American Pain Society
RESEARCH
EDUCATION
TREATMENT
ADVOCACY
PUBLISHED BY ELSEVIER

The Journal of Pain, Vol 13, No 6 (June), 2012: pp 524-531
Available online at www.jpain.org and www.sciencedirect.com

Original Reports

Analgesia Targeting IB4-Positive Neurons in Cancer-Induced Mechanical Hypersensitivity

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Abstract: Cancer patients often suffer from pain and most will be prescribed μ-opioids. μ-opioids are not satisfactory in treating cancer pain and are associated with multiple debilitating side effects. Recent studies show that μ and δ opioid receptors are separately expressed on IB4 (−) and IB4 (+) neurons, which control thermal and mechanical pain, respectively. In this study we investigated IB4 (+) and IB4 (−) neurons in mechanical and thermal hypersensitivity in an orthotopic mouse oral cancer model. We used a δ opioid receptor agonist and a $P2X_3$ antagonist to target IB4 (+) neurons and to demonstrate that this subset plays a key role in cancer-induced mechanical allodynia, but not in thermal hyperalgesia. Moreover, selective removal of IB4 (+) neurons using IB4-saporin impacts cancer-induced mechanical but not thermal hypersensitivity. Our results demonstrate that peripherally administered pharmacological agents targeting IB4 (+) neurons, such as a selective δ-opioid receptor agonist or $P2X_3$ antagonist, might be useful in treating oral cancer pain.

***Perspective:** To clarify the mechanisms of oral cancer pain, we examined the differential role of IB4 (+) and IB4 (−) neurons. Characterization of these 2 subsets of putative nociceptors is important for further development of effective clinical cancer pain relief.*

***Key words:** δ-opioid receptor (DOR), μ-opioid receptor (MOR), NGF, isolectin B4, cancer pain.*

Pain represents one of the worst symptoms for cancer patients and remains the most difficult to treat. Opioid analgesics (eg, morphine) that target μ opioid receptors (MORs) are the most common therapy for cancer pain. Although μ opioids are initially effective for cancer pain management, they are associated with undesired side effects, including opioid tolerance, physical dependence, nausea, respiratory depression, constipation, and immunosuppression.[11,14] There is no effective analgesic regimen for treating intractable cancer pain. As a result, patients often experience agonizing pain and resultant debilitation if tolerance to MOR agonists develops.

The contribution by δ opioid receptor (DORs) to cancer pain is not as well characterized as that of MORs. Some studies report that DOR agonists produce potent analgesia in several pain conditions including bone cancer pain in animal models.[9,15,30,56] Moreover, DOR agonists produce minimal side effects and do not lead to tolerance.[13,15,30] These characteristics make them a promising alternative for the treatment of cancer pain.

Recently it has been shown that MORs and DORs are expressed in different subsets of putative nociceptors that serve distinct functions.[46] DORs are located on IB4 (+) neurons and DOR agonists produce analgesia to mechanical pain with no effect on thermal pain. In contrast, MORs are expressed on IB4 (−) neurons and MOR agonists reduce heat pain without affecting mechanical pain. However, the complete segregation between MORs and DORs in mice has been challenged recently.[59] Nevertheless, IB4 (+) and IB4 (−) neurons differ in their neurochemical expression, sensitivity to neurotrophins, electrophysiological properties, and anatomical locations, which might ultimately lead to distinct functions.[18,21,35,41,52,53] IB4 (−) neurons express TrkA

Received August 2, 2011; Revised January 2, 2012; Accepted January 16, 2012.
This work was funded by NIH/NIDCR R21DE01856.
The authors have no conflicts of interest to declare.
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1526-5900/$36.00

doi:10.1016/j.jpain.2012.01.006

receptors that bind nerve growth factor (NGF), depend on NGF for survival, and express substance P (SP) and calcitonin gene-related peptide (CGRP).[4,36] In contrast, IB4 (+) neurons express receptors for glial cell line-derived neurotrophic factor (GDNF), depend on GDNF for survival, express $P2X_3$ receptors, and have poor expression of SP and CGRP.[3,5,8,22,37,58] Centrally, IB4 (+) neurons terminate predominantly in inner lamina II whereas IB4 (−) neurons terminate in lamina I and outer lamina.[36,51] Despite distinct characteristics of the 2 subsets, the specific function of IB4 (+) and IB4 (−) neurons is not known in oral cancer pain.

NGF, which primarily acts on IB4(−)/TrkA(+) neurons, plays a role in pancreatic cancer pain[62] as well as bone cancer pain.[23,34,47] Neutralizing NGF attenuates both spontaneous and movement-evoked pain in mice with both cancer.[23,34,47] Oral squamous cell carcinoma (OSCC), which is characterized by mechanical allodynia and is clinically distinct from bone cancer, also expresses high levels of NGF.[16,28,61] The limited antinociceptive effect of anti-NGF in our OSCC mouse paw model[61] implies that IB4 (−)/TrkA (+) neurons are not solely responsible for carcinoma-induced mechanical pain. The involvement of IB4 (+) neurons in oral cancer pain has never been studied. It is also unknown whether OSCC cells secrete neurotrophins from the GDNF family that can activate and sensitize IB4 (+) neurons. GDNF and neurturin are particularly of interest as they both maintain a subset of IB4 (+)/$P2X_3$ (+) neurons that do not express CGRP.[2,32] In addition, GDNF has been shown to reduce mechanical thresholds of IB4 (+) neurons.[7,38]

In the present study, we examined the role of IB4 (+) and IB4 (−) neurons in processing mechanical and thermal nociception following OSCC supernatant injection in mice. In addition, we evaluated whether pharmacological agents such as $P2X_3$ antagonists and DOR agonists are effective against OSCC-induced nociception.

Methods

Experimental Animals

42 male C57BL/6 mice (6–8 weeks old; Charles River Laboratories, Hollister, CA) were used in this study. They were exposed to a light-dark cycle (L:D 12:12-hour) and kept in a temperature-controlled room with food and water ad libitum. All procedures were approved by the New York University Institutional Animal Care and Use Committee.

Cell Culture

The human tongue SCC cell line, HSC-3 (ATCC, Manassas, VA), and human normal oral keratinocytes (NOK) were cultured at 37°C with 5% CO_2. Both cell cultures were grown to confluence and then washed to remove all unattached cells. The media for both SCC and NOK cell cultures were replaced with Defined Keratinocyte–Serum Free Media (SFM) and then further incubated for 72 hours prior to supernatant collection for behavioral testing and ELISA, as we previously described.[29]

Intrathecal Administration of IB4-Saporin

Two μL of IB4-saporin (SAP) (1.2 mg/mL, 53% saporin/mole IB4) or 3 μL unconjugated SAP (1 mg/mL) (Advanced Targeting Systems, San Diego, CA) was diluted with PBS to a total volume of 8 μL Mice were anesthetized with 2.5% isoflurane. With the use of a Hamilton syringe, IB4-SAP (n = 6 mice) or SAP (control, n = 6 mice) was injected into the subarachnoid space on the midline between the L4 and L5 vertebrae. All behavioral testing of IB4-SAP- and SAP-treated mice was performed 14 days after injection.

Supernatant Injection and Behavioral Testing

Fifty μL of supernatant from HSC-3 or NOK cells was injected in the midplantar right hind paw of each mouse (n = 6 in each group).[29] Our previous study demonstrated that HSC-3 supernatant induced mechanical allodynia for a duration of 3 or more hours.[29] In this current study, mechanical sensitivity was measured 1 hour postinjection using an electronic von Frey anesthesiometer (IITC Life Science, Woodland Hills, CA). The withdrawal-threshold was defined as the force (g) that was sufficient to elicit a withdrawal response. Six measurements were taken for each animal. Thermal sensitivity was measured using a paw thermal stimulator (Hargreaves' Apparatus; Department of Anesthesiology, UC San Diego, La Jolla, CA). Mice were placed in plastic chambers on a heated glass surface (25°C). A radiant heat source was focused on the hindpaw and latency to withdraw was measured as the average of 6 trials per animal taken ≥5 minutes apart. The cutoff latency was set at 20 seconds to avoid tissue damage. In all behavioral experiments, the observer was blind to the treatment groups.

Drugs

TNP-ATP is a selective P2X antagonist which potently blocks $P2X_1$, $P2X_3$, and heteromeric $P2X_{2/3}$ receptors. SNC80 is a highly selective agonist for DORs. Naltrindole (NTI) is a selective DOR antagonist. SNC80 (Tocris Bioscience, Ellisville, MI) was dissolved in sterile acidic (.2% HCl) saline solution. NTI (Sigma, St. Louis, MO), TNP-ATP (Sigma, St. Louis, MO), and NGF neutralizing antibody (Mab 256; R&D Systems, San Jose, CA) were mixed directly into 50 μL of cancer supernatant and injected into the right hind paw of the mice 1 hour prior to behavioral testing. 10 nmol of SNC80, .2nmol NTI, 2 μmol TNP-ATP, and 12.5μg anti-NGF were administered to each animal. Drug dosage was based on previous findings.[1,24,46]

IB4 Labeling

Animals were euthanized with 4% isoflurane and perfused with cold .1M PBS solution followed by 4% paraformaldehyde (PFA). The spinal cord was removed, postfixed in 4% PFA, and cryoprotected in sucrose gradient (20–50%) at 4°C. Serial frozen spinal cord sections (12 μm) were cut on a cryostat and thaw-mounted on