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Evaluation of pain-related behavior, bone destruction and effectiveness of fentanyl, sufentanil, and morphine in a murine model of cancer pain

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Abstract

The present study was conducted to evaluate the pain development and bone destruction during bone cancer growth in a murine model of bone cancer pain and to evaluate the analgesic efficacy of fentanyl, sufentanil, and morphine in this model. C3H/HeNCrl mice were inoculated into the intramedullary space of the femur with osteolytic NCTC 2472 fibrosarcoma cells, and followed during a 3-week period to assess pain behaviors (spontaneous lifting and limb-use during forced ambulation on rotarod) and bone destruction (parameters indicative of bone lesions determined by μCT-scans of the tumor-bearing bones) during bone cancer growth. The results showed that in this murine model of cancer-induced bone pain, behavioural manifestations of pain emerge in parallel with the progression of bone destruction. The subcutaneous administration of fentanyl (0.025–0.64 mg/kg), sufentanil (0.005–0.04 mg/kg), and morphine (2.5–40 mg/kg) on the test days 15 and 22 post-inoculation reduced pain-related behaviors in a dose dependent manner. A complete relief from pain-related behaviors was achieved with the following doses: ≥0.16 mg/kg fentanyl, 0.02 mg/kg sufentanil, and 20 mg/kg morphine. In conclusion, the results showed a clear link between tumor growth-induced bone destruction and behavioral pain manifestations, the latter was effectively controlled by the opioids fentanyl, sufentanil, and morphine.

Keywords: Bone cancer; Cancer pain; Fentanyl; Morphine; Sufentanil; Opioid; Murine model; Osteolysis; CT-scan; Trabecular bone structure

1. Introduction

Cancer pain resulting from bone metastatic infiltration and destruction remains difficult to treat and contributes significantly to increased morbidity and reduced quality of life of patients (Thurlimann and de Stoutz, 1996). The resulting symptoms of bone cancer-related pain consist generally of localized ongoing spontaneous pain, and more commonly, occur as a result of movement or weight-bearing of the affected limb (Mercadante, 1997). The pathogenic mechanism underlying bone cancer pain is not completely understood, but tumor metastasis often stimulates osteoclast activity resulting in bone resorption, which may result in microfractures due to weakened skeletal strength (Kanis et al., 1991; Mantyh et al., 2002). Such fractures, together with nerve root compression due to vertebral collapse and the release of soluble factors that may sensitize primary nociceptive afferents, are significantly contributing factors to pain associated with bone metastases (Portenoy and Lesage, 1999).

At present, the management of early bone pain involves the use of simple analgesics such as acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs), and weak opioids, but increasing doses are usually required as the cancer progresses. As the disease becomes more advanced and problematic, the management requires strong opioids and adjuvant drugs (Cherney, 2000), such as antidepressants and anticonvulsants, benzodiazepines, corticosteroids, and neuroleptic drugs (for review, see Mantyh et al., 2002). In cancer patients who do not receive adequate pain relief with oral or transdermal medications, options include nerve

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blocks, chemotherapy, hormone manipulation, radiation, and surgery. However, biphosphonates and systemic radioisotopes can be very helpful in the long term management of bone metastatic cancer pain. Although ongoing pain may respond relatively well to a variety of available therapies, breakthrough or movement-evoked pain can be problematic and remains particularly difficult to control even with high doses of opioids (Mercadante, 1997; Portenoy, 1999; Portenoy et al., 1999). The incomplete understanding of the mechanisms that drive bone cancer pain may be attributed in part to the lack of specific animal models to explore novel pharmacological treatment strategies based on advances in the knowledge of pain conditions other than those associated with cancer, such as neuropathic or inflammatory pain (Mantyh, 2002).

The development in recent years of a murine models of osteosarcoma able to produce pain behavior (Schwei et al., 1999) leads to a rapid knowledge progression of the pathophysiology of bone cancer pain compared to established models of neuropathic and inflammatory pain (Honore et al., 2000a; Luger et al., 2002). In this model, implantation of mouse osteosarcoma tumor cells into the intramedullary space of the femur results in significant bone destruction due to tumor growth and is associated with pain-related behaviors (Honore et al., 2000a; Schwei et al., 1999; Vermeirsch et al., 2004) similar to that observed in human patients with metastatic bone cancer pain. Moreover, this model may also be used to evaluate novel pharmacological approaches to treatment.

In this study we evaluated the pain-related behaviors in relation to bone destruction progression during bone tumor growth in a murine model of bone cancer pain. We therefore present here a quantitative evaluation over-time of pain behavior and bone destruction and tried to establish whether there was a link between tumor growth-induced bone destruction and behavioral pain manifestations. We thereafter evaluated whether the bone cancer-related pain behavior was effectively controlled by opioids such us: fentanyl, sufentanil, and morphine.

2. Methods

2.1. Cell inoculation and tumor induction

The mice were housed in a mouse facility that is fully compliant with the European policy on use of Laboratory Animals. Experimental protocols were approved by the Institutional Review Committee of Janssen Pharmaceutica (Beerse, Belgium), and met the European guidelines on animal experimentation.

Osteolytic murine sarcoma cells (NCTC 2472, American Type Culture Collection (ATCC), Rockville, MD, USA) were cultured in NCTC 135 medium (Invitrogen) containing 10% horse serum (Gibco) and passaged 2 times weekly according to ATCC guidelines. For their administration, cells were detached by scraping and then centrifuged at $1000 \times g$. The pellet was suspended in fresh NCTC 135 medium ($2.5\ 10^6$ cells/20 μl) and then used for intramedullary femur inoculation.

Male C3H/HeNCrl mice (25–30 g, Charles River, Sulzfeld, Germany) were used in all experiments. Induction of bone cancer was carried out as previously described (Vermeirsch et al., 2004). After induction of general anaesthesia, with xylazine (10 mg/kg i.p.) and ketamine (100 mg/kg i.p.) the left hind paw was shaved and disinfected with povidone–iodine followed by 70% ethanol. A superficial incision of 1 cm was then made over the knee overlaying the patella. The patellar ligament was then 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. Twenty μl of media (sham animals) or media containing tumor cells (approximately 2.5×10^6 cells) were then injected into the bone cavity using a syringe. To prevent leakage of cells outside the bone, the injection site was sealed with dental acrylic (Paladur, Heraeus Kulzer, GmbH, Wehrheim, Germany) and the wound closed with skin stitches.

2.2. Pain behavioral tests

Pain behaviors were evaluated in separate groups ($n=6$) of sham and bone tumor mice with confirmed hyperalgesia as assessed by spontaneous lifting behavior. Between 0 and 10% of mice did not develop tumors across various experiments performed. These mice showed little or no sign of pain behavior, and post-mortem examination revealed no sign of bone involvement or no tumor growth. Animals were behaviourally tested during a 3-week period prior to and 7, 12, 15, 18, and 22 days after tumor inoculation.

Body weight of the mice was recorded throughout the experimental period to get an idea on general health status. At the end of the experiment the femur of the left hind limb was sampled and used for μCT scanning as described in Vermeirsch et al. (2004).

2.2.1. Spontaneous lifting behavior

All tests were performed at room temperature during the light phase, and before each test animals were habituated to the laboratory room at least 30 min. To measure the spontaneous lifting, the animals were habituated in a transparent acrylic cylinder of 20 cm diameter put on an horizontal plan surface and thereafter observed during 4 min for spontaneous lifting behavior of the left hind paw.

2.2.2. Limb-use on rotarod

After spontaneous lifting behavior assessment, animals were immediately placed on a mouse rotarod (ENV-575M®, Med Associates Inc., GA, USA) at a speed of 16 rpm 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.

2.3. Evaluation of bone destruction

Bone analysis was carried out on left hind limbs in separate groups ($n=6$) of sham and tumor-bearing mice with confirmed hyperalgesia prior to and 7, 12, 15, 18, and 22 days following cell injection. Animals were sacrificed by euthanasia using CO_2 gas. The left hind limbs were dissected from the body and the femurs, including the surrounding tissues, were fixed in 10% phosphate-buffered formalin and transferred to a plastic cuvette filled with 70% ethanol for μCT processing.

Fixed mice left hind limbs were scanned using the Skyscan 1076 microtomograph system for small animal imaging (Skyscan 1076®, Skyscan, Aartselaar, Belgium). A standardized cone beam μCT-scan was performed of the left limb using a medium resolution (18 μm pixel size). Scans were processed and the reconstructed datasets for each bone were resampled using computer software (Ant, 3D-creator vs. 2.2 e, Skyscan, Aartselaar, Belgium) so that the medial axis of the bone was centrally oriented for each bone. Morphometric parameters were calculated by CT-analyser free software (CTanalyzer® vs. 1.02, Skyscan, Aartselaar, Belgium) either in 3D based on a volume model, or in 2D from crossection images taken immediately behind the proximal end of patellar trochlea, which covered 4-mm-long region of the distal femur. Measured parameters were expressed according to bone histomorphometry nomenclature (Parfitt et al., 1987). In the long distal femur, mean number of bone fragments (Obj.N), average bone fragment area (Av.Obj.Ar), bone volume (BV), trabecular bone surface fraction (BS/BV), trabecular thickness (Tb.Th), and trabecular number (Tb.N) were determined.

2.4. Drug treatment

The effects of fentanyl, sufentanil, and morphine were tested in this murine model of bone cancer pain in separate groups ($n=6$ per dose group). Animals with confirmed hyperalgesia, as assessed by spontaneous lifting, were behaviorally tested on days 15 and 22 after distal femur tumor inoculation before and 1 h after subcutaneous administration of vehicle, fentanyl (0.02–0.63 mg /kg), sufentanil (0.005–0.04 mg/kg), or morphine (2.5–40 mg/kg).

2.5. Statistics

The statistical analysis was performed by one-way ANOVA to compare behavioral measurements and bone parameters among the experimental groups. To compare behavioral measurements and bone parameters between sham and tumor-bearing animals, a Mann–Whitney U test was used. Results were considered statistically significant at $P<0.05$ (two-tailed). Data are expressed as mean±S.E.M. These analyses and correlations were made and plotted using GraphPad Prism® version 4 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Body weight gain

Body weight was measured systematically in sham and tumor-bearing groups. The average of the body weight before tumor cells or medium injection was the same in each group of animals (sham: 23.5±0.5 g, $n=6$; tumor: 23.8±0.8 g, $n=6$). The sham-operated mice exhibited a continuous increase in body weight throughout the 3-week observation period post-surgery. In tumor-bearing mice, the mean body weight exhibited no overt continuous increase in body weight throughout the 3-week observation period post-surgery. No statistical significance was observed between any of the sham-operated and tumor-bearing groups in terms of the average weight gain at any of the time points: 7 days after tumor inoculation (sham: 24.4±0.6 g, $n=6$; tumor: 24.8±0.5 g, $n=6$), 15 days after cell inoculation (sham: 24.9±0.5 g, $n=6$; tumor: 24.8±0.7 g, $n=6$), and 22 days after cancer cell inoculation (sham: 25.6±0.4 g, $n=6$; tumor: 24.5±0.8 g, $n=6$).

3.2. Spontaneous pain and limb-use on rotarod

Fibrosarcoma-implanted mice showed an increasingly robust induction of pain as the tumor progressed. In this study, the concentration of 2.5×10^5 tumor cells to induce locally bone cancer in mice. The time point for assessing bone destruction and testing drug efficacy were selected on the basis of extensive preliminary data (Vermeirsch et al., 2004).

In the group of mice that received cancer cells inoculation, a significant cumulative duration of the spontaneous lifting compared to sham-operated mice was evident by day 7 post-implantation (Fig. 1A). By day 15, the maximum of spontaneous lifting was reached and this persisted through day 22. No lifting was observed in the left hind paw of the sham group throughout the observation period and none of the mice of either group showed lifting of the hind paws prior to inoculation or vehicle injection.

To evaluate ambulation-induced pain behavior, mice were put on a rotarod and their limb-use was scored for 2 min. By day 7 after inoculation limb-use on the rotarod was significantly affected in tumor-bearing animals compared to sham-operated animals (Fig. 1B). The limb-use thereafter showed a tendency to gradually decrease through day 22 after injection of tumor cells. After day 15 of tumor cell injection, animals generally showed partial to substantial non-use of the tumor-bearing limb and were more affected

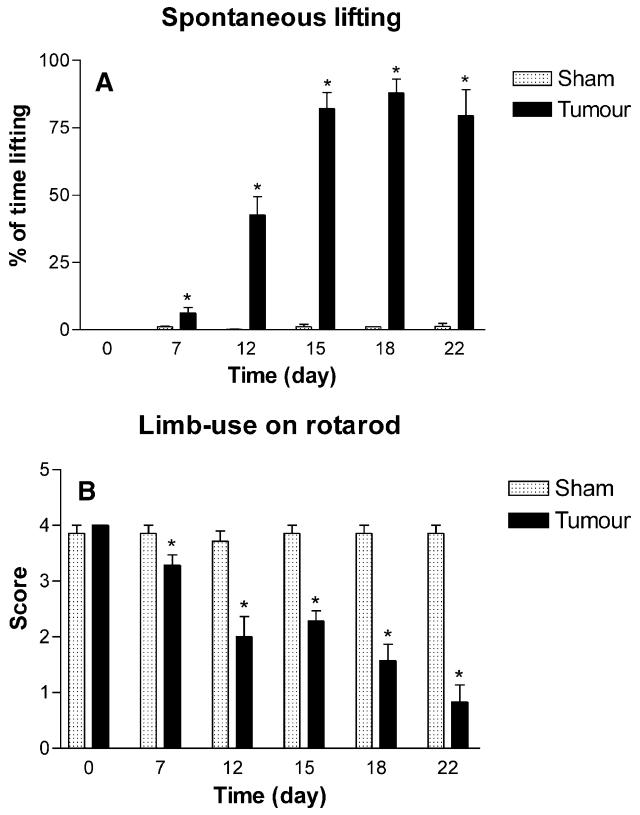

Fig. 1. Time-course of the development of spontaneous lifting and ambulatory limb-use on rotarod in sham and NCTC 2472 sarcoma-injected animals ($n=6$/group). Percentage of time lifting (A) and limb-use on rotarod scores (B) were measured in animal groups prior to and 7, 12, 15, 18, and 22 days after inoculation. Values are expressed as mean±S.E.M. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Mann–Whitney U test. *Significantly different from sham value.

by day 22. The mice of he sham group, injected with medium, demonstrated no reduction in limb-use when running on the rotarod throughout the observation period.

3.3. Evaluation of bone destruction

To evaluate tumor-induced bone destruction over time, μCT-scans of left femoral bones were taken prior to and 7, 9, 12, 15, 18, and 22 days after tumor or medium injection.

The μCT-scan technology allows to produce 3D images of bone structure. In our animal model of tumor-induced bone loss, the representative three-dimensionally reconstructed μCT images of the distal femur illustrated in Fig. 2 show a loss of trabecular bone and deterioration of the trabecular architecture following the implantation and growth of the tumor.

Injection of NCTC 2472 sarcoma cells induced a time-course dependent bone destruction along the distal femoral cortex and was clearly evident by day 12 in tumor-bearing animals compared to sham controls. By day 15, extensive bone lesions were seen which gradually progressed through day 22. Bone lesions consisted of osteolysis and new bone formation accompanied with cortical perforations and frequently destroyed trabeculae. In sham-operated animals, no evidence of bone destruction was present.

The quantitative analysis in the trabecular structure of the 4-mm-long region bellow the patella of distal bone femur revealed significant ($P<0.0001$) changes of the bone structure parameters in the tumor-bearing bones (Fig. 3). In a two-dimensional model based on cross section analysis, the evolution of mean number and the average area of bone fragments indicating bone osteolysis, progressed in a time-dependent manner. An increase of number of bone fragments and a decrease of bone fragment area were evident by day 12 after tumor inoculation (by 145% and 30%, respectively). In a three-dimensional analysis, a gradual increase in bone surface/bone volume ratio by day 12 (by 16%), as well as a decrease in trabecular thickness, trabecular number, and bone volume, which was significant ($P<0.01$) by day 12 (by 10%, 24% and 11%, respectively), demonstrated clearly the destruction of the trabecular bone structure by the tumor-induced process. The general loss of bone architecture due to tumor osteolysis resulted in an increase in bone surface to bone volume ratio of 16% at day 12 for the volume of interest containing cortical and trabecular bone and 105% by day 15 after tumor injection.

The biologic effect of the tumor on bones seems to be an elimination of the entire bone elements and not only thinning of the trabeculae. We noted that the pain behavior and bone destruction response was clearly time-dependent.

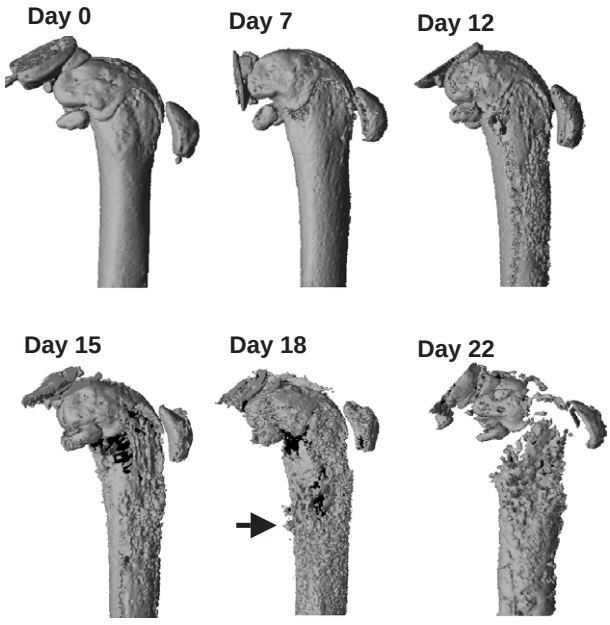

Fig. 2. Three-dimensional μCT re-construction images of the NCTC 2472 sarcoma-injected distal femur in different animal prior to and 7, 12, 15, 18, and 22 days after inoculation. In the animal prior injection (day 0), no bone destruction or bone formation was observed. At 12 (day 12) days after tumor injection, there was a clear bone destruction in tumor-bearing femoral bones. Note the extreme bone destruction with some new bone formation (arrow) in sarcoma-injected animals at day 15 (day 15) and 18 (day 18) after tumor implantation with pathological fractures frequently occurring by day 22 (day 22).

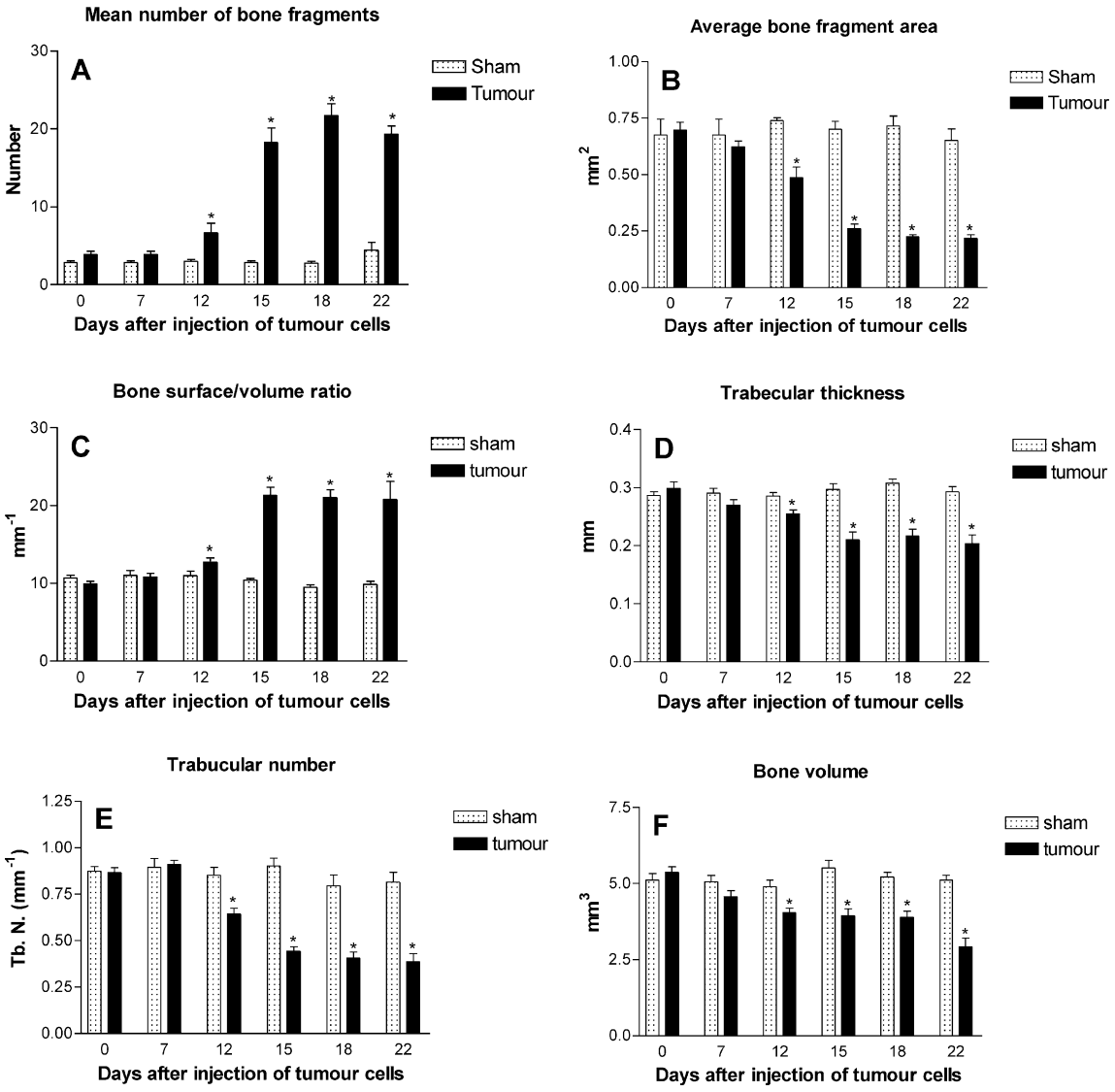

Fig. 3. Microcomputed tomography parameter measurements in the NCTC 2472 sarcoma-injected distal femur of different groups prior to and 7, 12, 15, 18, and 22 days after inoculation ($n=6$/group). The 4 μm region under the patella from distal left femurs in different groups was used for 2- and 3-dimensional analysis. In a 2-dimensional analysis, average of bone fragment area (Av.Obj.Ar, B) decrease and mean number of bone fragment (Obj.N, A) increase indicate bone osteolysis and fragmentation. In a 3-dimensional analysis, bone surface to volume ratio (BS/BV, C) increase indicates bone fragmentation in tumor-bearing animals, while decreased trabecular thickness (Tb.Th., D), and trabecular number (Tb.N., E), bone volume (BV, F) indicate reduction of bone thickness due to tumor-induced osteolysis in tumor-bearing animals. Values are expressed as mean±S.E.M. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Mann–Whitney *U* test. *Significantly different from sham value.

3.4. Correlation between cancer-related pain behavior and bone destruction

We knew from the present and previous studies (Sabino et al., 2002; Vermeirsch et al., 2004) that intramedullary injection of sarcoma cells into femur of mice resulted in a marked bone destruction response after tumor inoculation. To further delineate a potential relationship between all morphological and behavioural changes demonstrated so far, we examined the time dependence of their occurrence in distal bone femur of tumor or sham-injected animals. In this way, the loss of bone structure due to tumor osteolysis was found to be correlated with the pain behavior assessments (Figs. 4 and 5).

A fairly reasonable correlation was obtained when comparing the morphometric data (μCT-scan parameters) determined by 2D and 3D analysis with the spontaneous paw lifting obtained from individual animals. As shown in Fig. 4, increased spontaneous lifting behavior was highly correlated in a non-linear manner with an increased mean number of bone fragments and bone surface/volume ratio($R=0.95$ and 0.94, respectively; $P<0.0001$). Spontaneous lifting behavior was also highly correlated in a non-linear manner with a decreased average area of bone fragments and bone trabecular number ($R=0.9$ and 0.94, respectively; $P<0.0001$), and to a lesser degree with a decrease of trabecular thickness and bone volume ($R=0.82$ and 0.77, respectively; $P<0.0001$).

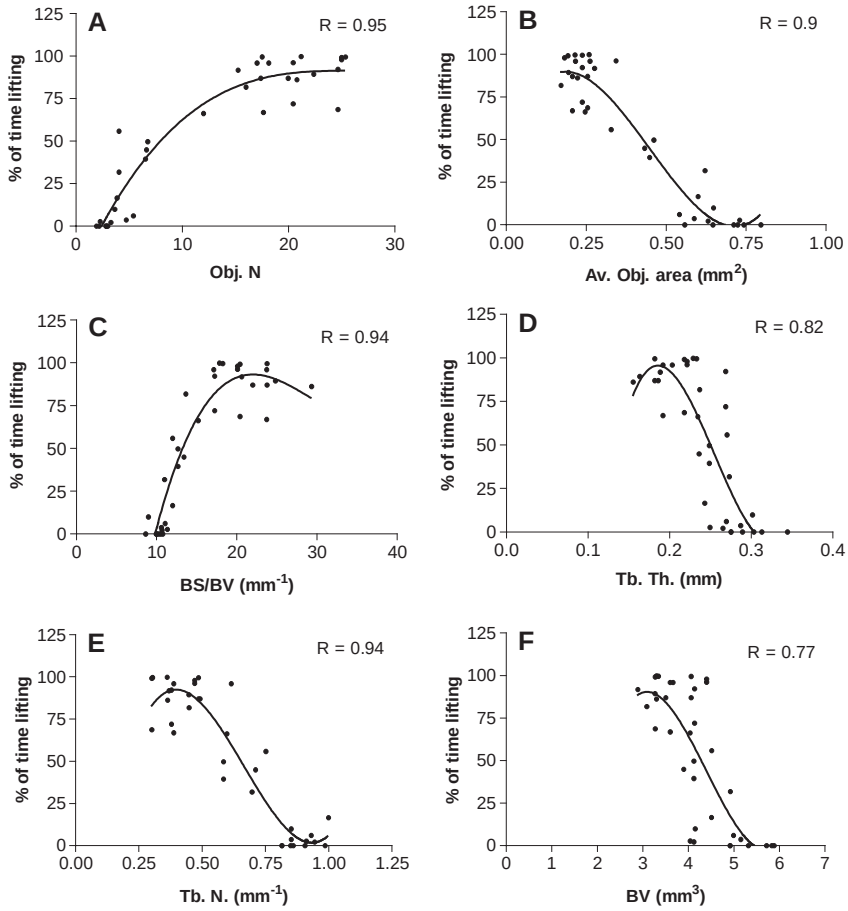

Fig. 4. Correlation between bone destruction parameters and bone cancer-related pain behavior in different groups ($n=6$/group) prior to and 7, 12, 15, 18, and 22 days after inoculation of the femur with NCTC 2472 sarcoma cells. Spontaneous lifting was plotted against μCT parameters, mean number of bone fragments (Obj.N.), bone surface to volume ratio (BS/BV) increase indicates bone fragmentation in tumor-bearing animals, while decreased average of bone fragment area (Avg.Obj.Ar) trabecular thickness (Tb.Th.), bone volume (BV), trabecular number (Tb.N.), and trabecular separation (Tb.Sp.) indicate reduction of bone thickness. Curvilinear polynomial functions were fitted by GraphPad software (for Panel A, $R=0.95$, $P<0.0001$; for Panel B, $R=0.9$, $P<0.0001$; for Panel C, $R=0.94$, $P<0.0001$; for Panel D, $R=0.82$, $P<0.0001$; for Panel E, $R=0.94$, $P<0.0001$; for Panel F, $R=0.77$, $P<0.0001$).

Results presented in Fig. 5 clearly show that paw-use impairment on rotarod at the level of all individual animals used to generate the data of Fig. 1B were correlated in a non-linear fashion to bone structure parameters. Fig. 5 shows that the paw impairment scores were indeed non-linearly correlated with a decrease of the average area of bone fragments, trabecular thickness, bone trabecular number, and bone volume ($R=0.83$, 0.87, 0.77, and 0.79, respectively; $P<0.0001$).

Paw-use impairment on rotarod was also correlated in a significant non-linear manner with an increased mean number of bone fragments and bone surface/volume ratio ($R=0.85$ and 0.77, respectively; $P<0.0001$).

3.5. Antinociceptive effects of fentanyl, sufentanil, and morphine

Sensitivity of the bone tumor-related pain behavior to fentanyl, sufentanil, and morphine was tested in separate groups of tumor-bearing animals. Mice were randomly distributed between three groups of 6 animals receiving a treatment on day 15 and 22 with vehicle, 0.2–0.63 mg/kg fentanyl, 0.005–0.04 mg/kg sufentanil, or 2.5–40 mg/kg morphine (Fig. 6). No significant differences in behavioral testing before drug treatment were observed between different groups (Mann–Whitney U test, exact 2-sided $p>0.05$). Animals were tested 1 h after subcutaneous administration of vehicle, fentanyl, sufentanil or morphine. In tumor-inoculated mice with confirmed hyperalgesia, treatment with fentanyl, sufentanil, and morphine was effective in reducing bone cancer pain-related behaviors whereas, tumor-bearing vehicle-treated mice showed signs of spontaneous pain and reduced limb-use while ambulating on the rotarod. As compared to lifting behavior before treatment, vehicle-treated animals showed no amelioration of pain behavior. In contrast fentanyl, sufentanil, and morphine-treatment induced a significant ($P<0.001$) dose-dependent decrease in duration of spontaneous lifting on days 15 and 22 in cancer cell-bearing mice. A complete relief from pain-related behavior was achieved with the following doses: ≥ 0.16 mg/kg fentanyl, ≥ 0.02 mg/kg sufentanil, and 20 mg/kg morphine.

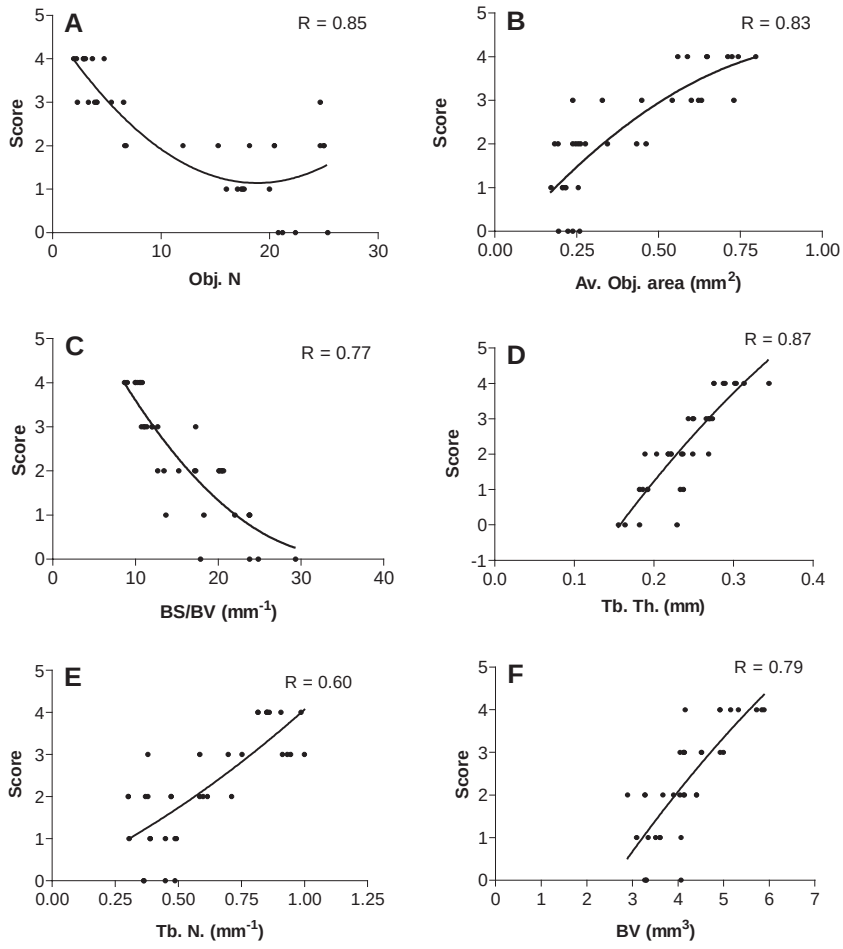

Fig. 5. Correlation between bone destruction parameters and bone cancer-related paw use impairment on rotarod in different groups ($n=6$/group) prior to and 7, 12, 15, 18, and 22 days after inoculation of the femur with NCTC2472 sarcoma cells. Rotarod scores were plotted against μCT parameters, mean number of bone fragments (Obj.N.), bone surface to volume ratio (BS/BV) increase indicates bone fragmentation in tumor-bearing animals, while decreased trabecular thickness (Tb.Th.), bone volume (BV), trabecular number (Tb.N.), and trabecular separation (Tb.Sp.) indicate reduction of bone thickness. Curvilinear polynomial functions were fitted by GraphPad software (for Panel A, $R=0.85$, $P<0.0001$; for Panel B, $R=0.83$, $P<0.0001$; for Panel C, $R=0.77$, $P<0.0001$; for Panel D, $R=0.87$, $P<0.0001$; Panel E, $R=0.60$, $P<0.0001$; Panel F, $R=0.79$, $P<0.0001$).

These mice also exhibited pain-induced impairment of their limbs as evidenced by reduction in limb-use during forced ambulation on rotarod. Acute administration of fentanyl, sufentanil, and morphine improved limb-use score compared to vehicle-inoculated groups (Fig. 7). The increase of limb-use was most important in the animals at doses of 0.163 mg/kg fentanyl, 0.02 mg/kg sufentanil, or 20 mg/kg morphine treatment ($P<0.05$).

4. Discussion

Bone cancer pain resulting from primary tumors or tumors that metastasize to bone is very common. Patients with this kind of pain may be difficult to treat (Mercadante, 1997; Mercadante and Arcuri, 1998, Portenoy et al., 1999).

Novel analgesics with greater efficacy are urgently needed for alleviation of cancer-related pain. However, the principal barrier for development of optimal analgesics for cancer pain has been the poor understanding of basic mechanisms that contribute to cancer pain. This situation is in part due to the lack of valid animal models of cancer pain.

Recently, a few animal models of bone cancer-related pain have been described (Schwei et al., 1999; Honore et al., 2000a; Wacnik et al., 2001; Medhurst et al., 2002; Walker et al., 2002) and most of these experimental models require injection or implantation of neoplastic cell into one specific bone, allowing, therefore, to easily perform behavioral pain tests (Lelekakis et al., 1999; Schwei et al., 1999; Honore et al., 2000a; Vermeirsch et al., 2004).

The murine model of bone cancer pain used in this study was developed by Schwei et al. (1999) and seems to share many characteristics of bone cancer pain in humans (Clohisy and Mantyh, 2003). This model is potentially useful in developing novel pharmacology treatment strategies and providing new insight into mechanisms through which malignant bone disease induces pain (Mantyh, 2002; Mantyh et al., 2002; Honore et al., 2000a).

The development of this model is of value in providing new insight into mechanisms through which malignant bone

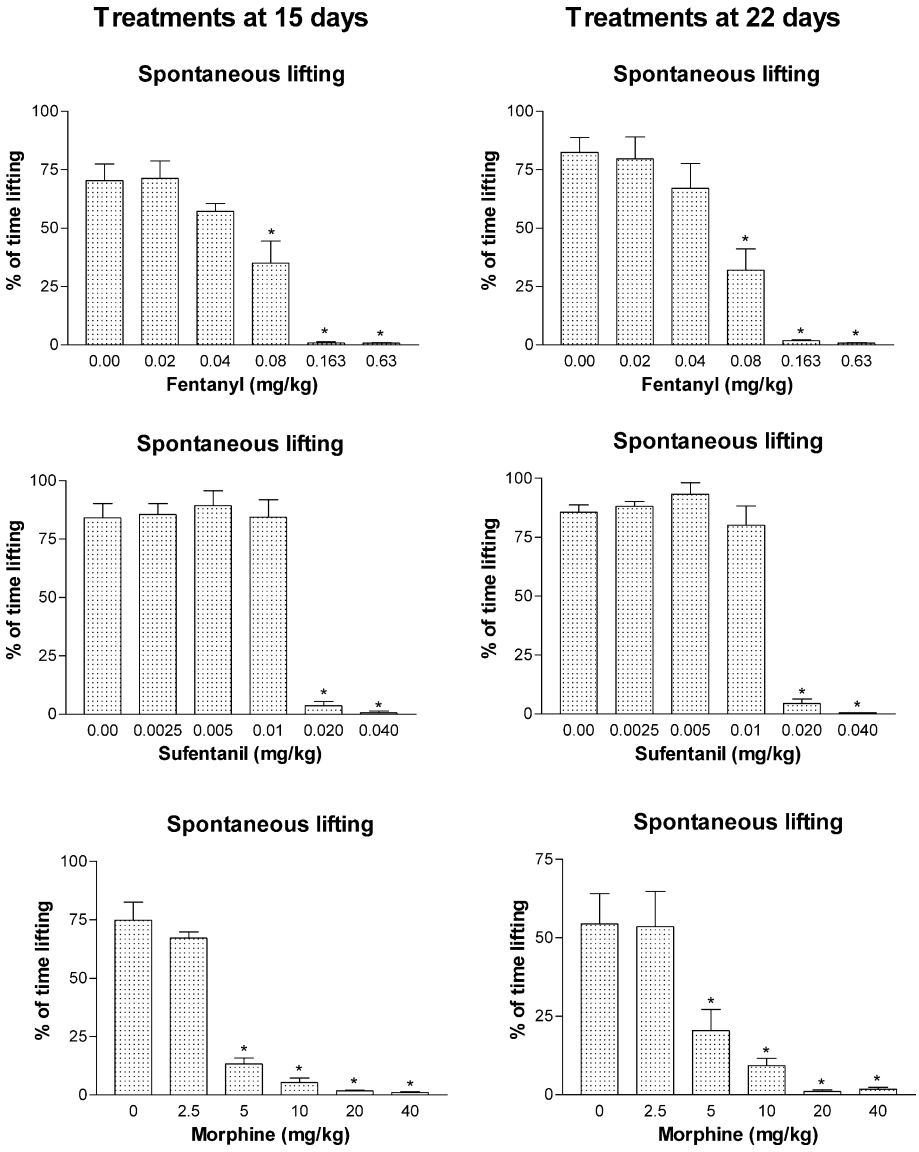

Fig. 6. Dose-dependent effect of fentanyl, sufentanil, and morphine on bone cancer-related spontaneous paw lifting behavior in NCTC 2472 sarcoma-injected animals (n=6/group). Percentage of time lifting was measured after 1 h in tumor-bearing animals treated subcutaneously with different doses of fentanyl (0.02–0.63 mg/kg s.c.), sufentanil (0.005–0.04 mg/kg s.c.), and morphine (2.5–40 mg/kg s.c.) on day 15, and 22 after tumor inoculation. Values are expressed as mean±S.E.M. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Mann–Whitney U test. *Significantly different from sham value.

disease induces pain and could lead to novel pharmacology treatment strategies (Mantyh, 2002; Mantyh et al., 2002; Honore et al., 2000a).

Histologic analyses of osteolytic tumors in experimental models consistently have demonstrated that osteoclasts are stimulated by osteolytic tumors and are responsible for cancer-induced osteolysis (Clohisy et al., 1996;Guise et al., 1996).

In addition, the tumor-induced osteoclast activity as well as secretion of various factors, such as prostaglandins, endothelins, epidermal growth factor, and cytokines, sensitize or excite primary afferent neurons. Receptors for many of these factors are expressed by primary afferent neurons with bone and may contribute to the severity and heterogeneity of bone cancer pain. However, recent results have shown that selective antagonism of EtA receptor or administration of anti-NGF antibody can produce pronounced antinociceptive effects in mice model of bone cancer pain without affecting the disease progression (Peters et al., 2004; Sevcik et al., 2005).

We have demonstrated that tumor growth following inoculation of NCTC 2472 sarcoma cells into medullary space of the distal femur in the mouse induces pain-related behavior and progressive insults to the bone. A gradual development of spontaneous pain over time was observed in tumor-bearing animals and, in parallel, there was decreased limb-use during forced ambulation on a rotarod.

Cancer pain associated with direct tumor involvement can be due to nerve injury of peripheral nerves, nerve plexuses, nerve roots or the spinal cord by compression or

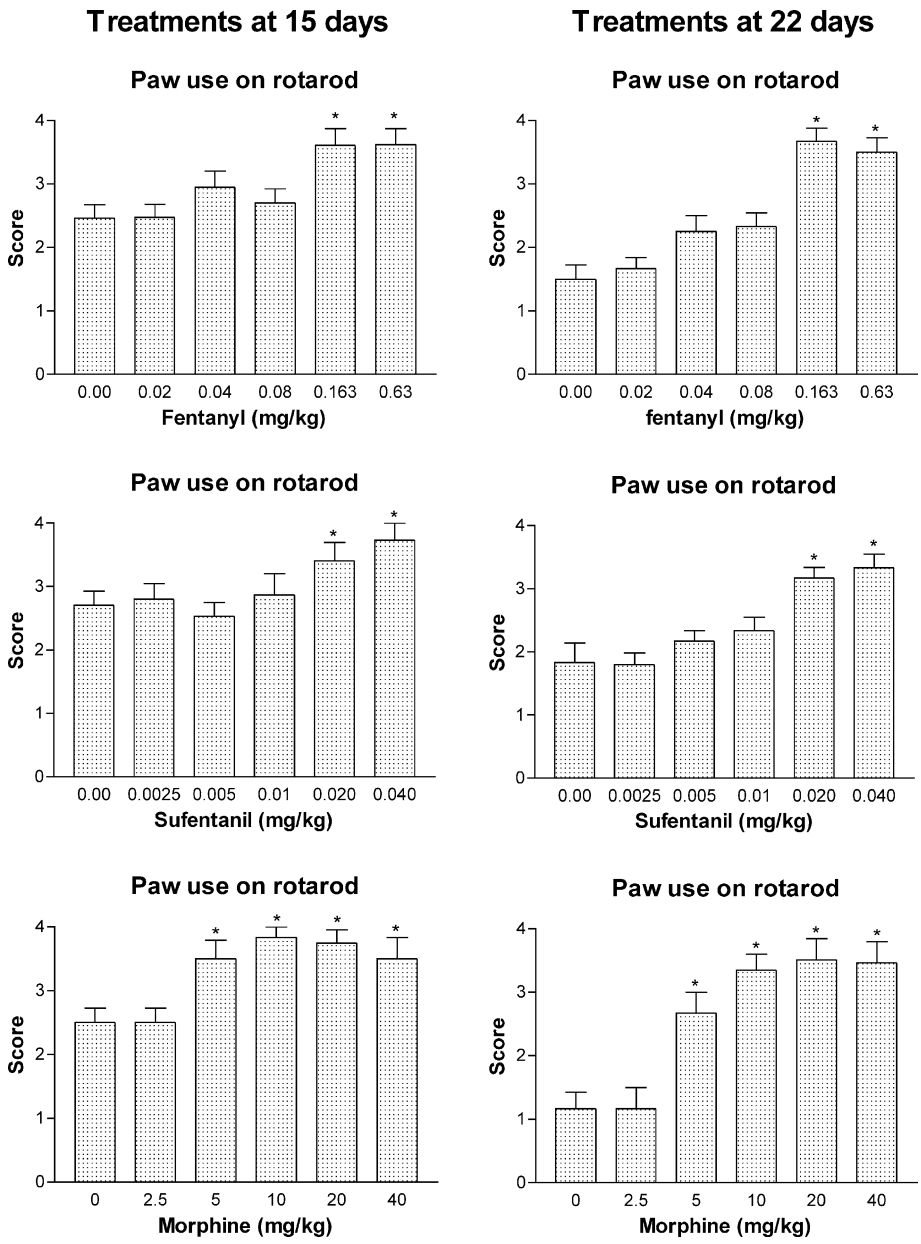

Fig. 7. Dose-dependent effect of fentanyl, sufentanil, and morphine on bone cancer-related paw-impairment behavior in NCTC 2472 sarcoma-injected animals ($n=6$/group). Limb-use on rotarod was scored after 1 h in tumor-bearing animals treated subcutaneously with different doses of fentanyl (0.02–0.63 mg/kg s.c.), sufentanil (0.005–0.04 mg/kg s.c.), and morphine (2.5–40 mg/kg s.c.) on days 15 and 22 after tumor inoculation. Values are expressed as mean ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA), followed by the Mann–Whitney *U* test. *Significantly different from sham value.

ischemia (Coleman, 1997; Portenoy and Lesage, 1999). In our model we observed that with the advancement of the tumor, spontaneous pain persisted.

In previous studies, bone destruction was assessed in a more limited way by assigning scores to radiographs of tumor-bearing bones (Schwei et al., 1999; Honore et al., 2000a). In the present study, the use of μCT-scan technology allows us to produce two- and three-dimensionally images, visualization of bone architecture, and produce 2D and 3D parameters of bone trabecular structure in an animal model for tumor-induced bone loss. This new technology offers a non-destructive approach to the study of the trabecular bone architecture in preclinical animal models of neoplastic bone disease. Significant differences in 2D and 3D morphometric parameters were found between the tumor-bearing bones and the sham-injected animals. The biologic effect of the tumor on bone structure seems to be an elimination of the entire bone elements and not only thinning of the trabeculae.

μCT-Scan bone morphology and pain-related behavioral measurements over-time showed clear significant correlations. Parameters assessing the degree of fragmentation of the bone, mean number of bone fragments, trabecular number, and bone surface to volume ratio, were highly correlated with tumor-induced pain behavior. We found also that decreased limb-use was highly correlated ($P<0.0001$) with the alteration in bone trabecular thickness, which

represents the strength and normal bone architecture. In the present study, the evaluation of the tumor on bone structure showed a small effect on trabecular thickness of the remaining trabeculae in the tumor-bearing bones compared with sham control bones and a large increase in bone surface/bone volume ratio. However, the biological effect of the tumor seems to be a destruction of the entire bone structure and not only thinning of the trabeculae. Furthermore, diseases that affect the bone structure and trabecular thickness are well known to cause pain and reduce mobility in humans, which could be reproduced in animal models.

The evaluation of bone architecture in pathologically altered bone is of great importance for the understanding of the relationship between bone structure and pain behavior. Furthermore, it is of great clinical importance to understand how tumor in bone affects the bone structure. In preclinical research numerous animal studies are necessary for the development of new therapeutic agents for bone cancer pain diseases.

Results from clinical and experimental data suggest that cancer-induced osteolysis contributes to bone cancer pain. Clinical trials and experimental studies in animals with biphosphonates or osteoprotegerin and analysis of bone metabolism in patients and animals with bone cancer suggest that cancer-induced bone resorption is linked to bone cancer pain (Coleman, 1998; Berruti et al., 1999; Lipton et al., 2000; Pelger et al., 1998; Honore et al., 2000b; Luger et al., 2001). These clinical and experimental investigations have not yet identified a direct cause-and-effect relation between cancer-induced bone loss and pain.

Our primary objective was to delineate the relationship between tumor-induced bone lesions and pain behavior. However, the efficacy of compounds for treating bone cancer pain should be evaluated over time at a severe stage of bone destruction. In addition, relating analgesic efficacy of compounds to bone destruction is an interesting tool to evaluate their potency for treatment of bone cancer pain. Moreover, the analgesic efficacy of compounds should be evaluated independent of prevention of metastasis or bone lesions. However, it is well known from the literature that agents, such as osteoprotegerin, which reduce bone destruction, even in advanced stages, reduce pain behaviors (Honore et al., 2000b; Honore and Mantyh, 2000; Luger et al., 2001).

The availability of this model has favoured the assessment of the analgesic efficacy rendered by the administration of the different drugs such as opiates (Luger et al., 2002; Vermeirsch et al., 2004), cannabinoids (Kehl et al., 2003), biphosphonates (Sevcik et al., 2004), COX-2 inhibitors (Sabino et al., 2002) and endothelin-type A receptor antagonists (Peters et al., 2004).

In tumor-inoculated mice, we demonstrated that acute treatment with fentanyl, sufentanil, and morphine was effective in reducing bone cancer pain-related behaviors. Fentanyl, sufentanil, and morphine-treatment induced a dose-dependent decrease in duration of spontaneous lifting and improved limb-use score on rotarod compared to vehicle-treated groups on both time points. Experimentally, it has been shown that the osteosarcoma cancer-related pain behaviors in a murine model, can be alleviated with morphine (Menéndez et al., 2003; Vermeirsch et al., 2004), although at rather high doses, as occurs in clinical settings.

In summary, we have demonstrated that tumor growth in mice inoculated with osteosarcoma cells is associated with cancer-related pain behavior and bone destruction. A good correlation could be obtained between the extent of morphological changes and spontaneous lifting pain behavior.

Fentanyl, sufentanil, and morphine were effective in this murine model of bone cancer pain, and all three opioids produced significant analgesic activity.

The mechanisms of tumor-induced pain are believed to be mediated by the similar factors in human and mice. Both are mediated by osteoclast activity-induced bone destruction, release of inflammatory and tumor factors, and tumor-induced injury to nerve fibres that innervate the bone (Mantyh et al., 2002).

The present data and results obtained in human patients (Clohisy and Ramnaraine, 1998) with bone cancer showed that pain tends to increase in relation to bone destruction induced by osteoclast activity. Consequently, the combination of new drugs targeting osteoclast activity-mediated bone destruction and analgesic products such as opioids might yield a therapeutic approach for treating this type of cancer pain.

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