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NGF blockade at early times during bone cancer development attenuates bone destruction and increases limb use

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Abstract

Studies in animals and humans show that blockade of nerve growth factor (NGF) attenuates both malignant and non-malignant skeletal pain. While reduction of pain is important, a largely unanswered question is what other benefits NGF blockade might confer in bone cancer patients. Using a mouse graft model of bone sarcoma, we demonstrate that early treatment with an NGF antibody reduced tumor-induced bone destruction, delayed time to bone fracture, and increased the use of the tumor-bearing limb. Consistent with animal studies in osteoarthritis and head and neck cancer, early blockade of NGF reduced weight loss in mice with bone sarcoma. In terms of the extent and time course of pain relief, NGF blockade also reduced pain 40-70% depending on the metric assessed. Importantly, this analgesic effect was maintained even in animals with late stage disease. Our results suggest that NGF blockade immediately upon detection of tumor metastasis to bone may help preserve the integrity and use, delay the time to tumor-induced bone fracture, and maintain body weight.

Keywords

NGF (nerve growth factor); bone cancer; weight loss; pain; time-to-fracture

Introduction

Cancer in bone tissue is typically the result of tumor metastases from a distant site, and cancers having the highest prevalence of bone metastases (lung, prostate, and breast) are the most frequently diagnosed cancers worldwide (1, 2). In the United States, in 2013, the estimated number of new cases of cancer in the lung (228,190), prostate (238,590) and breast (234,580) (3), combined with the known prevalence of bone metastasis in lung, prostate, and breast cancer (<36%, <90%, 65-75%, respectively)(4), results in the estimate that 460,000 new cases of bone metastases will be diagnosed. Once cancer metastasis to bone (CMB) occurs, it is usually incurable (unless the metastases are limited to very few sites). However, advances in cancer treatments continue to dramatically increase survival

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rates, thus patients with metastatic disease and suffering with malignant skeletal pain are living years to decades beyond their initial cancer diagnosis. Maintenance of the functional status of patients with CMB requires proactive management as CMB can cause excruciating bone pain, pathologic fractures, spinal cord and nerve compression syndromes, weight loss, derangements of calcium and phosphate homeostasis and decline in the ability to load and use tumor-bearing bones cannot be viewed individually but rather as interactive events ultimately contributing to increased morbidity, mortality, and diminished quality of life (2, 5).

In the past decade, progress has been made in understanding what drives CMB. Bisphosphonates and the RANK ligand inhibitor, Denosumab, have been approved for treatment of both pain and preventing bone fracture (6, 7). A therapy showing promise in blocking pain induced by CMB and non-malignant skeletal pain is inhibition of nerve growth factor (NGF) and its primary receptor, tyrosine kinase receptor type 1 (TrkA) (8-10). In breast, sarcoma, and prostate mouse models of CMB, anti-NGF significantly attenuates bone cancer pain (11-15). Additionally, in humans, anti-NGF attenuates moderate-to-severe skeletal pain due to osteoarthritis or low back pain (16, 17). While animal and human studies suggest that blockade of NGF/TrkA effectively alleviates malignant and non-malignant skeletal pain, most efforts at developing novel therapies to block or sequester NGF or TrkA were not initially developed to treat skeletal pain but rather to block/attenuate tumor growth and metastasis. Extensive *in vitro* studies show that NGF and/or TrkA drive the growth and metastasis of breast, ovarian, lung, pancreas, and prostate tumor cells (18-20). Moreover, *in vivo* studies show that anti-NGF inhibits ethylnitrosourea-induced carcinogenesis in mice and rats (21), and either anti-NGF or siRNA against NGF inhibits breast cancer tumor growth and metastasis in a mouse xenograft model (22).

In the present study we directly address these CMB patient issues by using a primarily osteolytic model of bone cancer which drives tumor-induced bone loss, bone fracture, loss of the use of the tumor-bearing limb, and weight loss. We explore whether early administration of anti-NGF can attenuate these pathological features. In addition we have modified and refined our bone disease progression and behavioral endpoints to more closely mirror endpoints used in human clinical studies in patients with CMB.

Materials and Methods

Surgical procedures and drug treatment

Mice—Experiments were conducted with adult C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) approximately 4-8 weeks old, weighing 25-30 g at time of tumor cell injection. Mice were housed in accordance with National Institutes of Health guidelines under specific pathogen-free conditions in autoclaved cages maintained at 22°C with a 12-hr alternating light/dark cycle and access to 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 (23) and were approved by the Institutional Animal Care and Use Committee at the University of Arizona (Tucson, AZ).Experiments were initiated with 90 mice, out of which a total of 85 mice were included for data analyses:naïve (n=8); sham surgery (n=13); NCTC 2472 (femur-injected) + vehicle (n=36); NCTC 2472 (femur

injected) + anti-NGF (n=24); NCTC 2472 + vehicle (muscle-injected) (n=4). To allow for careful attention to both surgical treatments and behavioral pain assessments/analyses, each experiment typically involved 12-16 mice, comprising of different experimental groups.

Cells—NCTC 2472 cells (ATCC® CCL11™, American Type Culture Collection, Manassas, VA), derived from a culture of subcutaneous areolar and adipose connective tissue (taken from a normal 82-day-old male C3H/HeJ mouse), aggressively stimulate osteoclast activity upon intramedullary femoral injection, and produce a bone histopathology resembling that found in human osteolytic bone cancer (24, 25). Authentication/testing carried out by ATCC confirmed the post-freeze viability, growth properties, morphology, absence of mycoplasma, species, sterility and development of sarcomas upon injection into mice. NCTC 2472 cells were stably transfected to express green fluorescent protein (GFP) to enable visualization. *In vitro* and *in vivo* tumor cell characteristics of GFP-transfected NCTC 2472 cells (growth rate, bone resorption rate, induction of bone cancer-related pain), were temporally, behaviorally, and physically identical to that of non-transfected NCTC 2472 cells (26). Upon thaw, GFP-transfected NCTC 2472 cells were cultured according to ATCC recommendations, passaged for at least three, but not more than 20 passages (less than three months), and verified mycoplasma-free before injection into mice. Additional information included in Supplemental Material.

Surgery—Injection of NCTC 2472 cells directly into the intramedullary space of the mouse femur was as previously described (13, 27-33). To prevent the patella from becoming displaced post-arthrotomy, muscles were secured back in position using a horizontal mattress suture. In addition, after surgery, animals were individually housed and allowed to recover for one week before being handled for behavioral and radiological assessment. Additional information included in Supplemental Material.

Anti-NGF Treatment—The anti-NGF sequestering antibody (mAb911), kindly provided by Dr. David Shelton (Rinat/Pfizer, San Francisco, CA), blocks the binding of NGF to both TrkA (tyrosine kinase receptor type 1, NTRK1) and p75 (neurotrophin receptor, LNGFR), and inhibits TrkA auto-phosphorylation (34). Anti-NGF has no effect on healthy bone (11-14, 35-37), its plasma half-life is five to six days in the mouse, and it does not appreciably cross the blood-brain barrier (38). In this study, the dose used (10 mg/kg, i.p.) was based on previous studies (11),and it was delivered starting at Day 7 post-cancer cell injection, and every five days thereafter.

Assessment of bone cancer disease progression and pain

Mice were assessed for bone cancer disease progression, functional status, and both spontaneous and movement-evoked pain, to measure endpoints that are clinically relevant to the patient with bone cancer (2, 9). Behavioral testing was performed on the same days as radiological assessment to enable comparison between pain behavior and bone destruction. Each method of behavioral assessment was performed by the same experimenter who was blinded to the drug treatments.

Radiology—High resolution X-ray images of cancer or vehicle-injected femurs were obtained several days before surgery (baseline), and immediately following weekly behavioral assessments, using a Faxitron MX-20 digital cabinet X-ray system (Faxitron/Bioptics, Wheeling, IL). Mice were lightly anesthetized with ketamine/xylazine (0.005 ml/g, 50 mg/10 kg, s.c.) to enable consistent placement of the animal for radiological assessment. Faxitron settings were optimized for radiological assessment of cortical or trabecular bone destruction. Animals were excluded from the study if a patella displacement was identified through radiography (see Supplemental Material, Fig. 1).

Bone Scoring—To quantify the extent of bone destruction, and to separately analyze disease progression at the distal and proximal aspects of tumor-bearing femora, a 10-point bone scoring method was used in which the distal and proximal halves of each femur were scored separately on a previously validated scale of 0 to 5 (28, 33), and then the scores for each femur half were summed (maximum possible score of 10). For each femoral aspect, bone scores were defined as: **0**, normal bone with no signs of destruction; **1**, small pits (1-3 in number) of bone destruction; **2**, increased pitted appearance (4-6 in number) and loss of medullary bone; **3**, loss of medullary bone and erosion of cortical bone; **4**, full thickness unicortical bone loss; **5**, full thickness bicortical bone loss and displaced skeletal fracture (Fig. 1).

Time-to-fracture—The radiographic endpoint "*time-to-fracture*" was defined as the time (day) following surgery when a fracture was identified through radiography.

Spontaneous nocifensive behavior—Mice were placed in small raised Plexiglas chambers (11.5 × 6.8 × 7.5 cm) with a wire grid floor, acclimated for 30 min (until cage exploration and major grooming activities ceased), and then their movements were videotaped from below using Sony Handycam DCR-SR68 cameras. Time spent in "nocifensive" behavior was assessed during a 5-min observation period (between minutes 15-20 of the filmed behavior). Nocifensive behavior was defined as: (a) full guarding (lifting the affected limb and holding it against its body), (b) reduced weight-bearing (affected limb is not completely held up against its body and some weight is borne on it), (c) tending to the affected limb (abnormal grooming behavior directed solely to affected limb), and (d) sporadic hopping (intermittent jumps without utilizing affected limb). See Supplemental Material for additional information, including videos of mice showing different types of spontaneous nocifensive behavior.

Limb Use (movement-evoked pain)—Limb use in an open field was assessed as previously described (28). The mouse was placed in the middle of a large Plexiglas box (40 × 50 × 20 cm) containing a mirror on the end facing the observer (for increased visibility of limb use), and observed while walking/running in a continuous motion over a two-minute period. Movements were categorized as: limping, guarding, flinching, non-use of limb, hunched posture, slow-to-get-up-and-go, walking slow, stiff/uneven gait, tilted stance, splayed limb, dragging of toes, and refusal to move. Movement of the affected limb was rated on the following scale: 0 = normal walking, +1 point assigned for each additional type of abnormal movement or posture observed (up to a total of 12).

Dynamic weight bearing—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). Data is presented as percent weight borne by ipsilateral hind limb of total weight borne by both hind limbs. Additional information included in Supplemental Material.

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, extra-femoral cancer cell growth, prolonged digestive abnormalities (e.g., diarrhea or vomiting for over three days), severe ulcerative dermatitis or infected tumors, and paralysis.

Statistical analysis

One-way ANOVA was used to compare behavioral and radiological bone scoring results between experimental groups. Significance level was set at $P < 0.05$.

Results

Characterization of bone cancer pain model

Experimental investigation in our optimized model involved first baseline behavioral assessment and X-ray analysis of naïve mice, followed by selection of animals with comparable values and their random assignment to treatment groups (vehicle or therapy). To monitor disease progression, high-resolution radiographs of the mouse femur were taken at various time points post-surgery. Radiological evidence of bone destruction presents as radiolucent areas, typically observed in the distal and proximal aspects of the femur at early time points of disease progression. As time and disease progress, multiple focal radiolucencies are accompanied by loss of medullary bone, erosion of cortical bone, and ultimately, fracture (Fig. 1).

At Day 0 (surgery), arthrotomies were performed, alternately between vehicle- and therapy-designated mice. Post-surgical pain, as exhibited by sham (HBSS-injected) mice, typically peaked at Day 3 following the arthrotomy, and approached baseline values by Day 7 post-surgery (Fig. 2). Spontaneous nocifensive behavior exhibited by NCTC 2472-injected mice was temporally differentiated into “surgical pain” and “bone cancer pain.” Post-surgical pain in NCTC 2472-injected mice was similar to that experienced by sham mice, peaking at Day 3 post-surgery and significantly lessened by Day 5 post-surgery. However, at Day 7 post-surgery, the observed profiles of nocifensive behavior for HBSS- and NCTC 2472-injected mice typically started to diverge, with tumor-bearing mice increasing in nocifensive behavior with time. At Days 10 and 14 post-surgery, nocifensive behavior exhibited by cancer cell-injected mice was approximately three and six times that of Day 7 post-surgery, respectively, and this rapid rise in cancer-induced bone pain extended to Day 18 post-surgery, at which time it was approximately eight-fold higher than that at Day 7 post-surgery. After Day 18 post-surgery, nocifensive behavior in NCTC 2472-injected mice steadily increased, although at a less rapid pace, and was approximately ten times that of

Day 7 post-surgery at the end of the study (Day 30). Collectively, these data demonstrated that our bone cancer model enabled the differentiation between surgical and cancer cell-induced bone pain, thereby providing an accurate assessment of cancer cell-induced pain and the efficacy of therapies targeted specifically to attenuate cancer-induced bone pain.

To demonstrate the importance of location of cancer cell growth in the development of pain behaviors, we included in our study a group of "muscle-injected" animals wherein NCTC 2472 cells were injected directly into the muscle adjacent to the femur. Despite the development of large tumor volumes, "muscle-injected" animals did not exhibit nocifensive behavior (Fig. 3).

Anti-NGF therapy attenuates sarcoma-induced bone destruction

To determine the effect of anti-NGF on NCTC 2472-induced bone destruction, mice were administered injections of either vehicle (phosphate-buffered saline, PBS) or anti-NGF in PBS (10 mg/kg, i.p.). Anti-NGF therapy was initiated at Day 7 post-surgery, and repeated every five days. At Days 7, 14, 21, and 28 post-cancer cell injection, mouse femurs were radiologically assessed for evidence of bone destruction. At Day 7, prior to the administration of the first dose of anti-NGF, minimal bone destruction was observed for both treatment groups. By Day 21 (after three doses of anti-NGF), a significant decrease in bone destruction was achieved with anti-NGF treated animals, and continued through Day 28 post-NCTC 2472 cell injection. Fig. 4A shows representative radiographs of the proximal aspects of mouse femurs of vehicle- and anti-NGF-treated mice at Day 28 post-NCTC 2472 cell injection, and the attenuating effect of anti-NGF on disease progression is striking. Whereas in vehicle-treated mice there was evidence of extreme bone destruction, this was not the case with the anti-NGF-treated mice. Mean bone scores for anti-NGF-treated mice at Days 21 and 28 were 4.0±0.5 and 5.7±0.4, respectively. In contrast, mean bone scores for vehicle-treated mice at Days 21 and 28 were significantly greater, 5.9±0.4 and 7.9±0.4, respectively (Fig 4B). The importance of the attenuating effect of anti-NGF on bone destruction in terms of overall functionality was demonstrated by the fact that at Day 28 post-NCTC 2472 injection, the percent of anti-NGF-treated mice with evidence of fracture was half that of vehicle-treated mice (Fig. 4C).

Anti-NGF therapy attenuates bone cancer-induced weight loss

Weight maintenance in cancer patients remains an important clinical challenge. In our pre-clinical model, a loss of 20% of baseline weight was a severe physiological event requiring removal from the study and euthanasia. Our data show that at Day 28 post-cancer cell injection, when there was significant disease progression and bone destruction, mice treated with anti-NGF therapy had only lost ∼2% of their baseline weight, whereas vehicle-treated mice had lost ∼6% of their baseline weight (Fig. 5).

Anti-NGF attenuates spontaneous and movement-evoked bone cancer pain behaviors

To determine the efficacy of anti-NGF in reducing bone pain in both early and late stage bone cancer disease, spontaneous nocifensive behavior exhibited by vehicle- and anti-NGF-treated mice was analyzed with respect to bone score (Fig. 6A). Mouse movements defined as "nocifensive" were full guarding, reducing weight-bearing and abnormal grooming of the

affected limb, and also sporadic hopping using the non-affected limb (see **Methods**, Supplemental Material videos). Spontaneous nocifensive behavior associated with a bone score of 0 was minimal for both treatment groups (approximately 25-30 sec, 8-10% of the 300-second assessment period), and was reflective of the pain experienced the week following cancer cell injection due to the surgical procedure. This pain was not NCTC 2472-induced bone cancer pain (Fig. 2, 4B).

Bone scores of 1-2, indicative of mild bone destruction and early stage bone cancer disease, in vehicle-treated mice were associated with a mean spontaneous nocifensive behavior of 195.0±5.3 sec. In contrast, in anti-NGF-treated mice, bone scores of 1-2 were associated with a mean spontaneous nocifensive behavior of 85.0±22.9 sec. Thus, anti-NGF treatment reduced pain in early stage bone cancer by approximately 56%. Bone scores of 3 and 4, indicative of moderate bone destruction and mid-stage bone cancer disease progression, in vehicle-treated mice were associated with mean spontaneous nocifensive behaviors of 203.8±13.5 and 255.3±17.6 sec, respectively. In anti-NGF-treated mice with similar bone scores, were associated with mean spontaneous nocifensive behaviors of 129.2±15.5 and 91.6±26.1 sec, respectively. Taken together, these data demonstrated that anti-NGF treatment reduced pain in mid-stage bone cancer disease by approximately 40-60%.

Bone scores of 5-6 and 7-10, indicative of severe to extreme bone destruction (with fracture), in vehicle-treated mice were associated with mean spontaneous nocifensive behaviors of 272.0±12.0 and 261.3±11.6 sec, respectively. In contrast, in anti-NGF-treated mice, bone scores of 5-6 and 7-10 were associated with mean spontaneous nocifensive behaviors of 93.0±32.4 and 70.4±25.6 sec, respectively. Taken together, these data show that anti-NGF treatment reduced the time spent in spontaneous nocifensive behavior in mice suffering with late stage bone cancer disease by approximately 70%.

The efficacy of anti-NGF in reducing bone pain in both early and late stage bone cancer disease was also revealed by analysis of spontaneous nocifensive behavior exhibited by vehicle- and anti-NGF-treated mice with respect to time (day) following cancer-cell injection (Fig. 6B). At Day 14 post-cancer cell injection, vehicle-treated mice had a mean spontaneous nocifensive behavior of 158.8±20.3 sec. This indicated that approximately 50% of the total 300-second assessment period the mice exhibited pain-related behaviors. In contrast, anti-NGF-treated mice had a mean spontaneous nocifensive behavior of 28.5±6.8 sec, illustrating that cancer-induced bone pain had been reduced six-fold. At Days 21 and 28 post-cancer cell injection, vehicle-treated mice had mean spontaneous nocifensive behaviors of 237.3±14.1 and 244.8±16.5 sec, respectively. However, at these same time points, anti-NGF-treated mice had significantly reduced mean spontaneous nocifensive behaviors of 114.8±14.8 and 102.9±24.3 sec, respectively. Taken together, these data show that at Days 21 and 28, when mice had severe to extreme bone destruction, anti-NGF treatment resulted in mice spending approximately 30-40% of the total 300-second assessment period in spontaneous nocifensive behavior as opposed to approximately 80% as in the case of vehicle-treated mice.

The ability of anti-NGF treatment to reduce pain-related behaviors in both early and late stage bone cancer disease was also revealed by analysis of dynamic weight bearing and limb

use in vehicle- and anti-NGF-treated mice with respect to time (day) following cancer-cell injection (Fig. 6C,D). Measurement of dynamic weight bearing in the mouse is equivalent to the human clinical measure of the ability to load the tumor-bearing bone. In vehicle-treated mice, the weight borne for the tumor-bearing limb progressively decreased over the course of the study; at Day 28 post-cancer cell injection the ability of the tumor-bearing hind limb to bear weight had been reduced by over 70% (Fig.6C). In contrast, anti-NGF treatment reduced bone cancer-induced pain enough that the animal was capable of preserving the ability of the tumor-bearing hind limb to bear weight. At Days 21 and 28 post-cancer cell injection, the percent weight borne by tumor-bearing hind limbs of vehicle-treated mice (18.4±2.4, 16.6±3.2, respectively) was significantly less than that of anti-NGF-treated mice (45.7±2.2, 46.6±2.3, respectively). Assessment of mouse limb use in an open field, comparable to assessment of gait, posture and the spontaneous use of the affected limb in humans, demonstrated that anti-NGF treatment prevented limb use impairment. A total of 12 different mouse movements or postures were included as evidence of limb impairment (see **Methods**). At Day 28 post-cancer cell injection, vehicle-treated mice had significantly more limb impairment, indicated by a higher limb use score (4.0±0.3) than anti-NGF-treated mice (2.2±0.2) (Fig. 6D).

Discussion

In the present study, we used a mouse model of sarcoma-induced bone cancer and showed that early treatment with anti-NGF reduced tumor-induced bone destruction, delayed time-to-fracture, and increased the use of the tumor-bearing limb. Additionally, as suggested in previous studies in animal models of osteoarthritis (39) and head/neck cancer (40), early blockade of NGF also reduced weight loss in animals with bone cancer. In terms of the extent and time course of pain relief, blockade of NGF reduced bone cancer pain by 40-70% depending on the pain measure being assessed, and this analgesic effect was maintained even in animals with late stage disease.

NGF and bone cancer disease progression

In the current study, NGF blockade delayed bone cancer disease progression; measured by time-to-fracture, tumor-induced bone destruction, and tumor-bearing limb use. What the specific mechanisms that maintain the integrity and use of bone remain unclear, but previous data in both older individuals and those with CMB have suggested that exercise and loading of the bone promotes bone health. Previous studies in human clinical trials in patients with moderate-to-severe osteoarthritis pain showed that anti-NGF attenuated this pain by 40-50%. However, within this group a small but significant number of patients receiving the highest dose of anti-NGF and naproxen required earlier-than-expected joint replacement. Whether this earlier-than-expected joint replacement was caused by overuse of the arthritic joints due to the robust pain relief provided by anti-NGF, or was a direct effect of blocking NGF on the maintenance and formation of adult bone remains unknown (10). However, in patients with CMB, fracture of the tumor-bearing bone is a highly undesirable event as active tumor-induced bone destruction makes stabilization and repair of the fractured bone problematic.

NGF and weight loss

A second major finding of the present study is that anti-NGF in this model of sarcoma-induced bone cancer reduced weight loss at late stage time points. This anti-NGF inhibition of weight loss confirms and extends what was previously reported in a rat model of Freund's induced osteoarthritis (39), and a mouse model of head/neck cancer (40), where anti-NGF markedly reduced weight loss. Currently, we do not know whether this inhibition of weight loss in the present study was due to blockade of a circulating anti-cachexia effect or reduction in pain/disease progression which allows the animal to maintain an appetite. Anti-NGF may indirectly affect weight loss by impacting the circulating levels of variety of cytokines (e.g., TNF-alpha, IL-6 and leptin) proposed to be involved in the regulation of body mass and cachexia. Chronic anti-NGF antibody treatment did not promote weight gain in normal rodents (39), thus the relationship between anti-NGF and weight is not straightforward. Given that opiates (commonly used to relieve cancer pain) have not been shown to reduce weight loss in patients (41), pain reduction and weight maintenance in the cancer patient appear to not be directly correlated. However, what is clear is that as CBM progresses, maintenance of body weight and muscle mass has an important impact on the functional status of the patient.

Pain induced by tumor growth in bone vs. muscle

A third aim of the present study was to examine whether tumor growth *per se* would be correlated with the extent of pain. To explore this possibility, we compared the pain induced by injection of NCTC 2472 cells into the bone versus the pain induced by injection of the same tumor cell line into the overlying muscle. Interestingly, while there was a clear increase in pain when tumor cells were injected, confined and grew within the bone, there was negligible pain following injection of NCTC 2472 cells into the muscle, despite the development of a tumor mass > 50× that in bone. These data extend previous data showing that it is not size, but rather location and specific nerve innervation, that influences tumor-induced pain (8, 9, 11, 42, 43).

Early vs. late administration of anti-NGF following cancer metastasis to bone

Current clinical trials are examining or considering examining the blockade of NGF/TrkA in terms of being an analgesic and not a disease modifying agent. However, the present data suggests that if anti-NGF can reduce tumor-induced bone destruction, time-to-fracture, weight loss, and CMB pain, and also maintain the functional status of patients, then early rather than late administration of anti-NGF following the initial diagnosis of CMB appears warranted. Currently, following diagnosis of CMB, where the initial diagnosis of CMB is frequently mild-to-moderate bone pain, patients usually are put on the WHO “analgesic ladder” for treatment of cancer pain which consists of NSAIDs alone, then NSAIDs + weak opiate, and finally NSAIDs + strong opiate. However, NSAIDs inhibit bone formation, and opiates have numerous side effects including nausea, dizziness, constipation and somnolence (9). In contrast, blockade of NGF or TrkA has remarkable efficacy in attenuating skeletal pain, and a total of 11,000 patients that received anti-NGF for non-metastatic skeletal pain had relatively few side effects (16).

One major concern with administering anti-NGF to humans with late stage disease bone cancer pain is that the patient typically will already be on NSAIDs + strong opiate, making it difficult to discern even an analgesic effect. However, in clinical trials if anti-NGF is given early (immediately upon diagnosis of CMB), and anti-NGF does have the positive effects on maintaining bone health, preventing bone fractures and weight loss, and reducing skeletal pain, early therapy with anti-NGF may significantly help in maintaining the functional status and quality of life of the patient. This would be especially true in a population of breast cancer patients who are young women (20–44 years old), and in the early or midstage of their careers and/or raising a family. As survival times of these young women with metastatic breast cancer have significantly increased (44), an important and unmet objective is to maintain their functional status and quality of life without the unwanted side effects of currently available therapies.

Pre-clinical vs. clinical measures of bone cancer disease progression and pain

Essential to the translational success of an emerging therapy is its validation in a pre-clinical animal model that incorporates endpoints directly translatable to those that can be used in human clinical trials. In the clinic, commonly-measured endpoints for bone cancer disease progression and functional status include radiological assessment of bone destruction and time-to-fracture (5), weight loss (45), evaluation of gait and weight-bearing (46, 47), and pain (48-50). As illustrated in Fig. 7, preclinical (mouse) measures incorporated in the present study to monitor disease progression, functional status, and pain closely mirror the human clinical measures used.

Limitations and conclusions of the study

To provide a clinically-relevant animal model of bone cancer pain, we originally developed and have recently optimized a mouse model in which mouse NCTC 2472 osteolytic sarcoma-producing cells are injected directly into the intramedullary space of the femur, following which the injection site is sealed with bone cement (13, 27-33). Surgical implantation of NCTC 2472 cells directly into the femoral intramedullary space enables a pre-clinical animal model that mirrors the development of bone cancer pain and bone remodeling in humans. The critical advantage of this method of bone cancer induction is that tumor cells are confined within the marrow space of the femur and prevented from invading surrounding soft tissue. However, this surgical procedure itself causes nocifensive behavior that is observed in the first week following surgery, and consequently, an important consideration in optimizing our bone cancer model was to ensure that an appropriate number of NCTC 2472 cells were injected such that assessment of sarcoma-induced bone cancer pain was not confounded by the presence of post-surgical (orthopedic) pain. Injection of too high a concentration of NCTC 2472 cells would induce significant tumor burden and bone destruction within a few days post-surgery and preclude differentiation between the pain caused by the arthrotomy from that of cancer-induced changes in bone remodeling.

Additional limitations of the current study are that the *in vivo* analysis was only performed using one tumor cell line, and that a single species and gender was used in the present study. However, previous work from our laboratory has investigated the effect of anti-NGF on bone cancer pain using implantation of breast cancer and prostate cancer cells (11, 12, 14,

15). These previous studies and the current study show anti-NGF effectively attenuates bone pain in all three mouse models of bone cancer pain.

Concerning the potential for observer bias in the analysis of pain behavior, we believe that due to the preventive measures taken in this work we have precluded, as much as humanly possible, the occurrence of subjective and biased pain measurements. Personnel measuring pain behaviors and analyzing x-rays were scrupulously blinded to the therapy treatments. Parameters for spontaneous nocifensive pain and limb use were specifically delineated prior to the start of this work, and videotaping of mice enabled careful (and repeat) analysis of limb movements. Use of a commercial, floor-instrumented dynamic weight bearing system enables objective measurement of weight borne by both ipsilateral and contralateral hind limbs.

The major conclusions of the present study are that early, sustained sequestration of NGF reduces tumor-induced bone destruction, delays time-to-fracture, increases use of the tumor-bearing limb, and reduces weight loss in animals with bone cancer. Blockade of NGF reduced bone cancer pain by 40-70% depending on the pain measure being assessed, and this analgesic effect was maintained even in animals with late stage disease. Given the potential effects of sequestration of NGF on disease progression, weight loss and pain, and the side effect profile observed with the 11,000 individuals who have received anti-NGF therapy to date (16), early rather than late blockade of NGF may improve the functional status and quality of life of patients with CMB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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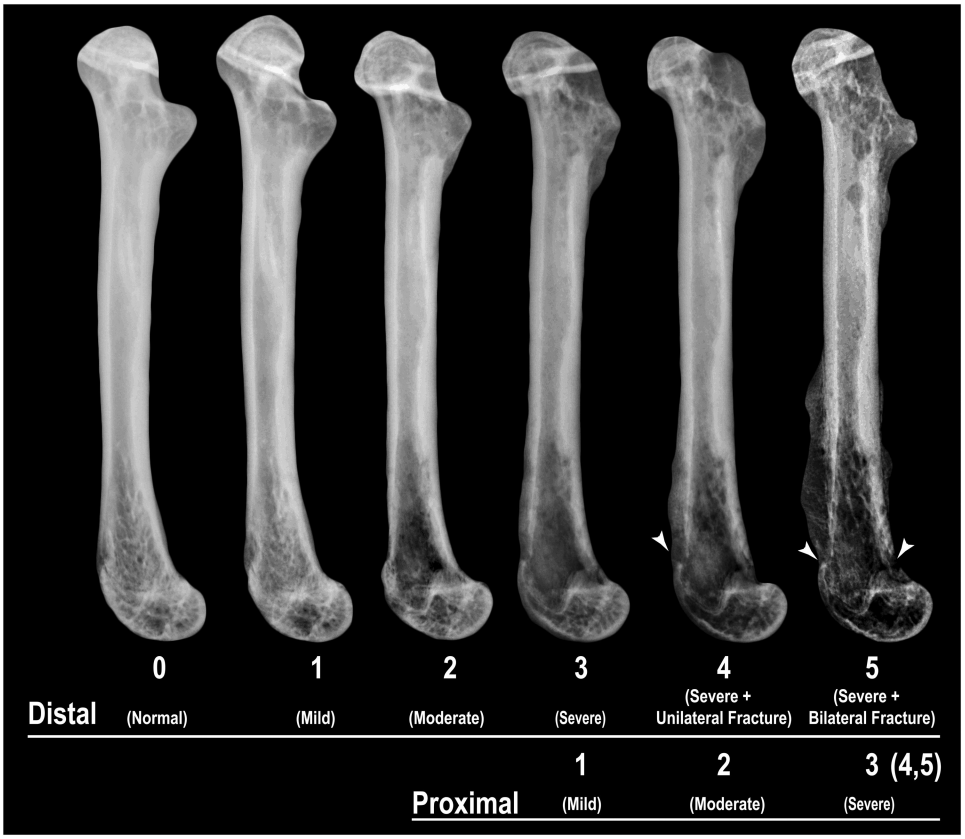

Figure 1. Sarcoma-induced bone destruction

High-resolution radiographs of the mouse femur illustrate progressive bone destruction following intramedullary injection of sarcoma cells. To measure the extent of bone destruction, each femur was divided into two equal-length aspects (distal and proximal), and bone destruction scores for each aspect were separately determined. For each femoral aspect, radiographs were scored on a 0-5 scale: **0 (normal)**, no signs of bone destruction; 1 (mild), small pits (1-3 in number) of bone destruction; **2 (moderate)**, increased pitted appearance (4-6 in number) and loss of medullary bone; **3 (severe)**, loss of medullary bone and erosion of cortical bone; **4 (severe + unilateral fracture)**, full thickness unicortical bone loss; **5 (severe + bilateral fracture)**, full thickness bicortical bone loss.

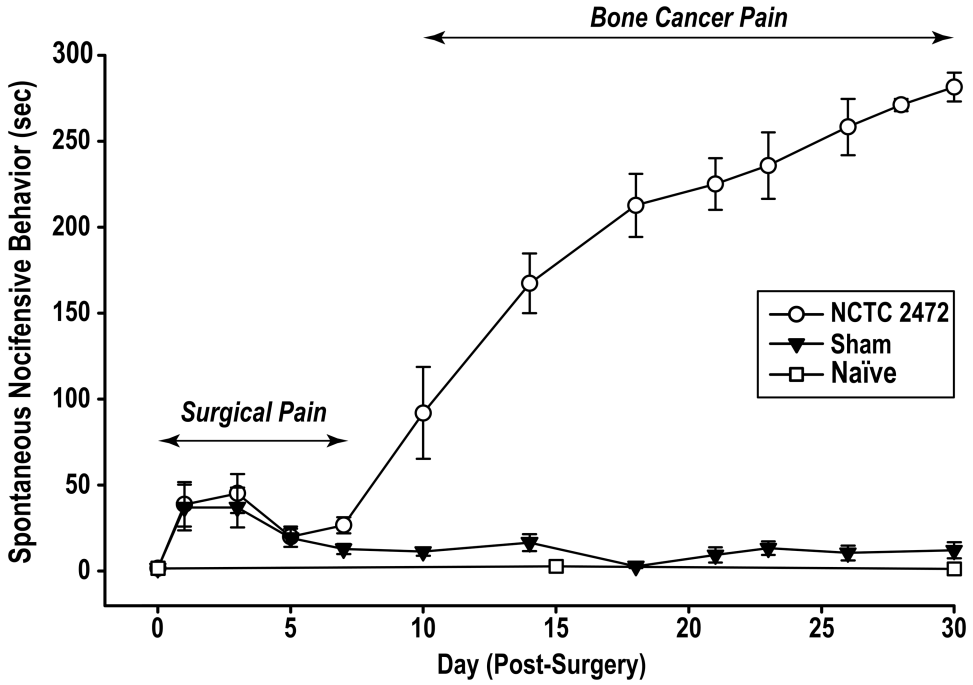

Figure 2. Nocifensive behavior over time in bone cancer model

Time-course of spontaneous pain-related behaviors following injection of either NCTC 2472 cells in Hanks balanced salt solution (HBSS) (open circles, n=36) or HBSS (sham) (inverted triangles, n=8) into the mouse femur show that spontaneous nocifensive behavior is temporally separated into “surgical (orthopedic) pain” (Day 1-7) and “bone cancer” pain (Day 7-30). Spontaneous nocifensive behavior exhibited by HBSS-injected (sham) mice show that by Day 7 post-surgery, spontaneous pain-related behaviors approach those exhibited by naïve mice (open squares, n=8). By Day 10 post-cancer-cell injection, time spent in nocifensive behavior by sarcoma-injected mice is easily differentiated from that of sham-injected mice. Error bars represent SEM.

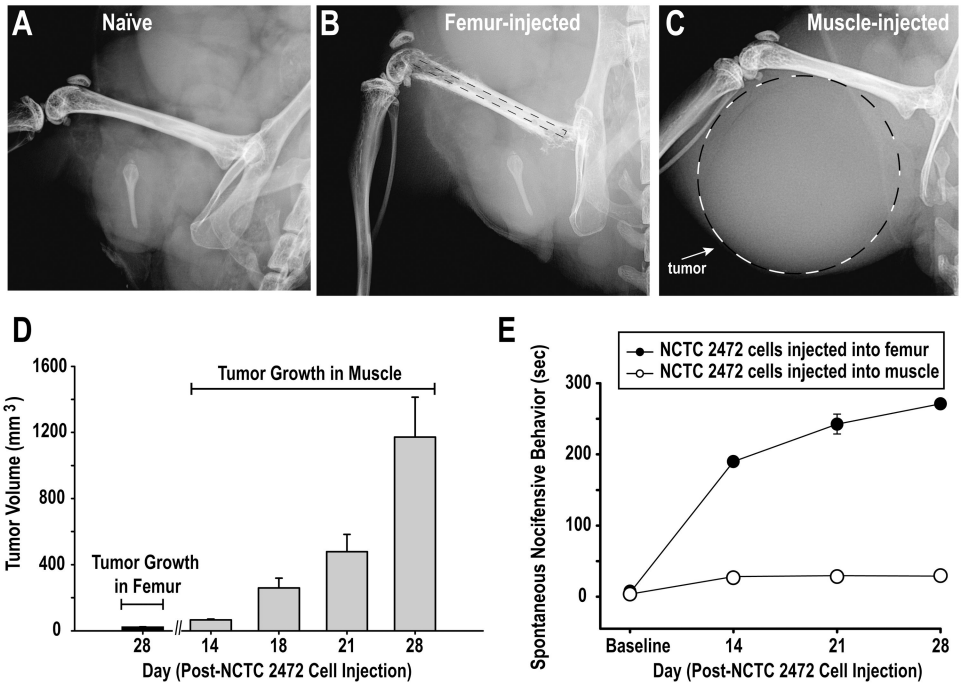

Figure 3. Tumor location (not size) influences cancer pain

To demonstrate that location of the tumor growth (within the bone or within the muscle adjacent but not involving the bone) is the critical factor in the intensity of bone cancer pain, experiments were performed in which murine NCTC 2472 sarcoma-producing cells were injected either directly into the intramedullary space of the mouse femur, or into the muscle directly adjacent to the femur. Representative high-resolution radiographs of a naïve mouse femur (**A**) a femur from a bone-injected (**B**), and a muscle-injected (**C**) mouse at Day 28 post-cancer cell injection revealed the greater tumor volume present in the muscle-injected mouse than in the femur-injected mouse. (**D**) Tumor volumes dramatically increased following cancer-cell-injection in the muscle-injected mice over time. (**E**) Despite harboring greater tumor volumes, the time spent in nocifensive behavior by muscle-injected mice (open circles, n=4) was significantly less than that exhibited by femur-injected mice (closed circles, n=6). Error bars represent SEM.

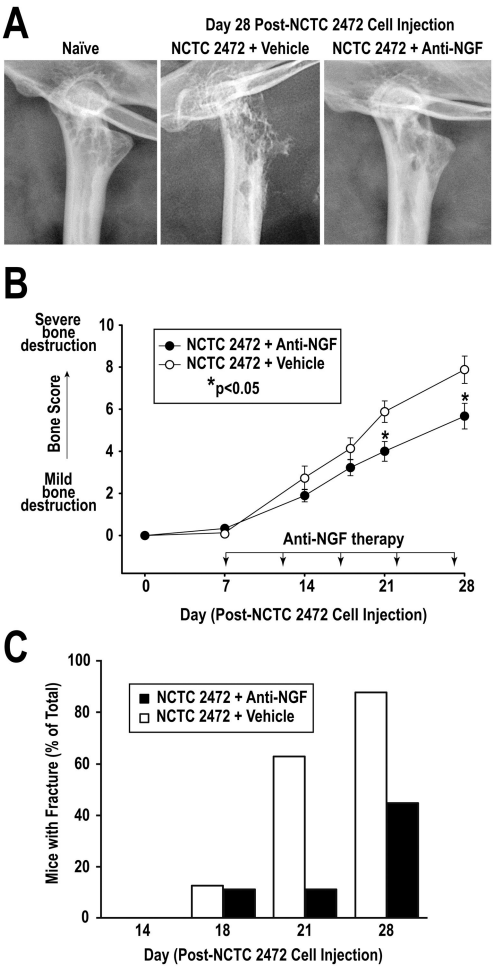

Figure 4. Anti-NGF reduces sarcoma-induced bone destruction

(**A**) Representative high-resolution radiographs of the proximal aspect of the femur of a naïve mouse, and of vehicle- and anti-NGF-treated mice at Day 28 post-cancer cell injection. Anti-NGF treatment attenuated sarcoma-induced bone destruction. (**B**) Anti-NGF significantly reduced the extent of bone destruction in therapy-treated mice (closed circles, n=24) compared to vehicle-treated mice (open circles, n=36) at Days 21 and 28 post-cancer cell injection. Error bars represent SEM; *p<0.05, one-way ANOVA. (**C**) Anti-NGF therapy resulted in an approximate 50% reduction of the number of mice with fractures at Day 28 post-cancer cell injection. Error bars represent SEM.

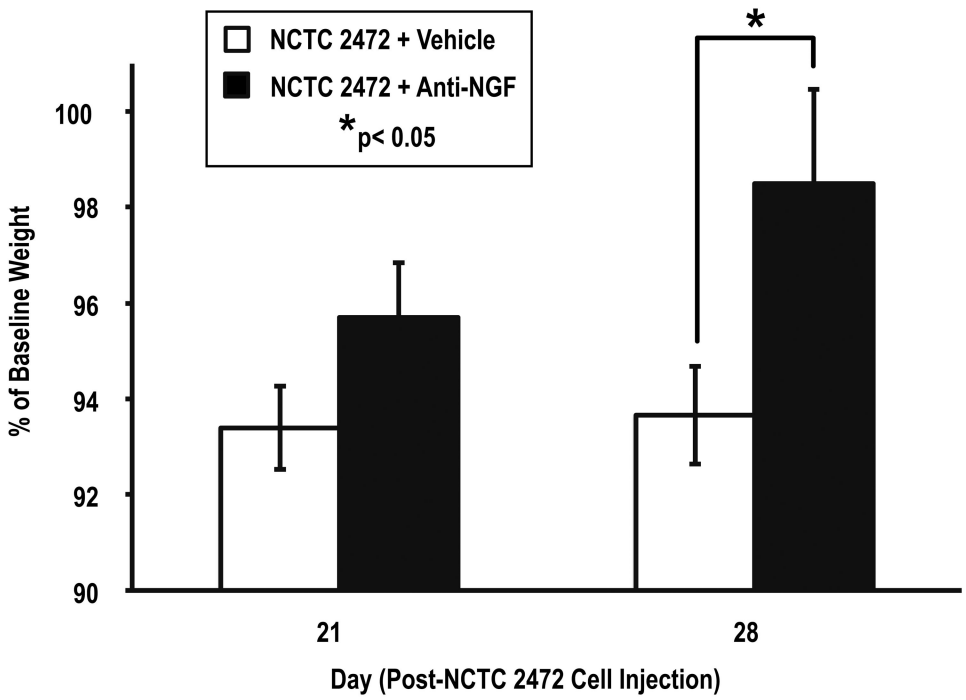

Figure 5. Anti-NGF therapy attenuates tumor-related weight loss

Mice treated with anti-NGF (closed rectangles, n=24) had significantly less weight loss than vehicle-treated mice (open rectangles, n=36) at Day 28 post-NCTC 2472 cell injection. Error bars represent SEM; *p<0.05, one-way ANOVA.

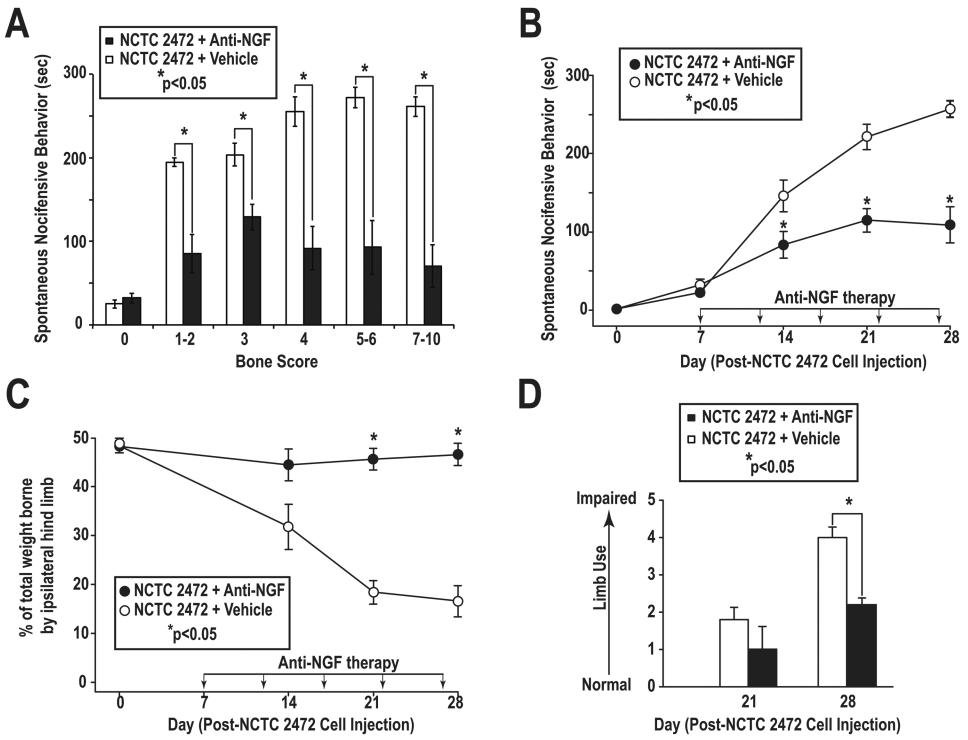

Figure 6. Anti-NGF attenuates spontaneous and movement-evoked bone cancer pain behaviors

(**A**) Time spent in spontaneous nocifensive behavior by anti-NGF-treated mice (closed rectangles) with bone scores of 1-2 (minimal bone destruction, early-stage bone cancer disease), 3-4 (moderate bone destruction), and 5-10 (severe to extreme bone destruction with fracture, late-stage bone cancer disease) was significantly reduced as compared to vehicle-treated mice (open rectangles). (**B**) Anti-NGF-treated mice (closed circles) spent significantly less time in spontaneous nocifensive behavior than vehicle-treated mice (open circles) at Days 14, 21, and 28 post-cancer cell injection. (**C**) Weight-bearing ability of tumor-bearing hind limbs of anti-NGF-treated mice (closed circles) was preserved throughout the study, whereas the weight-bearing ability of vehicle-treated mice (open circles) was significantly reduced at Days 21 and 28 post-cancer cell injection. (**D**) Impaired limb use in anti-NGF-treated mice (closed rectangles) was significantly reduced at Day 28 post-cancer cell injection, as compared to vehicle-treated mice (open rectangles). NCTC 2472 + vehicle, n=36; NCTC 2472 + anti-NGF, n=24. Error bars represent SEM; *p<0.05, one-way ANOVA.

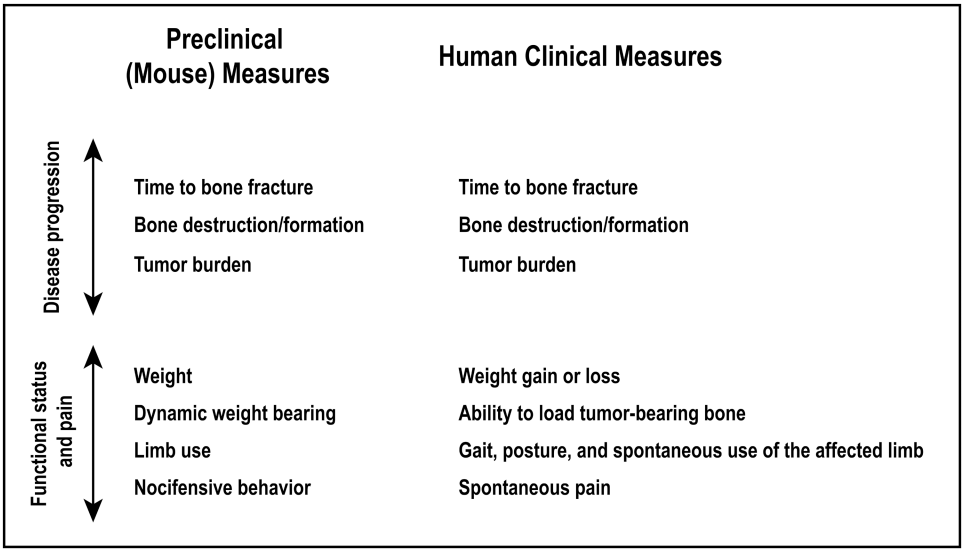

Figure 7. Preclinical and clinical measures of bone cancer disease progression, functional status, and cancer-induced bone pain

To facilitate the bench-to-beside translation of preclinical data examining the efficacy of a therapy targeting human bone cancer, it is critical that the pre-clinical animal model incorporate endpoints that are directly translatable to endpoints that can be used in human clinical trials. To that end, we have included in our bone cancer model a variety of preclinical measures of disease progression, functional status, and pain that might be used in human clinical trials involving cancer patients.