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Pharmacology of Cannabinoid Receptor Agonists and a Cyclooxygenase-2 Inhibitor in Rat Bone Tumor Pain

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Key Words
Bone tumor pain · Cannabinoid 1 and cannabinoid 2 receptor agonists · Cyclooxygenase-2 inhibitor · Spinal cord · Sprague-Dawley rat

Abstract
We evaluated the pharmacology of spinal selective cannabinoid (CB) receptor agonists and a cyclooxygenase-2 (COX-2) inhibitor on bone tumor pain. MRMT-1 tumor cells were injected into the tibia of female Sprague-Dawley rats. MRMT-1 tumor cells produced a bone tumor confirmed by radiologic and histological findings. Intrathecal CB1 (ACEA) and CB2 receptor (AM 1241) agonists and a COX-2 inhibitor (DuP 697) dose-dependently increased the withdrawal threshold. The calculated ED_{50} (nmol/l) values for ACEA, AM 1241 and DuP 697 were 0.007, 2.3 and 76.1, respectively. Reverse transcriptase polymerase chain reaction and Western blot showed that COX-2 mRNA and protein, but not CB1 or CB2 receptor, were increased in the spinal cords of rats with bone tumors. Spinal CB1 receptor and CB2 receptor agonists and COX-2 inhibitor may be useful in the management of bone tumor pain. Furthermore, CB2 receptor agonist may be more potent than CB1 receptor agonist and COX-2 inhibitor.

Introduction

One of the most common and devastating complications in cancer patients with bone metastases is bone pain [1]. Substances derived from tumor cells, inflammatory cells, and bone may be involved in the generation of bone tumor pain [2]. Additionally, neurochemical changes in the spinal cord appear to contribute to the biology of bone tumor pain [3, 4].

Pharmacological intervention is the main treatment for bone tumor pain. Because the use of conventional analgesics is limited by side effects such as constipation and gastrointestinal ulceration, there is a continuing need for the development of safer and more effective analgesics for bone tumor pain therapy.

One promising family of agents for the treatment of bone tumor pain is cannabinoids (CBs). CBs are a family of compounds named after the key active constituent of the marijuana plant, Δ9-tetrahydrocannabinol [5]. Previous studies have shown that spinal CB receptor agonists decreased carrageenan-induced thermal hyperalgesia and bone cancer pain [6, 7]. Recently, we demonstrated that intrathecal WIN 55,212-2, a CB receptor agonist, reduced bone-tumor-related pain behavior and that both CB1 and CB2 receptor antagonists reversed the effect at the spinal level [8].

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Several lines of evidence indicate that prostaglandins are involved in the process of nociceptive transmission [9]. Spinal DuP 697, a cyclooxygenase-2 (COX-2) inhibitor, inhibits formalin-induced inflammatory pain, and SC-236, another inhibitor, reverses mechanical allodynia in spinal nerve ligation [10, 11]. Although systemic COX-2 inhibitor attenuates bone cancer pain [12], a spinal effect remains to be determined.

The aims of this study were to compare the efficacy of intrathecal selective CB1 (ACEA) and CB2 (AM 1241) receptor agonists and a COX-2 inhibitor (DuP 697) in a murine model of bone tumor pain. Additionally, we assessed the levels of CB receptors and COX-2 in the spinal cord.

Methods

Animal and Bone Tumor Model

All procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University. Experiments were performed on female Sprague-Dawley rats weighing 180–200 g. Animals were housed in cages in standard facilities (22 ± 0.5°C, 12/12-hour light/dark cycle). Food and water were freely available in a vivarium.

Syngeneic MRMT-1 rat mammary gland carcinoma cells were cultured in media and then media or 1×10^5 tumor cells were injected into the medullary cavity of the right tibia to induce bone tumor according to a previously described method [8]. Under sevoflurane anesthesia, a 1/4 FG 0.5-mm round dental bur was used for intramedullary canalling. A 20-μl volume of culture medium (sham group) or tumor cells was injected into the intramedullary space. Only rats with marked allodynia (withdrawal threshold <5 g) after MRMT-1 tumor cell injection were enrolled for the study.

Implantation of Intrathecal Catheter

Three days after MRMT-1 tumor cell injection, an intrathecal catheter was placed according to a previously described method [13]. The catheter was passed caudally 8.5 cm from the cisterna magna to the lumbar enlargement. The outer part of the catheter was fixed at the scalp and plugged with wire. After surgery, only rats showing normal neurological function were used for the study, otherwise the animals were euthanized immediately.

Drugs

The following drugs were used: ACEA (Tocris Cookson, Bristol, UK), AM 1241 (Sigma-Aldrich, St. Louis, Mo., USA) and DuP 697 (Tocris Cookson). AM 1241 and DuP 697 were dissolved in dimethylsulfoxide and ACEA was dissolved in 10% ethanol. Intrathecal administration of these agents was performed using a hand-driven, gear-operated syringe pump. All drugs were delivered in a 10-μl volume of solution, followed by an additional 10 μl of normal saline to flush the catheter.

Assessment of Mechanical Allodynia

The withdrawal threshold was measured using the up-and-down method [14]. Von Frey filaments (0.4, 0.7, 1.2, 2.0, 3.6, 5.5, 8.5 and 15 g; Stoelting, Wood Dale, Ill., USA) were applied vertically to the plantar surface of the hind paw for 5 s while the hair was bent. Brisk withdrawal or paw lifting indicated a positive response. If a response was absent even at 15-gram pressure, this value was considered the cutoff value. Tests were performed in duplicate, with an approximately 3-min rest period between tests, and the average value was used.

Radiologic and Histologic Studies

Radiological and histological analyses were carried out following MRMT-1 tumor cell or medium injection. Fixed hind limbs were placed on Industrex X-ray film (Kodak, Rochester, N.Y., USA) and exposed to an X-ray source (Faxitron, Tucson, Ariz., USA) for 90 s at 50 kV, and films were processed in an automatic Optimax film processor. Ipsilateral hind limb tissues were fixed in 10% buffered formalin, decalcified in hydrochloric acid (CalciClear Rapid; National Diagnostics, Atlanta, Ga., USA), and embedded in paraffin. Sections (4 μm in thickness) were cut and stained with hematoxylin and eosin (HE) for routine histological evaluation.

Experimental Paradigm

Our previous study indicated that bone tumor pain peaked on day 10. Thus, we did experiments at this time [8]. The rats were placed the plastic cages and allowed to acclimate for a period of 20–30 min for the behavioral experiment. Animals were tested only once. All experiments were carried out by an observer blinded to the drug treatments.

Effects of Intrathecal ACEA, AM 1241 and DuP 697

To evaluate the effect of a selective CB1 receptor agonist (ACEA; 0.8, 2.7, 8.2 nmol/l, n = 25), a CB2 receptor agonist (AM 1241; 0.006, 0.02 and 0.06 nmol/l, n = 25) and DuP 697, a COX-2 receptor agonist (24.3, 72.90 and 243 nmol/l, n = 25), each agent was administered intrathecally. Measurement of the mechanical threshold was carried out before MRMT-1 tumor cell injection and this value was regarded as the baseline threshold. The withdrawal threshold measured immediately before intrathecal delivery of each drug was regarded as the control. The withdrawal threshold was determined at 15, 30, 60, 90, 120, 150, and 180 min after intrathecal administration. ED_{50} values (effective dose producing a 50% reduction of control withdrawal threshold) of ACEA, AM 1241 and DuP 697 were determined separately.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction Analysis

CB receptor subtype (CB1, CB2) and COX-2 mRNA expression were measured in the right dorsal spinal cord of sham rats (n = 5) and rats with bone tumor pain (n = 5) using reverse transcription polymerase chain reaction (RT-PCR). On day 10 after injection, the area of the right dorsal spinal cord from L4 to L6 was dissected and total RNA in the right dorsal spinal cord was isolated using the RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Isolated mRNA was reverse-transcribed using the Omniscript RT kit (Qiagen), following the manufacturer's instructions. Previously published primer sets were used for rat CB receptors [15] and COX-2 [16]. The RT-PCR conditions were standardized using PCR Premix (Bioneer, Daejeon, Korea) containing 1 × PCR buffer, 1.5 mmol/l $MgCl_2$, 250 μmol/l dNTPs, 10 pmol/l each of forward and reverse oligonucleotide primers coding for rat CB1, CB2 or COX-2, followed by 35 cycles of thermal cycling (94°C for 25 s, 59°C for 30 s, and 72°C for 1 min) for

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Fig. 1. Radiographs of the right tibia following medium (sham, **a**) or MRMT-1 tumor cell (**b**) injection. The images showed no change (**a**) or bone destruction caused by bone tumor (**b**).

CB1 and CB2 and 30 cycles of thermal cycling (94°C for 30 s, 58°C for 45 s, and 68°C for 1 min) for COX-2. Amplification of rat β-actin (GenBank accession No. NM_031144) was used as an internal and loading control. PCR products were separated by gel electrophoresis on a 1.5% agarose gel and then imaged using a LAS-3000 (Fujifilm Global, Tokyo, Japan).

Western Blot Analysis

CB receptors and COX-2 protein were measured in the right dorsal spinal cord of sham rats (n = 5) and rats with bone tumor pain (n = 5) using Western blot. On day 10 after injection, the right dorsal spinal cord from L4 to L6 was quickly removed and stored at –80°C. The spinal cord was extracted by homogenization in protein extraction solution (PRO-PREP™, Intron Biotechnology, Seoul, Korea), then resuspended in ice for 30 min and centrifugation at 13,000 rpm for 10 min. For the Western blot, 30 μg of protein was loaded and run on a 10% Tris-HCl sodium dodecyl sulfate polyacrylamide gel. Protein was electrotransferred to polyvinylidene difluoride membrane and then blocked with 5% nonfat dry milk in 20 mmol/l of Tris-buffered saline with 0.1% Tween-20. After blocking, the membrane was incubated with a 1:200 dilution of polyclonal primary antibodies against CB1 (sc-20754), CB2 (sc-25494) or COX-2 (sc-1747) from Santa Cruz Biotechnology (Santa Cruz, Calif., USA) in blocking solution overnight. The proteins were detected using horseradish peroxidase-coupled secondary antibody at a dilution of 1:3,000 in 5% nonfat dry milk. After washing 3 times, bands were detected using ECL Western blotting detection reagents (Santa Cruz Biotechnology) and then were imaged with LAS 3000 (Life Science, Fujifilm Global, Tokyo, Japan). The membrane was then stripped using a stripping buffer (Pierce, Wisc., USA), and reprobed with antibodies specific for β-actin (Santa Cruz). Densitometry was performed using Multi Gauge V3.0 (Life Science) chemiluminescence system and analysis software to determine the ratio between CB, COX-2 and β-actin.

General Behavior

To evaluate behavioral changes due to ACEA (n = 4), AM 1241 (n = 4) and DuP 697 (n = 4), additional rats received the maximum doses of agents used, and the animals were observed for 60 min after intrathecal administration. Motor function was assessed by examining the righting and placing-stepping reflexes. The former was evaluated by placing the rat horizontally with its back on the table, which normally gives rise to an immediate coordinated twisting of the body to achieve an upright position. The latter was assessed by drawing the dorsum of either hind paw across the edge of the table. Normally rats try to move the paw forward into a position for walking. Pinna and corneal reflexes were also evaluated and judged as present or absent.

Statistical Analysis

Data are expressed as the mean ± SEM. The time-response data are presented as the withdrawal threshold. Dose-response data are presented as the percentage of the maximum possible effect (MPE). The withdrawal threshold data from von Frey filament testing were converted to the percentage of MPE according to the following formula: % MPE = [(postdrug threshold – postinjured baseline threshold)/(cutoff threshold – postinjured baseline threshold)] × 100. The dose-response data were analyzed using repeated-measures or one-way analysis of variance with a post hoc Bonferroni correction. Dose-response lines were fitted using least-squares linear regression, and ED_{50} values with 95% confidence intervals were calculated using the method reported by Tallarida [17]. RT-PCR data were analyzed using an unpaired t test; $p < 0.05$ indicated statistical significance.

Results

Radiologic and Histologic Studies

Radiographic images taken after MRMT-1 tumor cell or medium injection are shown in figure 1. Bone destruction in the tumor cell-injected group indicated that tumors developed, but bone loss was not noted in the sham group. Histologically, tumor was noted in the tibial metaphysis and diaphysis. Tumor cells extended through the

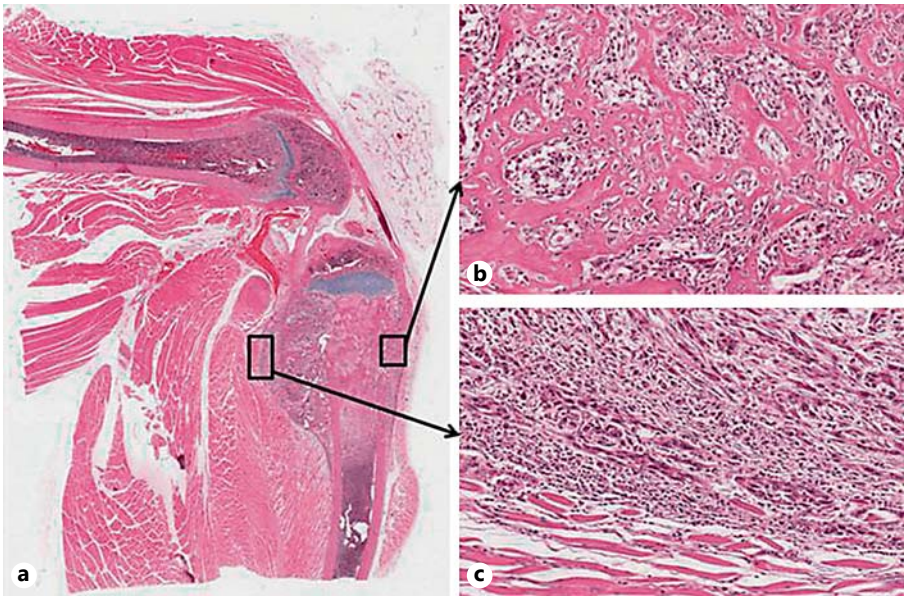

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Fig. 2. Histological section of tibia with HE staining following MRMT-1 tumor cell injection. Tumor is noted in the tibial metaphysis and diaphysis. Tumor cells are extending through the cortex and invading the adjacent muscle. **a** HE. ×1, scan view. Tumor is composed of hyperchromatic ovoid or plasmacytoid cells with osteoid formation. **b** HE. ×40. The tumor cells, showing epithelioid, plasmacytoid, or ovoid shape are invading adjacent skeletal muscle fibers. **c** HE. ×40.

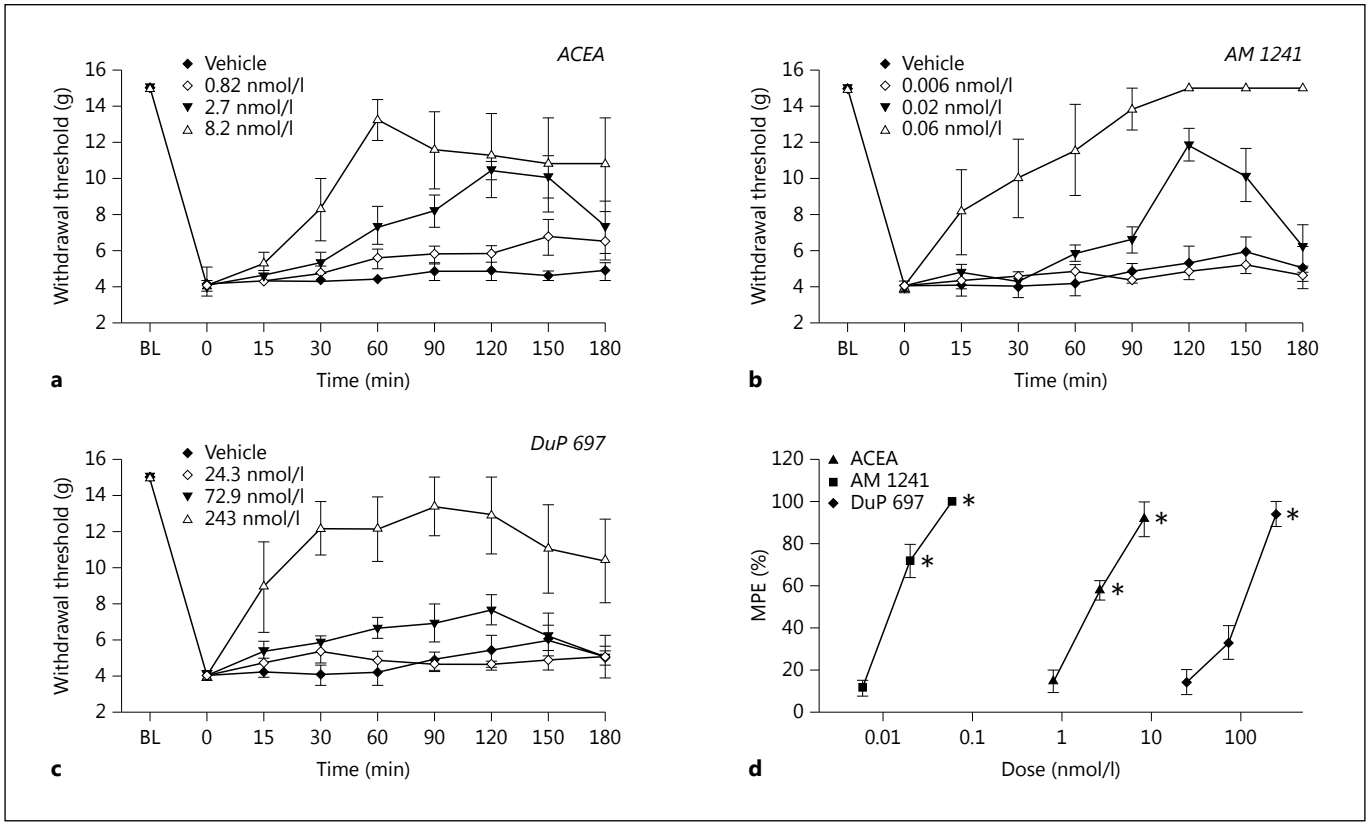

Fig. 3. Effect of intrathecal ACEA, AM 1241 and DuP 697 on hind paw withdrawal response to von Frey filaments after tumor cell injection. Data are presented as the withdrawal threshold (**a–c**) or the percentage of MPE (**d**). All values represent means ± SEM. BL = Baseline withdrawal threshold, measured before tumor cell inoculation. Three drugs were intrathecally administered at time 0. All of the three agents increased the withdrawal threshold in the injected paw dose-dependently. * $p < 0.05$, compared with vehicle.

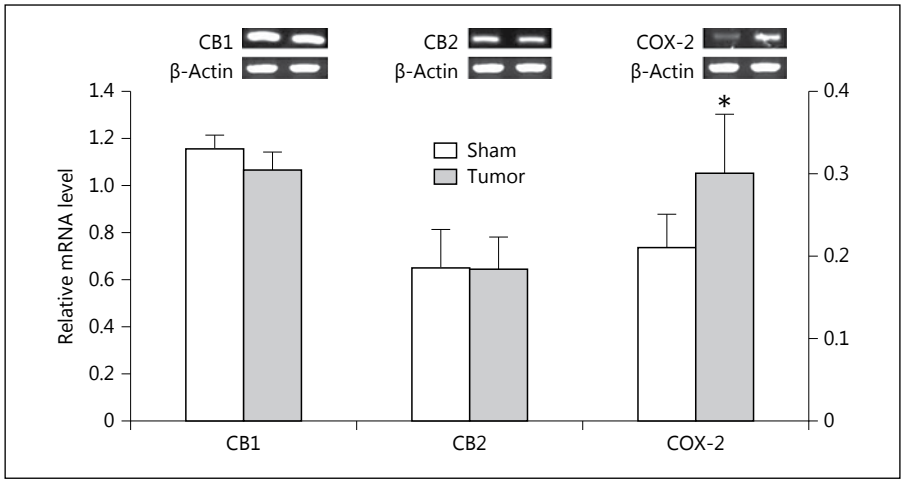

Fig. 4. CB1 and CB2 receptor and COX-2 mRNA expression in the spinal cord of sham rats and rats with bone tumor pain. RT-PCR amplification of CB1 (351 bp) and CB2 (481 bp) receptor and COX-2 (702 bp) mRNA from rat spinal cord. Products were analyzed on 1.5% agarose gel. The data are presented as relative mRNA level. Each bar represents the mean ± SEM. No difference was noted between sham and tumor-bearing rats in CB1 or CB2 receptor mRNA. COX-2 mRNA expression was increased in rats with bone tumor pain. * $p < 0.05$, compared with sham.

cortex and invaded the adjacent muscle (fig. 2a). Bone tumor was composed of hyperchromatic ovoid or plasmacytoid cells with osteoid formation (fig. 2b). Moreover, tumor cells, showing epithelioid, plasmacytoid, or ovoid shape, invaded adjacent skeletal muscle fibers (fig. 2c).

Effects of Intrathecal ACEA, AM 1241 and DuP 697

Intrathecal ACEA, AM 1241 and DuP 697 dose-dependently increased the withdrawal threshold in the tumor cell-injected paw and the percentage of MPE of ACEA, AM 1241 and DuP 697 was 91, 100 and 94, respectively (fig. 3). The calculated ED_{50} values with 95% confidence intervals of ACEA, AM 1241 and DuP 697 are shown in table 1. The rank order of potency (according to the ED_{50} values) was AM 1241 > ACEA > DuP 697. None of the three agents affected the withdrawal threshold in sham rats.

PCR Analysis

The rat spinal cord, analyzed by RT-PCR for the presence of CB1 and CB2 receptors and COX-2 mRNA using previously published primer pairs, yielded 351-, 481-, and 702-bp fragments from cDNA, respectively (table 2, fig. 4). After bone tumor development, the levels of CB1 and CB2 receptor mRNA expression were not altered ($p > 0.05$, fig. 4), whereas that of COX-2 mRNA was significantly increased, compared with that in sham rats ($p < 0.05$, fig. 4).

Western Blot Analysis

Western blotting indicated the relative abundance of CB1, CB2 and COX-2 protein in sham rats, and no significant differences in the levels of CB1 and CB2 protein expression were seen between sham and tumor-bearing rats. However, the level of COX-2 protein was significantly increased in tumor-bearing rats compared with sham rats ($p < 0.05$, fig. 5).

Table 1. ED_{50} value (nmol/l) with 95% confidence intervals (CI) of ACEA, AM 1241 and DuP 697

Drug	ED_{50} (95% CI)
ACEA	2.3 (1.8–2.9)
AM 1241	0.007 (0.005–0.008)
DuP 697	76.1 (54.7–106)

General Behavior

The righting and placing-stepping reflexes were normal after intrathecal administration of ACEA, AM 1241 and DuP 697 at maximum doses used in this study. Both pinna and corneal reflexes were present.

Discussion

In the current study, injection of MRMT-1 tumor cells induced bone tumors, confirmed by radiology and histology. Bone pain was also caused. These findings are in line with previous data [8].

The skeletal bone is a common site of metastases from tumors arising from breast and prostate [18]. Pain is the most frequent presenting symptom indicating tumor metastasis to bone [1]; it is both severe and difficult to treat, which eventually leads to a reduced quality of life [19].

Table 2. RT-PCR primer sequence of the rat CB1, CB2 and COX2 receptor

Subtype	Primer		Annealing temperature °C	Product size bp
CB1	forward reverse	CCT GGG CTG GAA CTG CAA CCG AAG ACG TCA TAC ACC ATG A	59	351
CB2	forward reverse	CGG CTT GGA GTT CAA CCC TA ACA ACA AGT CCA CCC CAT GAG	59	481
COX-2	forward reverse	TGA TGA CTG CCC AAC TCC CAT G AAT GTT GAA GGT GTC CGG CAG C	58	702

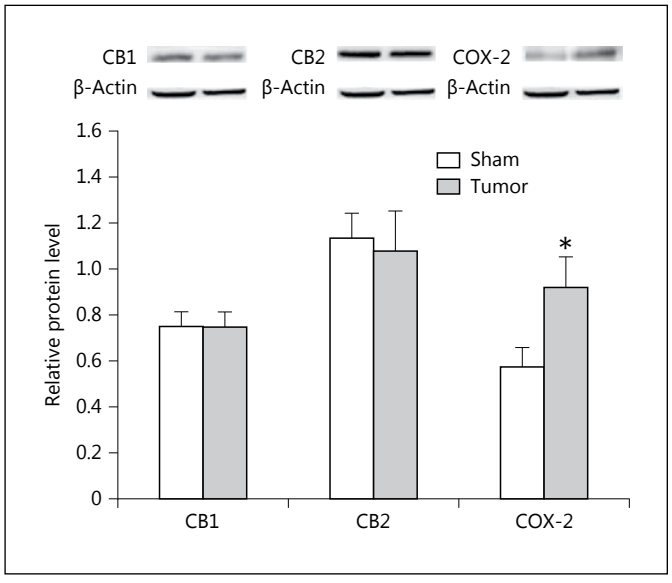

Fig. 5. CB1 and CB2 receptor and COX-2 protein expression in the spinal cord of sham rats and rats with tumor pain. Western blot analysis detected expected protein bands corresponding to the CB1 (63 kDa) and CB2 (45 kDa) receptor. The protein level was expressed as a ratio to β-actin. The data are presented as relative protein level. Each bar represents the mean ± SEM. No distinct differences were noted between sham and tumor-bearing rats in CB1 or CB2 receptor protein. COX-2 protein expression was increased in rats with bone tumor pain. * $p < 0.05$, compared with sham.

Thus, there is a continuing need for the development of effective analgesic therapies for bone tumor pain. However, unfortunately, the detailed mechanisms remain unclear despite extensive research on the basic neurobiology of bone tumor pain. On the other hand, it has been reported that several neurochemicals appear to be involved in bone tumor pain in the spinal cord [3, 4]. Moreover, animal models of bone tumor pain have been developed [8, 20]. These advances may accelerate the study of bone tumor pain.

In this study, the withdrawal threshold was dose-dependently increased by intrathecal ACEA (CB1 receptor agonist), AM 1241 (CB2 receptor agonist) and DuP 697 (COX-2 inhibitor).

Much evidence suggests that the CB system is involved in the modulation of various nociceptive conditions at almost all levels of pain transmission. In particular, CB receptors have been identified in the spinal cord and are subdivided into two subtypes: CB1 and CB2 [21]. Several studies indicated the antinociceptive effects of CB receptor agonists at the spinal level. Intrathecal nonselective CB receptor or CB1 receptor agonists decrease carrageenan-induced thermal hyperalgesia [6], formalin-induced nociception [22] and bone tumor pain [7, 8]. Intrathecal CB2 receptor agonists reduce peripheral nerve injury hypersensitivity [23] and bone tumor-induced thermal hyperalgesia and mechanical allodynia [24, 25]. However, the role of CB2 receptor on bone tumor pain has been conflicting according to the drug administration route. The effect of peripheral CB receptor agonist was mediated through both CB1 and CB2 receptors [26], while the effect of systemic agents was activation of CB1 receptor, but not CB2 receptor [27, 28]. At the spinal level, on the other hand, the nonselective CB receptor agonist attenuated vincristine-induced neuropathy and bone tumor pain and this antinociception was reversed by both CB1 and CB2 receptor antagonists [8, 29]. In this study, intrathecal administration of selective CB1 and CB2 receptor agonists increased withdrawal thresholds dose-dependently. Therefore, these findings jointly suggest that both CB1 and CB2 receptor subtypes may play important roles in the modulation of bone tumor pain particularly at the spinal level.

The role of prostaglandins, formed through the action of the two isoforms of the COX enzyme, COX-1 and COX-2, in the pathophysiology of pain is well documented [9]. Basically, COX-1 is expressed in a constitutive manner and is believed to play a more homeostatic role, whereas COX-2 is highly inducible in response to inflammatory stimuli [30, 31]. Additionally, spinal prostaglandins generate spinal hyperexcitability by enhancing synaptic transmission [32, 33]. Furthermore, COX-2 is expressed in the spinal cord [34]. Previous studies demonstrated the antinociception of spinal COX-2 inhibitors on inflammatory and neuropathic pain [10, 11]. There is little data about the effect of COX-2 inhibitors on bone tumor pain at the spinal level. In the present study, intrathecal DuP 697, a COX-2 inhibitor, reduced bone tumor pain dose-dependently. Therefore, these findings suggest that COX-2 is involved in the development of bone tumor pain in the spinal cord.

We examined whether the behavioral effects were accompanied by alteration of CB2 receptor expression. In the present study, RT-PCR and Western blot analysis detected CB1 and CB2 receptor in the spinal cord in both sham and bone tumor-bearing rats. However, the expression levels of CB receptor mRNA and protein did not differ significantly between tumor-bearing and sham rats. This finding was consistent with previous results. No difference was found in either CB1 or CB2 receptor mRNA expression levels in the spinal cord on the tumor side, compared with those on the opposite side [35]. Furthermore, both CB1 and CB2 protein levels in the spinal cord of rats with bone tumor pain were comparable to those of naïve animals [7, 24]. Therefore, it may be conceivable that the activation of a constitutive population of spinal CB receptors is sufficient to block such bone tumor-related pain. In addition, we demonstrated that the level of spinal COX-2 mRNA and protein expression was increased in rats with bone tumor pain, in comparison with sham rats. Previous studies indicated that COX-2 immunofluorescence was increased in the spinal cord of tumor-bearing or neuropathic animals [36]. Thus, it could be assumed that bone tumor pain directly increased the synthesis of spinal COX-2 mRNA.

The percentage of MPE of intrathecal ACEA, AM 1241 and DuP 697 was similar, which made it possible to compare their potency. Thus, the rank order of potency (according to the ED_{50} values) was AM 1241 > ACEA > DuP 697 at the spinal level. These observations suggest that spinal CB2 receptor may be more involved than CB1 receptor and COX-2 in the modulation of bone tumor pain. Therefore, CB2 receptor agonist may be more effective than CB1 receptor agonist and COX-2 inhibitor in the management of bone tumor pain in the spinal cord.

In terms of selectivity, ACEA and AM 1241 have a 2,000-fold higher or lower affinity for the CB1 receptor compared to the CB2 receptor [37, 38]. DuP 697 has an 80-fold higher affinity for COX-2 compared to COX-1 [39]. Thus, the antinociceptive effect of ACEA, AM 1241 and DuP 697 may be principally mediated by CB1 receptor, CB2 receptor and COX-2, respectively.

In conclusion, both CB1 and CB2 receptors may be actively involved in the modulation of bone tumor pain in the spinal cord. Spinal COX-2 may contribute to the development of bone tumor pain. Therefore, both spinal CB1 and CB2 receptor agonists, and COX-2 inhibitor may be a therapeutic option in bone tumor pain control. In addition, CB2 receptor agonist may be more effective than CB1 receptor agonist and COX-2 inhibitor.

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