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Effect of toad skin extracts on the pain behavior of cancer model mice and its peripheral mechanism of action

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ABSTRACT

The changes in thermal and mechanical hyperalgesia in paw cancer pain model mice and the action mechanism of toad skin extracts (TSE) was investigated. Eighty female mice were subcutaneously injected with saline or inoculated with H22 hepatoma cells in the right hind paw and administration with saline, vehicle, morphine and TSE. The pain behavior was recorded before treatment and at 0.5, 1.0, 1.5, 3 and 6 h after initial administration, and thereafter on the 2nd, 4th, 6th, and 8th day after administration. On the last day, samples were collected after the euthanasia for the detection of β-END, CRF, IL-1β, POMC, μ-OR, CD3+, CD8+ and CD4+ in sera and the tumor tissues. The results showed that TSE significantly increased the thresholds of thermal pain and mechanical pain, and upregulated the expressions of β-END, CRF, POMC, CD3+, CD8+ and μ-OR, and downregulated the expression of CD4+. These results indicate that TSE significantly relieved pain in cancer pain model mice and raised their pain threshold. In addition, TSE seems to play a prominent role in promoting the activity of tumor infiltrating lymphocytes (TILs, CD3+ and CD8+ T cells), and this immune-cell-derived peripheral analgesic pathway might have widespread potential for clinical use.

1. Introduction

Cancer pain, one of the most common symptoms of cancer, results from both the direct effects of the tumor and from the treatment. In 2016, 1,685,210 new cancer cases and an estimated 595,690 cancer deaths are projected to occur in the United States [1]. Malignant tumors such as those in lung, prostate, and breast can undergo skeletal metastases that could result in severe cancer-induced bone pain, which substantially reduces the quality of life in cancer patients [2]. Patients report that pain prevents them from concentrating or thinking and that pain creates difficulty in performing normal daily activities. Over one-third of patients describe pain related to cancer as distressing or even as an intolerable aspect of their cancer [3]. Thus, pain relief has to be taken into consideration when carrying out a comprehensive evaluation of each patient's cancer treatment strategy.

Although pain has been extensively studied, patient's cancer pain is still not well controlled. It has been repeatedly demonstrated that opioid is one of the most effective drugs for cancer pain currently [4–6]. Opioid drugs combined with opioid receptors to produce their analgesic effect after treatment in patients, and >80% of patients with cancer need to use opioids to improve or control pain. However, the accompanying side effects of opioid drugs such as tolerance, addiction, excitement, drowsiness, constipation, nausea, vomiting, and respiratory depression limit the further application [7–10]. Non-steroidal anti-inflammatory drugs (NSAID) are also used to manage painful metastases but they also have side effects such as cardiovascular risks [11] and gastrointestinal disturbances [12–13]. Growing evidence suggests that the neuroimmune response plays a pivotal role in the development and maintenance of cancer pain [14]. Immune cells have a clear role in peripheral analgesia, and studies have demonstrated a positive association between immune cell activation and opioid peptide content [15]. This immune-cell-derived peripheral analgesic pathway might have widespread potential for clinical use.

In recent years, Chinese medicine has played an important role in the treatment of cancer pain. It has been reported that 41–62% of cancer patients use Chinese medicine as a complementary or alternative medical therapy [16–17]. Oral ingestion of herbal medicines may cause nausea, vomiting or diarrhea, therefore external application may be an acceptable alternative route for the treatment of localized cancer-related pain. Clinical observations have shown that Chinese herbal medicines are very useful in the management of cancer pain [18–19].

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Dried toad skin, the dried skin of Toad *Bufo bufo gargarizans* Cantor, has many beneficial effects such as dissipating heat, flushing out toxins, reducing swelling, pain relief and anti-tumor therapy [20]. Studies have shown that toad skin extracts (TSE) functions as an anti-tumor and immunomodulatory agent [21]. An aqueous extract of the entire skin of *Bufo bufo gargarizans* Cantor had been widely used to treat carcinomas and alleviate cancer pain, especially the pain induced by hepatocarcinoma, gastric and bone cancer, with few toxic side effects [22–25].

Previous studies have demonstrated that cinobufacini, the main active ingredient in the TSE, significantly alleviates cancer pain in the model mice, an effect believed to be mediated by peripheral opioid receptors, as determined by the use of naloxone methiodide, which is a selective peripheral opioid receptor antagonist [26]. However, the mechanisms of action of many topically applied Chinese medicines remain largely unknown.

This research set out to evaluate the effects of TSE and to explore the mechanisms of its action in a cancer pain mice model, by assessing both thermal and mechanical hyperalgesia, and the expression of β-END, POMC, μ-OR, CD3+, CD8+ and CD4+. We hypothesized that the main mechanism in the analgesic effect of TSE may be by increasing the level of intracellular POMC and β-END, and therefore promote β-END binding to peripheral μ-opioid receptors (μ-ORs) in the tumor to modulate peripheral cancerous pain.

2. Materials and methods

2.1. Materials

Toad skin (batch number: 110506), purchased from Anhui Fu Chun Tang Chinese Herbal Medicine Co., Ltd., was pulverized and extracted by double-distilled water twice. The filter liquor was combined and reclaimed, followed by vacuum distillation and purification with ethanol (50 °C, −0.08 MPa) [27–28]. Vaseline was used as the binding agent in the preparation of the gel and azone was as a penetration enhancer. The extracts from 1 kg of dried toad skin was mixed with inert gel (vaseline and azone) and consisted of toad skin extracts 48%, vaseline 48% and azone 4%. And 14.8 g of a mixture was obtained.

Morphine Hydrochloride Injection (batch number: 110103-2) was produced by Shenyang No. 1 Pharmaceutical Co., Ltd. of the Dongbei Pharmaceutical Corporation; IL-1β ELISA kits were produced by Wuhan Boster Biological Engineering Co., Ltd.; CRF, β-END ELISA kits were produced by Shanghai YanHui Biological Technology Co., Ltd.; Goat anti-rabbit SP immunohistochemical kit, FITC-conjugated goat anti-rabbit IgG, rabbit anti-mouse POMC, β-END, μ-OR polyclonal antibody, rabbit anti-mouse CD3+, CD4+, CD8+ polyclonal antibody and DAPI, were produced by Beijing Biosynthesis Biotechnology Co., Ltd. The voucher specimens were deposited in the Third-Grade Pharmacological Laboratory on Chinese Medicine Approved by State Administration of Traditional Chinese Medicine, College of Medical Sciences, China Three Gorges University.

2.2. Animals

Female Kunming mice (weighing 18 ± 2 g) used in the present study were purchased from the Experimental Animal Institute of Hubei Disease Control Center (Wuhan, China). All mice were kept under specific pathogen-free and climate-controlled conditions (temperature 23 ± 3 °C, relative humidity 60 ± 5%) with 12-h light/dark cycles, individually housed in polystyrene cages containing wood shavings to minimize the possibility of painful contact with a hard surface and fed standard rodent chow and water ad libitum. They were fed in a nonstressful environment for at least one week prior to experiment. All experiments were conducted with the approval of the Animal Care and Use Committee of China Three Gorges University and were in accordance with the Guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Experimental design

64 mice of 80 female Kunming mice, which were administrated tumor cells, were randomly divided into 4 groups: model group (NS, i.p.), vehicle group (inert gel, topical administration), morphine group (morphine, 8 mg/kg/day, i.p.), and TSE group (TSE gel, 0.5 g/kg/day, topical administration), with 16 individuals in one group; the remaining 16 normal mice which were not administrated tumor cells but normal saline (NS) were selected as control group. The experiment mice were administrated homologous drug, respectively. To investigate the effects of the TSE gel on cancer-induced pain and its mechanisms, TSE gel (TSE group) or control inert gel (vehicle group) were evenly applied to the skin of tumor-bearing hind paw; mice in the control and model groups were administered NS, respectively, once daily lasting for 8 days. The pain behavior of each mouse was determined in the before and after treatment. On the last day of treatment the weight and pain behavior of mice were measured, and specimens were sampled for testing.

2.4. Establishment of the hind paw cancer pain model

H22 murine hepatoma cell line was purchased from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, and were cultured by the Immune Research Center at our university. And it was inoculated into the abdominal cavity of female Kunming mice continuous culture for three generations, and ascites were extracted after 7 days. The samples were washed with D-Hank's solution and centrifuged at 800 r/min for 5 min at 4 °C (2 cycles) and dyed with trypan blue to detect whether the survival rate ≥95%, and then calibrated at a concentration of 6×10^7 cells/mL and maintained on ice until inoculation.

To establish the hind paw cancer pain model, female Kunming mice in the control group were subcutaneously injected with 0.1 mL of normal saline (NS) into their right hind paw. The other mice were subcutaneously injected with an equivalent volume of H22 hepatoma cells into their right hind paw. The procedure was carried out aseptically and completed within 1 h.

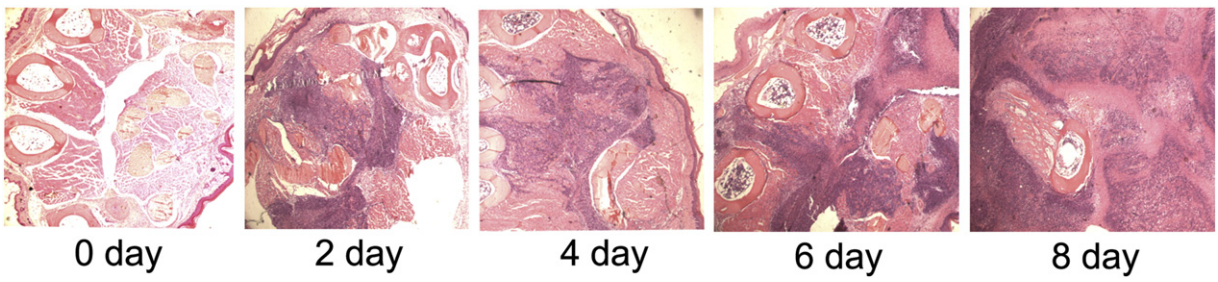

Fig. 1. The representative photomicrographs of mice right paws with hematoxylin and eosin on different days after injection of the cancer cell line H22 (×50). The right paw was found to have a large number of cancer cells on the 2th day after inoculation. There was a tendency for bone invasion in the right hind paw, which began on the 8th day after inoculation.