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COLOCALIZATION OF AROMATASE IN SPINAL CORD ASTROCYTES: DIFFERENCES IN EXPRESSION AND RELATIONSHIP TO MECHANICAL AND THERMAL HYPERALGESIA IN MURINE MODELS OF A PAINFUL AND A NON-PAINFUL BONE TUMOR

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Abstract—While spinal cord astrocytes play a key role in the generation of cancer pain, there have been no studies that have examined the relationship of tumor-induced astrocyte activation and aromatase expression during the development of cancer pain. Here, we examined tumor-induced mechanical hyperalgesia and cold allodynia, and changes in Glial fibrillary acid protein (GFAP) and aromatase expression in murine models of painful and non-painful bone cancer. We demonstrate that implantation of fibrosarcoma cells, but not melanoma cells, produces robust mechanical hyperalgesia and cold allodynia in tumor-bearing mice compared to saline-injected controls. Secondly, this increase in mechanical hyperalgesia and cold allodynia is mirrored by significant increases in both spinal astrocyte activity and aromatase expression in the dorsal horn of fibrosarcoma-bearing mice. Importantly, we show that aromatase is only found within a subset of astrocytes and not in neurons in the lumbar spinal cord. Finally, administration of an aromatase inhibitor reduced tumor-induced hyperalgesia in fibrosarcoma-bearing animals. We conclude that a painful fibrosarcoma tumor induces a significant increase in spinal astrocyte activation and aromatase expression and that the up-regulation of aromatase plays a role in the development of bone tumor-induced hyperalgesia. Since spinal aromatase is also upregulated, but to a lesser extent, in non-painful melanoma bone tumors, it may also be neuroprotective and responsive to the changing tumor environment.

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Abbreviations: GFAP, Glial fibrillary acid protein; IHC, immunohistochemical; NBF, neutral buffered formalin; PBS, Phosphate buffered saline; PID 7, post-implantation day 7; PID, post-implantation; WB, western blot.

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INTRODUCTION

Recently it has become evident that estradiol in males is responsible for a number of effects originally attributed to testosterone (de Ronde and de Jong, 2011). Recent work has shown that male rats exposed to acute foot shock have increased circulating estradiol (Lu et al., 2015). In addition to peripheral sources, estradiol is also synthesized *in situ* from testosterone by spinal tissue via the enzyme aromatase, where it appears to be biologically active only at the local tissue level (Simpson and Davis, 2001). In the quail, aromatase has been shown to be present in neurons in spinal cord laminae I–II. Chronic and systemic blockade of this enzyme with an aromatase inhibitor altered nociception within days (Evrard, 2006). Recently, aromatase was shown to be upregulated in the spinal cord of female rats with a central pain syndrome (Ghorbanpoor et al., 2014), but these investigators did not examine male animals. Localization of aromatase in the murine spinal cord and its potential role in chronic pain conditions including cancer pain has not yet been reported and is the topic of this report.

Studies with animal models of pain have suggested that the reaction of glia, including microglia and astrocytes, critically contributes to the development and maintenance of chronic pain. In particular, astrocyte activation in the spinal cord appears to be an important contributor to the chronic pain associated with inflammation (Ikeda et al., 2012), human immunodeficiency virus (HIV) (Shi et al., 2012), chemotherapy-induced neuropathy (Zhang et al., 2012; Ruiz-Medina et al., 2013) and tumor-induced pain (Geis et al., 2010; Yao et al., 2011; Ren et al., 2012). Activation may result in altered cell morphology, changes in receptor expression, or release of factors by glial cells, which ultimately enhance nociceptive transmission (Ren and Dubner, 2008). The literature remains controversial regarding whether astrocytes play a critical role in the development of cancer pain (Ren et al., 2012; Ducourneau et al., 2014; Hironaka et al., 2014). Moreover there are no studies in the literature that have examined whether differential expression of spinal aromatase is associated with the development or maintenance of cancer pain.

The major goal of this study was to examine the relationships among tumor-induced nociception, astrocyte activation and aromatase expression in the spinal cord in murine models of painful and non-painful bone cancer. In this initial study we examined male mice, since the only other study on aromatase in the mammalian spinal cord focused on female rats (Ghorbanpoor et al., 2014) and since as stated above estradiol in males is responsible for a number of effects originally attributed to testosterone. We hypothesized that: (1) mechanical and cold hyperalgesia would be evident in mice with the fibrosarcoma bone tumor, but not in mice with a melanoma bone tumor; (2) astrocyte activation would be greater in fibrosarcoma-bearing animals compared to non-painful melanoma-bearing animals; (3) aromatase would be upregulated in mice with the painful fibrosarcoma tumor and not in mice with the melanoma tumor; and that (4) giving an aromatase inhibitor would inhibit mechanical hyperalgesia by reducing aromatase expression.

EXPERIMENTAL PROCEDURES

Animals

A total of 81 male C3H animals that are syngeneic to fibrosarcoma cells and male B6C3F1/Cr (B6C3) male mice (an F1 cross between C3H/He and C57BL/6 strains) that are syngeneic to G3.26 melanoma cells were used for all experiments. All mice were 6–8 weeks old and were obtained from the National Cancer Institute (Bethesda, MD, USA). Mice were housed in small conventional boxes in a temperature- and humidity-controlled environment and maintained on a 12-h light/dark cycle with *ad libitum* access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Cell culture and preparation for implantation

Fibrosarcoma NCTC 2472 cells were obtained from the American Cell Culture Collection (Rockville, MD, USA) and were maintained in NCTC 135 Medium (Sigma–Aldrich, Munich, Germany). Melanoma G3.26 cells were obtained from Dr. Christopher W. Stackpole (New York Medical College, Vallhalla, NY, USA) and maintained in alpha Minimum Essential Medium (MEM) (Sigma–Aldrich, St. Louis, MO, USA) with 10% Fetal bovine serum (FBS) as previously described (Wacnik et al., 2001). MB-MDA 231 cells were a gift from Dr. Laura Mauro at the University of Minnesota. These cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) media (Gibco, Life technologies, Grand Island, NY, USA), 10% fetal bovine serum and 1% penicillin–streptomycin (Life technologies, Grand Island, NY). Cells were first washed with Phosphate buffered saline (PBS), trypsinized, pelleted and suspended in 5 mL of PBS for quantification using a hemacytometer. Following counting, all cells were re-pelleted and re-suspended in a volume of PBS for a final concentration of 2×10^5 fibrosarcoma cells per 10 μL or 1.5×10^5 melanoma cells per 10 μL. Cells were kept on ice and vortexed shortly before tumor implantation.

Tumor implantation

Implantation into the calcaneus bone of the hind paw was performed as previously described (Wacnik et al., 2001; Smeester et al., 2014). Briefly, mice were placed in an enclosed plexi-glas chamber and initially anesthetized with 3% isoflurane/3 L oxygen. Upon successful anesthetization, the flow rate was adjusted to a maintenance level of 2% isoflurane/1.5 L oxygen for the remainder of the implantation procedure. Cells were injected unilaterally into the left heel using a 29-gauge, sterile single-use needle attached to a 0.3-mL insulin syringe (Becton Dickenson, Franklin Lakes, NJ, USA) to manually bore into the hind paw calcaneus bone. Control mice underwent an identical procedure with the exception that they received injection of saline rather than tumor cells. Following implantation, animals were returned to their home cages and recovered on a heating pad. Animals showing any signs of dysfunction (e.g. problems with ambulation, lethargy or excessive bleeding) were removed from the study. This occurred in less than 1% of the animals used in this study.

Mechanical hyperalgesia

Tumor-induced mechanical hyperalgesia was tested using von Frey filaments (#3.61 – C3H mice and #1.65 – B6C3) (Smeester et al., 2012, 2013, 2014). These filaments produce forces of 400 mg and 8 mg respectively. Animals were placed under clear glass cups on a wire grid and allowed to acclimate for 30 min. Starting with the right hind paw, the numbers of positive responses out of a total of 10 applications were recorded. Baseline von Frey measurements were obtained prior to tumor implantation or saline control injection into the calcaneus. Subsequent von Frey measurements were on post-implantation (PID) days 3, 7, 10, and 14. Behavioral assessments were conducted at approximately the same time each day. The investigator performing the von Frey testing was blinded to the experimental condition of the animals being tested. As the tumor grew the investigator was no longer blinded to the experimental condition, but the analysis of the data was performed by another researcher blinded to the experimental conditions of the animals.

Cold plate hypersensitivity

A modified version of the cold plate procedure reported by Allchorne and colleagues (Allchorne et al., 2005) was utilized for all cold plate testing. Mice were placed on top of a Peltier cold plate (model LHP-1200CPV, TECA Corp., Chicago, IL, USA) in an enclosed container ($20 \times 16 \times 25\ cm^3$) maintained at 4 ± 0.1 °C where the number of licking or rapid shaking behaviors of the hind paw was recorded and quantified by trained observers (number of behaviors). A maximum cut off time of 2 min was observed for all cold plate testing to prevent tissue damage at lower temperatures. Each mouse was only tested once on any given test day to avoid any possible anesthetic or tissue damage effects that could be produced by repeated exposure to the cold surface.