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Research article

Nonviral vector plasmid DNA encoding human proenkephalin gene attenuates inflammatory and neuropathic pain-related behaviors in mice

Chunsheng Hu[a,b], Zhenzhen Cai[a], Yuxin Lu[a], Xiaochen Cheng[a], Qi Guo[a], Zuze Wu[a,b], Qinglin Zhang[a,*]

[a] Department of Experimental Hematology, Beijing Institute of Radiation Medicine, Beijing 100850, People's Republic of China
[b] College of Life Science and Bioengineering, Beijing University of Technology, Beijing 10024, People's Republic of China

HIGHLIGHTS

- Peripheral or spinal delivery of pVAX1-PENK alleviates CFA-induced pain for an extended period.
- Peripheral or spinal delivery of pVAX1-PENK attenuates SNI-induced nocifensive behaviors in mice.
- The analgesic effect of pVAX1-PENK is blocked by naloxone hydrochloride.

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ABSTRACT

Inflammatory pain and neuropathic pain are major clinical health issues that represent considerable social and economic burden worldwide. In the present study, we investigated the anti-nociceptive efficacy of delivery of human proenkephalin gene by a plasmid DNA vector (pVAX1-PENK) on complete Freund's adjuvant (CFA) induced inflammatory pain and spared nerve injury (SNI) induced neuropathic pain in mice. Mice were intramuscularly or intrathecally administered pVAX1 or pVAX1-PENK, respectively. Pain thresholds in the pVAX1-PENK treated mice were significantly higher at day 3, then reached a peak at day 7 and lasted until day 28 after gene transfer, and the analgesic effect of pVAX1-PENK was blocked with naloxone hydrochloride. In contrast, pVAX1 treated mice did not significantly improve pain thresholds. These results indicate that peripheral or spinal delivery of a plasmid encoding human proenkephalin gene provides a potential therapeutic strategy for inflammatory pain and neuropathic pain.

1. Introduction

Chronic pain is a vital characteristic of highly prevalent clinical problems, such as diabetic foot, sciatica, rheumatoid arthritis and postsurgical chronic pain. Optimal treatment and proper control of chronic pain refractory to currently available therapeutics remain a serious challenge. Drug therapy is one of the common strategies, but it is efficacious in only 10–60% of chronic pain patients and does not provide a long-term curative effect [1–3]. Furthermore, most of analgesia agents such as opioid and nonsteroidal anti-inflammatory drugs (NSAIDs) are limited by significant side effects, including addiction, abuse, tolerance, gastrointestinal constipation and toxicities [3]. This is the reason why the design of more effective therapeutic strategies with fewer side effect are considered as a critical point for the development for chronic pain relief.

Endogenous opioid peptides play a great function in control of pain perception, and produce a significant analgesic effect without adverse effects. Proenkephalin (PENK) gene yields both met- and leu-enkephalin (ENK), which modulate pain perception and can be found in primary and central neurons. The ENKs produce an analgesic effect by binding and activating δ and μ opioid receptors on both primary and central neurons. Although ENKs are powerful analgesics in animals and humans and proved to be safe, they are limited for clinical application because of their fast metabolism. Gene therapy is a novel and suitable strategy to enhance continuous production of endogenous opioid peptides for pain relief.

Non-viral vectors have been used for regenerating peripheral nerves after injury [4], and for improvement of pain-related behaviors in various pain models in rodents [5–9]. Naked plasmid is the

* Corresponding author at: Beijing Institute of Radiation Medicine, 27 Taiping Road, Haidian District, Beijing 100850, People's Republic of China.
E-mail address: qinglz@yahoo.com (Q. Zhang).

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simplest non-viral vector and is safe for delivery of a foreign gene into cells *in vivo*. The aim of this study was to test the antinociceptive effect of single direct injection of a plasmid vector encoding human PENK gene by peripheral (local injured muscle) or central (intrathecal) administration in a CFA-induced inflammatory pain model and spared nerve injury (SNI) induced neuropathic pain model in mice.

2. Experimental procedures

2.1. Preparation of plasmid

Plasmid pVAX1-PENK encoding human proenkephalin gene was constructed and conserved by our laboratory. The plasmid vectors (pVAX1-PENK, pVAX1) were prepared as described previously [10]. Briefly, plasmid production process included fed-batch fermentation, cell harvest, continuous alkaline lysis, separation of DNA from RNA on a Sepharose 6 Fast Flow column, selective supercoiled plasmid DNA purification with PlasmidSelect Xtra column and final refining with a Source 15Q column. The plasmid solution at 4 mg/ml concentration was obtained for further *in vivo* research.

2.2. Animals

Adult male C57BL/6J mice (18 ~ 22 g) obtained from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. were adapted to the laboratory for one week. The mice were housed in groups of five per plastic cage in a room with constant temperature (22 ± 2 °C) and relative humidity between 45% ~ 60% with a 12:12-h light-dark cycle. Animal study was approved by the Institutional Animal Care and Use Committee at Beijing Institute of Radiation Medicine and all experiments adhered to the ethical guidelines of the International Association for Study of Pain.

2.3. CFA-induced inflammatory pain model

To induce inflammatory pain, mice were anaesthetized with intraperitoneal injection of 40 mg/kg body wt. pentobarbital sodium and 10 μl of CFA (Sigma, MO, USA) suspension solution was injected into the plantar surface of left hind paw. For intramuscular injection, the treatment with a dosage of 100 μg pVAX1 (empty vector) or pVAX1-PENK (4 mg/ml) at the plantaris muscle began 30 min before CFA injection. For intrathecal injection, the treatment with a dosage of 20 μg pVAX1 or pVAX1-PENK began 30 min before CFA injection. For measurement of paw edema, ipsilateral and contralateral hindpaw volume were measured by plethysmometry (Model 7150, Ugo Basile, Varese, Italy) as described previously [11] before and 1, 3, 5, 7, 11, 14, 21, 28 days after CFA injection. The data obtained were expressed in ml. Paw edema was expressed as the percentage increase in the volume of the treated paw relative to that of the contralateral paw at each time.

2.4. SNI-induced neuropathic pain

SNI surgery was performed as previously described by Decosterd [12]. Briefly, mice were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg body w.t.), and three left distal branches (sural, common peroneal and tibial nerves) of the sciatic nerve were exposed. The common peroneal and tibial nerves were ligated with 5.0 silk and transected distal to the ligation, removing 2–4 mm of the distal nerve stump to prevent regeneration. Great care was taken to avoid damage the sural nerve. Following SNI operation, the mice were given 4 days of recovery. The pVAX1 or pVAX1-PENK was administered by intramuscular or intrathecal injection on postoperative day 5. For intramuscular injection, a dosage of 100 μg pVAX1 or pVAX1-PENK was injected into the muscle of the surgery site. For intrathecal injection, a dosage of 20 μg pVAX1 or pVAX1-PENK was injected into the cerebral spinal fluid.

2.5. Naloxone injections

The maximum anti-hyperalgesic effects of pVAX1-PENK were observed on day 7 after plasmid injections in CFA and SNI models. Mice treated with pVAX1 or pVAX1-PENK received intraperitoneal injection of naloxone hydrochloride (5 mg/kg body w.t.). The mechanical allodynia and thermal hyperalgesia were determined 15 min after injection of naloxone hydrochloride.

2.6. Behavioral testing

Mechanical allodynia was detected by von Frey hairs (Stoelting, Wood Dale, IL). The 50% withdrawal threshold was determined using the up-down method as described previously by Dixon and Chaplan [13,14]. Briefly, mice were allowed to adapt to a metal grid for 30 min on the morning of the test day. Starting with a von Frey filament of 0.04 g weight force, each filament was applied for 5 s. with sufficient force to cause slight bending against the paw. A positive response was defined as rapid withdrawal and/or licking of the paw immediately upon application of the stimulus, which was then followed by application of the next finer von Frey filament. When a negative response appeared, the next higher von Frey filament was applied. The cut-off of a 1.40 g filament was selected as the upper limited for testing, since stiffer filaments tended to raise the entire limb rather than to change the nature of the stimulus.

Thermal hyperalgesia was measured using a Hargreaves apparatus (plantar test apparatus; IITC, Woodland Hills, CA) recording the time of withdrawal from a radiant thermal stimulus directly under the hindpaw [15]. In brief, mice were placed in individual enclosures on a glass stage heated to 30 °C for at least 15 min. A radiant heat source was positioned underneath the glass plate directly under the hind paw. The time for mice to remove their paws in response to the heat source was recorded as the latency withdrawal period or at a 25 s cutoff time. Three independent measurements were made at 5 min intervals.

2.7. Human enkephalin quantification

On 3, 7, 14, 21 and 28 days after plasmid injection, mice in each group were sacrificed by decapitation. The tissue samples were rapidly removed and frozen in liquid nitrogen. Muscle or L3-5 lumbar segments of the spinal cord (50 mg) were homogenized in 500 μl RIPA lysis buffer (Beyotime, China) with protease inhibitor cocktails. The concentration of ENK was quantified by enzyme-linked immunosorbent assay using ELISA kit for human Leu-ENK (USCNK, Wuhan, China).

2.8. Statistical analysis

Behavior data were expressed as means ± S.E.M., differences between groups were assayed using a two-way ANOVA analysis of variance with Tukey post test. Molecular detection data were expressed as means ± S.D., differences between groups were assayed using an unpaired two-tailed *t*-test. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad software Inc., San Diego, CA). For all statistical tests, the significance level was set to $P < 0.05$.