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The sensitization of peripheral C-fibers to lysophosphatidic acid in bone cancer pain

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ABSTRACT

Aims: Lysophosphatidic acid (LPA) is released from injured tissue and cancer cells and is involved in the induction of neuropathic pain. The present study explores whether LPA plays a role in the development of osteocarcinoma-induced pain.
Main methods: The bone cancer model was established using the Walker 256 mammary gland carcinoma cell line, and cancer-related behavioral and physiological changes were observed using von Frey, X-ray and immunohistochemical methods. The role of LPA in the bone cancer model and related mechanisms were examined by using in vitro single fiber recording and western blot.
Key findings: Rats exhibited severe hyperalgesia 2 weeks after the cancer cell implantation. Several changes were observed at this time point including: ipsilateral dorsal root ganglion (DRG) neurons were labeled by injured neurons marker ATF3; LPA_1 receptor expression in DRG neurons was increased; sural C-fibers were more sensitive to LPA stimuli, and this response could be blocked by LPA receptor and substance P receptor antagonists.
Significance: These data indicate that LPA is involved in the induction of bone cancer pain through mechanisms of peripheral C-fibers sensitization. LPA and its downstream molecules possibly are promising therapeutic targets for treatment of cancer pain.

Introduction

Most of the patients with advanced cancer suffer from severe spontaneous and movement-induced pain (Mercadante and Arcuri 1998). With the establishment of animal models for various cancers including bone cancer, studies on mechanisms underlying cancer pain are rapidly progressing (Schwei et al. 1999; Mantyh et al. 2002). Several mechanisms are involved in cancer pain including direct mechanical stimuli, nerve injury-induced neuropathic pain, inflammation and cancer cell-released neurochemical stimuli (Schwei et al. 1999).

Lysophosphatidic acid (LPA) is a simple lysophospholipid which mediates a wide range of biological actions (Anliker and Chun 2004; Fukushima 2004; Rivera and Chun 2008). There is a high concentration of LPA in ascites and plasma of cancer patients. LPA is released from many kinds of cancer cells including ovarian, gastric, prostate and breast cancer cells (Xu et al. 1995; Fang et al. 2002; Boucharaba et al. 2004; Spiegel and Milstien 2005). These cancer cells may be the source of high LPA level in ascites (Fang et al. 2002). Abundant evidence shows that LPA is a key mediator in cancer development including cancer cell proliferation, survival and migration (Fang et al. 2002; Radeff-Huang et al. 2004; Guo et al. 2006; Ishdorj et al. 2008). Meanwhile, increasing studies have revealed that LPA also plays an important role in processing nociceptive information, particularly neuropathic pain (Renback et al. 1999; Inoue et al. 2004; Lee et al. 2005; Ueda 2006, 2008).

All five LPA receptor subtypes, LPA_{1-5}, are G protein-coupled receptors (Noguchi et al. 2003; Anliker and Chun 2004; Lee et al. 2006). Among the five subtypes, the LPA_1 receptor is the main subtype expressed in the dorsal root ganglion (DRG) (Inoue et al. 2004). Intraplantar injection of LPA into the hind limb of mice induced a dose-dependent nociceptive flexor response which was blocked by a substance P (SP) receptor antagonist (Renback et al. 1999). In isolated DRG neurons, TTX-resistant Na^+ current, an important transducer of nociceptive information, was increased after LPA treatment, whereas at the same time TTX-sensitive Na^+ current was suppressed by LPA (Lee et al. 2005). Moreover, LPA also induced demyelination of the peripheral nerve contributing to the induction of neuropathic pain via activation of the LPA_1 receptor (Ueda 2006, 2008).

However, whether cancer cell-released LPA is also involved in the peripheral mechanism of cancer pain is still unknown. By means of behavioral tests, immunohistochemistry and single fiber recording in vitro, the present study explores whether LPA participates in the development of bone cancer-induced pain mediated by the sural nerve adjacent to, but not directly innervating an osteocarcinoma in rats.

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Material and methods

Bone cancer model

Female Sprague–Dawley (SD) rats weighing 220 g were used (Experimental Animal Center, Fudan University, Shanghai, China). The detailed procedure for the Walker 256 rat mammary gland carcinoma cell culture and injection has been presented in our previous report (Mao-Ying et al. 2006). Carcinoma cells (10^8) in 4 μl PBS (cancer group) or 4 μl PBS alone (sham group) were injected into the left tibia cavity through the knee joint to induce the bone tumor in anesthetized rats. All experiments were conducted in accordance with the guidelines of the International Association for the Study of Pain (IASP) (Zimmermann 1983).

Mechanical pain hypersensitivity test

Rats were placed in the testing cage ($20\times10\times18$ cm^3) with a wire net floor with 0.5 cm distance between each line. After 30 min accommodation, von Frey hair stimulation was applied for 3 s with an ascending series of forces (1, 2, 4, 6, 8, 10, 15 and 26 g) to the lateral plantar surface of the ipsilateral hind paw. Each force was tested 5 times with a 10 min interval in between. Stimulation-induced leg lifting was defined as a positive response. The number of positive responses was recorded in each trial. The mechanical threshold was defined as the lowest force to induce at least 3 responses out of 5 stimulations. Testing sessions were performed on the day before injection and day 2, 4, 7, 10, 13, 16, 20 and 25 after cancer cell or sham injection.

Western blot

Rats were anesthetized with chloral hydrate (i.p. 400 mg/kg). L_{4-6} DRGs of sham and cancer group rats were collected at day 16 after cancer cell injection and homogenized in a lysis buffer containing protease inhibitor (Sigma). The protein concentrations were measured using a BCA assay (Pierce, Rockford, IL) and 3 mg protein was loaded on each lane. The protein samples were separated using SDS-PAGE (10%) and transferred to PVDF membranes. The membranes were blocked in 10% nonfat dry milk for 2 h at room temperature (RT) and incubated overnight with LPA_1 receptor (EDG-2) primary antibody (1:400; Santa Cruz Biotechnology) or mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10,000; Kangchen Bio-Tech), followed by incubation in horseradish peroxidase (HRP)-conjugated donkey anti-goat or a goat anti-mouse secondary antibody (1:3000, Santa Cruz Biotechnology) for 2 h at room temperature. Reactive bands were visualized in ECL solution (Pierce) for 1 min and exposed to X-films for 1–30 min.

Immunohistochemistry

Sham and cancer rats were given an overdose of urethane (2 g/kg, i.p.) on day 14 after cancer cell inoculation surgery and perfused intracardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). L_{4-6} DRGs were then removed, post-fixed in the same fixative for 4 h at 4 °C and immersed in a sucrose gradient from 10% to 30% in PB for 24–48 h at 4 °C for cryoprotection. Frozen 14 μm DRG sections were cut using a cryostat microtome and thaw-mounted onto gelatin-coated slides for further processing. The sections were blocked with 10% donkey serum in 0.01 M phosphate buffered saline (PBS, pH 7.4) with 0.3% Triton X-100 for 2 h at RT and incubated overnight at 4 °C with a primary antibody against activating transcription factor 3 (ATF3, polyclonal rabbit anti-rat, 1:200; Santa Cruz Biotechnology) in PBS with 1% normal donkey serum and 0.3% Triton X-100. Following three rinses for 15 min each in 0.01 M PBS, the sections were incubated in rhodamine-conjugated IgG (1:200, Jackson Immunolab) for 90 min at 4 °C and then washed in PBS. All sections were coverslipped with a mixture of 50% glycerin in 0.01 M PBS and then observed with a Leica fluorescence microscope. Images were captured with a CCD spot camera. Six rats were used for both groups, and for each rat, six sections of spinal L_{4-6} were randomly selected for quantitative evaluation. Then, averaged density for each rat was counted. The number of total cells and the number of ATF3-positive cells were respectively counted on each slide, and the percentage of ATF3-positive cells in total cells was calculated. The averaged percentage of ATF3-positive cells was compared between the two groups.

X-ray detection

Sham and osteocarcinoma (15 days after injection) rats were anesthetized with urethane (1.5 g/kg) before X-ray analysis. The left tibias were compared between groups. All pictures were taken using a Philips Digital Radiographer System in Fudan University Cancer Hospital.

Single unit recording in vitro

The organ bath used was the same as described in our previous study (Wei and Wang 2000). Rats were anesthetized with urethane (1.5 g/kg). The sural nerve and the accompanied popliteal artery and vein were isolated from the surrounding muscle and skin and placed into the main chamber continuously perfused with oxygenated HEPES buffer solution (in mM: 5 KCl, 1 $MgCl_2\cdot6H_2O$, 1.3 Na_2HPO_4, 2 $CaCl_2\cdot2H_2O$, 137 NaCl, 5.5 HEPES, 10 D-glucose; pH7.38 ± 0.02) at about 3 ml/min flow rate. Temperature of the chamber was maintained at 32 ± 1 °C by a heating system. A fine PE10 pipe was inserted into the popliteal artery for drug injection. The sural nerve was placed in a recording chamber and a thin nerve strand cut and isolated from the nerve trunk. The distal end was connected to the recording electrode (platinum wire, 30 μm). A slim piece of connective tissue was separated and connected to the other electrode as a reference electrode. Brushing and electrical stimulation on the skin were used to identify the receptive field and the type of fibers. Data acquired with Pulse software were analyzed with PowerLab 4/25 AD Instruments.

LPA was dissolved in 2.5% of MeOH to the concentration from 0.01 mM to 10 mM, while VPC32183 and WIN51708 were dissolved in saline. After each testing, the preparation was washed by 100 μl HEPES buffer solution and recovered for at least 30 min. Capsaicin (0.1 mM) was used at 1 h after all testing antagonists.

Statistical analysis

Data were analyzed using student's *t* test, χ^2 test and two-way ANOVA. The criterion of significance was set at * $p<0.05$, ** $p<0.01$, ***

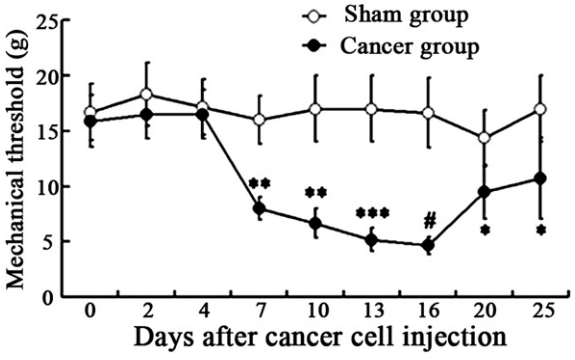

Fig. 1. Hypersensitization following bone cancer. The mechanical threshold to von Frey hair stimulation was examined at the day 2, 4, 7, 10, 13, 16, 20 and 25 after cancer cell injection. Data are expressed as the mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, # $p<0.0001$ vs. sham group.