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Analgesic effects of nonsteroidal antiinflammatory drugs, acetaminophen, and morphine in a mouse model of bone cancer pain

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Abstract
Purpose. Bone metastasis is one of the major causes of cancer-related pain, and not all bone cancer pain can be effectively treated. Recently, a mouse model of bone cancer pain was introduced. To test the analgesic effects of nonsteroidal antiinflammatory drugs on bone cancer pain, the authors examined the effects of oral administration of a cyclooxygenase-1 (COX-1) selective inhibitor (SC560), a COX-2 selective inhibitor (celecoxib), and a nonselective COX inhibitor (indomethacin) on bone cancer pain and compared these effects to the effect of orally administered acetaminophen and morphine.
Methods. An animal model of bone cancer pain was induced by injecting osteolytic murine sarcoma cells in the mouse femur. Drugs were administered orally 2 weeks after tumor-cell implantation, and the level of bone cancer pain was assessed 30, 60, 90, 120, and 180 min after drug administration.
Results. Oral administration of acetaminophen, indomethacin, and morphine, but not of SC560 or celecoxib, produced an analgesic effect on bone cancer pain. Co-administration of a subanalgesic does of morphine with acetaminophen enhanced the analgesic effect of acetaminophen.
Conclusion. These data suggest that bone cancer pain is effectively treated by oral administration of indomethacin, acetaminophen, and morphine and that the co-administration of acetaminophen and an opioid provides a beneficial effect when treating of bone cancer pain.

Key words Cancer pain · COX · Acetaminophen · Morphine

Introduction

The presence of bone metastases is the most common cause of cancer-related pain [1,2]. Bone metastases also cause bone fracture, hypercalcemia, and neurologic deficits and may deteriorate the quality of life in patients with prolonged survival [3].

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According to the World Health Organization (WHO) guidelines on cancer pain relief, nonsteroidal antiinflammatory drugs (NSAIDs) and acetaminophen are the recommended drugs for the first step on the analgesic ladder [4], and they are sometimes co-administered with morphine for the treatment of cancer pain. The guidelines in the management of NSAIDs or acetaminophen in the patient with cancer pain are largely empiric, drawn from clinical experience. Thus, it is important to understand the underlying mechanisms of tumor-induced bone cancer pain and to determine the precise mechanisms by which NSAIDs and acetaminophen reduce the level of bone cancer pain.

Prostaglandins (PGs) are thought to play an important role in nociceptive transmission at peripheral sites and in the spinal cord [5–7]. PGs are synthesized in tissues by cyclooxygenase (COX), which is an enzyme that catalyzes the conversion of arachidonic acid to PGs. Two COX forms have been characterized: COX-1 is constitutively expressed, whereas COX-2 is highly inducible in response to cytokines, growth factors, or other inflammatory stimuli [6]. Recently, a third, distinct COX isozyme, COX-3, was reported; it is a variant of COX-1 [8]. NSAIDs, such as aspirin and indomethacin, inhibit COX activity and elicit antiinflammatory and analgesic effects [6]; and most NSAIDs inhibit COX-1, COX-2, and COX-3 activity [8].

Acetaminophen produces an analgesic effect, but the mechanisms by which this is accomplished are not fully understood. Recently, acetaminophen has been found to act as a selective inhibitor of COX-3 but to have no effect on COX-1 or COX-2 [8]. However, Snipes et al. [9] reported that rat COX-3 does not have cyclooxygenase activity and does not have any effect on the inhibition of PG production by acetaminophen. Moreover, another acetaminophen-sensitive COX has been reported that is a variant of COX-2 [10].

Recently, an animal model of bone cancer pain, produced by injecting osteolytic murine sarcoma cells into

the mouse femur, has been developed [11]. In the present study, we investigated the analgesic effects of orally administered COX-1 selective inhibitor, COX-2 selective inhibitor, nonselective COX inhibitor, acetaminophen, and morphine in an animal model of bone cancer pain. We also examined the analgesic effect of co-administration of morphine with acetaminophen on bone cancer pain.

Material and methods

The following investigations were performed according to the protocol approved by the Institutional Animal Care Committee of Chiba University, Chiba, Japan.

Strain of mouse and injection of osteolytic cells

Experiments were performed on adult male C3H/HeJ mice approximately 5 weeks old and weighing 20–25 g (Japan SLC, Shizuoka, Japan). This strain was chosen for its histocompatibility with the NCTC 2472 tumor line [American Type Culture Collection (ATCC), Rockville, MD, USA], previously shown to form lytic lesions in bone after intramedullary injection [12,13]. The mice were housed in a vivarium with a 12-h alternating light-dark cycle and were given food and water ad libitum.

Tumor cells were maintained in NCTC135 medium containing 10% horse serum and passaged weekly according to ATCC instructions. A tumor cell injection protocol was followed as previously described by Schwei et al. [11]. Briefly, mice were anesthetized with sodium pentobarbital (50 mg·kg^{-1} i.p.), and a right knee arthrotomy was performed. Tumor cells, 10^5 in 20 µl of α minimum essential medium (αMEM; Sigma, St. Louis, MO, USA) containing 1% bovine serum albumin (BSA), were injected directly into the medullary cavity of the distal femur. To obtain control data, 20 µl of vehicle (αMEN containing 1% BSA) was injected into the medullary cavity of the distal femur.

Assessing the extent of bone destruction

The extent of bone destruction (osteolysis) in tumor-injected femur 2 and 3 weeks after implantation of tumor cells was radiologically assessed using standard X-ray film. According to the scale described by Schwei et al. [11], The loss of bone density was quantified on a scale of 0–3: 0, normal bone; 1, minor loss of bone in the medullary canal; 2, substantial loss of bone in the medullary canal with some destruction of the distal femur; and 3, substantial loss of bone in the medullary canal with major destruction of the distal femur.

Nociceptive test

Mice were placed in a clear plastic observation box (9 × 11 × 20 cm) with a wire mesh floor and allowed to habituate for a period of about 5 min. After acclimation, pain-related behaviors were induced by application of a von Frey monofilament (0.166 g) to the distal femur of the tumor cells-implanted paw (the site of implantation of the tumor cells) every second for 20 s (20 stimuli). The number of pain-related behaviors (characterized as guarding, strong withdrawal, fighting, and biting) was recorded.

Drugs and administration

The orally administered drugs were suspended in a methylcellulose (MC) 0.5% solution and administered in a volume of 0.5 ml. For this administration, a stainless-steel tube was inserted through the esophagus to the stomach of a restrained animal. The orally administered drugs were SC560 (10, 30, 100, and 300 mg·kg^{-1}), a COX-1 inhibitor (Pharmacia, Peapack, NJ, USA); celecoxib (10, 30, 100, and 300 mg·kg^{-1}), a COX-2 inhibitor (Pharmacia); indomethacin (0.1, 1, 10, and 100 mg·kg^{-1}), a nonselective COX inhibitor (Wako, Osaka, Japan); acetaminopohen (0.3, 3, 30, 300, and 3000 mg·kg^{-1}) (Sigma, St. Louis, MO, USA); or morphine hydrochloride (10, 13, 17.5, 30, and 100 mg·kg^{-1}) (Takeda, Osaka, Japan). Naloxone hydrochloride (10 mg·kg^{-1}) (Sigma) was dissolved in saline and was administered intraperitoneally.

Experimental protocol

A preliminary study performed in our laboratory revealed that severe pain-related behavior was induced by application of a von Frey filament 2 weeks after implantation of the tumor cells. Three weeks after implantation, radiological examination revealed a bone fracture at the site of implantation in all animals (Fig. 1) and it was not possible to test the level of pain induced by bone cancer itself 3 weeks after implantation. Thus, we examined the effect of drugs 2 weeks after implantation of the tumor cells. All animals were tested for pain-related behaviors before the injection of tumor cells. The animals were tested on day 14 after implantation of the tumor cells, before drug administration, and then 30, 60, 90, 120, and 180 min after drug administration. To verify that the analgesic effect of morphine was due to the interaction with an opioid receptor, the most effective dose of morphine (30 mg·kg^{-1}) was administered orally, followed 60 min later by intraperitoneal injection of naloxone 10 mg·kg^{-1}; the number of pain-related behaviors was measured 30 min after the naloxone administration. To examine the interaction of orally

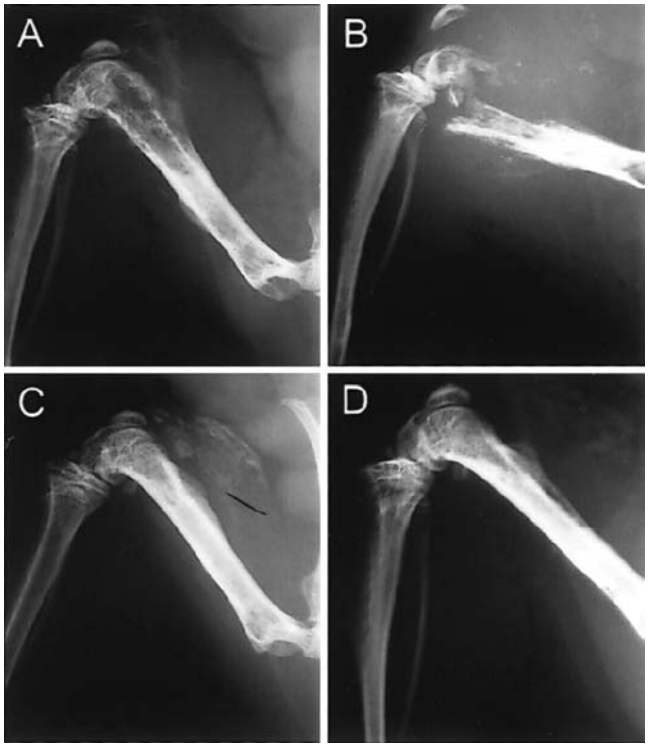

Fig. 1. Radiographs of the femurs injected with osteolytic cells (**A** 14 days after implantation, **B** 21 days after implantation) or 20 µl of α minimum essential medium containing 1% bovine serum albumin (**C** 14 days after the injection, **D** 21 days after the injection) to the medullary cavity. In the vehicle-injected mice, the radiographs show normal bone. **A** In the mice with implanted osteolytic cells, the radiograph shows substantial loss of bone in the medullary canal with some destruction of the distal femur 2 weeks after implantation. **B** Three weeks after implantation, the radiograph shows a bone fracture at the site of the tumor-cell implantation

administered morphine with orally administered acetaminophen, morphine 13 mg·kg^{-1} was co-administered with acetaminophen. Oral administration of morphine 13 mg·kg^{-1} had no analgesic effect in the present study. All animals were euthanized with an overdose of barbiturate after completion of all behavioral analyses.

Statistical tests

To determine whether the implantation of tumor cells induced significant bone cancer pain, we compared the preimplantation number of pain-related behaviors induced by application of the von Frey filament with the number of pain-related behaviors 2 weeks after the implantation with a *t*-test. The effect of vehicle injection into the medullary cavity was also analyzed with a *t*-text. To compare the baseline data (before drug administration) between groups, one-way analysis of variance (ANOVA) was used. The time-response data are presented as the mean number of pain-related responses (±SEM) induced by the 20 applications of a von Frey filament. For the dose-response analysis, the minimum number of pain-related responses during the entire time course was used. The use of the minimum number of pain-related responses allows us to examine the maximum drug effect despite the variation in the time course of drug absorption after oral administration. To analyze dose dependenc, one-way ANOVA with the Dunnett's test was used. To analyze the effect of naloxone on the analgesic effect of morphine, the paired *t*-text was used. A simple linear regression analysis was used for the interaction study of morphine and acetaminophen. To compare the slopes and elevation of the regression lines, we used a *t*-text [14].

Wherever appropriate, results are expressed as the mean ± SD. Critical values that reached a $P < 0.05$ level of significance were considered statistically significant.

Results

Before the implantation of tumor cells the number of pain-related behaviors was 1.5 ± 1.7 ($n = 132$), and 2 weeks after the implantation the number was 17.2 ± 2.5. The implantation of osteolytic cells significantly increased the number of pain-related behaviors ($P < 0.001$, *t*-text). In the vehicle (20 µl of αMEN containing 1% BSA)-injected mice, the number of pain-related behaviors before the vehicle injection was 0.6 ± 0.9 ($n = 5$) and the number 2 weeks after vehicle injection was 2.6 ± 2.1. There is no difference between the number of pain-related behaviors before the vehicle injection and that 2 weeks after the vehicle injection ($P > 0.05$, *t*-text).

Radiological evaluation of bone destruction showed that 2 weeks after the implantation of osteolytic cells all of the animals had a bone destruction score of 2 and that 2 weeks after vehicle injection into the medullary cavity all of the animals had a bone destruction score of 1 (Fig. 1). Three weeks after osteolytic cell implantation, all of the animals had a bone destruction score of 3 and that 3 weeks after vehicle injection all of the animals had a bone destruction score of 1 (Fig. 1).

No difference was apparent between the predrug number of pain-related behaviors induced by the 20 applications of the von Frey filament in each group (data not shown, $P > 0.05$ by one-way ANOVA), suggesting that all the groups had the same level of bone cancer pain before drug administration.

Oral administration of acetaminophen decreased the minimum number of pain-related responses in a dose-dependent manner at doses between 0.3 and 300 mg·kg^{-1}, and the dose-response curve of acetami-

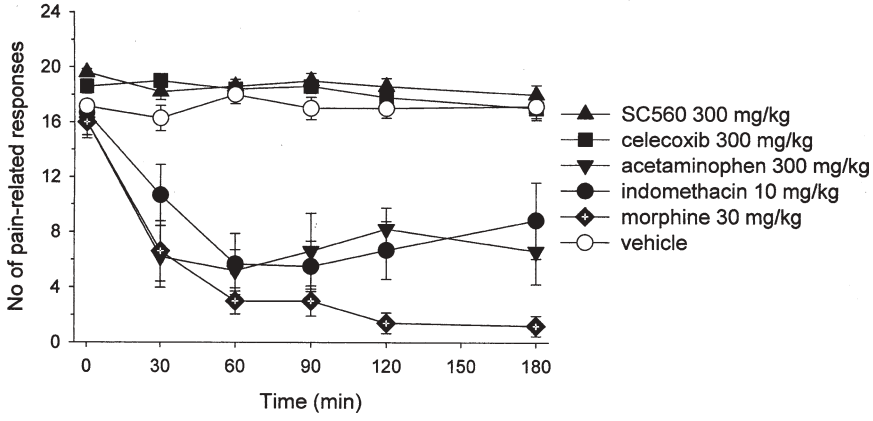

Fig. 2. Effect of oral administration of SC560 300 mg·kg^{-1}, celecoxib 300 mg·kg^{-1}, acetaminophen 300 mg·kg^{-1}, indomethacin 10 mg·kg^{-1}, morphine 30 mg·kg^{-1}, and vehicle (0.5% methylcellulose) on the time course of the number of pain-related behaviors induced by 20 applications of a 0.166-g von Frey filament to the distal femur of the tumor cells-implanted paw. SC560 300 mg·kg^{-1} and celecoxib 300 mg·kg^{-1} are the highest doses applied in the present study. Indomethacin 10 mg·kg^{-1}, acetaminophen 300 mg·kg^{-1}, and morphine 30 mg·kg^{-1} are the most effective doses of each drug. Drugs were administered orally 14 days after tumor-cell implantation. The number of pain-related responses is plotted versus the time after drug administration. Each line represents the group mean and SEM of five or six mice

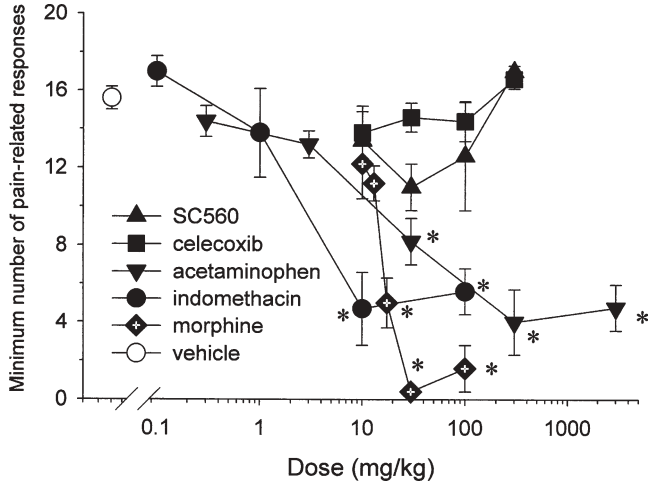

Fig. 3. Dose-response curves for oral administration of SC560, celecoxib, acetaminophen, indomethacin, morphine, and vehicle (0.5% methylcellulose) representing the minimum number of pain-related behaviors for each drug. Drugs were administered orally 14 days after tumor-cell implantation. Each point represents the mean and SEM of five or six mice. Acetaminophen, indomethacin, and morphine, but not SC560 or celecoxib, significantly decreased the minimum number of pain-related behaviors in a dose-dependent manner. * $P < 0.05$ compared with the vehicle-treated mice

nophen showed limited efficacy, with a plateau effect at an acetaminophen dose of 3000 mg·kg^{-1} ($P < 0.005$ by one-way ANOVA) (Figs. 2, 3). Oral administration of indomethacin decreased the minimum number of pain-related behaviors in a dose-dependent manner at doses between 0.1 and 10 mg·kg^{-1}, and the dose-response curve of indomethacin showed limited efficacy, with a plateau effect at a dose of 100 mg·kg^{-1} ($P < 0.001$, one-way ANOVA) (Figs. 2, 3). Oral administration of either SC560 or celecoxib had no effect on the minimum number of pain-related responses at doses between 10 and 300 mg·kg^{-1} ($P > 0.05$, one-way ANOVA) (Figs. 2, 3). Oral administration of morphine decreased the minimum number of pain-related responses in a dose-dependent manner at doses between 10 and 30 mg·kg^{-1}, and the dose-response curve of morphine showed limited efficacy, with a plateau effect at a dose of 100 mg/kg ($P < 0.01$, one-way ANOVA) (Figs. 2, 3).

In the naloxone study, oral administration of morphine 30 mg·kg^{-1} reduced the number of pain-related behaviors 60 min after morphine administration; this effect of morphine was completely antagonized with naloxone 10 mg·kg^{-1} (number of pain-related behaviors: before morphine 19 ± 1.4; 60 min after morphine 5.3 ± 1.3; after naloxone 19 ± 1.0; $P < 0.001$, paired *t*-test).

Oral administration of morphine 13 mg·kg^{-1} had no effect on the minimum number of pain-related responses when compared with the vehicle-treated mice ($P > 0.05$ by ANOVA) (Fig. 3). Co-administration of morphine 13 mg·kg^{-1} with acetaminophen shifted the dose-response curve of acetaminophen to the left in a parallel fashion ($P < 0.05$ by *t*-test) (Fig. 4).

Discussion

The present study clearly demonstrated that oral administration of indomethacin, but not SC560 or

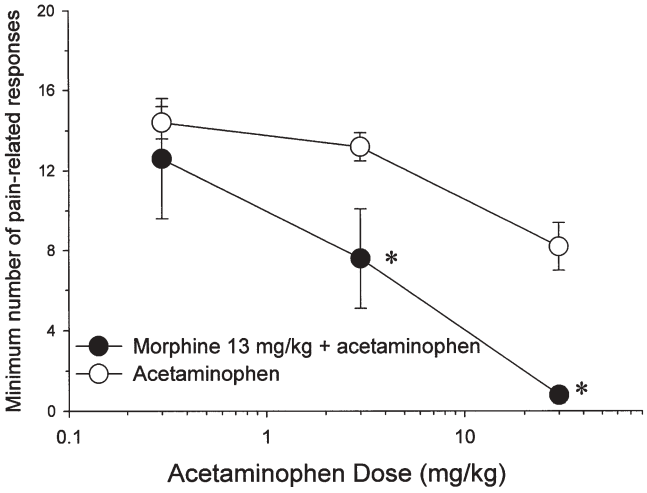

Fig. 4. Dose-response curve for oral co-administration of acetaminophen with morphine 13 mg·kg^{-1}, representing the minimum number of pain-related behaviors. Drugs were administered orally 14 days after tumor-cell implantation. Each point represents the mean and SEM of five mice. For comparison, a dose-response curve of acetaminophen is also presented. Co-administration of morphine 13 mg·kg^{-1} with acetaminophen significantly shifted the dose-response curve of acetaminophen to the left ($P < 0.005$ by t-test)

celecoxib, attenuated the number of pain-related behaviors, induced by application of a von Frey filament, in a dose-dependent manner. As mentioned above, SC560 is a COX-1 selective inhibitor, celecoxib is a COX-2 selective inhibitor, and indomethacin is a non-selective COX-1 and COX-2 inhibitor. The data indicated that the inhibition of either COX-1 alone or COX-2 alone dose not produce an analgesic effect on bone cancer pain.

There are two possible mechanisms by which indomethacin produces an analgesic effect in the bone cancer pain model: The first is that inhibition of both COX-1 and COX-2 produce an analgesic effect on bone cancer pain, and the second is that an analgesic effect of indomethacin on bone cancer pain is mediated by the same mechanism by which acetaminophen achieves that effect.

As mentioned earlier, the mechanisms used by acetaminophen to produce an analgesic effect are not fully understood. Chandrasekharan et al. [8] suggested that inhibition of COX-3 is the primary central mechanism by which acetaminophen produces an analgesic effect. On the other hand, Kis et al. [15] reported that COX-3 does not have any cyclooxygenase activity and that acetaminophen acts against COX-2, but not COX-1 or COX-3, to inhibit PG production. Simmons et al. [10] demonstrated that acetaminophen inhibited a COX that is a variant of COX-2. Although the precise mode of action of acetaminophen is not clear, the data indicated that orally administered acetaminophen significantly reduces the number of pain-related behaviors induced by application of a von Frey filament in a dose-dependent manner.

Radiographic examination showed that severe destruction of the distal femur had occurred 2 weeks after implantation of tumor cells. On the other hand, 2 weeks after intramedullary injection of vehicle, there was only slight loss of bone in the medullary canal of the distal femur, a change that may be due to the reaction against knee arthrotomy. Moreover, the implantation of tumor cells, but not the vehicle injection, increased the number of pain-related behaviors 2 weeks after injection. This strongly suggests that the implantation of tumor cells caused bone destruction of the distal femur and produced bone cancer pain 2 weeks after implantation.

In the present study, we estimated the level of bone cancer pain by the number of pain-related behaviors induced by the application of von Frey filaments to the distal femur of the tumor cells-implanted paw. As shown in Fig. 1, significant destruction occurred at the distal femur of the tumor cells-implanted paw. Although spontaneous pain is the main symptom of human metastatic bone tumor, spontaneous pain is a subjective experience, and it is difficult to measure spontaneous pain objectively in an animal model. Even if spontaneous pain can be measured, we think that the data on spontaneous pain may have less objectivity than that of pain induced by application of a von Frey filament. Therefore, the authors investigated the relevant pain-related behavior induced by mechanical stimuli to the site of bone destruction. Guarding, strong withdrawal, fighting, and biting behaviors were used as pain-related behaviors evoked by application of the von Frey filament. Each behavior may represent a different level of pain felt by the animals. In the animal study, it is impossible to predict the response to stimulation by the of von Frey filament. Moreover, the animals do not always show the same response to the same stimulation. Thus, it is impossible to control the pain level felt by the animals. In the present study, we applied the same stimulation to induce pain behavior and counted the number of pain-related behaviors to quantify the level of pain.

It is possible that the ineffectiveness of the oral administration of either SC560 or celecoxib was due to the small doses of these agents applied in this study. It had been reported that oral administration of SC560 10 mg·kg^{-1} produced maximal inhibition of platelet TxB2; and this effect of SC560 on platelet TxB2 was equivalent to that observed with indomethacin 10 mg·kg^{-1} [16]. Moreover, the inflammatory reactions induced by intradermal injection of anti-chicken egg albumin immunoglotubin G (IgG) and chicken

egg albumin have been reported to be significantly attenuated by the oral administration of SC560 (1–100 mg·kg^{-1}). These data suggested that 300 mg·kg^{-1} doses of SC560 are bioactive and that the dose is enough to produce an analgesic effect. Oral administration of celecoxib produced an analgesic effect in the rat formalin test at doses of 3–100 mg·kg^{-1} [17]. This suggests that oral administration of celecoxib 300 mg·kg^{-1} is enough to produce an analgesic effect. Thus, we believe that the doses of either SC560 or celecoxib applied in the present study were adequate to examine the roles of COX-1 and COX-2, respectively.

The effect of COX-2 selective inhibitor on bone cancer pain is unclear. Sabino et al. [18] reported that NS-398, a COX-2 inhibitor, reduced the number of spontaneous flinches and improved limb use, but it did not improve guarding during forced ambulation. Walker et al. [19] and Medhurst et al. [20] reported that celecoxib had no effect on bone cancer pain in the rat. They estimated the level of bone cancer pain by applying the von Frey monofilament to the plantar surface of a hind paw. These data and ours suggest that bone cancer pain is maintained not by a single mechanism but by various mechanisms.

Oral administration of morphine attenuated the number of pain-related behaviors induced by the application of a von Frey filament in a dose-dependent manner at doses between 10 and 30 mg·kg^{-1}; and this effect of morphine was antagonized by naloxone. Thus, the analgesic effect of morphine is mediated by activation of the naloxone-sensitive opioid receptor. Oral administration of morphine 13 mg·kg^{-1} did not produce an analgesic effect, but when morphine 13 mg·kg^{-1} and acetaminophen were co-administered orally, the morphine significantly shifted the dose-response curve of acetaminophen to the left in a parallel fashion. These data suggest that a subanalgesic dose of morphine significantly enhanced the analgesic effect of acetaminophen, suggesting that co-administration of opioid and acetaminophen produces a useful analgesic effect in patients with bone cancer pain. It has been proposed that opioids produce an analgesic effect within the midbrain periaqueductal gray (PAG) matter by inhibiting GABAergic inhibitory influences on neurons that form part of the descending antinociceptive pathway [21]. Vaughan et al. [22] demonstrated that mu-opioid inhibition of GABAergic synaptic transmission is mediated by modulation of presynaptic, dendrotoxin-sensitive potassium conductance coupled via a phospholipase A_2–arachidonic acid–12-lipoxygenase pathway. This opioid inhibition is potentiated by COX inhibitor, especially COX-1 inhibitor, but not by COX-2 inhibitor, presumably because more arachidonic acid is available for enzymic conversion to 12-lipoxygenase products [23]. The precise mechanisms by which a subanalgesic dose of morphine enhances the analgesic effect of acetaminophen are not clear, but it is possible that acetaminophen potentiates opioid inhibition of GABAergic synaptic transmission in the PAG matter and produces a synergistic analgesic effect. COX-1 inhibitors may potentiate opioid inhibition of GABAergic synaptic transmission more strongly than COX-2 inhibitors and may produce a better synergistic analgesic effect than COX-2 inhibitor when COX-1 inhibitor is co-administered with morphine.

Conclusions

We demonstrated that oral administration of indomethacin, acetaminophen, and morphine produced a profound analgesic effect on bone cancer pain. Co-administration of a subanalgesic dose of morphine with acetaminophen enhances the analgesic effect of acetaminophen. These data suggest a potential new therapeutic approach to treating bone cancer pain.

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