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The impact of the opioids fentanyl and morphine on nociception and bone destruction in a murine model of bone cancer pain

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Abstract

Chronic pain resulting from metastasis into skeleton of certain neoplastic diseases remains poorly understood and relatively resistant to analgesic treatment. Opioids are the principal axis in drug therapy for this type of pain, especially at the end stage of cancer. Our aim was to examine whether, fentanyl as well as morphine, two potent analgesic opioids commonly used to treat cancer pain, would inhibit pain and bone lesion-related responses in a murine model of bone cancer pain. Repeated administration of equianalgesic doses of fentanyl (0.16 mg/kg s.c. once a day) and morphine (20 mg/kg s.c. once a day) initiated at day 1 (prophylactic treatment) or at day 7 (curative treatment) after tumor cell inoculation in the femoral cavity consistently decreased bone pain symptoms and tumor growth-induced bone destruction (micro-CT bone structure parameters). Both fentanyl and morphine treatments resulted in clear antinociceptive properties as well as reductions in cancer cell-induced bone lesions.

The present results demonstrate that fentanyl, and to some lesser degree morphine, has potential benefits in the treatment and development of bone cancer pain. As such, chronic administration of high doses of certain opioids like fentanyl may have clinical utility in the management of bone cancer pain.

Keywords: Fentanyl; Morphine; Bone cancer pain model; Hyperalgesia; Murine model; Bone structure

1. Introduction

Metastasis to bones is a common feature of malignant tumors, and is associated with significant complications including severe pain, skeletal fractures, bone marrow suppression, hypercalcemia, and an overall reduced quality of life (Coleman, 2002).

Approximately 60–90% of cancer patients suffer from chronic pain in the course of their disease and many of them don't receive adequate pain relief even from conventional therapies (Meuser et al., 2001). In general there are two types of pain in patients with bone cancer. The first type is known as ongoing pain and is usually described as a dull aching or throbbing pain that increases in severity over time (Mercadante, 1997). The second type of bone cancer pain, known as movement-evoked, breakthrough, or episodic pain, emerges frequently over time, is more acute in nature, and often occurs as spontaneous and intermittent exacerbations of pain or by movement of the cancerous bone (Portenoy et al, 1999a).

In patients and rodent models the pain generally tends to increase in relation to tumor involving bone destruction (Coleman, 1998). The severity of pain is positively correlated with the extent of bone destruction and ongoing osteoclast activity (Mercadante and Arcuri, 1998; EL Mouedden and Meert, 2005). Tumor-induced bone resorption plays a role in driving bone cancer pain but other mechanisms, such as the release of various pro-nociceptive factors by tumor and/or inflammatory cells may also be involved in the pathology of bone cancer-related pain (Payne, 1997).

Currently, the available therapies are focused on eliminating tumor proliferation, reducing tumor-induced bone loss, intervening surgically to stabilize painful bones infiltrated with skeletal metastases, blocking nerves, stimulating spinal cord,

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and administrating powerful pain medications (Miguel, 2000). Treatment regimens can include monotherapy or combinations of nonsteroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase-2 (COX-2) inhibitors, chemotherapy, radiotherapy, calcitonin, nitrogen-containing bisphosphonates, antidepressants and anticonvulsants, benzodiazepines, corticosteroids, neuroleptic drugs, and opioids.

Opioids, acting via G-protein coupled membrane receptors, induce analgesia and relieve ongoing cancer pain (Radbruch et al., 2001) but need to be increased substantially to high doses for a sufficient block of breakthrough pain, with an increased risk for the occurrence of unwanted side effects (Portenoy et al, 1999b; Levy, 1996). Long-term opioid use can be associated with gastro-intestinal side effects as well as tolerance, physical dependence, and sometimes addiction.

The role of opioids is not limited to their antinociceptive action. They are found in several peripheral tissues acting as negative or positive regulators of cellular processes.

Opioids such as morphine have been shown to enhance tumor growth and might be tumor-promoting in mice and rats (Lewis et al., 1984; Ishikawa et al., 1993 Gupta et al., 2002), but the underlying molecular mechanisms remain unclear. Recently many researchers have demonstrated that opioids may trigger the apoptotic death of widely ranging cell types, and inhibit cell proliferation and tumor growth in cancer animal models (Harimaya et al., 2002; Sasamura et al., 2002; Tegeder et al., 2003). They have proposed a protective role for opioids against tumor growth and metastasis, especially through induction of apoptosis in tumoral cells.

The reasons for these conflicting results are unclear. It has been suggested that "atypical" opioid binding sites might be involved in tumor suppression because in some studies, the antiproliferative effects of opioids were not antagonized by naloxone (Gupta et al., 2002; Maneckjee and Minna, 1992; Kugawa et al., 1998; Hatzoglou et al., 1996). Furthermore, it has become clear that opioids differ amongst themselves in terms of opioid receptor interactions and functional outcomes (Meert and Vermeirsch, 2005; Adriaensen et al, 2003; Meert, 1996).

The development of optimal analgesics for cancer pain has been hampered by the lack of understanding of basic mechanisms that contribute to cancer pain. The development of animal models of bone cancer pain is providing new and important information regarding mechanisms underlying cancer pain. Therefore, the present study was set up to determine whether chronic treatment with fentanyl and morphine, two potent mu-opioid agonists widely used in clinic, would affect pain and the tumor growth-induced bone lesions in a murine model of bone cancer pain.

2. Materials and methods

2.1. Animals

Male mice (C3H/HeNCrl, body weight 25–30 g, Charles River, Sulzfeld, Germany) were housed in a mouse facility in accordance with institutional guidelines of the Belgian ethical committee and under the supervision of the authorized investigators. Food and water were freely available. All procedures were performed in compliance with the Belgian and European guidelines. Experimental protocols were approved by the Institutional Review Committee of Janssen Pharmaceutica (Beerse, Belgium), and met the guidelines published in a Guest Editorial in Pain on ethical standards for investigations of experimental pain in animals (Zimmermann, 1983).

2.2. Cell culture

Osteolytic murine sarcoma cells (NCTC 2472, American Type Culture Collection (ATCC), Rockville, MD, USA) originally derived from connective tissue tumor in a C3H mouse were cultured in NCTC 135 medium containing 10% horse serum (Gibco, life Science, Belgium) and passaged 2 times weekly according to ATCC guidelines. The cells (2.5×10^5 cells) suspended in medium were locally implanted into the medullar space of the mouse left femur of the unilateral hind paw in a volume of 20 μL.

2.3. Tumor induction in the left femur

For tumor induction, the mice were injected with 2.5×10^5 NCTC 2472 cells as described previously (El Mouedden and Meert, 2005). Briefly, animals were anesthetized by inhalation of a combination of isoflurane/air (1.5%, 0.5 L/min) and the left knee of mice was bent and placed facing the experimenter, shaved and disinfected with povidone-iodine followed by ethanol 70%. A minimal skin incision was made and the patellar ligaments were cut, exposing the condyles of the distal femur. A 23-gauge needle was inserted at the level of the intercondylar notch and the intramedullary canal of the femur to create a cavity for injection of the cells. 2.5×10^5 separated single NCTC 2472 cells in ~20 μL were injected into the distal end of the femur. Sham control groups were injected with 20 μL of medium. To prevent leakage of cells outside the bone, the injection site was sealed with dental acrylic (Paladur, Heraeus Kulzer, GmbH, and Wehrheim, Germany). The surgical procedure was finalized by stitching the skin of the knee.

2.4. Nociceptive tests

Nociceptive behaviors were evaluated in different groups ($n=10$) of cancer cell and vehicle loaded mice with or without drug. Mostly testing was done the day before surgery and on day 0, 7, 9, 12, 14 and 15 or 17 and 18 after tumor inoculation.

2.4.1. Spontaneous paw lifting behavior

Spontaneous lifting of the hind paws was measured as described by El Mouedden and Meert (2005). Briefly, animals were habituated to the laboratory room at least 30 min before testing. Thereafter, behavioral observations were performed after placing and habituating animals in a transparent acrylic cylinder of 20 cm diameter put on the surface of a glass plate. During a 4 min period, spontaneous lifting behavior of the left

hind paw was recorded electronically to the nearest 0.1 s. Data were expressed as % withdrawal time over total session time. 0% was the normal value observed in most non-operated and sham-operated animals.

2.4.2. Limb-use on rotarod

After spontaneous lifting behavior assessment, animals were immediately placed on a mouse rotarod (ENV-575M®, Med Associates Inc., Georgia, US) at a speed of 16 rounds per min for 2 min and limb-use during forced ambulation was scored: 4 = normal; 3 = limping; 2 = partial non-use of left hind paw; 1 = substantial non-use of left hind paw; 0 = non-use of left hind paw.

Body weight of the mice was also recorded throughout the experimental period to get an idea on general health status.

2.5. Micro-CT images and micro-architectural quantification

At the end of the experiment, the femur of the left hind limb was sampled and used for μCT scanning as described by El Mouedden and Meert (2005). Bone architectural analysis was performed on longitudinal sections of the control or sarcoma left distal femur from 10 animals per group 15 or 18 days following tumor implantation. Limbs were fixed in 10% phosphate-buffered formalin and transferred to a plastic cuvette filled with 70% ethanol. The fixed distal femur of the mice were micro-CT scanned using a high resolution X-ray micro-CT system for small animal imaging SkyScan-1076 (Skyscan 1067®, Skyscan, Aartselaar, Belgium). After standardized reconstruction, the datasets for each bone were resampled using computer software (Ant, 3D-creator vs. 2.2e, Skyscan, Aartselaar, Belgium) so that the medial axis of the bone was centrally oriented for each bone. Scans were processed and a two- and three-dimensional morphometric analysis was performed on a 4 mm femur bone segment at proximal end of the patellar trochlea using free software (CTanalyzer vs. 1.02, Skyscan, Aartselaar, Belgium). Measured parameters were expressed according to bone histomorphometry nomenclature (Parfitt et al., 1987). The mean number of bone fragments (Obj. N.), average bone fragment area (Av. Obj. Ar), the relative bone surface, the trabecular thickness (Tb. Th) (Hildebrand and Ruegsegger, 1997), and bone porosity (cavity/perforation, %) (Odgaard and Gundersen 1993) were determined for each group and compared to femur of saline-treated mice.

2.6. Drugs and treatment schedules

Based on the previous obtained results on the growth of femur-implanted cancer cells and the efficacy of acute opioids (El Mouedden and Meert, 2005; Vermeirsch et al., 2004) several experimental conditions were selected in the various experiments.

In the first experiment, evaluating a prophylactic treatment with opioids, animals were randomly divided into groups treated subcutaneously with 0.16 mg/kg fentanyl, 20 mg/kg morphine or saline from day 1 to day 14 onwards.

Sham-operated animals (controls) were injected with saline. The doses of the opioids were selected on the basis of showing equipotent antinociceptive efficacy in various parameters after acute administration in 15 days old tumor-bearing mice. Behavior testing (intrinsic paw lifting) took place in the morning on days 0 (baseline) and 7, 9, 12 14 and 15 after cell inoculation. Drug injections were given in the afternoon after behavioral testing. On day 15, also the paw use on the rotarod was tested before sampling the inoculated left hindpaw.

In the second experiment, a curative treatment of fentanyl and morphine was evaluated. To do so, different tumor inoculated animal groups ($n=10$) were again treated subcutaneously with 0.16 mg/kg fentanyl, 20 mg/kg morphine or saline from day 7 to day 17 onwards. Sham-operated controls received saline injections. In the same condition, the effects of naloxone on fentanyl were studied. To do so, other sarcoma animal groups ($n=10$) received 10 mg/kg naloxone or 0.16 mg/kg fentanyl plus 10 mg/kg naloxone. On day 18 after inoculation, behavioral testing (spontaneous paw lifting and paw use on the rotarod) was performed and limbs were collected for scanning.

2.7. Statistical analysis

For all experiments, groups of 10 animals per condition were used. Data are presented as mean±SEM values. A Mann–Whitney U test (which corrections for multiple testing) was used to compare behavioral measurement and bone parameters between saline and drug-treated animals. Results were considered statistically significant at $p<0.05$ (two-tailed).

3. Results

3.1. Body weight

All animals ($n=10$ per treatment group) displayed no obvious signs of distress during the 15 or 18 days observation period in both treatment paradigms (initiated at day 1 or 7 days after tumor inoculation). No significant differences were observed between any of the groups in terms of the average weight gain between 7 days (saline: 22.6±0.4 g; fentanyl: 22.0±0.4 g; morphine: 20.4±0.3 g) and 15 days after tumor inoculation (saline: 22.9±0.5 g; fentanyl: 23.4±0.7 g; morphine: 21.6±0.3 g) when treatment was initiated at day 1 after procedure. Also no differences were observed between any of the groups in terms of the average weight gain between 7 days after tumor inoculation (saline: 24.7±0.7 g; fentanyl: 24.5±0.5 g; morphine: 25.8±0.6 g) and 18 days after tumor inoculation (saline: 24.2±0.4 g; fentanyl tumor: 24.3±0.2 g; morphine: 23.5±1.5 g) when the treatment was initiated 7 days after tumor inoculation.

Globally, mice that had been treated with saline and inoculated with cancer cells or vehicle, exhibited no overt continuous increase in body weight throughout the 15 or 18 days observation period post-surgery in both treatment schedules. Mice that received chronic fentanyl treatment also showed no overt signs of body weight increase. However, a

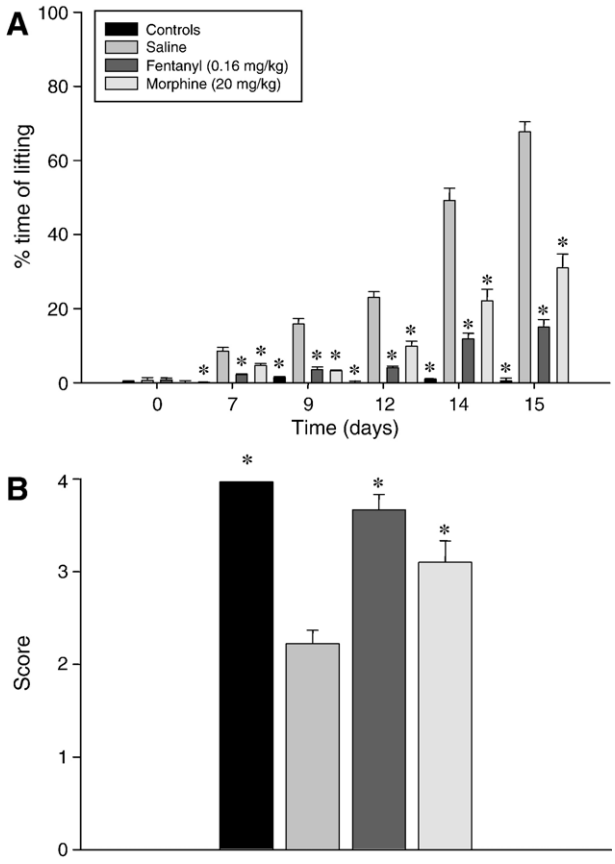

Fig. 1. Effect of fentanyl (0.16 mg/kg s.c.) and morphine (20 mg/kg s.c.) chronic treatment initiated at the day 1 after tumor inoculation (prophylactic treatment) on the development of spontaneous lifting and ambulatory limb-use on rotarod in NCTC2472 sarcoma-injected animals ($n=10$/group). Percentage of time lifting (A) were measured at different time points in sham (controls) and tumor-bearing animals treated subcutaneously with chronic administration of saline, fentanyl (0.16 mg/kg s.c.), or morphine (20 mg/kg s.c.) on days 0, 7, 9, 12, 14, and 15 after tumor inoculation. Limb-use scores on rotarod (B) were measured in animal groups 15 days after tumor inoculation. Values are expressed as mean±SEM. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Mann–Whitney *U* test. *Significantly different from sham value.

small subpopulation of the morphine-treated mice exhibited small but not significant weight losses.

3.2. Anti-nociceptive effects of repetitive fentanyl and morphine treatment

In the first series of experiments, we examined the effects of daily injection of fentanyl and morphine for 2 weeks on tumor growth-induced pain-related behavior and bone lesions. As shown in Fig. 1A, repeated administration of fentanyl (0.16 mg/kg s.c. once daily) or morphine (20 mg/kg s.c. once daily) initiated at day 1 after tumor inoculation significantly decreased spontaneous paw lifting over the different observation days ($p<0.05$). Over the entire observation period, morphine was somewhat less effective than fentanyl. By day 15, fentanyl and morphine significantly reduced paw lifting (expressed as % withdrawal time over total session time) from a saline baseline value of 67.73±2.68 to 15.06±1.96 and 30.98±3.77, respectively. Sham-operated controls did show almost no lifting at all (<1% time of lifting).

To evaluate the effects of fentanyl and morphine on ambulation-induced pain behavior, paw use on the rotarod was scored at day 15 (Fig. 1B). Both fentanyl and morphine significantly improved limb-use scores on the rotarod from 2.22±0.15 in the saline controls to 3.66±0.17 and 3.1±0.23, respectively. The sham-operated controls had a normal paw use at 15 days after surgery.

In the second series of experiments the effects of fentanyl and morphine were evaluated on day 18 after inoculation, when daily subcutaneous treatments were given from day 7 to day 17 post-surgery. Using this treatment schedule, both fentanyl and morphine reduce the spontaneous paw lifting (Fig. 2A) and improved paw use on the rotarod (Fig. 2B). In terms of spontaneous lifting, fentanyl and morphine decreased paw lifting (expressed as % withdrawal time over total session time) from 72.45±8.34 in the saline controls to 43.3±11.74 and 44.67±12.0, respectively. These values remained significantly different ($p<0.001$) from sham control animals. In terms of paw use on the rotarod (Fig. 2B), the opioids

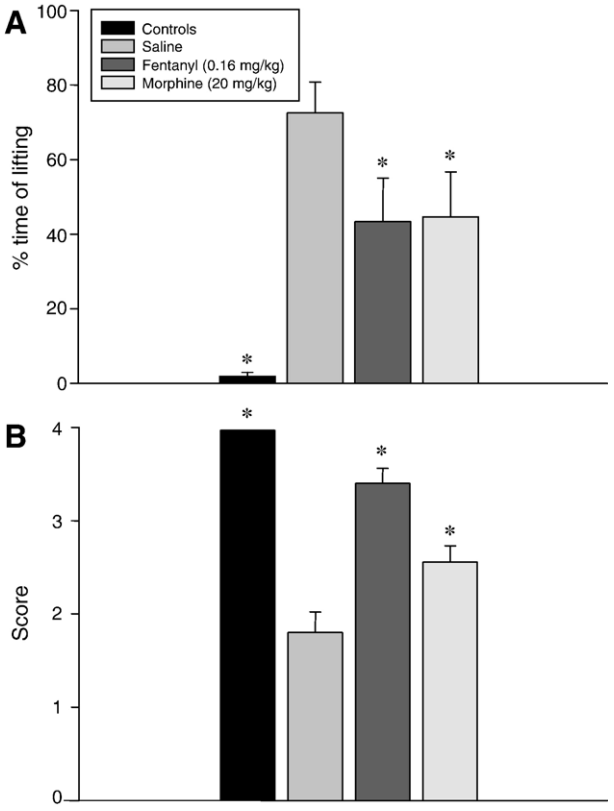

Fig. 2. Effect of fentanyl (0.16 mg/kg s.c.) and morphine (20 mg/kg s.c.) treatment initiated at the day 7 after tumor inoculation (prophylactic treatment) on the development of spontaneous lifting and ambulatory limb-use on rotarod in NCTC2472 sarcoma-injected animals ($n=10$/group). Percentage of time lifting (A) and ambulatory paw use scores on rotarod (B) were measured at day 18 in sham (controls) and tumor-bearing animals treated subcutaneously with chronic administration of saline, fentanyl (0.16 mg/kg s.c.), or morphine (20 mg/kg s.c.). Values are expressed as mean±SEM. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Mann–Whitney *U* test. *Significantly different from sham value.

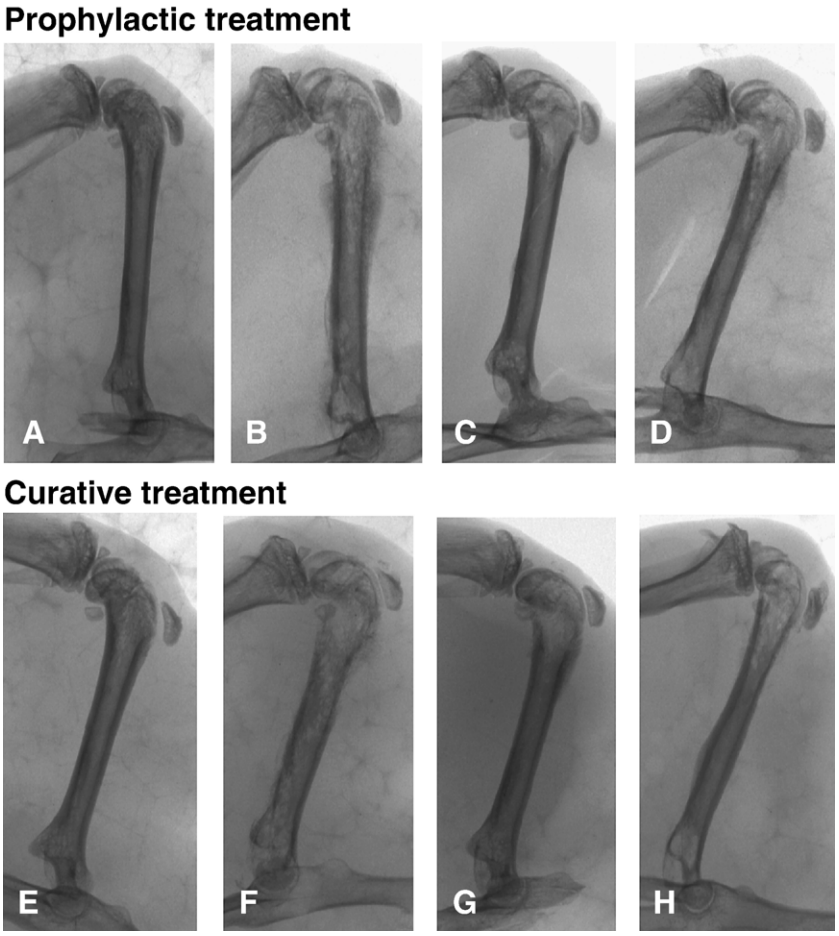

Fig. 3. Radiographs of mice distal femurs 15 days (prophylactic treatment) and 18 days (curative treatment) after inoculation with medium (controls) or NCTC 2472 osteosarcoma cells. (A, E) control animals (B, F) saline-treated group, (C, G) 0.16 mg fentanyl treatment group (D, H) 20 mg morphine treatment group. In control animals, no bone destruction or bone formation was observed. In saline-treated tumor inoculated animals, there was clear bone destruction in tumor-bearing femoral bones. Fentanyl-treated animals showed a consistely less effect on bone destruction. Morphine inhibits but at a lesser extent the tumor-induced bone degradation as compared to fentanyl-treated animals.

improved the scores from 1.8±0.22 for saline-treated animals to 3.4±0.16 and 2.55±0.18 for fentanyl and morphine respectively.

3.3. Effects of fentanyl and morphine administration on tumor-induced bone lesions

To determine if repeated administration of fentanyl and morphine influences tumor-induced bone destruction, photo-radiographs and μCT-scans of left femoral bones were taken on day 15 (experiment 1: prophylactic treatment) and day 18 (experiment 2: curative treatment) after tumor inoculation. Representative examples of the radiographic pictures of femurs from sham controls and the different tumor cell treated animals are presented in Fig. 3. The photoradiographs showed that in sham non-sarcoma mice (Fig. 3A, E) the femurs exhibited no bone lesions. In sarcoma saline-treated group (Fig. 3B, F), strong bone remodelling was observed at the tumor implantation site, resulting in cortical destruction. Treatment of tumor-bearing mice with fentanyl resulted in reduced bone resorption compared to sarcoma saline-treated animals (Fig 3C–G). In the fentanyl-treated group, osteolytic lesions were less observed and the metaphysic of long bone exhibited high bone density reflecting inhibition of bone resorption. Radiographs from morphine-treated mice revealed also less osteolytic lesions than saline-treated animals but apparently more than fentanyl-treated mice (Fig. 3D–H).

In order to better profile these observations, micro-CT scans were combined with 3D-bone image reconstruction techniques and quantifications were performed. In order to demonstrate that a prophylactic treatment with fentanyl had a stronger normalizing impact on bone structure versus saline but also morphine-treated mice, all bone 3D-images of the treated animals from experiment 1 are presented (Fig. 4). As seen here, several of the fentanyl-treated animals show normal bone structures.

The tumor-bearing femurs from saline-treated sarcoma mice had extensive bone destruction and loss of trabecular bone along the distal femoral cortex (Fig. 4). Bone lesions observed in saline-treated group consist of osteolysis and new bone formation accompanied with cortical perforations and frequently destroyed trabeculae. The 3D-image analysis of the fentanyl

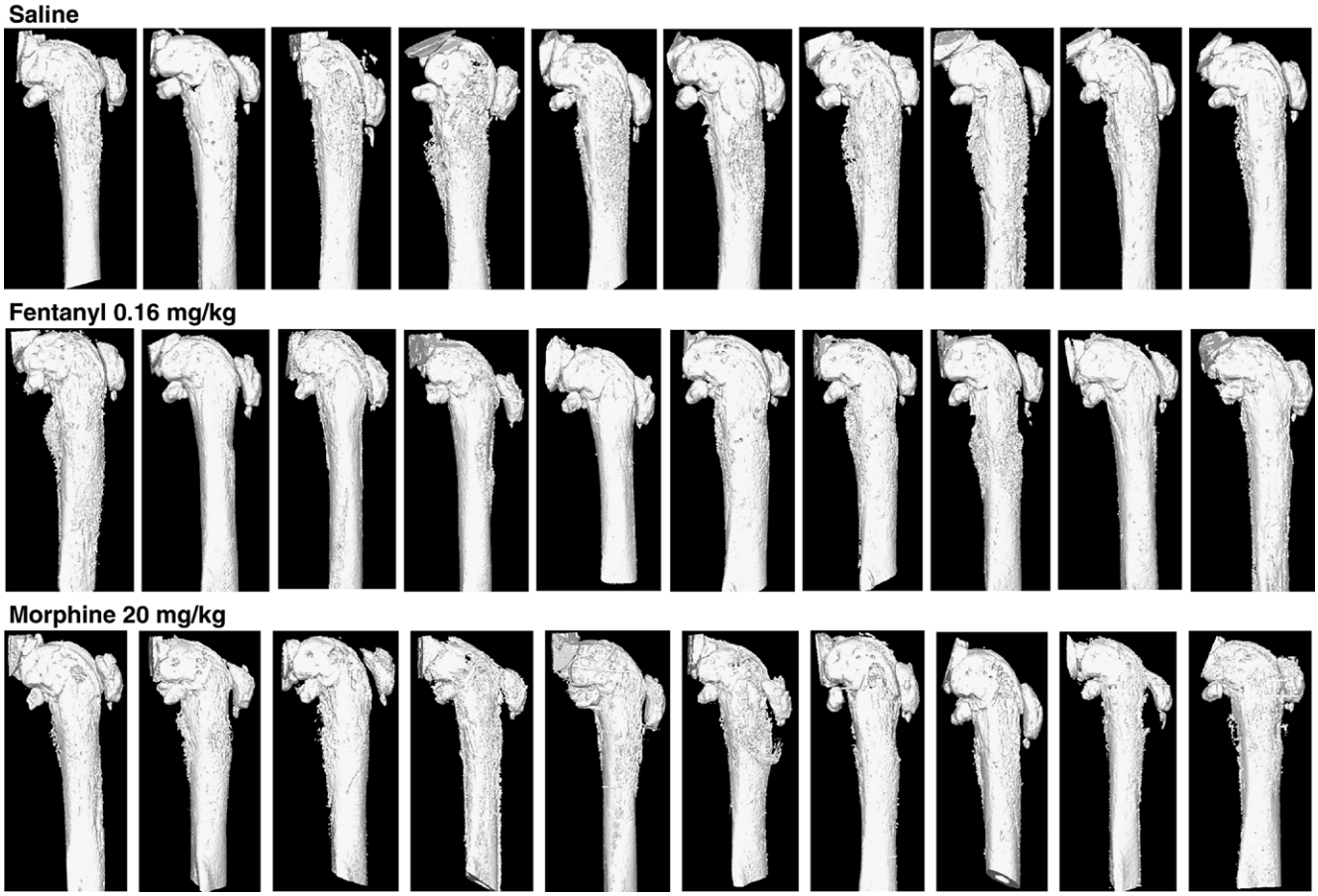

Fig. 4. Suppression of the tumor growth-induced bone destruction by repeated analgesic doses of fentanyl (0.16 mg/kg s.c.) and morphine (20 mg/kg s.c.). Three-dimensional μCT reconstruction images of the medium and NCTC2472 sarcoma-injected distal femur in different animal treated with saline, 0.16 mg fentanyl, and 20 mg morphine for 15 days (prophylactic treatment) after tumor inoculation. In the control animal, no bone destruction or bone formation was observed. In saline-treated tumor inoculated animals, there was clear bone destruction in tumor-bearing femoral bones. Fentanyl-treated animals showed a less effect of tumor-induced bone destruction. Morphine was less efficient in reducing tumor-induced bone degradation.

treatment group showed a great inhibition of cancer-induced bone destruction (Fig. 4). Chronic administration of morphine was better than saline but seems to be less effective than fentanyl in reducing bone lesions (Fig. 4).

The quantitative analysis, in the trabecular micro-architectural structure of the 4 mm region below the patella of distal bone femur, revealed significant ($p<0.05$) changes of the bone structure parameters in the tumor-bearing saline-treated bones versus sham controls (Table 1). In the two-dimensional model based on cross section analysis, fentanyl-treated mice initiated at day 1 after inoculation, exhibited a significant decreases in the mean number of bone fragments down to 45.2±5.7% ($p<0.05$) and the bone surface to 78.6±6.6% ($p<0.05$) as compared to saline tumor-treated group. Repeated administration of fentanyl further induced a significant increase in the average bone fragment area (140.1±13.6%, $p<0.05$), trabecular thickness (116.1±5.9%, $p<0.05$), and bone porosity (140.8±6.2%, $p<0.05$) parameters when compared to saline-treated tumor group. These effects of fentanyl on trabecular structure indicate reductions of bone osteolysis and the preservation of bone microstructure, observed at both the cortical and trabecular levels, compared to femur of saline-treated group (Table 1). These results are consistent with the histological appearance of the femur.

In the group of sarcoma mice treated 2 weeks prophylactically with 20 mg/kg morphine, the bone lesions were reduced as compared to the saline sarcoma group, but to a lesser extent than with the fentanyl-treated animals (Table 1). Morphine reduced significantly the mean number of bone fragments down 59.9±8.2% ($p<0.05$), and the relative bone surface to 75.5±3.9% ($p<0.05$) of the saline-treated tumor group. Morphine also induced an increase in the trabecular thickness (115.5±6.0%, $p<0.05$), and bone porosity (129.3±5.8%, $p<0.05$) as compared to the saline-treated tumor group. The average bone fragment area (105.9±13.5%, $p>0.05$) was not significantly changed.

For experiment 2, with treatment schedules started at day 7, comparable results were obtained (Table 2). Here fentanyl, as compared to saline-treated sarcoma mice, decreased the mean number of bone fragments down to 23.0±2.8% ($p<0.05$) and the bone surface down to 79.4±6.9% ($p<0.05$) and increased the average bone fragment area (258.3±33.3%, $p<0.05$),

Table 1
2D parameters of mice distal femur trabecular bone architecture

	Obj. N	Av. Obj. Ar (mm^2)	Tb. Th (mm)	BS (mm^2)	Po (%)
Controls	4.50±0.17*	0.589±0.05*	0.211±0.01*	47.53±2.19*	30.76±5.11*
Saline	14.31±1.48	0.222±0.04	0.168±0.02	97.89±6.86	20.20±0.96
Fentanyl	6.47±0.81*	0.311±0.03*	0.195±0.01*	76.96±6.47*	28.44±1.26*
Morphine	8.57±1.17*	0.235±0.03	0.194±0.01*	73.94±3.8*	26.12±1.18*

Microcomputed tomography measurements in the medium (controls) and NCTC 2472 sarcoma-injected distal femur of saline, fentanyl (0.16 mg/kg s.c.), or morphine (20 mg/kg s.c.) treated groups (n=10/group) from day 1 to day 14 after inoculation. The animals were sacrificed on day 15 after inoculation and sarcoma-limbs were collected and fixed. The 4 mm region under the patella from distal left femurs in different groups was used for analysis. In a 2-dimentional analysis, number of bone fragments (Obj. N.), average of bone fragment area (Av. Obj. Ar), relative bone surface (BS), trabecular thickness (Tb. Th.), and bone porosity (cavity/perforation, %). Values are expressed as mean±SEM. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Mann–Whitney U test. *Significantly different from sham value.

trabecular thickness (154.5±18.18%, $p<0.05$), and bone porosity (154.7±32.5%, $p<0.05$). Comparably, morphine reduced the mean number of bone fragments down to 75.6±10.1% ($p<0.05$), and the relative bone surface to 83.8±11.5% ($p<0.05$) of the saline sarcoma group, while increasing the average bone fragment area (200.0±50.0%, $p<0.05$) and the trabicular thickness (145.5±18.2%, $p<0.05$). The bone porosity (105.3±8.9%, $p>0.05$) was not significantly changed.

3.4. Antagonism of the effects of fentanyl with naloxone in the bone cancer model in mice

To determine whether the effects of fentanyl were mediated by opiate receptor interactions, we tested whether naloxone could reverse the behavioral and histological effects of fentanyl. To do so, 10 mg/kg naloxone, 0.16 mg/kg fentanyl or the combination was injected daily over 10 days in animal groups (n=10) starting from day 7 after tumor cell inoculation. Repeated fentanyl administration resulted in reductions in pain behavior (Fig 5A–B) as compared to saline-treated animals. In sarcoma-inoculated animals, fentanyl reduced both spontaneous lifting (43.3±11.74%; $p<0.05$ vs. sarcoma+saline; 72.45±8.34; Fig. 5A) and paw use on rotarod (3.4±0.16; $p<0.04$ vs. sarcoma+saline; 1.8±0.22; Fig. 5B) although these reductions were not at the sham control levels.

Importantly, in the sarcoma mice, repeated administration of a combination of fentanyl (0.16 mg/kg s.c.) and naloxone (10 mg/kg s.c.) reduced spontaneous lifting (40.7±16.2%; $p<0.05$ vs. sarcoma+saline; 72.5±8.3; Fig. 5) and an increased of paw use on rotarod (3.4±0.18; $p<0.04$ vs. sarcoma+saline; 1.8±0.2; Fig. 5).

Naloxone by itself was inactive and values comparable to the saline group were obtained (73.08±6.06%; $p<0.05$ vs. sarcoma+saline; 72.5±8.3; Fig. 5). No statistically significant effects were noted when comparing the pain behaviors of fentanyl-treated sarcoma mice with mice receiving a combination of fentanyl and naloxone (Fig. 5).

NCTC 2472 causes new bone formation with a pronounced periosteal reaction. Representative examples of the 3D-images appearance of control and treated femurs are shown in Fig. 6. 3D-images of fentanyl (0.16 mg/kg s.c.) treated animals (Fig. 6C) show denser bone with a smaller perimeter. Importantly the femurs from mice treated with the combination of fentanyl and naloxone (Fig. 6E) show dense bone and decreases in the periosteal reaction. The decrease in bone lesions upon treatment with fentanyl alone (Fig. 6C) or the fentanyl+naloxone combination (Fig. 6E) can be clearly seen in the comparison with the control animal (Fig. 6A). Naloxone alone doesn't alter the tumor-induced bone lesions in sarcoma mice compared to that in saline-treated sarcoma animals (Fig. 6B, D).

2D analysis using the micro-architectural structure of the 4 mm region below the patella of distal bone femur demonstrated that the administration of naloxone (10 mg/kg) alone was not effective in tumor lesions inhibition compared to saline-treated sarcoma group (Table 3).

The combination of fentanyl (0.16 mg/kg) and naloxone (10 mg/kg) decreased tumor-induced bone lesions to that observed with fentanyl alone. However, the combination of fentanyl (0.16 mg/kg) and naloxone (10 mg/kg) significantly decreased the mean number of bone fragments (20.1±3.3%, $p<0.05$), and the relative bone surface (70.2±10.2%,

Table 2
2D parameters of mice distal femur trabecular bone architecture

	Obj. N	Av. Obj. Ar (mm^2)	Tb. Th (mm)	BS (mm^2)	Po (%)
Controls	3.96±0.19*	0.54±0.07*	0.25±0.01*	51.62±2.23*	32.69±4.97*
Saline	26.68±2.92	0.12±0.02	0.11±0.01	103.63±3.41	13.49±2.48
Fentanyl	6.13±0.76*	0.31±0.04*	0.17±0.02*	82.28±7.20*	20.87±4.39*
Morphine	20.17±2.71*	0.24±0.06*	0.16±0.02*	86.83±11.87*	14.21±1.20

Microcomputed tomography measurements in the medium (controls) and NCTC 2472 sarcoma-injected distal femur of saline, fentanyl (0.16 mg/kg s.c.), or morphine (20 mg/kg s.c.) treated groups (n=10/group) from day 7 to day 17 after inoculation. The animals were sacrificed on day 18 after inoculation and sarcoma-limbs were collected and fixed. The 4 mm region under the patella from distal left femurs in different groups was used for analysis. In a 2-dimentional analysis, number of bone fragments (Obj. N.), average of bone fragment area (Av. Obj. Ar), relative bone surface (BS), trabecular thickness (Tb. Th.), and bone porosity (cavity/perforation, %). Values are expressed as mean±SEM. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Mann–Whitney U test. *Significantly different from sham value.

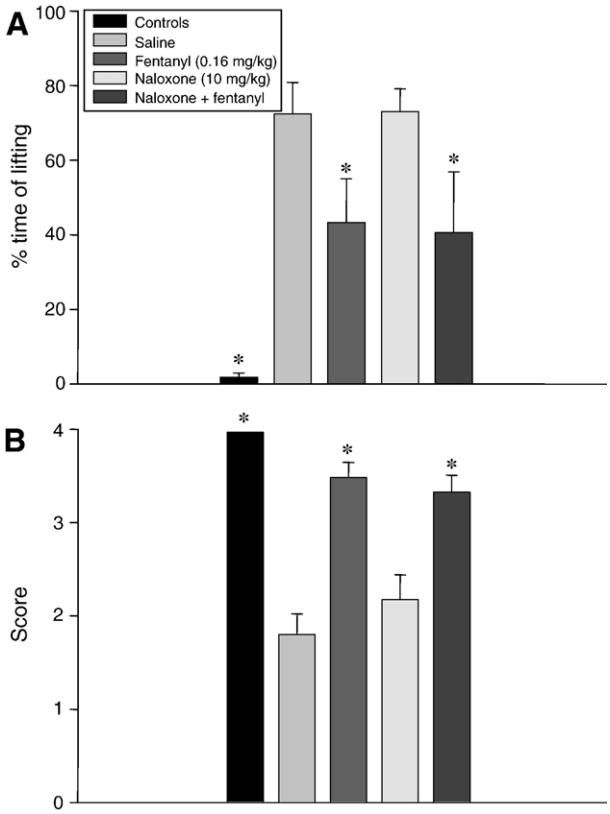

Fig. 5. Effect of repetitive coadministration of naloxone and fentanyl on the development of spontaneous lifting and ambulatory limb-use on rotarod in NCTC2472 sarcoma-injected animals (n=10/group). Percentage of time lifting (A) and limb-use scores on rotarod (B) were measured at day 18 after procedure in sham (controls) and tumor-bearing animals treated with subcutaneous administration of saline, fentanyl (0.16 mg/kg s.c.), naloxone (10 mg/kg s.c.) or coadministration of fentanyl (0.16 mg/kg s.c.) and naloxone (10 mg/kg s.c.). Values are expressed as mean±SEM. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Mann–Whitney U test. *Significantly different from sham value.

$p<0.05$), and induced a significant increase in average bone fragment area (291.7±41.6%, $p<0.05$), trabecular thickness (155.9±18.92%, $p<0.05$), and bone porosity (174.1±51.2%, $p<0.05$) compared to femur of saline-treated sarcoma group (Table 2).

5. Discussion

Using an established model of bone cancer pain, we demonstrated that pain symptoms became apparent by day 7 post-inoculation and highly important by day 15 post-inoculation (El Mouedden and Meert, 2005). In addition, fentanyl or morphine at low doses (fentanyl 0.04 mg/kg; morphine 2.5 mg) did not affect the hyperalgesia (El Mouedden and Meert, 2005). These findings suggest the importance of the severity of pain at this stage. At present, the mechanisms of the late stage of cancer pain are poorly understood. However, the data suggesting that tumor growth induces extensive bone destruction raises the possibility that any factors involved in tumor-induced bone resorption are responsible for pain in the late stage of cancer. Although we do not exclude the involvement of neuropathic component, the nociception may be augmented by the production and release of proinflammatory agents from in vivo tumor mass changing with the tumor growth may be involved in the appearance of cancer pain. These agents such as cytokines, adenosine, bradykinin, serotonin, and prostanoids can alter or sensitize neural transmission and create a chronic state. In this context, some studies have shown that naive mice given an intraplantar injection of the tumor mass extract showed a marked hyperalgesia and spontaneous licking behavior (Zhang et al., 2001).

Despite these challenges, advances in our understanding of the pathophysiology of the cancer pain problems have been made which in turn have led to new pharmacotherapeutic options for managing chronic pain. Eradication of the tumor is usually approached with chemotherapy and radiotherapy management. Unfortunately, more than 50% of patients who undergo radiation treatment and obtain pain relief will experience a relapse of pain equivalent to pre-treatment levels (Tong et al., 1982).

Opiates are used to relieve ongoing cancer pain (Radbruch et al., 1996) but need to be increased enormously to high doses for a sufficient block of breakthrough pain, with an increased

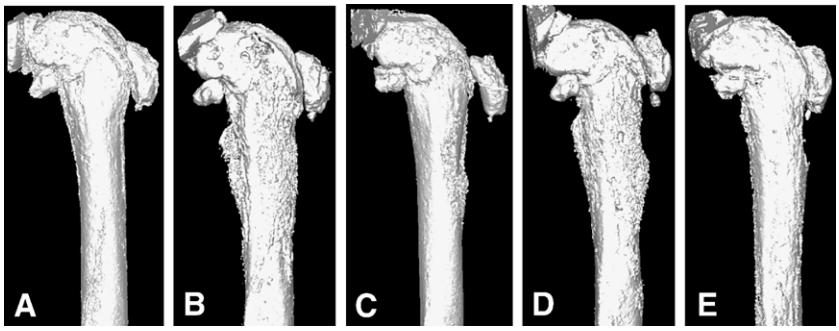

Fig. 6. Suppression of the tumor growth-induced bone destruction by repeated coadministration analgesic doses of fentanyl (0.16 mg/kg s.c.) and naloxone (10 mg/kg s.c.). Three-dimensional μCT reconstruction images of the medium and NCTC2472 sarcoma-injected distal femur in different animal treated with saline, 0.16 mg/kg fentanyl alone, 10 mg/kg naloxone, and combination of 0.16 mg/kg fentanyl alone, 10 mg/kg naloxone initiated 7 days after tumor inoculation. Animals were sacrificed 18 days after procedure. In the control animal, no bone destruction or bone formation was observed. In saline-treated tumor inoculated animals, there was clear bone destruction in tumor-bearing femoral bones. Fentanyl-treated animals showed a concisely less effect of tumor-induced bone destruction. Naloxone alone is inefficient in reducing tumor-induced bone degradation. Combination of fentanyl and naloxone showed less effect of tumor-induced bone lesions.

Table 3
2D parameters of mice distal femur trabecular bone architecture

	Obj. N	Av. Obj. Ar (mm^2)	Tb. Th (mm)	BS (mm^2)	Po (%)
Controls	3.96±0.19*	0.54±0.07*	0.25±0.01*	51.62±2.23*	32.69±4.97*
Saline	26.68±2.92	0.12±0.02	0.11±0.01	103.63±3.41	13.49±2.48
Fentanyl	6.13±0.76*	0.31±0.04*	0.17±0.02*	82.28±7.20*	20.87±4.39*
Naloxone	24.54±3.42	0.11±0.18	0.12±0.03	98.79±9.47*	15.48±1.91
Fentanyl+naloxone	5.77±0.88*	0.35±0.05*	0.17±0.02*	72.77±10.54*	23.51±6.93*

Microcomputed tomography measurements in the medium (controls) and NCTC 2472 sarcoma-injected distal femur of saline, fentanyl (0.16 mg/kg s.c.), naloxone (10 mg/kg s.c.), or co-administered fentanyl (0.16 mg/kg s.c.) and naloxone (10 mg/kg s.c.) treated groups (n=0/group) from day 7 to day 17 after inoculation. The animals were sacrificed on day 18 after inoculation and sarcoma-limbs were collected and fixed. The 4 mm region under the patella from distal left femurs in different groups was used for bone structure analysis. In a 2-dimentional analysis, number of bone fragments (Obj. N.), average of bone fragment area (Av. Obj. Ar), relative bone surface (BS), trabecular thickness (Tb. Th.), and bone porosity (cavity/perforation, %). Values are expressed as mean±SEM. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Mann–Whitney U test. *Significantly different from sham value.

risk and the occurrence of unwanted side effects (Portenoy et al, 1999b; Levy, 1996). Long-term opioid use can be associated with side effects such as tolerance, physical dependence, and addiction. However, development of tolerance and physiological dependence in pharmacotherapy of many medical conditions is not unique to the opioids. Furthermore, it should be noted that although there are in fact many addicts of various drugs in the world, there is clear evidence that a non-addict who is using an opioid for a medical condition is inextremely unlikely to become an addict.

Opioid such as morphine, are documented to enhance tumor growth and reduces survival of tumor-bearing rats (Lewis et al., 1984) and their tumor-promoting activity resulted in mice after an injection with leukemia or sarcoma cells (Ishikaw et al., 1993). This was suggested to be due to an inhibition of the cellular immune response and suppression of immune systems (Ishikaw et al., 1993; Gaveriaux et al., 1995). Moreover, morphine was shown to promote the growth of breast cancer xenografts in nude mice by increasing angiogenesis (Gupta et al, 2002). These findings raising the possibility that immoderate use of opioids may aggravates cancer and could strength the attitude of physicians to avoid the prescription of potent opioid analgesics. Opioid therapeutic failure is due also not only to the development of opioid-resistant pain syndromes like neuropathic pain or the worsening of the intensity of pain, but also to the under treatment of the patient pain caused by clinician deficiencies of knowledge of opioid therapy or patient fear of side effects and addiction to the opioids. On the other hand, many studies have demonstrated that opioids such as methadone, morphine, and buprenorphine inhibit the growth of tumor cells in vitro and in cancer animal models (Harimaya et al., 2002; Sasamura et al., 2002, Tegeder et al., 2003). As such there is a clear conflict in the literature in the outcome and use of opioids in cancer models.

In the present study, repeated administration of analgesic doses of fentanyl and morphine decreased significantly symptom behaviors indicative of pain and also improved mobility as assessed by limb-use on rotarod test. In addition, fentanyl treatment and morphine at a lesser extent decreased consistently the progression of bone lesions. This observation was confirmed by the quantification of micro-architecture parameters by micro-CT scanner analysis. Interestingly, tumor lesions were also inhibited by the combination of fentanyl and naloxone, whereas naloxone itself had no significant effect. One would expect that fentanyl effects are antagonized with naloxone, an opioid receptor antagonist. However, the inhibition of the tumor induces bone destruction by fentanyl demonstrated in the present study as well as the tumor growth inhibition by morphine was mostly not antagonized by naloxone (Maneckjee and Minna, 1992; Hatzogolou et al., 1996; Kugawa et al., 1998; Gupta et al., 2002; Tegeder et al., 2003).

The observed fentanyl effects on tumor lesions, which were not antagonized with naloxone, suggest the possibility of using fentanyl in cancer therapy to relieve cancer pain but also to reduce bone tumor burden. Since naloxone was able to prevent neuronal toxicity (Mao et al., 2002) and failed to antagonize fentanyl inhibition of bone destruction, chronic high dose fentanyl plus low dose naloxone treatment might be a therapeutic approach that possibly combines favorable tumor bone lesion reduction with reduced side effects such as neuronal toxicity.

Since fentanyl in particular and opioids in general have been widely reported as potent analgesic drugs for skeletal pain complications associated with bone cancer metastasis, it is interesting that the effect of chronic opioid on bone cancer pain and on osteolysis has not well been elucidated. The osteolytic metastases is associated with release of osteolytic mediators by tumor cells, bone degradation, release of growth factors from degraded bone, enhanced tumor cell growth factors from degraded bone, enhanced tumor cell growth, and further release of osteolytic mediators. Opioids can interfere with one of these mechanisms such inhibition of bone resorption. In this context, biphosphonates agents are used as promising tools to manage skeletal metastases and pain-related behavior. Consequently, the combination of biphosphonates and fentanyl might yield a therapeutic approach for treating bone cancer with biphosphonate that should be assessed in this murine model of bone cancer to evaluate a therapeutic value in treating bone cancer pain.

Another presumed mechanism of opioids on osteolysis is opioids analgesia and pain relief-induced reduction of the tumor burden (Sasamura et al., 2002). Data supporting this hypothesis were the blockade of pain signals by sciatic neurectomy which reduced the tumor growth and metastasis (Sasamura et al., 2002). Our findings confirm that specific mu-opioids such as morphine and fentanyl, decreases tumor burden and pain associated behaviors.

Another explanation for the anti-tumor effects of fentanyl or morphine is that opioids act directly to inhibit tumor cell growth. The anti-tumor effects of opioids observed in vivo can be partly explained by an inhibitory effect exerted by these compounds on the proliferation and survival of a variety tumor cells themselves including those of breast cancer (Kawase et al., 2002; Kugawa et al., 2000; Hu et al., 2002; Mao et al., 2002). In our experimental model, we have also demonstrated that fentanyl and mophine induced a decrease in cell proliferation together with the induction of cell death in NCTC 2472 oeteosracoma cells (data not shown). In addition, we have demonstrated the expression of mu-opioid receptors as well as signs of apoptosis in NCT2472 cells (data not shown).

Hence, it is not known whether the observed tumor-promoting and/or tumor suppressing effects of opioids are mediated through the well-characterized opioid-mediated intracellular signaling pathway that involves activation of a PTX-sensitive inhibitory G protein (Gi) (Standifer and Pasternak, 1997), inhibition of adenylyl cyclase, and decrease of cAMP levels and thereby inhibition of protein kinase A. The anti-tumoral effects of opioids were suggested to depend on the abundance of opioid receptors. At first sight, this appears illogical because the growth-inhibitory effects of morphine occurred primarily at concentrations where Gi was no longer active. This suggests that possibly the uncoupling of Gi, rather than its activation, may be the initiating event that ultimately leads to cell death. This idea is supported by the finding that neuronal apoptosis induced by morphine was associated with morphine tolerance, which is known to be caused by opioid receptor desensitization and uncoupling of the Gi protein (Whistler et al., 1998; Pei et al., 1995).

Other studies have demonstrated recently that psychological stress enhances the liver metastasis of colon tumor, in part by suppressing cellular immunity in mice (Wu et al., 2000). There is also evidence suggesting that various stresses promote tumor growth and metastasis (Giraldi et al., 1989; Kanno et al., 1997). The suppression of immune functions such as decrease in natural killer activity was claimed to be involved (Holden and Ben). In this context, pre- and postoperative administration of an analgesic dose of morphine attenuates the surgery-induced increase in metastasis (Page et al., 1993). In that case, the attenuation was suggested to be caused by prevention of surgery-induced increases in plasma corticosterone concentration (Page et al., 1998). With these findings taken into account, the present results suggest that sufficient relief from pain by medication of fentanyl and morphine in cancer patients is needed to improve quality of life and anti-tumor efficacy. Further studies are needed to determine the mechanisms of fentanyl-induced inhibition of the growth and metastasis of tumor cells.

Beyond induction of tumor cell apoptosis, opioids can inhibit tumor growth via other mechanisms such as inhibition of cell growth, inhibition of cell adhesion and spreading, and inhibition of tumor cell invasion. However, the precise mechanism by which opioids inhibits tumor growth of bone tumors is still unknown. Currently the data are conflicting and whether or not opioids process anticancer effects are still controversial.

Taken together, these data demonstrate clearly that chronic treatment with fentanyl after osteosarcoma implantation not only prevents the development of osteolytic lesions but also reduced pain-related behavior. However, there are many unanswered questions regarding the mechanisms by which fentanyl treatment decreases bone cancer lesions. Future use of the experimental system described herein and in vitro cell culture should accelerate the pace of discovery regarding the cellular mechanisms through which fentanyl and opioids in general decrease bone cancer lesions.

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