Regional Anesthesia and Pain

Ketamine and N-acetylaspartylglutamate peptidase inhibitor exert analgesia in bone cancer pain

[La kétamine et un inhibiteur N-acétylaspartylglutamate peptidase exercent une analgésie sur la douleur du cancer des os]

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Purpose: Not all bone cancer pain can be effectively treated with current therapies. In the present study, the effects of ip administration of α -2 agonists (dexmedetomidine and clonidine), N-methyl-D-aspartate (NMDA) antagonists (MK-801 and ketamine), an N-acetylaspartylglutamate peptidase inhibitor (ZJ-43), and morphine were examined in a mouse bone cancer pain model.

Methods: A bone cancer pain model was produced by injection of murine sarcoma cells into the medullary cavity of the distal femur. To estimate the level of bone cancer pain, the number of pain-related behaviours induced by repeated applications of a von Frey monofilament (0.166 g) to the site of tumour cells implantation was counted. Drugs were administered two weeks after the implantation.

Results: Morphine produced a significant analgesic effect ($P < 0.001$). The α -2 agonists produced analgesic effects ($P < 0.001$) with an efficacy similar to that of morphine, but only at doses that produced severe sedation. MK-801 had only limited analgesic effects, while ketamine produced an analgesic effect ($P < 0.001$) with the same efficacy as morphine. ZJ-43 (100 mg·kg⁻¹) had a significant analgesic effect ($P < 0.05$) and the effect of ZJ-43 was antagonized by the selective group II metabotropic glutamate receptor (mGluR) antagonist.

Conclusion: These data suggest that α -2 agonists produce an analgesic effect only at a sedative dose and that ketamine, but not MK-801, is associated with an analgesic response without overt side effects. The effect of ZJ-43 is mediated by activating group II mGluRs.

Objectif : Les douleurs du cancer des os ne sont pas toujours soulagées par les traitements habituels. Dans la présente étude, les effets de l'administration ip d'agonistes α -2 (dexmédétomidine et clonidine), d'antagonistes N-méthyl-D-aspartate (NMDA) (MK-801 et kétamine), d'un inhibiteur de N-acétylaspartylglutamate peptidase (ZJ-43) et de morphine ont été examinés dans un modèle de douleur du cancer des os chez la souris.

Méthode : Un modèle de douleur du cancer des os a été produit en injectant des cellules de sarcome d'Ewing dans la cavité médullaire distale du fémur. Pour évaluer le niveau de douleur, nous avons calculé le nombre de mouvements reliés à la douleur induite par l'application répétée d'un monofilament von Frey (0,166 g) au site de la tumeur implantée. Les médicaments ont été administrés deux semaines après l'implantation.

Résultats : La morphine a produit une analgésie significative ($P < 0,001$). Les agonistes α -2 ont produit une analgésie ($P < 0,001$) similaire à celle de la morphine, mais seulement à des doses produisant une sédation importante. Le MK-801 a eu un effet limité et la kétamine a produit une analgésie aussi efficace que celle de la morphine ($P < 0,001$). Le ZJ-43 (100 mg·kg⁻¹) a eu un effet analgésique significatif ($P < 0,05$) contré par l'antagoniste sélectif des récepteurs de glutamate métabotrope (mGluR) de groupe II.

Conclusion : Les agonistes α -2 produisent un effet analgésique à des doses sédatives seulement et la kétamine, mais non le MK-801, est associée à une réaction analgésique sans effets secondaires apparents. L'effet du ZJ-43 origine de l'activation des mGluR de groupe II.

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THE presence of bone metastases is the most common cause of cancer related pain.¹ The World Health Organization (WHO) has recommended the use of non-steroidal anti-inflammatory drugs as the first step on the analgesic ladder and the use of weak and strong opioids as the second and the third steps² but, at present, many patients still have severe bone cancer pain which worsens their quality of life.³ Given the development of tolerance to opioids and secondary effects of these drugs, it is important to identify and characterize new drug therapies for the management of bone cancer pain.

Alpha-2 adrenergic receptors and glutamate receptors are reported to be involved in transmission of nociceptive information. Systemic administration of an α -2 receptor agonist, such as dexmedetomidine, has been reported to produce analgesic and sedative effects.⁴ Two types of glutamate receptors, an ionotropic receptor and a metabotropic receptor, have been reported to be involved in pain perception.^{5,6} Ionotropic glutamate receptors are classified into three subtypes; α -amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate receptors, whereas metabotropic glutamate receptors (mGluR) are classified into eight subtypes, mGluR1-8. The mGluRs are classified into three subgroups. Group I (mGluR1 and mGluR5) are coupled to polyphosphoinositide hydrolysis. Group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8) are negatively coupled to cyclic AMP (cAMP) levels. Spinal application of an NMDA receptor antagonist produces an analgesic effect in both inflammatory and neuropathic pain models.^{7,8} It has been suggested that Group II mGluRs play an inhibitory role.⁹ It has also been reported that the peptide neurotransmitter N-acetylaspartylglutamate (NAAG; reviewed in Neale *et al.*)^{10,11} is released from spinal cord cells by depolarizing stimuli and selectively activates mGluR3.¹² Intrathecal administration of NAAG peptidase inhibitors produces an analgesic effect in both inflammatory pain models and in neuropathic pain models.¹³⁻¹⁶ Taken together, these data support the hypothesis that α -2 adrenergic receptor agonists, NMDA receptor antagonists, and NAAG peptidase inhibitors may have some analgesic effects on bone cancer pain. At present, however, the efficacy of these drugs on bone cancer pain has not been examined comprehensively.

Recently, an animal model of bone cancer pain, produced by injecting osteolytic murine sarcoma cells into the mouse femur, has been developed.¹⁷ It has been reported that systemic administration of morphine produces an analgesic effect in this model, although

the potency of morphine appears to be lower in the bone cancer pain model compared to the inflammatory pain model.^{18,19} This suggests the existence of a different mechanism underlying bone cancer pain *vs* inflammatory pain. In the present study, the authors investigated the analgesic effects of *ip* administered α -2 receptor agonists dexmedetomidine and clonidine, NMDA receptor antagonists MK801 and ketamine, and the NAAG peptidase inhibitor ZJ-43, and compared the analgesic effect of these drugs with that of morphine in an animal model of bone cancer pain.

Methods

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

Experiments were performed on adult male C3H/HeJ mice, approximately five weeks old, 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). A tumour cell injection protocol was performed as described previously by Schwei *et al.*¹⁷ Tumour cells, 10^5 in 20 μ l of α minimal essential medium (α MEM, Sigma, St. Louis, MO, USA) containing 1% bovine serum albumin were injected directly into the medullary cavity of the distal femur under pentobarbital anesthesia.

Mice were placed in a clear plastic observation box (9 cm $\times$ 11 cm $\times$ 20 cm) with a wire mesh floor. After a period of acclimatization, pain-related behaviours were induced by iterative applications of a von Frey monofilament (0.166 g) to the site of tumour cell implantation every second for 20 sec (20 stimuli). To estimate the level of bone cancer pain, the number of pain-related behaviours was recorded. The behaviours were characterized as either guarding, strong withdrawal, or fighting and biting.

The *ip* administered drugs were dissolved in saline and administered in a volume of 0.5 mL. The drugs were morphine (Takeda, Osaka, Japan), naloxone (10 mg $\cdot$ kg⁻¹; Sigma), dexmedetomidine (an α -2 agonist, Abbott, Peapack, NJ, USA), clonidine (an α -2 agonist, Sigma), idazoxan (an α -2 antagonist, Sigma), ketamine (an NMDA antagonist, Sigma), MK801 (an NMDA antagonist, Research Biochemicals Incorporated, Natick, MA, USA), ZJ-43 (an NAAG peptidase inhibitor), and LY341495 (a highly selective Group II mGluR antagonist, Tocris Cookson Ltd., Bristol, UK). ZJ-43 was synthesized in our laboratory as described previously.²⁰ To obtain control data, vehicle was administered *ip*.

FIGURE 1 Response to *ip* administration of an opioid agonist [morphine (MOR)], N-methyl-D-aspartate (NMDA) receptor antagonists (MK801 and ketamine), α -2 agonists (clonidine (CLO) and dexmedetomidine (DEX)), NAAG peptidase inhibitor (ZJ-43) and vehicle (saline) on the number of pain related behaviours induced by 20 applications of a 0.166 g von Frey filament to the site of tumour implantation. The effect of naloxone (NAL) on morphine analgesia, the effect of idazoxan (IDA) on clonidine and dexmedetomidine analgesia, and the effect of LY341495 on ZJ-43 analgesia are also presented. Naloxone *ip* was administered 10 min after morphine administration and idazoxan *ip* was administered 10 min after administration of dexmedetomidine or clonidine. LY341495 *ip* was administered 30 min before the ZJ43 administration. Ordinate: number of pain related behaviours induced by 20 applications of 0.166 g von Frey filament to the site of tumour implantation. Abscissa: time after drug administration. Each line represents mean $\pm$ SEM of measurements made in five mice.

Our previous study revealed that severe pain-related behaviour occurred two weeks after the implantation of tumour cells. Three weeks after the implantation of tumour cells, radiological examination revealed a bone fracture at the site of implantation in all animals.²¹ We examined the effect of drugs two weeks after the implantation of tumour cells. The investigator responsible for counting the number of pain-related behaviours evoked by application of a von Frey monofilament was blind to the drug treatment of each animal. All animals were tested for pain-related behaviours before

the injection of tumour cells. At day 14 post-tumour cell implantation, morphine (2.5, 5, 10, 20, 40 and 80 mg·kg⁻¹), dexmedetomidine (10, 20, 30 and 60 μ g·kg⁻¹), clonidine (0.1, 0.25, 0.5 and 1 mg·kg⁻¹), MK-801 (0.1, 0.2, and 0.4 mg·kg⁻¹), ketamine (5, 10, 20, 40 and 80 mg·kg⁻¹) and ZJ-43 (10, 30, 100, 170 and 300 mg·kg⁻¹) were administered *ip*. Four to six mice were treated at each dose of each drug. Only one dose of each drug was tested per mouse. Animals were tested before drug administration and five, ten, 15, 30, 45 and 60 min after the drug administration. To deter-

FIGURE 2 Dose-response curves for the effect of *ip* administered morphine, MK801, ketamine, clonidine (CLO), dexmedetomidine (DEX) and ZJ-43 on the minimum number of pain related behaviours between ten and 60 min after drug administration. * $P < 0.05$ as compared with the vehicle response. Each line represents mean $\pm$ SEM of measurements made in four to six mice.

mine if the analgesic effects of either morphine or ketamine were due to interactions with an opioid receptor, the most effective dose of morphine ($80 \text{ mg} \cdot \text{kg}^{-1}$, $n = 5$) or ketamine ($40 \text{ mg} \cdot \text{kg}^{-1}$, $n = 5$) was administered *ip*, followed ten minutes later by naloxone $10 \text{ mg} \cdot \text{kg}^{-1}$, and the number of pain-related behaviours was measured five, 20, 35 and 50 min after the naloxone administration. To confirm that the analgesic effect of either clonidine or dexmedetomidine was due to the interaction with an α -2 receptor, the most effective dose of either clonidine ($1 \text{ mg} \cdot \text{kg}^{-1}$, $n = 5$) or dexmedetomi-

dine ($60 \text{ } \mu\text{g} \cdot \text{kg}^{-1}$, $n = 5$) was administered *ip*, followed ten minutes later by idazoxan $100 \text{ mg} \cdot \text{kg}^{-1}$, and the number of pain-related behaviours was measured five, 20, 35 and 50 min after the idazoxan administration. To test the hypothesis that the analgesic effect of ZJ-43 was mediated by the activation of mGluR3, LY-341495 $1 \text{ mg} \cdot \text{kg}^{-1}$ was administered *ip* 30 min before the *ip* injection of ZJ-43 ($n = 5$) and the tumour implanted paw was tested at five, ten, 15, 30, 45 and 60 min after ZJ-43 administration. To obtain control data, naloxone $10 \text{ mg} \cdot \text{kg}^{-1}$ ($n = 5$), idazoxan $10 \text{ mg} \cdot \text{kg}^{-1}$ ($n = 5$) or LY-

341495 1 mg·kg⁻¹ ($n = 5$) were administered *ip* and the tumour implanted paw was tested five, ten, 15, 30, 45 and 60 min after the drug administration. All animals were euthanized with an overdose of barbiturate after completion of all behavioural analyses.

To determine whether the implantation of tumour cells induced significant bone cancer pain, we compared the number of pre-implantation pain-related behaviours with responses observed two weeks after the implantation using a *t* test. To compare the base-line data (before the drug administration) between groups, one-way analysis of variance (one-way ANOVA) was used. For the dose-response analysis, the minimum number of pain related behaviours between ten and 60 min after the drug administration was used. Use of the minimum number of pain related behaviours allowed us to examine maximum drug effect independently from the variation in the time course of analgesic effect of each drug. To analyze the dose-dependency, one-way ANOVA with Dunnett's test was used. In the antagonist study (naloxone and idazoxan), the paired *t* test was used. To analyze the effect of LY-341495 on the analgesic effect of ZJ-43, the *t* test was used.

Wherever appropriate, results are expressed as mean $\pm$ SD in the text, and as mean $\pm$ SEM in the figures. A *P* value < 0.05 was considered statistically significant.

Results

Intraperitoneal administration of MK801 0.4 mg·kg⁻¹, but not doses of 0.1 and 0.2 mg·kg⁻¹, resulted in agitation. Accordingly, we were unable to perform nociceptive testing at the higher dose. Vigilance of the animals was depressed by *ip* administration of dexmedetomidine 30 and 60 μ g·kg⁻¹ and clonidine doses of 0.25, 0.5 and 1 mg·kg⁻¹, indicating sedation.

Before tumour cell implantation, the number of pain-related behavioural responses was 1.3 ± 1.7 ($n = 191$), whereas two weeks after implantation, the number of responses increased to 18.8 ± 1.1 ($P < 0.001$). No difference was apparent between the pre-drug number of pain related behavioural responses in each group (data not shown, $P > 0.2$) demonstrating that the groups had similar degrees of bone cancer pain before the drug administration.

Morphine decreased the minimum number of pain-related responses in a dose-dependent manner, over a dose range from 2.5–80 mg·kg⁻¹ as compared with vehicle treated mice ($P < 0.001$ by one-way ANOVA, Figures 1, 2). The effect of morphine was completely antagonized by naloxone 10 mg·kg⁻¹ ($P < 0.01$ by paired Student's *t* test, Figure 1). Naloxone 10 mg·kg⁻¹ had no effect on the minimum number

of pain-related behaviours as compared with vehicle treated mice ($P > 0.4$, Figure 1).

Dexmedetomidine was associated with a decrease in the minimum number of pain related behaviours in a dose-dependent manner over a dose range from 10–60 μ g·kg⁻¹ as compared with vehicle treated mice ($P < 0.001$ by one-way ANOVA, Figures 1, 2). Clonidine also decreased the minimum number of pain related behaviours in a dose-dependent manner over a dose range from 0.1–1 mg·kg⁻¹ as compared with vehicle treated mice ($P < 0.001$ by one-way ANOVA, Figures 1, 2). The effects of both dexmedetomidine 60 μ g·kg⁻¹ and clonidine 1 mg·kg⁻¹ were antagonized by idazoxan 100 mg·kg⁻¹ ($P < 0.005$ by paired test, Figure 1). Idazoxan 100 mg·kg⁻¹ alone had no effect on the number of pain related behaviours as compared with vehicle treated mice ($P > 0.1$ by *t* test, Figure 1).

MK801 0.2 mg·kg⁻¹ decreased the minimum number of pain related behaviours as compared with vehicle treated mice ($P < 0.05$ by one-way ANOVA, Figures 1 and 2), although absolute differences were small. Ketamine decreased the minimum number of pain related behaviours in a dose-dependent manner over a dose range from 5 to 40 mg·kg⁻¹ as compared with vehicle treated mice ($P < 0.001$ by one-way ANOVA, Figures 1, 2). The analgesic effect of ketamine 40 mg·kg⁻¹ was not antagonized by naloxone 10 mg·kg⁻¹ ($P > 0.2$ by paired *t* test, data not shown).

ZJ-43 100 mg·kg⁻¹ decreased the minimum number of pain related behaviours as compared with vehicle treated mice ($P < 0.05$ by one-way ANOVA, Figures 1, 2). There was no significant effect at doses of 10, 30, 170 or 300 mg·kg⁻¹ of ZJ-43 (Figure 2). The analgesic effect of ZJ-43 100 mg·kg⁻¹ was completely antagonized by pre-treatment with LY341495 1 mg·kg⁻¹ ($P < 0.01$ by *t* test, Figure 1). LY341495 1 mg·kg⁻¹ had no effect on the minimum number of pain related behaviours as compared with vehicle treated mice ($P > 0.1$ by *t* test, Figure 1).

Discussion

This study demonstrates that *ip* injection of morphine and α -2 agonists (dexmedetomidine and clonidine) decreases the minimum number of pain related behaviours in a dose-dependent manner in a mouse bone cancer pain model. A significant decrease of the minimum number of pain-related behaviours was achieved with morphine 20–80 mg·kg⁻¹. This dose range of morphine is similar to that previously reported as being effective in blocking bone cancer pain-related behaviour in a mouse bone cancer pain model.^{18,19} The effects of morphine were antagonized completely in response to naloxone, and the effects of the α -2

agonists were completely antagonized by idazoxan. These data support the concept that the action of morphine is mediated via activation of naloxone-sensitive opioid receptors, and that the effects of α -2 agonists are mediated via activation of idazoxan-sensitive α -2 receptors.

Both morphine and α -2 agonists decreased the minimum number of pain related behaviours to 0, suggesting that the efficacy of morphine is similar to that of the α -2 agonists. However, the maximum analgesic effects of α -2 agonists were observed only at doses that produced sedative effects, while morphine produced its maximum analgesic effect at a dose without observable sedation. Cortinez *et al.*²² reported that dexmedetomidine is not as potent an analgesic as the opioids, and that the difference in the quality of analgesia between dexmedetomidine and opioids may be a reflection of the sedative properties of dexmedetomidine.

MK801 0.2 mg·kg⁻¹ decreased the minimum number of paw withdrawal responses, but this effect was small, and a higher dose of MK801 was associated with agitation. Hao and Xu²³ reported, using a neuropathic pain model, that systemic administration of MK-801 0.1 mg·kg⁻¹ produced no analgesic effect, and that the drug achieves analgesic efficacy only at a dose (0.25 mg·kg⁻¹) associated with severe motor impairment. These data suggest that systemic MK-801 produces analgesia only at a dose associated with clinically important side effects. In contrast, ketamine produces analgesia in a dose-dependent manner at doses between 5 and 40 mg·kg⁻¹. Ketamine 40 mg·kg⁻¹ decreases the minimum number of pain related behaviours to 0, similar to the response observed with morphine, and without any overt behavioural effects. This analgesic response is not antagonized by naloxone. It has been reported that MK-801 produces a different neuronal response in a rat neuropathic pain model, compared to ketamine.²⁴ It is possible that the different effects of ketamine and MK-801 on bone cancer pain may relate to the characteristics of their voltage-dependent blockade of the channel associated with the NMDA receptor.²⁴

At a dose of 100 mg·kg⁻¹, ZJ-43 decreased the minimum number of pain related behaviours. ZJ-43 is a NAAG peptidase inhibitor and NAAG is an mGluR3 agonist.¹² Inhibition of NAAG peptidase activity is expected to result in increased activation of group II mGluR, particularly mGluR3, due to accumulated NAAG, and results in reduced pain perception. The effect of ZJ-43 was completely blocked by the pretreatment with LY341495, a potent group II mGluR antagonist. These findings support the hypothesis that inhibition of NAAG peptidase activity increases the

activation of group II mGluRs to produce an analgesic effect.

At the higher doses of ZJ-43 (170 and 300 mg·kg⁻¹), the analgesic effect of ZJ-43 was not detectable. We previously reported that intrathecal administration of 2-(phosphonomethyl)pentanedioic acid, a NAAG peptidase inhibitor, produces an analgesic effect in the rat formalin test and carrageenan test, with a bell-shaped dose-response curve.^{13,14} We do not know the mechanisms underlying the dose-response curves for these two structurally different NAAG peptidase inhibitors in these pain models. It has been reported that a high concentration of NAAG acts as a NMDA receptor agonist.^{25,26} However, recent papers provide conflicting conclusions as to the efficacy of NAAG as an agonist, antagonist or partial agonist at the NMDA receptor.^{27,28}

Morphine, α -2 agonists or ketamine, but not ZJ-43, reduced the number of pain related behaviours to 0. Thus, the efficacy of ZJ-43 was not as great as that of morphine or ketamine, but it remains to be seen if ZJ-43 produces side effects comparable to those associated with the other two drugs. These NAAG peptidase inhibitors represent a novel approach to bone cancer pain management that may be of therapeutic value.

In the present study, the number (on average five) of animals per group was chosen for the dose-response study of each drug, on the basis that a previous study²¹ revealed that five animals per group is a suitable number for a dose-response study using this bone cancer pain model.

We used the number of pain related behaviours induced by application of von Frey filament to the site of tumour cell injection to estimate the level of bone cancer pain. Although spontaneous pain is the main symptom of metastatic bone tumour, pain increases with pressure at the area of involvement.³ Spontaneous pain may be moderate, but may also be exacerbated by different movements or changes in body position, such as standing or walking (incident pain), because of pressure to the site of bone tumour. Incident pain is much more difficult to manage as compared with spontaneous pain.³ It has been reported that, in this mouse bone cancer pain model, spontaneous pain related behaviour is much more sensitive than limb use during forced ambulation.¹⁸ Thus, we choose the number of pain related behaviours induced by application of the von Frey filament to estimate the level of pain.

In conclusion, *ip* administration of an opioid receptor agonist, α -2 agonists, and a NAAG peptidase inhibitor produce an analgesic effect in a mouse bone cancer pain model. Administration of ketamine and a

NAAG peptidase inhibitor may present a novel and effective approach to the management of bone cancer pain.

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