Substance P and Beta Endorphin Mediate Electroacupuncture Induced Analgesic Activity in Mouse Cancer Pain Model

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(Received May 21, 2008; Accepted with revisions May 5, 2009)

ABSTRACT:
Cancer pain impairs the quality of life of cancer patients, but opioid analgesics can not only cause inhibition of respiratory function, and constipation, but also other significant side effects such as addiction and tolerance that further decrease quality of life. Thus, in the present study, the effects of electro-acupuncture treatment (EA) on mechanical allodynia were examined in cancer pain mouse model. In order to induce neuropathic cancer pain model, S-180 sarcoma cells were inoculated around the sciatic nerve of left legs of Balb/c mice. The mass of S-180 cancer cells embedded around sciatic nerve in a time course was confirmed by Magnetic Resonance Imaging (MRI) scanning. Mechanical allodynia was most consistently induced in mouse sarcoma cell line S-180 (2 x 10^6 sarcoma cells) treated group among all groups. EA stimulation (2Hz) was daily given to ST36 (Zusanli) of S-180 bearing mice for 30 min for 9 days after S-180 inoculation. EA treatment significantly prolonged paw withdrawal latency from 5 days after inoculation as well as shortened cumulative lifting duration from 7 days after inoculation compared with tumor control. In addition, the overexpressions of pain peptide substance P in dorsal horn of spinal cord were significantly decreased in EA treated...
Introduction

Pain is a very frequent problem in cancer patients. Analgesic ladder for cancer-related pain provided by the WHO involves progressing from non-opioid (e.g., acetaminophen, ibuprofen) to weak opioid (e.g., codeine), to strong opioid (e.g., morphine, fentanyl) intervention for pain relief [12]. Unfortunately, opioid analgesics often not only produce poor pain relief against neuropathic cancer pain but also induce adverse side effects such as hormone (e.g., ACTH, cortisol, LH and testosterone) secretion, neurotransmitter (e.g., nicotine, adenosine, GABA and cholecystokinin) release, feeding, gastrointestinal motility, and respiratory activity [8]. Thus, safe and effective complementary therapies for cancer pain were suggested recently [8].

Generally, of three types of somatic, visceral and neuropathic pain of cancer pain, neuropathic pain is a main factor due to compression or infiltration of peripheral nerves by malignant tumors in cancer patients [18]. Neuropathic pain resulting from nerve injury is characterized by spontaneous pain, allodynia (the perception of normally innocuous stimuli as painful) and hyperalgesia (an increased sensitivity to painful stimuli). However, animal model for neuropathic cancer pain still remains unclear regarding cancer cell and animal type.

Acupuncture has a long history, but its scientific evaluation has been begun rather recently. Acupuncture treatment or electro-acupuncture has been applied for a wide range of symptoms and has produced some benefits. Especially, electro-acupuncture at acupoint ST36 has been reported to relieve pain and reduce inflammation and cerebral ischemia [9,13]. Early scientific work on manual and electrical stimulation on ST36 was carried out by Omura, Y [1-3].

In the present study, our experiments were performed to evaluate the effects of electro-acupuncture treatment on mechanical allodynia in the mouse model of neuropathic cancer pain using S-180 sarcoma cells and elucidated antianalgesic mechanism in dorsal horn of spinal cord of mice by immunohistochemistry for c-Fos, substance P and dynorphin A and enzyme immunoassay (EIA) for beta endorphin in blood and brain of mice.

Materials and Methods

Animals

Male BALB/c mice weighing 25-30 g were purchased from Daehan Bio Link. The animals were kept and maintained under laboratory conditions of temperature, humidity, and light. Mice were maintained on a 12:12h dark-light cycle with food and water ad libitum.

Cell Culture
S-180 sarcoma cells (ATCC CCL-8) were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY) with 100 ml/l heat inactivated (30 min at 56 °C) fetal bovine serum, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C in 50 ml/l CO₂.

First Experiment
Neuropathic Cancer Pain Model
To determine the optimal number of S-180 cells that can induce neuropathic cancer pain model, three different cell numbers (1x10⁷(n=3), 5x 10⁶(n=3), and 2x 10⁶(n=3)) of S-180 cancer cells were inoculated into the muscular tissue in the immediate vicinity of the nerve near the trochanter, immediately distal to where the posterior biceps semitendinosus branches off the common sciatic nerve. Thereafter, neuropathic cancer pain was comparatively monitored in S-180 treated groups.

MRI Scanning
To confirm the tumor mass around sciatic nerve by anatomical examination, MRI scanning was performed. On day 10, 16 and 24 after inoculation, mice of each group were sacrificed and scanned around sciatic nerve by MRI.

2nd Experiment

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Fig.1: Experimental Scheme for EA treatment to neuropathic cancer pain model.

Neuropathic Cancer Pain Model
Based on 1st experiment, neuropathic cancer pain model, we decided number of S-180 cells that can induce neuropathic cancer pain model, 2x 10⁶ of S-180 cancer cells were inoculated into the muscular tissue in the immediate vicinity of the nerve near the trochanter, immediately distal to
where the posterior biceps semitendinosus branches off the common sciatic nerve.

Fig. 2: Neuropathic cancer pain model

**EA Treatment**

EA treatment was applied to the EA group only. Stainless steel needle with 0.3 mm diameter was inserted with a depth of 5 mm into the unilateral acupuncture point ST36(Zusanli) located 0.5cm below fibular head of hinder leg in mouse and stimulated with intensity of 2 Hz (<3 mA) for 30 min. The levels of EA treatment were based on those in a previous report [9,11]. The proximal end was soldered to a wire that was connected to one of the output channels of an electric stimulator, PG-306(YoungMok, Japan). As shown Fig.2, the ST36 (Zusanli) acupoint is located 5 mm below and lateral to the anterior tubercle of the tibia. Electrical stimulation was applied to ST36 point using two outlets via two needles. An electrical pulse with a voltage of 3-5V, a duration of 0.25 ms and a frequency of 2 Hz was delivered from an EA stimulator. The intensity of the stimulation was determined to be minimum voltage to cause moderate muscle contraction.
Fig. 3: ST36 acupuncture points used in Neuropathic cancer pain model
Acupuncture point ST36 in both human and mouse

Behavioral Test (Mechanical von Frey test)
During behavior test, all mice were divided into three groups such as Tumor control group (n=8), EA Treated group (n=8) and Normal group (n=8). All mice were placed on the wire mesh platform which was fixed in a transparent plexiglass chamber (20 x 10 x 5 cm). This study was performed based on a modified protocol [11]. Behavior assessment was performed on Day 1, 3, 5, 7 and 9 after tumor inoculation. The series of von Frey hairs was applied from below the wire mesh platform to the plantar surface of the left hind paw. The hind paw withdrawal threshold was determined using von Frey hairs weighing from 0.4g to 4g. Behavioral test using von Frey hair to the hind paw of mice was carried out five times in 5 s intervals. A withdrawal response was considered valid only if the hind paw was completely removed from the wire mesh platform.

Fig. 4: Behavioral Test (Mechanical von Frey test)
Behavior Observation and Mechanical Allodynia Measurement von Frey.
On days 3, 10, 14, and 24 after tumor injection surgery, the paw withdrawal was determined by von Frey hair filament.

Spontaneous Pain Test
All mice of three groups were observed for mechanical allodynia signs as a spontaneous pain on Day 3, 5, 7 and 9 after tumor inoculation. Spontaneous pain test was performed in all mice of three groups placed in a clear plastic chamber with wire grid floors at room temperature. After approximately 1h acclimatization, the cumulative duration of hind paw-lifting of each mouse was measured for 10 min. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic anaesthesiometer, IITC Life science, Woodland Hills, CA, USA) adapted with a 0.5mm² polypylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point characterized by the withdrawal of the paw followed by clear lifting and flinching behavior.
The lifting of the paw as a part of grooming behavior was not taken into account.

**Immunohistochemistry**

The specimens of spinal cord dorsal horn of mice were sectioned on cryostat as 40 μm coronal sections between L3-L5. The sectioned tissues were rinsed in phosphate buffered saline with tween 20(PBST) about 3 times before use. 1X PBST contains 3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4. Primary antibody was diluted with blocking solution (Vector Laboratories, Burlingame, CA) and tissues were incubated with antibodies against substance P (1:50) purchased from Abcam Ltd. (Cambridge, UK) for 48 h at room temperature under constant agitation. After rinsing in PBS, the sections were incubated for 2 h with the biotinylated rabbit anti-serum (Vector Laboratories, Burlingame, CA) that was diluted to 1:200 in PBST containing 1% normal goat serum. The sections were placed in the Vectastatin™ Elite ABC reagent (Vector Lab., UK) for 1 h. After further rinsing in PBS, the tissues were developed using diaminobenzadine as a chromogen with nickel intensification. These slides were air-dried, cover-slipped and then observed under a light microscope (Carl Zeiss, Germany).

**Enzyme Immunoassay**

Blood samples were collected (1 ml) into the lavender vacutainer tubes containing EDTA. The tubes were gently rocked several times immediately after collection of blood for anti-coagulation. Blood was transferred from the lavender vacutainer tubes to centrifuge tubes containing aprotinin (0.6 TIU / ml of blood) and gently rocked several times to inhibit the activity of proteinases. The blood was centrifuged at 1,600 x g for 15 min at 4 °C and plasma collected. Brain tissues were grinded using Teflon Homogenizer in 2 ml lysis buffer (10 mM Tris-HCl, pH 7.4) and centrifuged at 12,000 x g for 15 min at 4 °C and supernatant was collected. Plasma and brain samples were stored at -20 °C prior to EIAs and brought to 4 °C. Samples were acidified with an equal amount of buffer A (250 μl), centrifuged at 17,000 x g for 20 min at 4 °C and equilibrated using SEP-COLUMN (CA, USA) containing 200 mg of C18 (Code RK-SEPCOL-1) by washing with buffer B (1 ml, once) and followed by buffer A (3 ml, 3 times). The acidified plasma solution was added to the pre-treated C-18 SEP-COLUMN. The column was slowly washed with buffer A (3 ml, twice). The peptide was slowly eluted with buffer B (3 ml, once), collected into a polystyrene tube and evaporated to dryness. The contents of beta endorphin were measured by direct beta endorphin EIA kit from Phoenix Pharmaceuticals (CA, USA).

**Results**

**Morphological changes of S-180 tumor mass around sciatic nerve and induction of neuropathic cancer pain**

As shown in Fig. 5, S-180 cells grow rapidly and embedded around sciatic nerve in a time course, which was confirmed by MRI scanning. On Day 9 after inoculation, the sciatic nerve was partially embedded by S-180 tumor mass and on Day 24, the sciatic nerve was almost surrounded by S-180 tumor mass. As shown in Fig. 6, among three groups (1x10⁶, 5x 10⁶ and 2x 10⁶ injected groups), neuropathic cancer pain was most steadily induced in 2x 10⁶ injected group 2 days after inoculation, suggesting the suitable cell number is 2x 10⁶ cells to induce neuropathic cancer pain.
Fig. 5: MRI scans of S-180 tumor mass around the sciatic nerve. After inoculation of S-180 tumor cells around the sciatic nerve, MRI scan was performed. (A) On inoculation day  (B) 10 days after inoculation  (C) 16 days after inoculation  (D) 24 days after inoculation.

![MRI scans of S-180 tumor mass around the sciatic nerve.](image)

![Graph showing von Frey hair threshold.](image)
Fig. 6: S-180 implantation around sciatic nerve induced neuropathic cancer pain according to cell number in a time course. Withdrawal latency of left hind paws was measured every 2 days until 17 days after inoculation. Values are expressed means ± SE. Statistically significant differences compared with control by student's $t$ test (* $p<0.05$, ** $p<0.01$).

Effect of EA treatment on neuropathic cancer pain
As shown in Fig. 7, EA treatment significantly attenuated paw lifting latency induced 3 days after inoculation by von Frey test. As shown in Fig. 8, hind paw-lifting in tumor control became apparent compared with normal group from Day 5 after tumor inoculation and the cumulative paw-lifting duration reached to the peak on Day 9 showing all mice in tumor control group showed slight foot drop in the left hind limb. On the contrary, EA treatment significantly reduced cumulative lifting duration compared with untreated tumor control.

![Graph](image_url)

Fig. 7: EA treatment increased paw withdrawal latency compared with untreated tumor control. Paw withdrawal latency was measured every 2 days until 9 days after inoculation. Statistically significant differences compared with normal control by student's $t$ test (* $p<0.05$).
Fig. 8: EA treatment reduced cumulative lifting duration of paw compared with untreated tumor control. Cumulative lifting duration of the left hind paws was measured every 2 days until 9 days after inoculation. Statistically significant differences compared with normal group by student's t test (* p<0.05).

**Effect of EA treatment on substance P and beta endorphin**

Nine days after inoculation, immunohistochemistry was performed with antibody of substance P in sections of spinal cord dorsal horn of mice. As shown in Fig. 9, substance P was overexpressed in tumor control without inoculation compared with normal control, suggesting that tumor mass can activate neuropathic pain related proteins. On the contrary, EA treatment for 9 days effectively reduced the expressions of pain peptide substance P in the dorsal horn section of spinal cord of mice compared with untreated tumor control. To confirm the analgesic mechanism of EA treatment, EA was performed to measure the level of beta endorphin in blood and brain tissues of mice. As shown in Fig. 10A, the level of beta endorphin in blood of tumor control was significantly increased up to 2.8754 ±0.0278 ng/ml compared with that of normal group, 1.3236 ± 0.0041. On the contrary, EA treatment significantly increased the level of beta endorphin up to 4.355 ± 0.2972 ng/ml more than tumor control, 2.8754 ± 0.0278 ng/ml. Consistently, as shown in Fig. 10B, in brain tissue of mice, the level of beta endorphin in tumor control was significantly increased up to 4.0115 ± 0.3848 ng/ml compared with that of normal group, 2.668 ± 1.069 ng/ml. In contrast, EA treatment significantly increased the level of beta endorphin up to 9.0847 ± 0.5901 ng/ml more than tumor control, 4.0115 ± 0.3848 ng/ml.
Fig. 9: Representative photographs of coronal section showing SP expression in the spinal cord. Photographs (200 X) illustrate SP immunoreactive neurons in the mouse superficial dorsal horn (SDH) of L3-5 levels. (A) Normal group, (B) Control group, (C) EA treated group. Arrows indicate SP positive cells.
Fig. 10: EA treatment increased the level of beta endorphin in blood and brain compared with untreated tumor control. (A) level of beta endorphin in blood (B) level of beta endorphin in brain. Values of beta endorphin are expressed as means ± SE. # p < 0.05 ### and p < 0.001 compared to Normal values. * p < 0.05 and . *** p < 0.001 compared with tumor control.

Discussion

Pain is an important symptom in cancer patients. The prevalence of pain depends on tumor type and varies from 5% in patients with leukemia to 52% in patients with lung cancer. The causes of pain are tumor itself by bone invasion, compression of the spinal cord or neural structures and pressure on hollow organs [17]. Thus, in the current study, we set up the neuropathic cancer mouse model by inoculation of S-180 tumor cells around the sciatic nerve of mice tumor mass. MRI scanning revealed the tumor size and position around sciatic nerve of mice. Ten days after inoculation, the tumor mass was shown half around the sciatic nerve and on Day 24 after inoculation, S-180 tumor cells embedded most of gluteal area induced the neuropathic pain by compression of the sciatic nerve [19].

Behavior test using von Frey hairs showed tumor mass of S-180 cells significantly induced a paw hind lifting from 3 days after inoculation and prolonged cumulative lifting duration as a spontaneous pain 5 days after inoculation up to 9 days after inoculation, suggesting that neuropathic cancer pain mouse model was properly set up for cancer pain assessment. In contrast, Shimoyama’s cancer model that was produced by inoculation Meth-A sarcoma cells to the vicinity of the sciatic nerve [10] showed that hind paw-lifting, a behavioral sign of spontaneous pain, was at maximum on Day 18 after inoculation of Meth-A sarcoma cells to the vicinity of the sciatic nerve. Therefore, our cancer pain model may induce neuropathic cancer pain more rapidly and consistently within ten days after S-180 cell inoculation compared with Shimoyama’s cancer model. These data strongly suggest that our cancer model can be applied for evaluation of in vivo cancer pain control efficacy in a short time.

To confirm the roles of pain related peptides during acupuncture induced analgesic activity,
immunohistochemistry for substance P and enzyme immunoassay for beta endorphin in blood and brain of mice were performed in spinal cord dorsal horn of mice. On Day 9 (peak paw-lifting), Substance P is a neuropeptide involved in the transmission of pain impulses from peripheral receptors to the central nervous system. It belongs to the tachykinin neuropeptide family [7]. EA treatment downregulated the substance P expression [15], while substance P was overexpressed in the dorsal horn of our tumor control on Day 9 after inoculation [5].

Endorphins are endogenous opioid polypeptide released in the pituitary gland and the hypothalamus during strenuous exercise and excitement. Although the role of plasma beta endorphin in pain regulation is unclear, plasma beta endorphin levels have been reported to correlate inversely with pain levels in cancer pain [4]. In the current study, unexpectedly beta endorphin was released ~ 2 fold in blood and brain of tumor control much more than in normal group. The beta endorphin that is released into the blood cannot enter the brain in large quantities because of the blood-brain barrier [18]. On the contrary, EA treatment significantly increased the level of beta endorphin compared with tumor control. These data support involvement of the endorphin system in neuropathic cancer pain model.

In summary, the mass of S-180 cancer cells was embedded around sciatic nerve as shown by a time course of MRI scanning. Mechanical allodynia was most consistently induced in S-180 (2 x 10^6) treated group among all groups. EA treatment significantly prolonged paw withdrawal latency as well as shortened cumulative lifting duration compared with tumor control. In addition, the overexpression of pain peptide substance P in dorsal horn of spinal cord was significantly decreased in EA treated group compared with tumor control on Day 9 after inoculation. Furthermore, EA treatment effectively increased the concentration of beta endorphin in blood and brain of mice more than tumor control. Taken together, S-180 cancer pain model can be a consistent and short time animal model and also EA treatment can be an alternative therapeutic method for cancer pain via decreased substance P and increased beta endorphin.

Recently, a more accurate location of ST 36 in humans was described in detail by Omura, Y. et al [16]. True ST 36 has been shown with manual acupuncture to be much superior to traditional ST 36. It is often used by Omura for the treatment of cancer by the normal cell telomere-increasing effects of True ST 36 by press needle stimulation of 200 press-release repetitions for 4 times a day (which increases normal cell telomere to over 500 ng BDORT units, which in turn reduces the cancer cell telomere practically to 0 and inhibits cell division of the cancer cells). Although in humans, the distance between Omura’s ST 36 and traditional ST 36 is anywhere between 6 - 12 mm, in animals the distance is so small that they are nearly indistinguishable. This has to be evaluated in future research.

**Acknowledgements**

This work was supported by Medical Research Center (MRC) grant (R13-2007-019-00000-0).

**References**


