Etanercept attenuates thermal and mechanical hyperalgesia induced by bone cancer

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Received September 3, 2015; Accepted January 20, 2017

DOI: 10.3892/etm.2017.4260

Abstract. Bone cancer pain commonly occurs when tumors originating in the breast, prostate or lung metastasize to long bones, spinal vertebrae and/or the pelvis. However, the underlying mechanisms of bone cancer pain remain largely unknown. The present study aimed to determine the role of spinal tumor necrosis factor-α (TNF-α) in the development of bone cancer pain. Osteosarcoma NCTC 2472 cells were implanted into the femoral intramedullary space of C3H/HeJ mice to establish a bone cancer model. Resulting pain-related behaviors, namely spontaneous foot lifting, paw withdrawal mechanical threshold and paw withdrawal thermal latency were observed prior to inoculation and on days 3, 5, 7, 10 and 14 thereafter. Reverse transcription-quantitative polymerase chain reaction was also performed to assess the levels of TNF-α mRNA within the spinal cord. In addition, the effects of the TNF-α antagonist etanercept on TNF-α levels and pain behaviors were evaluated. It was observed that the levels of TNF-α mRNA in the spinal cord were significantly higher in tumor-bearing mice 10 days post-inoculation, which was accompanied by increases in spontaneous flinching, mechanical hyperalgesia and thermal hyperalgesia, relative to control mice. Etanercept attenuated the bone cancer-induced increase in TNF-α and pain-related behaviors. These results suggest that etanercept may be a potential therapeutic for the treatment of bone cancer pain.

Introduction

The metastasis of tumors into bone tissue is a common clinical event and typically leads to intractable pain and bone destruction (1). A number of types of cancer have a predisposition to metastasize to bone and induce bone destruction. This most commonly occurs in patients with breast, lung and prostate cancer, in which a metastatic rate of ~80% is observed (2). Astrocytes and microglia in the spinal cord serve crucial roles in the production and maintenance of pain. Upon activation, glial cells release a range of neuroexcitatory substances including pro-inflammatory cytokines (3) and D-serine (4) that potentiate the transmission of pain by neurons. Pro-inflammatory cytokines are typically produced in a cascade and have been demonstrated to participate in the development of allodynia and hyperalgesia in rat and mouse animal models of pain (5). Tumor necrosis factor (TNF) is considered to be a key pro-inflammatory cytokine during pain nociception, due to its early generation and stimulation of target cells to produce numerous other cytokines within a complex cascade, including interleukin (IL)-1β and IL-6 (6).

Within bone tumors, TNF-α has been identified as a key factor in the development and maintenance of cancer-related pain (7). In turn, the TNF-α antagonist etanercept has demonstrated efficacy in reducing pain and osteoclast-mediated osteolysis in a clinical research and murine model of bone cancer pain (8-10). The results of this clinical research involving two patients one with a diagnosis of non-small cell lung cancer and the other with a diagnosis of breast cancer metastases to bone, that had treatment-refractory pain due to cancer, has suggested that targeted administration of etanercept in anatomical proximity to a site of bone metastasis may provide rapid, substantial and prolonged pain relief (8). However, it remains unknown whether TNF-α in the spinal cord is involved in sensitization of the central nervous system to bone cancer pain. Therefore, the current study used a bone cancer model established in mice to determine if spinal cord TNF-α contributes to bone cancer-related pain, by evaluating levels of TNF-α mRNA in the spinal cord and pain-related behaviors. Furthermore, the efficacy of etanercept in attenuating bone cancer pain was evaluated.

Materials and methods

Cell culture. The osteosarcoma cell line NCTC 2472 [American Type Culture Collection (ATCC), Manassas, VA, USA, lot no. 2087787] was used in the current experiments. Cells were incubated in NCTC-135 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% horse

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Key words: tumor necrosis factor-α, bone cancer pain, etanercept

serum (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) at 37˚C in 5% CO_2, according to ATCC recommendations.

Animal treatment. Animal experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications no. 80-23, revised 1996) and were approved by the Ethics Review Board for Animal Studies of Nanjing University (Nanjing, China). The number of animals used and their suffering were minimized. A total of 150 male C3H/HeJ mice (Model Animal Research Center of Nanjing University), 4-6 weeks old and weighing 20-25 g were used. Mice were housed in a temperature-controlled (21±1˚C) room under a 12/12 h light/dark cycle with food pellets and water provided *ad libitum*. The mice were divided into three groups (n=8 in each group): i) NCTC 2472 cell-treated; ii) NCTC 2472 cell + etanercept-treated; and iii) a control group.

In accordance with a previous method by Schwei *et al* (9), 1% pentobarbital sodium (50 mg/kg, Sigma-Aldrich, Merck KGaA) in normal saline was intraperitoneally injected into mice. A minimal skin incision was subsequently made on the right hind leg and the patellar ligaments were cut to expose the condyles of the distal femur. A 23-gauge needle (Jiangsu Zhengkang Medical Equipment, Changzhou, China) was used to perforate the cortex at the level of the intercondylar notch, then ~$2.5x10^5$ NCTC 2472 cells in 20 μl α-Minimal Essential Medium (Thermo Fisher Scientific, Inc.) was injected unilaterally into the intramedullary cavity of the femur with a syringe. The injection site was sealed with Paladur dental acrylic (Heraeus Kulzer GmbH, Hanau, Germany) to prevent leakage of cells from the bone. The femurs of control mice were inoculated with 20 μl α-MEM alone. All mice were housed in the same room and conditions to minimize stress associated with novel environmental cues. For the behavior experiments, etanercept (100 μg in 0.5 ml saline, Amgen, Inc., Thousand Oaks, CA, USA) was intraperitoneally injected into mice prior to femur inoculation, and on days 3 and 6 thereafter.

Pain-related behaviors. Mice were given 30 min to acclimatize prior to each test. Spontaneous foot lifting (SFL), paw withdrawal mechanical threshold (PWMT) and paw withdrawal thermal latency (PWTL) were measured prior to femur inoculation and on days 3, 7, 10 and 14 thereafter. All tests were performed during the light phase of the mice light/dark cycle.

SFL. Mice were individually placed in a clear plastic chamber (60x30x15 cm) and the number of mice that accompanied lifting of their ipsilateral hind foot with aversive behavior (e.g., foot shaking/licking) was measured over 5 min. Foot lifting associated with exploratory behavior, locomotion and body repositioning was excluded.

PWMT. Mechanical hyperalgesia was assessed by applying von Frey filaments (Stoelting, Co., Wood Dale, IL, USA) to the right hind paw, as described by Chaplan *et al* (10). Mice were placed in individual transparent plexiglass compartments (10x10x15 cm) on a metal mesh floor (graticule: 0.5x0.5 cm) and mechanical threshold was measured using a set of von Frey filaments (0.16, 0.4, 0.6, 1.0, 1.4 and 2.0 g). Filaments were poked vertically into the plantar surface of the right hind paw with sufficient force to cause slight bending of the filament, then held for 6-8 sec. Brisk withdrawal or paw flinching were considered to be positive responses. PWMT was determined by using sequentially larger and smaller filaments, corresponding to an increase and decrease in stimulus force, respectively (the 'up-and-down' method). Each mouse was tested 5 times per stimulus strength with a 10 min interval between consecutive stimuli. The minimum von Frey filament that evoked ≥3 positive responses was regarded as the PWMT.

PWTL. Thermal hyperalgesia was assessed by measuring PWTL to radiant heat, according to a protocol by Hargreaves *et al* (11). Mice were placed on a 3 mm-thick glass floor in individual transparent plexiglass compartments (10x10x5 cm). A BME410A radiant thermal stimulator (Institute of Biological Medicine, Academy of Medical Science, China) was focused onto the plantar surface of the right hind paw through the glass floor. Characteristic lifting or licking of the right hind paw were considered to be the nociceptive endpoints of the test and the time taken to reach the endpoint was defined as the PWTL. A 20 sec cut-off time was used to avoid tissue damage. Each mouse was tested 5 times with a 5 min interval between consecutive stimuli.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). On days 7, 10 and 14 after establishment of the bone cancer model, mice were decapitated and the L3-L5 lumbar spinal cord segments were removed immediately, frozen in liquid nitrogen and stored at -80˚C until use. Total RNA was isolated and purified using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. The concentration of RNA was adjusted to ~1 μg/μl prior to use. RT was performed at 37˚C for 15 min, 85˚C for 5 sec, and 4˚C for 7 sec using a PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). A total of 2 μl cDNA in a 20 μl reaction system and a Power SYBR-Green Master mix (Thermo Fisher Scientific, Inc.) was used for PCR in a StepOnePlus system (Applied Biosystems, Thermo Fisher Scientific, Inc.). TNF-α and β-actin, which was used as an internal standard, were amplified using the following primers (GenScript, Piscataway, NJ, USA): TNF-α, forward, 5'-GGCAGGTCTACTTTGGAG TCATTGC-3' and reverse, 5'-ACATTCGAGGCTCCAGTG AATTCGG-3'; β-actin, forward, 5'-GAGACCTTCAACACC CCAGC-3' and reverse, 5'-CCACAGGATTCCATACCAA-3'. PCR amplification was performed at 94˚C for 5 min, followed by 30 cycles at 94˚C for 30 sec, 60˚C for 30 sec and then 72˚C for 15 sec. This was succeeded by a melt step from 60 to 94˚C in increments of 0.3˚C (held at 15 sec each) for subsequent melt curve analysis. Relative expression was calculated using the ΔΔCq method provided by the StepOnePlus system (Applied Biosystems) and optimized with a standard curve to confirm specificity (12). Levels of TNF-α were normalized to β-actin. Three repetitions were performed per sample. A sample lacking template DNA was used as negative control. Following PCR, 5 μl amplified cDNA was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Statistical analysis. Data are expressed as the mean ± standard deviation. Results of the SFL, PWMT and PWTL assays were analyzed by repeated measures analysis of variance (ANOVA)

to determine if differences were significant at each time point. Two-way ANOVA followed by Fisher's Least Significant Difference post hoc test was used for multiple group comparisons of TNF-α mRNA levels. All analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) and P<0.05 was considered to indicate a statistically significant difference.

Results

Etanercept attenuates bone cancer-induced SFL. In tumor-bearing mice, the rate of SFL (Fig. 1) was significantly elevated 7 days after establishment of the bone cancer pain model, relative to control mice at the same time point (P<0.05; 4.13±0.83 vs. 1.13±0.64, respectively). The SFL rate of tumor-bearing mice was further elevated by day 14 post-operation, relative to controls (P<0.05; 10.63±1.41 vs. 0.88±0.35, respectively). By contrast, the rate of SFL of tumor-bearing mice treated with etanercept did not significantly differ to that of control mice by days 7 (2.37±0.68) and 10 (2.02±0.72) post-operation. In addition, treatment with etanercept significantly reduced the increased SFL rate of tumor-bearing mice on days 10 and 14 post-operation (both P<0.05; 2.02±0.72 and 4.75±1.66, respectively). However, SFL rate in etanercept-treated tumor-bearing mice remained significantly higher than that in control mice on day 14 post-operation (P<0.05).

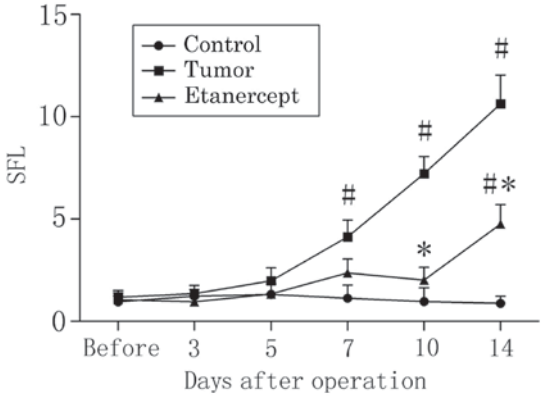

Figure 1. Effect of etanercept on bone cancer-induced SFL. Following establishment of a femur cancer pain model in mice, the rate of SFL was measured over 14 days. SFL rate was significantly elevated in tumor-bearing mice on days 7-14 in a time-dependent manner, while etanercept treatment significantly attenuated bone cancer-evoked SFL on day 14. Data are expressed as the mean ± standard deviation (n=8 per group). #P<0.05 vs. control group and *P<0.05 vs. tumor group at the same time points. SFL, spontaneous foot lifting.

Etanercept attenuates bone cancer-induced thermal hyperalgesia. The PWTL of the right hind limb to radiant heat stimulation in tumor-bearing mice was significantly decreased by day 7 post-operation, relative to control mice (P<0.05; 14.6±0.7 vs. 18.2±0.7, respectively; Fig. 2). Relative to controls, this significant decrease in the PWTL of tumor-bearing mice was consecutively enhanced on days 10 (P<0.05; 12.8±0.5 vs. 17.6±0.6) and 14 (P<0.05; 10.4±0.9 vs. 18.2±0.7) post-operation, suggesting that tumor-bearing mice became increasingly sensitive to the noxious heat stimulus. In addition, treatment with etanercept significantly reversed the reduced PWTL of tumor-bearing mice on days 7 (17.8±0.6 vs. 18.2±0.7), 10 (17.0±0.6 vs. 17.6±0.6) and 14 (16.9±0.5 vs. 18.2±0.7) post-operation (all P<0.05), indicating that bone cancer-induced thermal hyperalgesia was attenuated by etanercept.

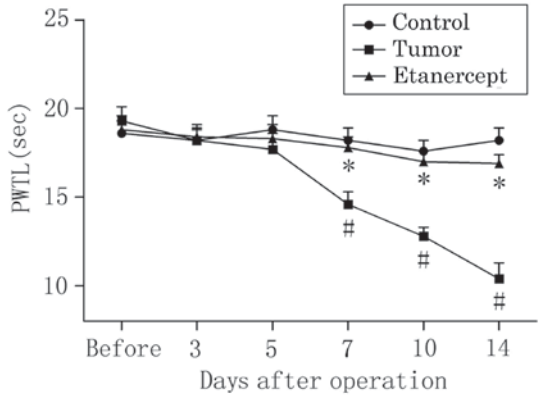

Figure 2. Effect of etanercept on bone cancer-induced thermal hyperalgesia. In a femur cancer mouse model, the latency of foot withdrawal to a noxious heat stimulus was measured over 14 days. PWTL of tumor-bearing mice significantly decreased by day 7 following femur inoculation, and further decreased until day 14. Treatment with etanercept significantly alleviated bone cancer-induced sensitivity to a heat stimulus in tumor-bearing mice on days 7-14 post-femur inoculation. Data are expressed as the mean ± standard deviation (n=8 per group). #P<0.05 vs. control group and *P<0.05 vs. tumor group at the same time points. PWTL, paw withdrawal thermal latency.

Etanercept attenuates bone cancer-induced mechanical allodynia. The PWMT of the right hind limb to stimulation by von Frey filaments in tumor-bearing mice was significantly decreased by day 10 post-operation, relative to control mice (P<0.05; 1.02±0.23 vs. 1.78±0.28), and further decreased by day 14, relative to controls (P<0.05; 0.42±0.07 vs. 1.92±0.21; Fig. 3). Similarly, significant decreases in the PWMT of mice treated with etanercept were observed on days 10 (1.22±0.18 vs. 1.85±0.27) and 14 (0.95±0.25 vs. 1.92±0.21), relative to controls (both P<0.05); however, etanercept treatment significantly elevated the lowered PWMT of tumor-bearing mice on day 14 (P<0.05). This suggests that etanercept attenuated bone cancer-induced mechanical allodynia, though at a different time point to its attenuation of thermal hyperalgesia.

Elevated TNF-α transcription in the spinal cord during bone cancer is attenuated by etanercept. As depicted in Fig. 4, the level of TNF-α mRNA in the L3-L5 lumbar spinal cord segments in tumor-bearing mice did not significantly differ by day 7, when compared to that in control mice (1.14±0.09 vs. 0.96±0.06, respectively). β-actin was used as an internal standard. However, relative to control mice, levels of TNF-α in tumor-bearing mice gradually elevated to significant levels by day 10 (1.09±0.07 vs. 2.54±0.15) and 14 (1.11±0.08 vs. 4.21±0.38) post-operation (both P<0.05). In addition, treatment with etanercept significantly reversed the elevated levels of TNF-α in tumor-bearing mice on days 10 (1.31±0.08) and 14 (1.22±0.12; both P<0.05).

Discussion

In the present study, a mouse model of bone cancer pain was successfully established by inoculating osteosarcoma cancer

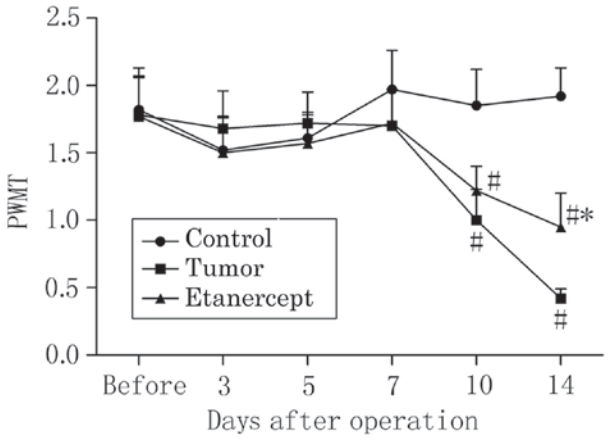

Figure 3. Effect of etanercept on bone cancer-induced mechanical allodynia. The rate of foot withdrawals in response to repeated mechanical stimuli were recorded in a femur cancer mouse model over 14 days. The PWMT of tumor-bearing mice was significantly decreased on days 10 and 14 post-femur inoculation in a time-dependent manner. Similar results were observed in etanercept-treated tumor-bearing mice; however, the PWMT of tumor-bearing mice was significantly increased by etanercept treatment on day 14 post-inoculation. Data are expressed as mean ± standard deviation (n=8 per group). $^{\#}$P<0.05 vs. control group and *P<0.05 vs. tumor group at the same time points. PWMT, paw withdrawal mechanical threshold.

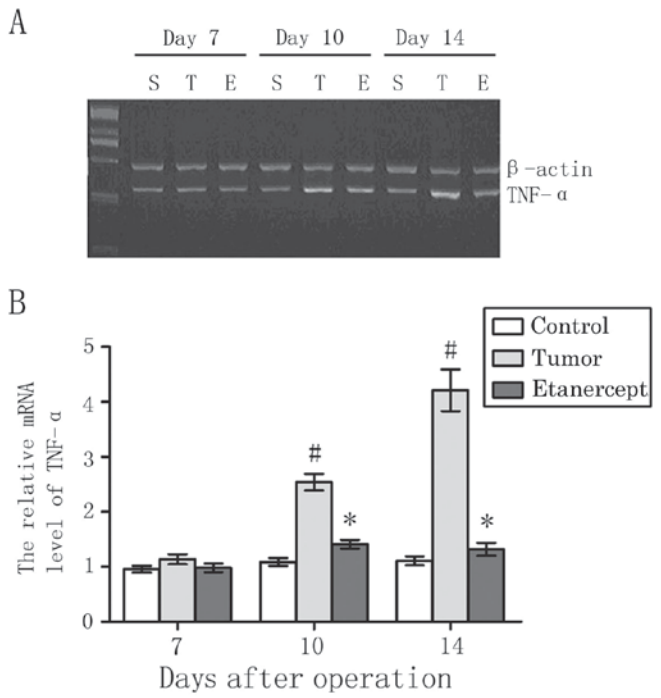

Figure 4. Effect of etanercept on the expression of spinal TNF-α during bone cancer. Levels of TNF-α mRNA in the L3-L5 lumbar spinal cord segments of a femur cancer mouse model were measured by reverse transcription quantitative polymerase chain reaction. (A) Optical density of PCR products run on a 2% agarose gel and stained with ethidium bromide. β-actin was used as an internal standard. (B) The mRNA levels of TNF-α relative to β-actin. Elevated levels of TNF-α in the spinal cord of tumor-bearing mice on days 10 and 14 post-femur inoculation were significantly reduced by pretreatment with etanercept (n=8 per group). $^{\#}$P<0.05 vs. control group and *P<0.05 vs. tumor group at the same time points. TNF-α, tumor necrosis factor-α.

cells into the femoral intramedullary space. Results demonstrated that bone cancer induced progressive mechanical and heat hyperalgesia, along with increased levels of TNF-α mRNA in the spine. Furthermore, it was observed that pain-related behaviors and elevated TNF-α levels were attenuated by the TNF-α antagonist etanercept, indicating that TNF-α in the spine serves a key role in the generation and development of bone cancer pain.

TNF-α has been demonstrated to participate in peripheral and central nociception in a number of pain models, including those for inflammatory (13) and neuropathic pain (14). However, neurochemical changes observed in the spinal cord and sensory neurons of mice due to cancer pain are distinct from those induced by inflammatory or neuropathic conditions (15). Previous *in vitro* studies have indicated that TNF-α within tumors contributes to peripheral sensitization of bone cancer-related pain. Specifically, it has been observed that TNF-α produced by cancer cells may be responsible for stimulation of osteoclast activity, which in turn serve a key role in bone destruction and hyperalgesia (16,17). Proinflammatory cytokines are typically released from activated microglia and astrocytes (18) and subsequently bind to cognate receptors expressed by neurons. In electrophysiological studies, it has been demonstrated that injection of exogenous proinflammatory cytokines over the spinal cord region enhances nociception, by increasing neuronal excitability in response to noxious stimuli following the cytokine injection (19). The current study indicated that TNF-α in the spinal cord also contributes to the generation of bone cancer pain. Collectively, these results indicate that nociceptive neurons innervating the tumor area are sensitized during bone cancer, and that sensory neurons in the spinal cord may be activated by TNF-α originating from spinal microglia and astrocytes.

Several studies have suggested that TNF-α inhibitor may be ineffective in alleviating hypersensitivity if it is administered long after the development of hyperalgesia (20-22). Therefore, the present study intraperitoneally administered etanercept to mice prior to establishment of the bone cancer model and on days 3 and 6 thereafter, when pain-related behaviors had not yet been observed. Results demonstrated that this treatment procedure alleviated hyperalgia and attenuated the increased levels of TNF-α, thus indicating the key role of TNF-α in initiating a positive feedback cascade of proinflammatory cytokines.

TNF-α interacts with two distinct membrane receptors, TNF receptor (R)-1 and TNFR2. The effects of TNF-α occur through its direct action on neurons via TNFR1 or through its facilitation of macrophage accumulation in dorsal root ganglion via a TNFR2-mediated pathway (23). In a sciatic nerve chronic constriction injury (CCI) model of neuropathic pain, intra-operative epineural administration of neutralizing antibodies against TNFR-1 was demonstrated to reduce mechanical allodynia and thermal hyperalgesia (24), while administration on day 4 post-CCI reduced thermal, but not mechanical hyperalgesia (25). Constantin *et al* (25) also observed that TNFR2 knockdown attenuated heat hyperalgesia in tumor-bearing mice, while TNFR1 knockdown had less of an effect, suggesting that TNFR2 serves the primary role in the generation of tumor-induced heat hyperalgesia. In the current study, both mechanical allodynia and thermal hyperalgesia were alleviated in tumor-bearing mice treated with etanercept; however, the effect of etanercept on thermal

hyperalgesia appeared earlier and more substantial. This suggests that TNF-α may participate in bone cancer-related pain through different signaling pathways, which warrant further investigation.

In conclusion, the present study demonstrated that the proinflammatory cytokine TNF-α within the spine was upregulated in a mouse bone cancer model, and may have contributed to nociceptive signal processing in the development of bone cancer pain. In addition, it was observed that neutralization of TNF-α had a significant beneficial effect on bone cancer-induced mechanical allodynia and thermal hyperalgesia, thus suggesting that administration of etanercept in the early stages of bone cancer may be a potential therapeutic strategy for the prevention and treatment of cancer-related pain.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. 81171047, 81300950, 81371207 and 81300951).

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