 dorsal root ganglia isolated from 28-day sham or post sarcoma-injected animals (A and B, respectively) were immunostained for expression of TrkA (red, D) and P2X3 (green, E). The number of neurons that express both P2X3 and TrkA (yellow, C) represent a very small population of neurons in the dorsal root ganglia (6.8% sham, 4.7% sarcoma, F) (these dual immunostained neurons do not necessarily correspond to neuronal cell body size). Note that there was no significant change in the percentage of sensory neurons expressing either P2X3, TrkA, or co-expressing P2X3 and TrkA in animals with CIBP vs. sham control animals (F). Mean±SEM plotted.

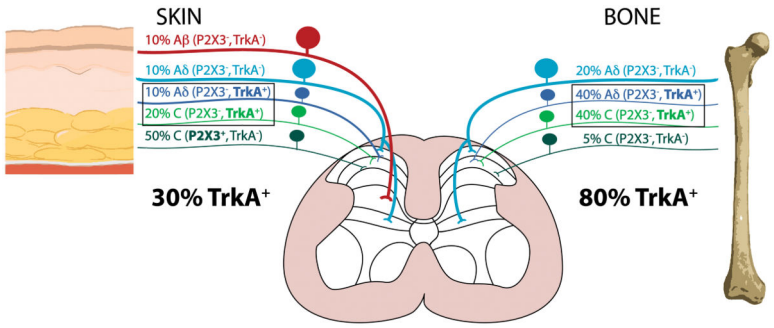

Figure 4. Schematic illustrating the approximate percentage and types of TrkA and P2X3 expressing sensory nerve fibers that innervate the skin vs. bone

The skin is innervated by thickly myelinated A-beta fibers (TrkA−, P2X3−), thinly myelinated A-delta fibers (TrkA−, P2X3−), unmyelinated peptide-rich C fibers (TrkA+, P2X3−) and unmyelinated peptide-poor C fibers (TrkA+, P2X3+). In contrast, the bone appears to completely lack any A-beta sensory nerve fibers and to be predominantly innervated by thinly myelinated A-delta fibers (TrkA+, P2X3−) and unmyelinated C fibers (TrkA+, P2X3−). In skin and bone there is also a small proportion (5% of the total) of unmyelinated C fibers (TrkA−, P2X3−). The percentages and types of sensory nerve fibers innervating the skin were estimated using data from previous studies (Bennett et al., 1996; Lu et al., 2001; Ambalavanar et al., 2005; Zylka et al., 2005) and for bone from the present and previous studies (Nakajima et al., 2008; Sugiura et al., 2008; Jimenez-Andrade et al., 2010). Note that the skin is heavily innervated by P2X3+ but not TrkA+ nerve fibers, where as the majority of sensory nerve fibers in bone are TrkA+ but not P2X3+.