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Epigallocatechin-3-gallate attenuates bone cancer pain involving decreasing spinal Tumor Necrosis Factor-α expression in a mouse model

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ABSTRACT

Tumor metastasis to bone often elicits a wide array of symptoms, in which pain is a significant factor in catastrophic complications of bone cancer. The complete understanding of bone cancer-related pain is still unknown, while several pathophysiological components have been suggested, from tumor-stimulated osteolysis, nerve compression, stimulations of ion channels, and locally generated inflammatory cytokines. In particular, it has been shown that pro-inflammatory cytokine TNFα-mediated actions are necessary for the development of bone cancer pain. As a member of catechin family in green tea extracts, EGCG (Epigallocatechin-3-gallate) can reduce excess free radicals and attenuate overactive inflammatory signaling including TNFα. In addition, EGCG or its related molecules have been used to control neuropathic pain in various preclinical settings. However, its potential use in bone cancer-caused pain has not yet been reported. Here we show that treating a mouse model of bone cancer by EGCG, results in a dramatic reduction in pain behavior and a significant decrease of TNFα expression within the spinal cord of tumor-bearing mice. Thus, this study reveals an anti-nociceptive role for EGCG in the progression of pain caused by tumor bone metastasis, and highlights a potential scheme by using anti-TNFα as a therapeutic option for osteolytic pain.

1. Introduction

As one of the most common organs for tumor metastasis, the bone complications contribute significantly to patient's quality of life during cancer progression [1,2]. By local expansion and extravasation followed by chemotactic movement through lymphatic or blood circulation, primary cancer cells can develop at a distant bone site. As an early indicator of cancer presence or recurrence, pain in patients with bone cancer represents a debilitating and severe clinical problem [2,3]. Meanwhile, the mechanisms of bone cancer-caused pain are ill-defined. It is possible that the osteolysis stimulated by cancer metastasis plays a priming role, and thereafter pain might be produced by a combination of bone microstructure collapse, stretching of the periosteum nerve, and activation of nociceptors by locally-released neurochemical factors [3–6]. However, there are significant questions remained for developing effective treatments for chronic cancer pain in the skeleton. There has been a lack of correlation between the presence of pain with the type, number and size of tumor, and pain can even be generated in the absence of fracture [3,7]. Moreover, there is no well-accepted knowledge on the neurochemical mechanisms underlying the development of cancer pain [3,4,6,8]. Finally, there is a remarkable heterogeneity in the type and location of sensory and sympathetic innervations in the bone, which could play distinct functions during the pathogenesis of bone cancer pain [3,6].

Bone cancer related complications are often led by coordination and interactions between cancer cells and the adjacent bone cell behaviors. For instance, RANKL (Receptor Activator of NF-κB Ligand) expressed in osteoblastic cells as a critical factor for osteoclast differentiation, also stimulates migration of breast cancer cells as a chemokine for bone metastasis [9]. Similarly, pro-inflammatory cytokine TNFα (Tumor Necrosis Factor, can be generated by either cancer cells or bone cells) may promote both the osteolytic effect caused by enhanced bone resorption [10,11], and tumor progression by increased tumor growth and angiogenesis [12,13]. In addition, several lines of evidences have suggested that TNFα signaling also play critical functions in the contexts of bone cancer related pain [4,14,15], and antagonism of TNFα may be a useful therapeutic strategy in the relief of hyperalgesia in bone malignancy [16].

EGCG (Epigallocatechin-3-gallate), a main active component of green tea, has been frequently used as an antioxidant and anti-inflammatory agent [17] in a variety of diseases such as suppressing cancer progression, preventing metabolic disorders, protecting brain functions and modulating immune responses. EGCG can block a wide array of inflammatory stimuli including TNFα actions [18] and intracellular NF-κB activation [18]. Importantly, there has been emerging evidence that EGCG may have protective effects on pain or neuronal injures [19–22], such as thermal hyperalgesia, peripheral nerve damages, and diabetic neuropathy. Whether or not EGCG could also extend

Abbreviations: EGCG, Epigallocatechin-3-gallate; TNFα, Tumor Necrosis Factor α.

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its beneficial actions in relieving skeleton pain in tumor metastasis is still an open question. In order to examine the anti-inflammatory functions of EGCG, and dissect its potential effects on bone cancer-related pain involving TNFα inflammatory signaling, we treated cancer-bearing mice by a various doses of EGCG and measured their pain behaviors and the levels of TNFα in the spinal cord. Our data show that EGCG can significantly reduce bone cancer-induced hyperalgesia, potentially by inhibiting the generation of TNFα signaling in the spinal cord.

2. Materials and methods

2.1. Mouse model of bone cancer

The animals used in the study were male C57/BL mice at the age of 8–10 weeks (approximately 20 to 30 g of body weight). Mice were housed in a vivarium at 22 °C with a twelve-hour alternating light/dark cycle and given food and water ad libitum. The mouse bone cancer model was established using MC57G fibrosarcoma cells as described in [16]. Briefly, proliferating cancer cells cultured in vitro were harvested and prepared as single-cell suspensions at 10^6 cells/20 μl in PBS. An arthrotomy was performed in anesthetized mice by exposing the condyles of the distal femur. The cells (20 μl of each) were then injected through a drill hole in the intramedullary space of the right femur. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (revised 2012). The protocol was approved by the Committee on the Ethics of Animal Experiments of The Second Hospital of Shandong University. All surgery was performed under sodium pentobarbital anesthesia (50 mg/kg, intraperitoneally injection), and all efforts were made to minimize suffering.

2.2. Experimental groups

Animals were randomly divided into the following groups: sham + veh (with femoral drill operation and PBS by intrafemoral injections without cancer cells, n = 8); sham + 1% DMSO (with femoral drill operation, PBS by intrafemoral injections without cancer cells and 1% DMSO by intraperitoneally injection, n = 8); tumor (with femoral drill operation and cancer cells by intrafemoral injections, n = 8); tumor + EGCG (with femoral drill operation, cancer cells by intrafemoral injections and different doses of EGCG treatment by intraperitoneally injection, n = 8 for each dose); tumor + veh (with femoral drill operation, cancer cells by intrafemoral injections and 1% DMSO). In this study, EGCG doses were 10, 25, 50 and 100 mg/kg according to the preliminary experiments. In this range of doses, the animals behaved normally and did not lose weight.

2.3. EGCG administration

EGCG dissolved in DMSO was diluted by saline at various concentrations as indicated (final DMSO concentration less to 1%). In the experiments, cancer-bearing mice were treated either by vehicle (1% DMSO only) or different doses of EGCG (10, 25, 50 and 100 mg/kg) through daily intraperitoneal injection (i.p.) during the first week after the surgery.

2.4. Pain behavior examination

Pain behavior examination was performed based on the report [16] and briefly described as below. First, ongoing pain behavior measured as the spontaneous guarding time and flinching numbers were recorded simultaneously during a 2-min observation after an acclimation period of 30 min. Guarding was defined as the time the hind paw was held aloft, while flinches were the number of times the animals held the limb aloft. Second, movement-related pain behaviors were measured as limb use during normal ambulation and guarding during forced ambulation on a rotarod. A score scale of 0 to 4 was used for normal limb use during spontaneous ambulation as follows: (0) no use of the hind limb, (1) partial non-use of the hind limb in locomotor activity, (2) limp and guarding behavior, (3) pronounced limp, and (4) normal use. A score scale of 0 to 5 was rated for forced ambulatory guarding in rotarod-trained mice with 400-second time on the rotarod after the surgery. (0) normal use, (1) some limp, but not pronounced, (2) pronounced limp, (3) pronounced limp and prolonged guarding of limb, (4) partial non-use of the limb, and (5) complete lack of use of the limb. Third, tactile hypersensitivity was measured by withdrawal thresholds to von Frey filaments on the plantar surface of the ipsilateral hind paw.

The values of individual test were determined at the day 4, 8, 11, 15 and 18 after cancer cell injection, and then normalized to the baseline values in a double-blinded fashion. In this observed time window, the pain in cancer-bearing animals increased to a peak, then decreased and kept nearly unchanged.

2.5. Western blotting

Mice were sacrificed and lumbar spinal cord segments (approximately around L4 segment) were removed and snap-frozen by liquid nitrogen [23]. The protein lysates generated from the tissue homogenate was cleared by a centrifugation at 12,000 rpm for 10 min at 4 °C, and then quantitated by BCA protein assays kit (Haoran Biotech., Shanghai, China). The same amount of total protein (50 μg) was run on SDS-PAGE (12%) and subsequently transferred to PVDF membranes (Millipore Corporation, CA, USA). The blots after blocking (5% nonfat milk for 1 h at room temperature) were incubated with goat anti-TNFα antibody (1:400, Santa Cruz Biotech., CA, USA) overnight at 4 °C. The membrane was washed twice with TBST buffer, followed incubation by HRP conjugated secondary antibody (1:200, Life Technology, USA) for 1 h at room temperature. Then, the membrane was detected with an ECL-based imaging system (Santa Cruz Biotechnol., CA, USA). GAPDH was used as a loading control.

2.6. Statistics

All data in graphs are expressed as the means ± SEM as indicated. The two-way ANOVA followed by Tukey's test was used for Figs. 1 and 2. The one-way ANOVA followed by Tukey's test was used for Fig. 3. The one-way ANOVA followed by Tukey's test was used in Fig. 4. The comparisons with $^{*}p < 0.05$ and $^{\#}p < 0.01$ (versus the controls as indicated) considered as significant.

3. Results

3.1. Characterization of pain behavior in bone cancer-bearing mice

Before the pain behavior tests, we checked the general situation of the tumor-bearing mice after operation. The tumor-bearing mice experienced some weight loss at d8–d18 when compared to control mice, but weight loss was not significant when compared to sham-operated mice (data not shown). Besides, the tumor-bearing mice underwent similar locomotor activity when compared to sham-operated mice. We first evaluated the pain behaviors of tumor-bearing mice generated by the intra-femur inoculation of fibrosarcoma. Compared to sham-operated mice, significant decreases in the withdrawal threshold (increased tactile sensitivity, Fig. 1a), and increases in both the guarding time and the numbers of flinching (enhanced ongoing pain behavior, Fig. 1b and c) were evidently found in tumor-bearing mice, suggesting an enhanced pain perception. Similarly, movement-related pain behaviors were stimulated in the disease groups versus the vehicle groups, shown by dramatic decreases in the free walking behavior (less limb use during normal ambulation, Fig. 1d) and increases in the forced

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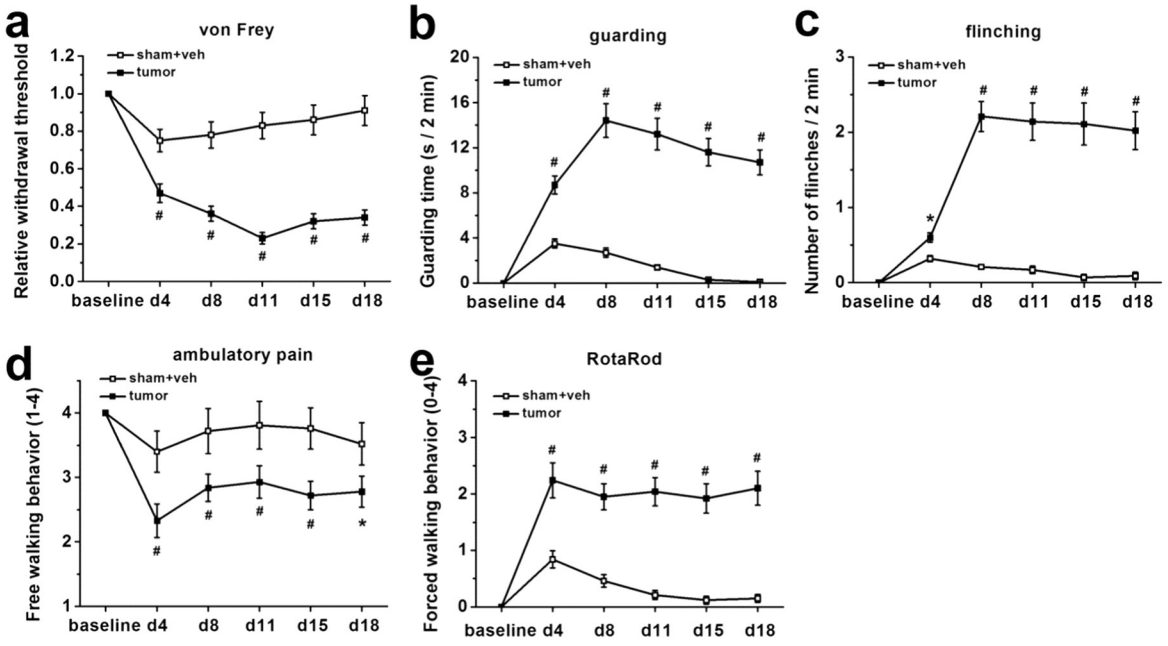

Fig. 1. Pain behavior of tumor-bearing mice and sham mice, including tactile hypersensitivity measured by withdrawal thresholds to von Frey filaments on the plantar surface of the ipsilateral hind paw (a), ongoing pain behavior measured as the guarding time (b) and the number of flinches (c) during a 2-min observation period, and movement-related pain behavior measured as limb use during normal ambulation (d) and guarding during forced ambulation on a rotarod (e). N = 8 in all experimental groups. Data were presented as mean ± SEM, $^{*}p < 0.05$ and $^{\#}p < 0.01$ vs sham + veh group.

walking behavior (increased guarding during forced ambulation on a rotarod, Fig. 1e). Here, rotarod was used to examine the forced ambulatory pain [16,24]. Starting from the day 4 after the surgery, the hyperalgesia was readily induced in the cancer-bearing mice and sustained through to the end of observation at the day 18. Interestingly, the progression of ongoing pain behavior and tactile hypersensitivity were found relatively slow with the maximum around the day 8–11, while the movement-related pain behaviors were rapidly developed to the maximum at the day 4. All pain behaviors were maintained throughout the whole time. Although it is difficult to discern between pain caused by cancer cells and pain induced by femoral drill, the results could reflect indirectly the differences. The pain caused by femoral drill usually decreased after the peak at d4, while the pain caused by cancer lasted much longer if the pain caused by femoral drill was deducted from the tumor group.

3.2. Effects of EGCG on pain behavior of cancer-bearing mice

We next investigated whether the anti-inflammatory agent EGCG could protect the hyperalgesia induced by bone cancer. We performed daily injections of EGCG during the first week right after the tumor inoculation using various doses at 10, 25, 50 and 100 mg/kg, respectively. The choice of the doses in the present study is mainly based on the preliminary experiments. The animals subjected to those doses were

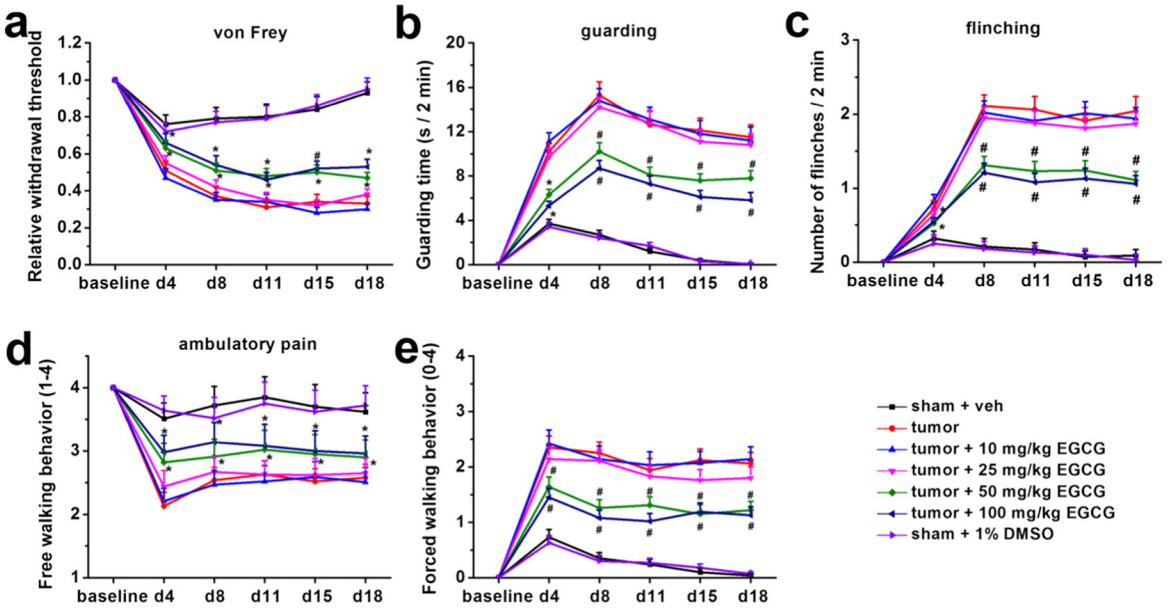

Fig. 2. Effects of different doses of EGCG (10, 25, 50 and 100 mg/kg, i.p. injection daily during the first week after operation) on pain behavior in tumor-bearing mice. Increased withdrawal threshold (a) and free walking behavior (d), decreased guarding time (b) and number of flinches (c) and forced walking behavior, could be observed in tumor-bearing mice after treated with 50 or 100 mg/kg EGCG. N = 8 in all experimental groups. Data were presented as mean ± SEM, $^{*}p < 0.05$ and $^{\#}p < 0.01$ vs tumor-bearing group without treatment.

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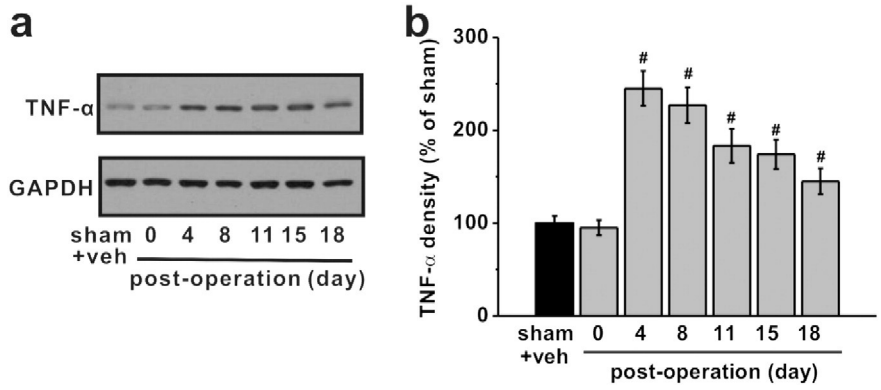

Fig. 3. TNF-α expression within the spinal cord in the tumor-bearing mice at different indicated time post-operation (n = 8 for each group at every time point). (a) Western blot analysis of TNF-α expression, GAPDH as a loading control. (b) Statistical analysis of relative optical density when normalized to sham + veh group. Data were presented as mean ± SEM, $^{\#}p < 0.01$ vs sham + veh group.

safe and behaved normally. In cancer free mice (sham operation) there is no effect of all doses of EGCG when compared to sham + 1% DMSO group or sham + veh (with operation and PBS) group (data not shown). However, compared to tumor-bearing mice, high doses of EGCG (50 and 100 mg/kg) significantly reduced the pain behavior in tumor-bearing mice in all tests used for pain behaviors, including increased the withdrawal threshold (Fig. 2a) and free walking behavior (Fig. 2d), decreased guarding time (Fig. 2b), numbers of flinches (Fig. 2c) and forced walking behavior (Fig. 2e). By contrast, lower doses of EGCG (10 and 25 mg/kg) have minimal effects, suggesting that a considerable concentration threshold of drugs needs to be reached in order to prevent the generation of pain behavior induced by bone cancer. Moreover, the protection of EGCG at high doses in our study is not complete. There are still significant increases of pain response in EGCG-treated mice comparing to the pain behavior in sham + veh mice (Fig. 2). Worthy of mention, no significant differences were found in the pain behaviors between sham + veh group and sham + 1% DMSO group. Taken together, the dose-dependent effects of EGCG on relief of hyperalgesia in bone cancer mouse model indicated that the underlying pathways to the pathogenesis of pain behavior induced by cancer could be compromised by a subsequent administration of the anti-inflammatory diet supplement EGCG.

3.3. TNFα expression is induced by bone cancer in the spinal cord

Although spinal cord TNFα is widely believed to play an important role in the development of inflammatory and neuropathic pain, its role in bone cancer-related pain was revealed just recently [16,23]. In this study, we focus on the relationship between TNFα and bone-cancer induced pain to address the potential inflammatory signaling that could be the target of EGCG in modulating the pain behavior in cancer-bearing mice. We examined the level of TNFα expression in the spinal cord, a major processing component of central nervous system that receives neuronal signals from peripheral nerves. Consistent

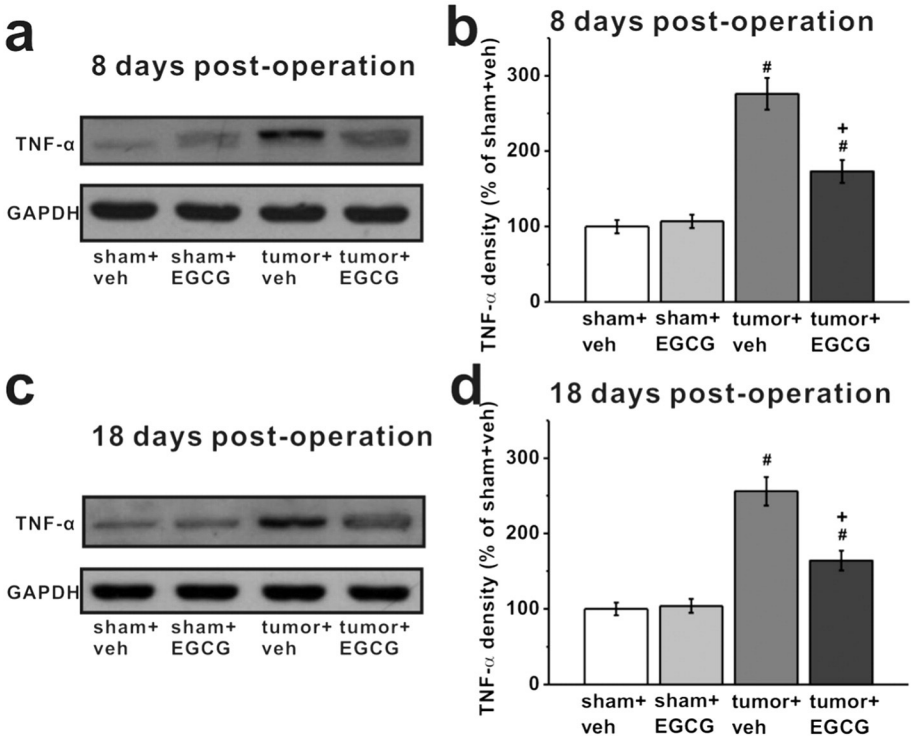

Fig. 4. 100 mg/kg EGCG decreased TNF-α expression within the spinal cord in the tumor-bearing mice (n = 8 for each group at every time point). Western blot analysis of TNF-α expression and the statistical analysis of the relative optical density in different experimental groups at day 8 (a, b) and at day 18 (c, d) post-operation. GAPDH is a loading control. Data were presented as mean ± SEM, $^{\#}p < 0.01$ vs sham + veh group and $^{+}p < 0.01$ vs tumor + veh group.

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with previous findings [23], we found the protein level of TNFα in the spinal cord was enhanced if received intra-femur tumor inoculation (Fig. 3a and b). Notably, the increases were rapidly developed and peaked on the day 4 (more than two fold compare to the controls) and then sustained but progressively attenuated throughout to the day 18, at which the levels were still elevated (around 50% increases) comparing to the levels of sham + veh group. Thus, the increased expression of TNFα correlates the progression of animal pain behaviors in the mouse bone cancer model.

3.4. EGCG reducing TNFα expression of the spinal cord in cancer-bearing mice

To determine whether the anti-nociceptive effects of EGCG could involve TNFα expression during bone cancer, we investigated the levels of TNFα in cancer-bearing mice when treated with 100 mg/kg EGCG. We chose two time points for the analysis. One is the day 8 when all pain behaviors examined were at peak in cancer-bearing mice (Fig. 1), and another is the longest observation day 18 in the study. Importantly, EGCG reduced the spinal cord expressions of TNFα at the both time points (Fig. 4, partially rescued since remained elevated compared to the sham groups). Our findings indicated that the changes of pain behaviors in cancer-bearing mice treated by EGCG could be caused by the lower amounts of TNFα expression in the spinal cord.

4. Discussion

Our studies provide evidence that an intra-femur inoculation of cancer can generate pain signals in the central nerve system, resulting changes in the physiological pain behaviors. These changes were correlated to a sustained elevation of TNFα expression in the spinal cord. The administration of EGCG immediately following the operation significantly ameliorated both the pain behavior and the increased expression of TNFα. This finding may suggest a bidirectional communication between the central nerve systems and peripheral bone metastasis through TNFα signaling, in which EGCG can partially block.

The reduction of pain behaviors in cancer-bearing mice treated with EGCG highlights the importance of neuroinflammation in the pathogenesis of pain behavior induced by bone cancer. While it is relatively clear that the afferent sensory neuron can penetrate the mineralized bone, bone marrow or periosteum [5–7,15], the drivers of bone cancer pain are still elusive. It has drawn extensive interest that many inflammation mediators were found in bone cancer. Generated by cancer cells (tumor cells or associated macrophages), these secreted pro-inflammatory factors (such as TNFα, interleukin-1, prostaglandins ref) play pivotal roles in inducing bone resorption [25–27]. By eliciting osteoclast expansion, differentiation and activation, osteolytic tumor can trigger pain by altering the innervated bone microenvironment, such as decreased pH values and destructed bone micro architectures [28,29]. One the other hand, inflammation can directly affect pain behaviors, either by exciting the afferent nerve fibers in peripheral sites or remodeling the organization of the spinal cord in the central nerve system [4,29,30]. For example, TNFα is a central player in inflammation [10]. It has been previously shown that a local production of TNFα can be related to tumor-induced pain perception [31–33], and TNFα signaling inhibition attenuated experimental hyperalgesia [31,34,35]. Similarly, a study using TNFα receptor KO mice has demonstrated that TNFα signaling is indispensable for the progression of nociception induced by bone cancer, including tactile hypersensitivity and guarding behavior [16]. Consistent with these findings, we also observed a dramatic increase of TNFα expression in the spinal cord after the establishment of bone cancer (Fig. 3), implicating a role of TNFα in the central sensitization that is frequently seen in persistent pain in cancer patients. Along with preclinical studies on the anti-nociceptive activity of TNFα antagonists for pain induced by tumor metastasis [36–39], these data support an important therapeutic strategy for treating the chronic pain in patients with cancer bone metastasis by targeting inflammatory mediators.

Based on this rationale and the findings of previous research studies, using EGCG could be an excellent option for treating bone cancer pain. First, besides its anti-cancer functions, EGCG or green tea extracts have positive effects on bone health [40], which could directly benefit the patients with bone metastasis. It has been reported that tea drinking could improve bone mineral density (BMD) in osteoporosis patients or postmenopausal women, and decrease the risk of hip fractures in elderly people [40–42]. The next, green tea polyphenols have been used on the animal models of neuropathic pain, including diabetic neuropathy [43], chronic constriction injuries of the sciatic nerve [20], muscular pain in chronic fatigue syndrome [22], or the pain induced by spinal nerve ligation [44]. In addition, EGCG can block the voltage-gated sodium channels in primary hippocampal neurons in vitro [45], and restore the diminished glutamate decarboxylase (GAD) 65-expressing neurons in the brainstem nucleus raphe magnus (NRM) that correlate with hyperalgesia in adenomyosis [46]. Taken together, these lines of evidence are consistent with a protective role for EGCG as a putative treatment for neuronal injures through suppressing the devastating inflammation cascades. What is not yet known is the pathophysiological importance of the anti-inflammatory effects of EGCG in the hyperalgesia stimulated by bone tumor.

Our results indeed demonstrated a potential application of EGCG in this scene. By the daily intraperitoneal injections of EGCG at 50 or 100 mg/kg to cancer-bearing mice, we observed a significant amelioration of pain behavior in all tests used (Fig. 2). These dose-dependent effects are consistent with the previous reports on the in vitro anti-inflammatory actions of EGCG, including reduction of NF-κB activation, TNFα cytokine production, or MAPK pathway stimulation. Specifically, the concentration of 50 mg/kg has been successfully used for anti-nociception effects in animal models such as chronic thermal hyperalgesia [19] and chronic fatigue syndrome [22]. Moreover, it has been reported that the various levels of EGCG can activate cells differently, even in the opposite directions. For instance, a recent study has shown that EGCG at a low concentration increases, whereas at higher concentrations decreases osteogenic differentiation of alveolar bone cells [47]. It appears that the different routes of drug administration could also potentially affect the pharmacokinetics, thus resulting in distinctive effects of EGCG in animal experiments.

The cellular and molecular mechanisms of EGCG diminishing bone cancer-induced neuropathic pain can be complicated, and our data pointed to an important correlation with the blockade of TNFα expression in the spinal cord (Fig. 4). Decreases of TNFα expression may be indicative a less inflammatory insult in the peripheral sites, or a reduction of reorganization response in the central, both of which may be derived from the known functions of EGCG, such as suppressing oxidative stress or inhibiting NF-κB-dependent pathways [17]. In addition, there could be non TNFα-mediated pathways that were targeted by EGCG in our model. In fact, there was a discrepancy between the observed induction of TNFα expression and the severity of pain behavior in our time course study. The spinal cord TNFα expression has been declined progressively from its peak at the day 4 after the surgery, while the pain behaviors were all sustained through the end of observation day 18, suggesting a TNFα-independent mechanism. For instance, the extent of tumor progression needs to be addressed in mice treated with EGCG. Given the widely recognized anti-cancer functions, EGCG possibly represses bone cancer pain by simply diminishing cancer load in the bone. In addition, EGCG has known functions in protecting neuron cell apoptosis and enhancing cell survival by inducing neuron growth factors such as BDNF (Brain-derived neurotrophic factor) and GDNF (Glial cell-derived neurotrophic factor), or increasing the number of neuron stem cells [48,49]. Finally, there were still substantial pain developed even at the highest concentration of EGCG used, the potential mechanisms require further investigation in the future in developing novel drugs for treating bone cancer pain in coordination with EGCG.

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5. Conclusion

In conclusion, our studies have shown that EGCG can ameliorate the neuropathic pain caused by bone cancer. The pain behaviors were found reduced in mouse models of bone cancer treated by EGCG at 50 or 100 mg/kg concentrations. The elevated expression of TNFα has been observed in the spinal cord of disease animals, and partially diminished by the treatment of EGCG. The similar extents of reduction by EGCG in both TNFα expression and severity of pain behaviors were also found, implicating a strong correlation. Therefore, besides the effects in reducing bone resorption stimulated by osteolytic tumor, EGCG or other TNFα antagonists may be used to prevent the bone cancer pain.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

None.

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