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Peripheral Cannabinoids Attenuate Carcinoma Induced Nociception in Mice

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Abstract

We investigated the cannabinoid receptor (CB₁) agonists Win55,212-2 (non-selective) and AM1241 (CB₂ selective) and the peripheral receptor (CB₁) in carcinoma-induced pain using a mouse model. Tumors were induced in the hind paw of female mice by local injection of a human oral squamous cell carcinoma (SCC). Significant pain, as indicated by reduction in withdrawal thresholds in response to mechanical stimulation, began at four days after SCC inoculation and lasted to 18 days. Local administration of Win55,212-2 (10 mg/kg) and AM1241 (10 mg/kg) significantly elevated withdrawal thresholds, indicating an antinociceptive effect. Ipsilateral expression of CB₁ protein in L5 DRG was significantly upregulated compared to ipsilateral L4 DRG and in normal tissue. These findings support the suggestion that cannabinoids are capable of producing antinociception in carcinoma-induced pain.

Keywords

cannabinoids; cancer; pain; cancer mouse model; oral cancer

Introduction

Cancer pain remains poorly understood and there are no effective therapies. Mechanical hyperalgesia secondary to carcinoma, due to its intensity and impairment of function, is debilitating. Seventy-five to ninety percent of terminal cancer patients cope with opiate-resistant pain related to tumor progression.[28,29,36,39] Eighty-five percent of cancer patients experience severe pain in their final days.[43]

Cancer pain is classified into three syndromes: somatic, visceral and neuropathic. Somatic cancer pain is caused by tumor invasion of connective tissues, bones and muscles. Visceral

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cancer pain is caused by invasion into visceral organs. Neuropathic cancer pain is caused by peripheral or central nervous system damage due to released inflammatory cytokines that sensitize neurons.[37] Carcinoma-induced pain is not related to tumor size and small carcinomas produce severe pain.[6] These observations suggest that carcinoma pain is primarily of neuropathic origin and is characterized by mechanical hyperalgesia.

Mechanical hyperalgesia secondary to carcinoma is poorly responsive to opioids, and tolerance rapidly develops.[25,26,33] Cannabinoids are analgesic in patients with neuropathic pain [12,13,20,24,34] and show promise in cancer pain.[32] Cannabinoids activate two receptors types: cannabinoid receptor 1 and 2 (CB₁ and CB₂, respectively).[27,31] CB₁ and CB₂ contribute to analgesia. CB₁s are localized in the spinal dorsal horn, periaqueductal grey [9, 11] and dorsal root ganglion (DRG).[24,40] In neuropathic pain, cannabinoids act at central and peripheral nerve CB₁s [20,34], and at CB₂s on keratinocytes.[18,20] Cannabinoid's analgesic action in cancer pain is less clear.[2,10,19] In a murine bone sarcoma pain model, systemic cannabinoids act through CB₁. [15,21] However, the role of peripheral CB₁ and CB₂ receptors in soft tissue carcinoma pain is not known. We hypothesize that cannabinoid agonists are analgesic with carcinoma induced pain and that the site of action is within the tumor microenvironment. To study soft tissue carcinoma pain, we produce a mouse model by injecting human oral squamous cell carcinoma (SCC) into the hindpaws which leads to mechanical hyperalgesia.[42] Oral SCC reproducibly produces mechanical hyperalgesia in mice and humans. The mouse model can be used to test for analgesics.[6,42] We sought to determine whether peripheral cannabinoid agonists attenuate mechanical hyperalgesia in a carcinoma mouse model.

Methods

2.1. Cell culture

A human oral SCC cell line (ATCC, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium (DMEH-21), 10% fetal bovine serum, fungizone (0.5×), penicillin-streptomycin (1×), non-essential amino acids (1×), and sodium pyruvate (1×).

2.2. SCC paw model

The cancer pain mouse model was produced using adult (4-5 weeks old, 20-25 g) female *Foxn1^{nu}*, athymic mice as previously described.[42] Mice were housed in a temperature-controlled room on a 12:12 h light cycle (0600-1800 h light), with unrestricted access to food and water; estrous cycles were not monitored. All procedures were approved by UCSF Committee on Animal Research. Researchers were trained under the Animal Welfare Assurance Program. Mice were injected either with squamous carcinoma cells (SCC group) or cell culture media (sham operated). Both groups were anesthetized by intraperitoneal injection of Avertin® (0.015 ml of a 2.5% solution/g body wt). SCC injections consisted of 1.0×10^6 tumor cells in 50 μ l of Dulbecco's modified Eagle's medium (DMEM) into the plantar surface of the right hind paw. The sham-operated group received injections of the cell culture media.

2.3. Behavioral testing for the SCC paw model

Behavioral testing was performed between 14:00 and 16:00 h (during the light phase) and quantitative assay guidelines were used as described previously.[42] Mice were placed in a plastic cage with a wire mesh floor which allowed access to the paws. Fifteen minutes were allowed for cage exploration prior to testing. The mid-plantar right hind paw, or the tumor-front on the hind paw toward the later stages of tumor development was tested. Paw withdrawal thresholds were determined in response to pressure from an electronic von Frey anesthesiometer (2390 series, IITC Instruments, Woodland Hills, CA). The amount of pressure

(g) needed to produce a paw withdrawal response was measured three times on each paw separated by 3 minute intervals. The three tests were averaged for each paw for that day. The SCC and sham injected groups were tested at 4, 7, 9, 11, 14, 16, and 18 days post-injection.

2.4. Win55,212-2 and AM1241 administration and pain behavioral testing

A non-selective (Win55,212-2) or a selective (AM1241) cannabinoid agonist was administered prior to paw withdrawal testing. Testing was performed at 20 days following oral SCC hindpaw inoculation. The cannabinoid agonist was injected directly into the mid-plantar hind paw at the site of greatest tumor development with a 30 gauge beveled needle. 10 mg/kg of either Win55,212-2 or AM1241 was diluted in 15 μ l DMSO. A control group of mice with SCC paw tumors received 15 μ l of DMSO (vehicle) injection in the same manner. Paw withdrawal testing was performed: (1) 15 minutes before drug or control injection, and (2) 15, 30, 60, 90, 180 and 1440 minutes post drug or control injection.

2.5. Immunofluorescence

Mice received a lethal dose of pentobarbital (100 mg/kg, intraperitoneal), and were fixed with intracardiac PBS (10ml) perfusion, pH 7.4, room temperature followed by an ice-cold fixative (20 ml, 4% paraformaldehyde and 0.14% picric acid in 0.1 M phosphate buffer, pH 7.4). The DRG and lumbar spinal cord were extracted. Tissue was postfixed and cryoprotected in 30% sucrose. Ten μ m sections were cut after embedding in Tissue-Tek (Fisher Scientific, Inc., Hampton, NH) and plated on superfrost plus slides (Fisher Scientific, Inc., Hampton, NH). Sections were washed three times with PBS and incubated with an affinity purified rabbit CBr1 C-terminal antibody (1:1000) in PBS/Triton X-100 with 1% normal donkey serum (NDS) at 4°C overnight (14-16 hrs). Sections were incubated with anti-rabbit Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS/Triton with 1% NDS for 2 hrs. Sections from ipsilateral L4 and L5 DRG were processed simultaneously. The slides were visualized on a Nikon Eclipse E600 microscope using epifluorescence. The images were captured with a RT Spot Camera and Software (Diagnostics Instruments, Inc., Sterling Heights, MI).

2.6 CBr1 expression measurements

The colored fluorescent images of ipsilateral L4 and L5 DRG were converted to grayscale using RT Spot Software (Diagnostics Instruments, Inc., Sterling Heights, MI). The fluorescence emitted by each DRG cell body was quantified by Scion Image software as the average gray value per pixel in the selected DRG cell body (Alpha version 4.0.3.2, Scion Corporation, Frederick, MD). The gray value per pixel ranges between 0 and 256, with higher values indicating higher intensities of fluorescence. A value of 256 indicates that all of the pixels in the selected image are expressing maximum gray value. Therefore, to prevent the skewing of data by using absolute values, we calculated the fluorescence values as a percentage of 256. Only DRG neurons that did not overlap with other cells and had a visible nucleus were used for image analysis.

2.7 Statistical Analysis

A one-way analysis of variance (ANOVA) with a Bonferroni Multiple Comparisons post-test was used to compare the withdrawal threshold of the SCC and sham mice over 18 days. The same test was used to compare the percent change of withdrawal threshold of the SCC inoculated mice before and after drug or control injection. A paired two-tailed T-test was used to compare the intensity of immunofluorescence of L4 and L5 in the SCC inoculated to the sham control.

Results

3.1. Paw withdrawal in the SCC mouse model

The withdrawal thresholds for the SCC and sham group were compared. Mean paw withdrawal thresholds were significantly reduced in the SCC mice on all days of behavioral testing (Figure 1). The mean paw withdrawal thresholds of the SCC inoculated mice and the sham group prior to inoculation were 4.21 ± 0.22 g and 4.48 ± 0.45 g, respectively. The mean paw withdrawal thresholds of the SCC inoculated and sham group 14 days after inoculation were 1.84 ± 0.5 g and 4.94 ± 0.85 g.

3.2. Intra-tumor cannabinoid agonist administration and behavioral testing

We tested the effect of peripheral administration of the non-selective CBr agonist Win55,212-2 and CBr2 selective agonist AM1241 on paw withdrawal thresholds. Win55,212-2 significantly elevated paw withdrawal thresholds of SCC-inoculated paws at 15, 30, 60, 90 and 180 minutes after inoculation relative to vehicle control (Figure 2). Thirty minutes after injection of Win55,212-2 the mean paw withdrawal thresholds was 3.43 ± 1.36 g. AM1241 (10 mg/kg) significantly elevated paw withdrawal thresholds of SCC-inoculated paws at 15 minutes after inoculation relative to vehicle control (Figure 2). Thirty minutes after injection of AM1241 the mean paw withdrawal thresholds was 3.02 ± 1.1 g. Recovery to baseline was observed by 90 minutes after administration of AM1241 and 24 hours after administration of Win55,212-2.

3.3. CBr1 immunofluorescence in L4 and L5 DRG of SCC mice

To determine the effect of carcinoma on CBr1 expression in the DRG of the spinal nerves innervating the tumor site CBr1 immunofluorescence in the ipsilateral L4 and L5 DRG of SCC mice were compared to sham mice. There was no significant difference in CBr1 immunofluorescence of the L4 DRG (Figure 3). L5 DRG immunofluorescence in the SCC group was $20.40 \pm 7.89\%$ and significantly greater than the sham group at $12.22 \pm 3.01\%$ (Figure 4).

Discussion

In this study synthetic cannabinoids WIN55,212-2 (non-selective) and AM1241 (CBr2 selective) both significantly attenuate mechanical hyperalgesia in a carcinoma pain mouse model. Intra-tumor administration of WIN55,212-2 significantly elevated nociceptive thresholds for 180 minutes. While WIN55,212-2 is nonselective, its antinociceptive action is primarily through CBr1.[22,35,38] CBr1 inhibits glutamatergic transmission between primary nociceptive afferents and second-order neurons in the dorsal horn.[44] Kehl et al. found that the antinociceptive effects of systemic cannabinoids on osteolytic sarcoma induced nociception were mediated via CBr1.[21] CBr1 are expressed at central and peripheral nerve terminals and in keratinocytes after being synthesized in DRG.[16] However, only peripheral CBr1 on nociceptors contribute to antinociception in inflammatory and neuropathic pain models.[1] CBr2 are found on immune cells [31,41] and keratinocytes [15,16]. CBr2 on keratinocytes mediates antinociception via opioid release.[17,18] CBr2 stimulates β -endorphin release from keratinocytes, leading to antinociception through μ -opioid receptors. We therefore investigated a CBr2 selective agonist in the mouse cancer pain model. We found that intra-tumor administration of AM1241, a CBr2 selective agonist, significantly elevated nociceptive thresholds but for a shorter time than the nonselective agonist. We did not measure paw withdrawal following agonist administration into the contralateral paw as a control. However, two previous studies have demonstrated an antinociceptive effect of local administration of Win55,212-2 in rats with carrageenan-evoked hyperalgesia and neuropathic pain.[10] Intraplantar administration of AM1241 is antinociceptive in inflammatory hyperalgesia in the rat.[14] In these three studies contralateral intraplantar administration had no antinociceptive

effect on the paw being tested confirming a local antinociceptive effect with the cannabinoid agonists. CBr2 activation inhibits cytokine release and might contribute to antinociception. [8] However, the target cells of CBr2-mediated immunosuppression are unclear. The athymic mice we used have suppressed cell-mediated immunity. Their humoral immunity is partially intact and it is possible that cytokines are released by B cells or neutrophils. However, these cells do not infiltrate the carcinoma in the mouse model.[42] Therefore, CBr2 mediated antinociception in the athymic mouse model is likely mediated via release of opioids by keratinocytes.

Our results suggest that cannabinoids attenuate carcinoma mediated hyperalgesia via CBr1 on peripheral primary afferents and CBr2 on keratinocytes. While CBr1 and CBr2 are expressed in skin cancer, it is unknown whether activation of cannabinoid receptors in malignant keratinocytes produces antinociception.[3] Cannabinoids regulate tumor cell growth and apoptosis; however, significant apoptosis only occurs 3 days after injection of cannabinoid. [3] Our antinociceptive measurements were performed within twenty-four hours of cannabinoid administration and it is unlikely that its antitumor activity contributes to antinociception. Our findings differ from the osteolytic fibrosarcoma hyperalgesia mouse model where the antinociceptive effect was mediated via CBr1. Fibrosarcoma and SCC are histologically distinct and the nociceptive mediators that they produce likely differ in concentration and type. We evaluated the analgesic effect of local cannabinoid administration, while the authors using the fibrosarcoma model evaluated systemic administration. We used a selective CBr2 agonist while they used a non-selective agonist with a CBr1 inhibitor.

Our mouse cancer pain model is produced by injecting human oral SCC into the hindpaw. [42] Thresholds for withdrawal were significantly diminished in the SCC paws, but not in sham paws. The paw is innervated by spinal nerves from L4 and L5 DRG. [7,23] We investigated whether carcinoma induced pain produces a change in L4 and L5 DRG CBr1 expression. Animals with paw SCC tumors expressed significantly elevated levels of CBr1 in the L5 DRG, but not in the L4 DRG. These differences could be due to the location of nerve endings relative to the cancer within the paw. In a neuropathic pain rodent model the uninjured nerve exhibited increased CBr1 expression while the injured nerve revealed no significant change.[30] Lack of cancer infiltration of an L5 afferent could account for its increase in CBr1 immunofluorescence. Understanding the changes and mechanism of neuronal receptor expression in carcinoma pain states will elucidate new targets for cancer pain therapy.

Systemic cannabinoids produce sedation and catalepsy due to CBr1 activation. [21] We tested whether a local CBr2 agonist produces antinociception. Our findings suggest that a peripheral CBr2 agonist could provide relief for cancer patients. Cannabinoids also potentiate the analgesic effects of morphine and prevent tolerance.[4,5] These desirable effects of cannabinoids show promise for management of cancer pain and may lead to improved analgesic therapy.

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Figure 1.

Mean percent change in paw withdrawal threshold relative to baseline reading prior to SCC or sham inoculation. Sham displayed no change over 18 days. The SCC mice demonstrated a significant decrease on all days (*, days 4, 7, 14, 16, 18 $p < 0.01$; days 9, 11 $p < 0.05$).

Figure 2.

Antinociceptive effect of cannabinoid agonist. Intra-tumor injection of non-selective CBr agonist (Win55,212-2) produced significant (*, $p < 0.05$) increase in paw withdrawal threshold at 15, 30, 60, 90, and 180 minutes compared to DMSO (drug vehicle). Recovery to baseline was observed by 24 hours. Intra-tumor injection of the selective CBr2 agonist (AM1241) produced significant (*, $p < 0.05$) increase in paw withdrawal threshold at 15 minutes.

Figure 3. Immunofluorescence staining of CBr1 in L4 DRG of (A) SCC injected mice and (B) sham mice using Texas Red conjugated antibodies (20× magnification). Analysis of immunofluorescence revealed no significant difference in neuronal CBr1 expression (arrow).

Figure 4. Immunofluorescence staining of CBr1 in L5 DRG of (A) SCC injected mice and (B) sham mice using Texas Red conjugated antibodies (20 × magnification). Analysis of immunofluorescence revealed an increase in neuronal CBr1 expression (arrow) in SCC ($20.40 \pm 7.89\%$) versus sham ($12.22 \pm 3.01\%$) ($p=0.0471$).