A Blocking Antibody to Nerve Growth Factor Attenuates Skeletal Pain Induced by Prostate Tumor Cells Growing in Bone

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Abstract

Prostate cancer is unique in that bone is often the only clinically detectable site of metastasis. Prostate tumors that have metastasized to bone frequently induce bone pain which can be difficult to fully control as it seems to be driven simultaneously by inflammatory, neuropathic, and tumorigenic mechanisms. As nerve growth factor (NGF) has been shown to modulate inflammatory and some neuropathic pain states in animal models, an NGF-sequestering antibody was administered in a prostate model of bone cancer where significant bone formation and bone destruction occur simultaneously in the mouse femur. Administration of a blocking antibody to NGF produced a significant reduction in both early and late stage bone cancer pain--related behaviors that was greater than or equivalent to that achieved with acute administration of 10 or 30 mg/kg of morphine sulfate. In contrast, this therapy did not influence tumor-induced bone remodeling, osteoblast proliferation, osteoclastogenesis, tumor growth, or markers of sensory or sympathetic innervation in the skin or bone. One rather unique aspect of the sensory innervation of bone, that may partially explain the analgesic efficacy of anti-NGF therapy in relieving prostate cancer--induced bone pain, is that nearly all nerve fibers that innervate the bone express trkA and p75, and these are the receptors through which NGF sensitizes and/or activates nociceptors. The present results suggest that anti-NGF therapy may be effective in reducing pain and enhancing the quality of life in patients with prostate tumor--induced bone cancer pain. (Cancer Res 2005; 65(20): 9426-35)

Introduction

The most frequent presenting symptom of prostate metastasis to the skeleton is bone pain (1). Pain originating from these bony metastases usually increases in intensity with the evolution of the disease and is commonly divided into three categories: ongoing pain, spontaneous breakthrough (incident) pain, and movement-evoked breakthrough pain (2, 3). Ongoing pain, which is the most frequent initial symptom of bone cancer, begins as a dull, constant, throbbing pain that increases in intensity with time and is exacerbated by use of involved portions of the skeleton (4). As bone cancer progresses, intermittent episodes of extreme pain can occur spontaneously, or more commonly, after weight-bearing or movement of the affected limb (4). Of these types of pain, breakthrough pain is the most difficult to control, as the dose of opioids required to control this pain are usually significantly greater than that needed to control ongoing pain and are often accompanied by significant unwanted side effects such as sedation, somnolence, respiratory depression, and constipation (4, 5).

In an effort to develop mechanism-based therapies that attenuate bone cancer pain due to tumors, such as those from the prostate, which commonly metastasize to the skeleton and induce both bone formation and bone destruction, canine prostate tumor cells were injected and confined to the intramedullary space of the femur of nude mice. Significant tumor-induced, pain-related behaviors were first observed at 9 days following injection of prostate tumor cells and continued to increase until 19 days post injection, at which time the mice were euthanized. Similar to humans with skeletal pain due to prostate tumors that have metastasized to bone, there was significant bone formation and bone destruction. This newly formed “woven” bone tended to isolate individual tumor-bearing compartments, which, when viewed radiologically, present the characteristic scalloped appearance seen in the bones of patients with prostate tumor metastases to the skeleton (6).

Previous studies have shown that nerve growth factor (NGF) plays a significant role in the generation of pain and hyperalgesia in a variety of acute and chronic pain states in rodents (7–9). NGF expression is enhanced in injured and inflamed tissue, where it is expressed by activated macrophages and neutrophils which can directly activate and/or sensitize primary afferent neurons that express the NGF receptors, trkA and/or p75 (10, 11). NGF also has been shown to induce hyperalgesia associated with mast cell degranulation (12). Previous studies have also suggested that NGF may be involved in the survival and proliferation of some tumor types (13). In the present report, we examine whether a blocking antibody to NGF may be therapeutically useful in attenuating bone pain, tumor growth, and tumor-induced bone remodeling due to a prostate tumor, which simultaneously induces significant bone formation and bone destruction.

Materials and Methods

Mice. Experiments were done on a total of 89 adult male athymic nude mice (8-10 weeks old, Harlan Laboratories, Madison, WI), weighing 20 to 32 g. The mice were housed in accordance with the NIH guidelines under specific pathogen-free conditions in autoclaved cages maintained at 22°C with a 12-hour alternating light and dark cycle and were given autoclaved food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.
Culture and injection of tumor cells. Canine prostate carcinoma (ACE-1, Dr. Thomas J. Rosol, Ohio State University) cells were maintained and injections of tumor cells were done as previously described (14). Following induction of general anesthesia with sodium pentobarbital (50 mg/kg, i.p.), an arthrotomy was done exposing the condyles of the distal femur. Hank’s buffered sterile saline (20 μL, Sigma Chemical Co., St. Louis, MO; sham, n = 7) or Hank’s containing 10⁵ ACE-1 cells (20 μL, ACE-1, n = 60) was injected into the intramedullary space of the left mouse femur and the injection site was sealed with dental amalgam (Dentsply, Milford, DE), followed by irrigation with sterile filtered water. A 19-day post injection end point was used, as this is the point when the tumor is still confined to the bone and there is maximal presentation of cancer-related pain behaviors and tumor-induced bone remodeling. Sham mice were used for control analysis for behavioral experiments and bone histology/immunohistochemistry, as naïve mice were not significantly different from sham, both behaviorally or histologically, 9 days post tumor injection.

Characterization and treatment with anti–nerve growth factor antibody. Previously, the sequestering antibody, anti-NGF, (monoclonal antibody 911, Rinat Neuroscience Corporation, Palo Alto, CA) was characterized and shown to be effective in blocking the binding of NGF to the trkA and p75 NGF receptors and inhibiting trkA autophosphorylation (15). Anti-NGF binds both human and rodent NGF, and does not bind to other members of the neurotrophin family (15). Pharmacokinetic experiments in rodents indicate a terminal half-life of 5 to 6 days in plasma after i.v. injection in the mouse (16). To assess the effect of the anti-NGF therapy on pain-related behaviors, tumor growth, bone formation, and bone destruction, the anti-NGF antibody was administered (three 10-μg/kg injections, i.p. at days 7, 12, and 17 post-sham or ACE-1 injection) when tumor-induced bone remodeling was first evident ensuring a high concentration of antibody throughout the duration of the study. As in previous experiments, using C3H/C2 mice (14), the dose regime used in the present experiment caused no adverse effects such as hypoglycemia in naive mice, as assessed by thermal and mechanical sensitivity experiments. The general health of the mice was closely monitored using food consumption and body weight as general health indicators throughout the experiments.

Mice were randomly placed into treatment groups receiving either injections of sterile saline (ACE-1 + vehicle, n = 21; 1.4 μL/kg, i.p.) or anti-NGF antibody, (ACE-1 + anti-NGF, n = 9; 10 mg/kg, i.p.). For behavioral comparison of anti-NGF antibody to morphine sulfate, mice were given an acute dose of morphine sulfate 15 minutes prior to behavioral testing (naïve, n = 6; sham, n = 7; ACE-1 + vehicle, n = 7; ACE-1 + anti-NGF, n = 7; ACE-1 + morphine sulfate 10 mg/kg, s.c., n = 8; ACE-1 + morphine sulfate 30 mg/kg, s.c., n = 8). For thermal and mechanical sensitivity testing and the assessment of hindpaw skin innervation, naïve mice were divided into two treatment groups receiving either sterile saline (naïve + vehicle, n = 8; three injections, i.p. at days 7, 12, and 17) or anti-NGF antibody (naïve + anti-NGF, n = 8; three 10-μg/kg injections, i.p. at days 7, 12, and 17).

Euthanasia and processing of tissue. Mice were euthanized and perfused 19 days post tumor injection. Following radiologic examination, the femurs and hindpaw skin were processed for immunohistochemical analysis as previously described (14, 17). Femoral sections were stained with tartrate-resistant acid phosphatase (TRAP) and H&E to visualize the histologic features of normal bone marrow, tumor, osteoclasts, osteoblasts, and macrophages. Skin sections were stained with antibodies raised against calcitonin gene–related peptide (CGRP), neurofilament 200 (RT97), and tyrosine hydroxylase (TH).

Radiographical analysis of bone. Radiographs (Faxitron X-ray Corp., Wheeling, IL) of dissected femora were obtained at the day 19 end point to assess the extent of bone formation and bone destruction. Images were captured on Kodak Min-R 2000 mammography film (Eastman Kodak Co., Rochester, NY; exposure settings: 7 seconds, 21 kVp). Analysis of bone density was used to assess the extent of tumor-induced bone remodeling radiographically of whole bone images at 5× magnification. Tumor and non–tumor-bearing femurs (naïve + vehicle, sham + vehicle, ACE-1 + vehicle, and ACE-1 + anti-NGF; n = 8 for each group) were analyzed using ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, MD) as previously described (18). Briefly, blank radiograph films and a standard step tablet (Eastman Kodak) were used to develop a calibration curve. ImageJ was used to measure absorbance and subsequently converted to transmission as follows: transmission = 1/[anti-log₁₀(Optical Density)]. Given data are determined from a negative image, transmission is directly proportional to bone density. An HP ScanJet 7400c (Hewlett Packard, Palo Alto, CA) scanner was used to capture subsaturation femoral radiographs and readings were recorded in duplicate from each femora. Results are presented as normalized transmission mean ± SEM.

Histologic analysis of osteoblasts, osteoclasts, and macrophages, tumor growth and bone remodeling. Osteoblast proliferation was analyzed by quantifying the number of osteoblasts in contact with regions of both tumor-induced new bone formation contained within the femur and cortical bone throughout the entire diaphyseal intramedullary space for naïve, sham-injected, and tumor-bearing mice. Diaphyseal intramedullary space was defined as the area between the distal and proximal trabeculae and was selected for quantification as the predominant active bone remodeling occurs in this region. Osteoblasts were identified as those cells in direct contact with the newly advancing bone matrix that display typical cuboidal or columnar epithelial cell and are connected to one another via thin processes identifiable at high magnification (200× or greater; ref. 19). Results are presented as the number of osteoblasts/mm² of diaphyseal intramedullary space. Osteoclast proliferation was determined by quantifying the number of TRAP-stained osteoclasts at the bone/tumor interface and at the normal marrow/bone interface for naïve, sham-injected, and ACE-1-injected mice on TRAP-stained femoral sections throughout the diaphyseal intramedullary space. Osteoclasts are histologically differentiat- ed cells appearing as TRAP-stained and which are closely associated with regions of bone resorption. These cells are multinucleate and are found in Howship’s lacunae along the cortical and trabecular bone (19). Macrophage proliferation was determined by quantifying the number of TRAP-stained cells that were dispersed throughout the tumor and normal marrow not associated with the endosteal surface of the mineralized bone. Macrophages within the bone become activated due to tumor-released factors that stimulate the cells, and the cellular appearance of these activated macrophages is marked by their highly irregular surface, multiple lamellipodia, and phagocytic vacuoles (19). Results are expressed as the mean number of osteoblasts per square millimeter or macrophages per square millimeter of diaphyseal intramedullary space, respectively.

Femora containing ACE-1 cells were imaged using bright-field microcopy on a Nikon E600 microscope equipped with a SPOT II digital camera using SPOT image capture software (Diagnostic Instruments, Sterling Heights, MI). The total area of intramedullary space and the percentage of intramedullary space occupied by tumor, bone formation, and remaining hematopoietic cells was calculated using Image Pro Plus v3.0 software (Media Cybernetics, Silver Spring, MD; ref. 20). Bone formation was analyzed using the same H&E-stained femora sections used to quantify tumor growth viewed under polarized light to identify regions of woven and lamellar bone formation. Results are presented as a percentage of total intramedullary area.

Quantification of sensory fibers in bone and skin. The number of sensory nerve fibers in bone was determined as previously described (14, 21). CGRP-immunoreactive (CGRP-IR) nerve fiber counts were done on six femoral sections per mouse and included only fibers >30 μm in length. Results are presented as the number of CGRP-IR fibers counted per total section area. Quantification of epidermal innervation density was done on four randomly selected plantar hindpaw skin sections per mouse as previously described. The total number of CGRP, TOH, and RT97-IR nerve fibers were counted using a 20× objective. Counting criteria were established to count only single intraepidermal fibers and not multiple branches of the same fiber, as previously described (17). Results are given as the mean number of intraepidermal nerve fibers per millimeter.

Behavioral analysis. Mice were tested for pain-related behaviors both prior to and at 7, 9, 11, 13, 15, 17, and 19 days following ACE-1 or sham injections to assess the efficacy of the anti-NGF antibody over the progression of the disease. Briefly, ongoing nociceptive behaviors were
evaluated by measuring the time spent spontaneously guarding and flinching over a 2-minute observation period. The number of flinches was defined as lifting the tumor-injected limb while stationary and time spent guarding was defined as the length of time the tumor-injected limb was held aloft (22).

Following a 15-minute acclimation period, thermal and mechanical sensitivity were measured in naïve and naïve + anti-NGF mice to assess whether the normal pain threshold responses were altered with anti-NGF treatment. Thermal sensitivity was measured using a Thermal Paw Stimulator (University of California, San Diego, San Diego, CA) as previously described (14). Mechanical sensitivity was measured using a previously validated method (14, 23).

RT-PCR analysis of mRNA levels of nerve growth factor in the prostatic ACE-1 cell line. Total RNA from dog brain tissue samples or ACE-1 prostate cells were prepared using the RNAsesy micro kit (Qiagen, Valencia, CA), and the RNA was quantified using Ribogreen reagent (Molecular Probes, Eugene, OR). Two-step RT-PCR was done using the TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA). The RNA was reverse-transcribed using random hexamers, and the cDNA was amplified using a primer/probe set specific for NGF (AACAGGACTCA-CAGGGACCA, CGGCCTTGGTCCTCAAAAGAA, and AATGGTCACCTCTCC-CAGCACCATCA). The samples were analyzed in duplicate from the reverse-transcription step and normalized to total RNA input.

Statistical analysis. The Statview computer statistics package (SAS Institute, Inc., Cary, NC) was used to perform statistical tests. One-way ANOVA was used to compare behavioral results, bone histologic results, and immunohistochemical measures among the experimental groups. For multiple comparisons, Fisher’s protected least significant difference post hoc test was used. Significance level was set at P < 0.05. The individual investigator responsible for behavior, immunohistochemical analysis, and scoring bone remodeling was blind to the experimental situation of each animal. Results are presented as mean ± SEM.

Results

Anti–nerve growth factor therapy attenuated bone cancer pain to a greater extent than morphine sulfate but did not affect baseline thermal or mechanical thresholds. Ongoing pain was analyzed by measuring spontaneous guarding and flinching over a 2-minute time period. ACE-1 + vehicle mice showed a greater time spent guarding (7.7 ± 0.8 seconds, day 19) as compared with the sham + vehicle controls (0.6 ± 0.3 seconds, day 19; Fig. 1A). Additionally, ACE-1 + vehicle mice exhibited an increased number of flinches (11.9 ± 1.2, day 19) as compared with sham + vehicle controls (1.0 ± 0.4, day 19; Fig. 1B). Administration of anti-NGF in ACE-1-injected mice significantly attenuated spontaneous guarding (1.2 ± 0.4 seconds, day 19) as compared with ACE-1 + vehicle mice (Fig. 1A). Anti-NGF treatment also significantly reduced spontaneous flinching in ACE-1-injected mice (2.1 ± 0.7, day 19) as compared with ACE-1 + vehicle (Fig. 1B). In preliminary studies, no significant behavioral differences or side effects were observed between sham-operated controls receiving either vehicle or anti-NGF (results not shown).

Anti-NGF therapy had no effect on either normal thermal response (10.2 ± 0.4 seconds, day 19) as compared with naïve + vehicle (11.2 ± 0.4 seconds, day 19; Fig. 1C) or normal mechanical response (5.4 ± 0.3 g, day 19) as compared with naïve + vehicle (5.2 ± 0.4 g, day 19; Fig. 1D).

Mice were tested to compare the efficacy of morphine sulfate to the anti-NGF antibody in reducing bone cancer–related pain behaviors. Behavioral assessment on days 11 and 19 post-tumor injection revealed that ACE-1 + vehicle mice showed statistically longer time guarding of the injected limb (6.0 ± 1.0 and 7.6 ± 1.2 seconds, days 11 and 19, respectively) compared with sham + vehicle mice (0.4 ± 0.2 and 0.6 ± 0.3 seconds, days 11 and 19, respectively; Fig. 1E). ACE-1 + vehicle also showed a significantly larger number of flinches of the injected limb (8.6 ± 1.2 and 11.7 ± 1.7, days 11 and 19, respectively) compared with sham + vehicle mice (0.7 ± 0.3 and 1.0 ± 0.4, days 11 and 19, respectively; Fig. 1F). Ongoing guarding was significantly reduced by either chronic treatment with anti-NGF (2.1 ± 1.1 and 1.4 ± 0.4 seconds, days 11 and 19, respectively), acute 10 mg/kg morphine sulfate (3.5 ± 0.3 and 4.0 ± 0.5 seconds, days 11 and 19, respectively) or acute 30 mg/kg morphine sulfate (2.2 ± 0.3 and 2.0 ± 0.4 seconds, on days 11 and 19, respectively), as compared with ACE-1 + vehicle mice (Fig. 1E). Ongoing flinching was also significantly reduced by either chronic treatment with anti-NGF (3.4 ± 1.7 and 2.6 ± 0.6, days 11 and 19, respectively), acute 10 mg/kg morphine sulfate (5.6 ± 0.5 and 6.8 ± 0.7, days 11 and 19, respectively) or acute 30 mg/kg morphine sulfate (3.6 ± 0.5 and 3.5 ± 0.7, days 11 and 19, respectively), as compared with ACE-1 + vehicle mice (Fig. 1F).

Anti-NGF therapy significantly attenuated the bone cancer–related pain behaviors more effectively than acute 10 mg/kg morphine sulfate. No differences in terminal weights were observed between sham + vehicle (27 ± 1 g), ACE-1 + vehicle (27 ± 1 g), and ACE-1 + Anti-NGF (26 ± 1 g) mice. In these studies, no significant side effects, such as ataxia, illness, or lethargy, were observed between mice receiving either vehicle or anti-NGF.

Anti–nerve growth factor therapy had no effect on markers of disease progression or tumor-induced bone formation. The effects of anti-NGF therapy on bone formation and destruction, tumor growth (Fig. 2), and osteoclast proliferation (Fig. 3) were examined 19 days post-tumor injection (Table 1). Sham-injected mice did not show significant bone remodeling (Fig. 2A), osteoclast proliferation throughout the entire intramedullary space (Fig. 3A) or tumor cells (Fig. 2D), as assessed by radiologic, TRAP, and H&E analysis, respectively, when compared with ACE-1-injected mice. In ACE-1 + vehicle mice, there was extensive bone formation, but nearly equivalent destruction as observed and characterized by multifocal diaphyseal bridging and radiolucencies (Fig. 2B), marked increase in the number of osteoclasts (Fig. 3B) and osteoblasts throughout the diaphyseal intramedullary area and the tumor had filled most of the intramedullary space (Fig. 2E). Treatment of tumor-bearing mice with anti-NGF from day 7 post-tumor injection resulted in no significant change in bone remodeling (Fig. 2C), reduction in ACE-1-induced osteoclast (Fig. 3C) or osteoblast proliferation throughout the diaphyseal intramedullary area or tumor growth as compared with ACE-1 + vehicle mice (Fig. 2F).

Nineteen days following tumor injection, ACE-1 + vehicle mice displayed an increase in macrophages as compared with sham + vehicle control mice. Anti-NGF treatment of ACE-1-injected mice did not significantly alter macrophage infiltration, as seen in the ACE-1 + vehicle mice (Table 1).

Anti–nerve growth factor therapy has no observable effect on sensory or sympathetic innervation in bone or skin. Thiny myelinated or unmyelinated peptidergic sensory nerve fibers (CGRP-IR), large myelinated sensory fibers (RT97-IR), and noradrenergic sympathetic nerve fibers (TH-IR) were analyzed in the ACE-1-injected femora or the hindpaw plantar skin by immunohistochemistry using antibodies raised against CGRP, RT-97, and TH, respectively. CGRP-IR nerve fibers were found throughout the entire bone (periosteum, mineralized bone, bone marrow and tumor) of the ACE-1 + vehicle and ACE-1 + anti-NGF mice (Fig. 4) as well as in naïve + vehicle mice or naïve + anti-NGF mice. There was no significant difference between the intensity or density of

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CGRP-IR fibers in ACE-1 + vehicle and ACE-1 + anti-NGF hindpaw skin samples (Fig. 5C and D). Similarly, there was no significant difference between the intensity or density of CGRP-IR fibers in naïve + vehicle and naïve + anti-NGF hindpaw skin samples (Fig. 5A and B). Differences in the density and intensity of RT97-IR and TH-IR fibers were also undetectable in naïve + vehicle and naïve + anti-NGF–treated mice. There were no significant observable differences between the intensity or density of CGRP, RT97 or TH-IR fibers in the skin samples of ACE-1 + vehicle and ACE-1+ anti-NGF versus the naïve + vehicle and naïve + anti-NGF–treated mice (Table 1).

Expression of nerve growth factor mRNA by ACE-1 cells as compared with dog brain. In order to determine whether the ACE-1 tumor cells were a possible source of NGF, five independent samples of ACE-1 cells grown in culture were assessed for their level of NGF mRNA by RT-PCR. These levels were compared with those found in the brain. ACE-1 cells in vitro do not contain NGF mRNA at levels detectable by current RT-PCR techniques. NGF mRNA in brain crossed the detection threshold at cycle 35.2 of a 40-cycle experiment, whereas the ACE-1 samples failed to cross the threshold after 40 cycles. Therefore, NGF expression in the ACE-1 samples is at least 27.8-fold less than expression in brain.

Discussion
Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer-related deaths in men in the U.S. (24). Whereas tumor metastasis to bone is common in lung, breast, and prostate cancers, prostate cancer is unique in that bone is often the only clinically detectable site of metastasis and prostate tumors tend to be primarily bone-forming rather than bone-destroying (25, 26). The most common presenting symptom indicating that tumor cells have metastasized to the skeleton is bone pain (4, 27). Although bone is not a vital organ, prostate...
metastasis to bone is a major cause of morbidity as it usually results in anemia, increased susceptibility to infection, pain, and bone fractures—all of which compromise the patient's survival and quality of life (27). Once tumor cells have metastasized to bone, the tumor cells induce significant bone remodeling that is correlated with an ongoing pain and is usually described as dull in character, constant in presentation, and gradually increasing in intensity with time (4). As bone remodeling progresses, acute pain is frequently observed if the remodeled bone is moved or if pressure is applied to it (28). Breakthrough pain, which is an intermittent episode of extreme pain, can occur spontaneously or more commonly by weight-bearing or movement (2, 3). In humans, the extent of bone remodeling is correlated with the severity of the pain.

Currently, the treatment of pain from bone metastases involves the use of different complementary approaches including radiotherapy, chemotherapy, bisphosphonates, and analgesics (4). However, bone cancer pain is one of the most difficult of all persistent

Figure 2. Anti-NGF had no effect on tumor burden or tumor-induced bone remodeling. Sham mice, given vehicle (A), show no radiographically or histologically (H&E; D) apparent bone destruction at day 19, whereas ACE-1 + vehicle mice (B and E) and ACE-1 + anti-NGF mice (C and F) showed significant tumor growth and bone remodeling when examined radiologically and histologically. Boxes, radiographed regions (A-C) which are enlarged in the corresponding H&E-stained sections (D-F); H, hematopoietic cells; T, tumor; WB, ACE-1-induced woven bone formation; bar, 1.5 mm.

Figure 3. Anti-NGF therapy did not significantly reduce tumor-induced osteoclastogenesis. TRAP-stained images of sham + vehicle (A), ACE-1 + vehicle (B), and ACE-1 + anti-NGF (C) illustrate that osteoclast proliferation occurs in this model along regions of tumor-induced bone remodeling and an increase in the number of osteoclasts per square millimeter of diaphyseal intramedullary area in both the anti-NGF and vehicle-treated mice as compared with sham + vehicle and naïve + vehicle mice was observed. Sham + vehicle mice (A) present osteoclast numbers, morphology, and macrophages which are not significantly different from naïve mice. There is no observable difference in histologic appearance of the osteoclasts along the tumor/bone interface or macrophages throughout the tumor when anti-NGF-treated mice (C) are compared with vehicle-treated mice (B). Arrows, osteoclasts; arrowheads, macrophages; MB, mineralized bone; H, hematopoietic cells; T, tumor; bar, 50 μm.
NGF treatment would arise in the three most well-characterized of anti-NGF. The most likely origin of any side effects due to anti-NGF is so difficult to control is that bone metastases are generally not limited to a single site and the analogies that are most commonly used to treat bone cancer pain, the nonsteroidal anti-inflammatory drugs and opioids, are limited by significant adverse side effects and the complex nature of interactions between neuropathic and inflammatory mediators (29). For example, the nonselective nonsteroidal anti-inflammatory drugs could cause intestinal bleeding, whereas some selective cyclooxygenase-2 inhibitors, although causing less bleeding, have significant cardiovascular and renal safety issues (30, 31). Opioids are effective in attenuating bone cancer pain, but these frequently cause constipation, sedation, nausea, vomiting, and respiratory depression (32). Because prostate tumors metastasize primarily to bone, and not the other vital organs such as lung, liver, or brain, patients with prostate tumor metastases tend to live for a significant period (on average, 55 months) beyond their initial diagnosis (33), making it essential that new therapies be developed that can be administered over years and control bone pain without the side effects commonly encountered with high-dose opiates. Although the studies reported here are relatively short-term rodent studies, no signs of adverse events were observed with administration of anti-NGF. The most likely origin of any side effects due to anti-NGF treatment would arise in the three most well-characterized NGF-responsive cell populations, sympathetic, peptidergic sensory, and basal forebrain cholinergic neurons. There was no observable change in sensory or sympathetic innervation density of the skin or bone or in tactile or thermal sensitivity in this or other studies using this antibody (14). The IgG used to inhibit NGF would not be expected to cross the blood-brain barrier under normal circumstances, making it unlikely that there would be adverse events due to anti-NGF in the central nervous system. Longer-term human studies will be required to further clarify any adverse event profile possibly due to anti-NGF therapy.

Previously, we and others have attempted to develop novel mechanism-based therapies to treat bone cancer pain using a variety of tumor types implanted into the rat or mouse bone (34–36). Although these studies have provided insight into the mechanisms that generate and maintain bone cancer pain, the tumor cells that were employed were primarily osteolytic or bone-destroying in nature (34–36). A major question is whether these studies will accurately predict the mechanisms that generate the bone pain driven by tumor cells, such as prostate, that are primarily bone-forming rather than bone-destroying resulting from an upset in the balance between osteoblastic or osteolytic activity in the tumor-bearing femur (37). For this reason, we focused on the ACE-1 canine prostate tumor cells, as when these cells are implanted into the femur of nude mice, significant new “woven” bone or in tactile or thermal sensitivity in this or other studies using this antibody (14). The IgG used to inhibit NGF would not be expected to cross the blood-brain barrier under normal circumstances, making it unlikely that there would be adverse events due to anti-NGF in the central nervous system. Longer-term human studies will be required to further clarify any adverse event profile possibly due to anti-NGF therapy.

Table 1. Histologic, radiologic, and immunohistochemical quantification of bone remodeling, tumor progression, and hindpaw skin innervation in anti-NGF and vehicle-treated ACE-1 mice

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<tr>
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<th>Naïve + vehicle</th>
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<td><strong>Bone histomorphometry</strong></td>
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<td>Osteoclasts (OC #/mm²)</td>
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<td>% Normalized transmission ((Optical Density)/(naïve transmission) × 100%</td>
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NOTE: Anti-NGF therapy did not have a significant effect on measures of bone histomorphometry as compared with vehicle-treated mice. Tumor-injected mice treated with either vehicle or anti-NGF therapy showed a significant increase in the number of osteoclasts, osteoblasts, and macrophages throughout the diaphyseal intramedullary space as compared with sham + vehicle or naïve + vehicle mice (per mm²). The percentage of tumor cells and hematopoietic cells remaining were not significantly reduced in ACE-1-injected mice treated with anti-NGF therapy. Tumor-induced bone formation as a percentage of intramedullary space occupied was not reduced with anti-NGF therapy as compared with ACE-1 + vehicle mice. All histomorphometric variables in tumor-injected mice were significantly elevated from both sham + vehicle and naïve + vehicle mice. Normalized transmission values for ACE-1-injected mice were not significantly elevated from either sham or naïve + vehicle mice. There was no statistical difference in the number of CGRP, TH, or RT97-IR (neurofilament 200) epidermal fibers/mm in hindpaw skin between naïve + vehicle, sham + vehicle, ACE-1 + vehicle, or ACE-1 + anti-NGF–treated mice. Results presented include mean ± SEM.

*P < 0.05 versus naïve.

†P < 0.05 versus sham + vehicle (one-way ANOVA, Fisher’s protected least significant difference).
bone is formed in local compartments, giving the bone a unique scalloped appearance similar to what is observed in humans with prostate tumor metastases to bone (6). Nearly equivalent normalized transmission values suggest that ACE-1-induced woven bone formation has a decreased mineral content as compared with lamellar bone which is consistent with human prostate tumor-induced bone remodeling (38). The concurrent bone destruction and formation in the present model is quite distinct from that observed in tumors such as sarcoma or breast, where the tumor was primarily osteolytic as bone destruction predominates (39).

One of the findings of the present study was that administration of anti-NGF was not only highly efficacious in reducing both early and late stage bone cancer pain-related behaviors, but that this reduction was greater than that achieved with acute administration of 10 or 30 mg/kg of morphine sulfate. In light of these findings, a critical question is: what are the mechanisms that contribute to the efficacy of anti-NGF in blocking prostate tumor-induced bone pain? One important concept that has emerged over the past decade is that in addition to NGF being able to directly activate sensory neurons that express the trkA receptor, NGF modulates expression and function of a wide variety of molecules and proteins expressed by sensory neurons that express the trkA or p75 receptor, which include: neurotransmitters (substance P and CGRP), receptors (bradykinin R), channels (P2X3, TRPV1, ASIC-3, and sodium channels), activating transcription factors (ATF-3), and structural molecules (neurofilaments and the sodium channel anchoring molecule p11; refs. 40–47). Additionally, NGF has been shown to modulate the trafficking and insertion of sodium channels such as Nav 1.8 (44) and TRPV1 (45) in the sensory neurons as well as modulating the expression profile of supporting cells in the dorsal root ganglia and peripheral nerve, such as nonmyelinating Schwann cells and macrophages (48). Therefore, anti-NGF therapy may be particularly effective in blocking bone cancer pain as NGF seems to be integrally involved in the up-regulation, sensitization, and disinhibition of multiple neurotransmitters, ion channels, and receptors in the primary afferent nerve and dorsal root ganglia fibers that synergistically increase nociceptive signals originating from the tumor-bearing bone. Importantly, anti-NGF therapy did not significantly influence tumor-induced bone remodeling, osteoblast proliferation, osteoclastogenesis, tumor growth, or markers of sensory or sympathetic innervation in the skin or bone, suggesting anti-NGF–induced

Figure 4. Anti-NGF therapy did not influence the density of CGRP-IR sensory fibers in the tumor femur. There was no observable difference in the levels of immunofluorescence or density of CGRP-IR fibers between ACE-1 + vehicle mice (A) and ACE-1 + anti-NGF mice (B) at day 19. Also, note that there was maintenance of CGRP-IR fibers with anti-NGF therapy. H&E-stained serial sections are included for orientation reference (A and C, B and D). T, tumor; bar, 50 μm.
disease modification or denervation of the sensory/sympathetic innervation were not the primary mechanism(s) by which anti-NGF exerted its analgesic effects in the present model of bone cancer pain. The above data may suggest that anti-NGF should be an excellent anti-hyperalgesic, yet in other pain models, such as the Brennan incision model (49), anti-NGF seems to block only certain components of the pain state (for example, thermal but not mechanical hyperalgesia).

One rather unique aspect of the sensory innervation of bone, which may explain the analgesic efficacy of anti-NGF therapy in relieving prostate tumor–induced bone pain, is that unlike skin, nearly all nerve fibers that innervate the bone express CGRP (20). Previous studies have shown that nearly all CGRP-IR fibers coexpress trkA and p75, which are the receptors by which NGF sensitizes and/or activates nociceptors (50, 51). In both rodents and humans, fibers from primary afferent sensory neurons that innervate bone marrow, mineralized bone, and periosteum (52), are CGRP-IR fibers with few, if any, of the nonpeptidergic IB4/RET-IR nerve fibers being present (21). Within the marrow and mineralized bone, sensory fibers are generally associated with the blood vessels (21), and nearly all of these fibers express CGRP (21). Thus, the great majority of sensory fibers that innervate the bone are CGRP/trkA-IR fibers. If the sensitization and activation of these fibers is blocked by anti-NGF therapy, there would not be another population of nociceptors, such as the nonpeptidergic IB4/RET-IR nerve fibers, to take their place in signaling nociceptive events in the tumor-bearing bone.

It is interesting to speculate the cellular source of NGF which is normally involved in driving pain in the present model given the in vitro observation that ACE-1 cells express undetectable levels of NGF mRNA or protein. Previous studies conducted primarily in the skin have shown that macrophages and neutrophils express high levels of NGF mRNA and protein and are a major source of NGF that drives inflammatory pain following tissue injury (53). Athymic nude mice lack T lymphocytes, but have macrophages and other immune and inflammatory cells maintained, and it is these cells, as in the skin, that could be an important source of NGF which is driving the ACE-1 prostate tumor–induced bone pain. Previous data concerning the receptor type that is important in driving NGF-induced bone cancer pain has shown that the anti-NGF
antibody used in the present study inhibits the interaction of NGF with both the trkA and p75 neurotrophin receptor (15). NGF is capable of causing acute hyperalgesia in p75 knock-out mice, showing that trkA is sufficient to mediate the NGF effect in this setting (54). However, it is known that the p75 receptor is critical for NGF regulation of bradykinin receptors (55). In the complex setting of a growing tumor in the bone, with the accompanying presence of multiple inflammatory mediators, it is not clear whether it is the interaction of NGF with trkA, p75, or both, that is important for the behavioral and histologic effects seen here.

Whether anti-NGF therapy can inhibit prostate tumor–induced bone pain in humans remains to be determined. The mechanism of tumor-induced bone pain is thought to be similar in mouse and human as both are mediated by excessive bone remodeling, release of allogenic inflammatory and tumor products. Previous studies have shown the presence of nerve fibers in bones of rats (52), cats (56), dogs (57), and horses (58) in addition to humans (59), and this pain mechanism could also be mediated by tumor-induced injury to nerve fibers that innervate the bone. If anti-NGF therapies can block tumor-induced bone pain, retain their analgesic efficacy with disease progression, and provide significant opioid-sparing action without significant unwanted side effects, they may provide significant advantages over the analgesics that are currently used to treat bone cancer pain. Given the relatively long and increasing survival times in patients with prostate metastasis to bone, anti-NGF therapy may be particularly useful in reducing the pain and improving the quality of life in this patient population.

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References


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