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THE RELATIONSHIP OF BONE-TUMOR-INDUCED SPINAL CORD ASTROCYTE ACTIVATION AND AROMATASE EXPRESSION TO MECHANICAL HYPERALGESIA AND COLD HYPERSENSITIVITY IN INTACT FEMALE AND OVARIECTOMIZED MICE

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Abstract—Recently, our group established a relationship between tumor-induced spinal cord astrocyte activation and aromatase expression and the development of bone tumor nociception in male mice. As an extension of this work, we now report on the association of tumor-induced mechanical hyperalgesia and cold hypersensitivity to changes in spinal cord dorsal horn GFAP and aromatase expression in intact (INT) female mice and the effect of ovariectomy on these parameters. Implantation of fibrosarcoma cells produced robust mechanical hyperalgesia in INT animals, while ovariectomized (OVX) females had significantly less mechanical hyperalgesia. Cold hypersensitivity was apparent by post-implantation day 7 in INT and OVX females compared to their saline-injected controls and increased throughout the experiment. The decrease in mechanical hyperalgesia in OVX females was mirrored by significant decreases in spinal astrocyte activity in laminae I-II, III-IV, V-VI and X and aromatase expression in laminae V-VI and X in the dorsal horn of tumor-bearing animals. Administration of the aromatase inhibitor letrozole reduced tumor-induced hyperalgesia in INT females only suggesting that the tumor-induced increase in aromatase expression and its associated increase in spinal estrogen play a role in the development of bone tumor-induced hyperalgesia. Finally, intrathecal (i.t.) administration of 17β-estradiol caused a significant increase in tumor-induced hyperalgesia in INT tumor-bearing females. Since i.t. 17β-estradiol increases tumor pain and ovariectomy significantly decreases tumor pain, as well as spinal aromatase, estrogen may play a critical role in the spinal cord response to the changing tumor environment and the development of tumor-induced nociception.

Key words: astrocytes, estrogen, aromatase, letrozole, tumor pain, spinal cord.

INTRODUCTION

Localized estrogen synthesis plays a significant physiological role in tissue-specific functions (Cui et al., 2013). In addition to peripheral sources, estradiol is also synthesized in situ from testosterone by the enzyme aromatase in spinal cord tissue, where it appears to be biologically active only at the local tissue level (Simpson and Davis, 2001). Our group has recently shown that aromatase is present in astrocytes and not neurons in the mouse spinal cord (O'Brien et al., 2015). In mice, chronic and systemic blockade of this enzyme with an aromatase inhibitor altered nociception.

Studies with animal models of pain have suggested that the reaction of glia, including microglia and astrocytes, contributes critically 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), 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). Aside from the recent work from our laboratories in male mice, (O'Brien et al., 2015) 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 a murine model of painful bone cancer in

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Abbreviations: i.t., intrathecal; INT, intact; NBF, neutral-buffered formalin; OVX, ovariectomized; s.c., subcutaneously.

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intact (INT) and ovariectomized (OVX) females. In the experimental design, we mirrored that used in a previous study performed in male mice (O'Brien et al., 2015) in order to examine potential sex differences in nociception and aromatase expression. Moreover, we compare our results to the only other study of aromatase in the mammalian spinal cord, which focused on the effects of spinal cord injury on aromatase expression in female rats (Ghorbanpoor et al., 2014). We hypothesized that: (1) mechanical and cold hyperalgesia would be evident and comparable to previous male mice findings in INT females; (2) astrocyte activation would be greater in INT tumor-bearing females versus OVX animals; (3) aromatase would be up-regulated in tumor-bearing INT mice and not in OVX tumor-bearing animals; (4) administration of an aromatase inhibitor would reduce mechanical hyperalgesia in INT, but not OVX females and (5) intrathecal (i.t.) administration of 17β-estradiol would significantly increase pain in INT tumor-bearing mice. We found that implantation of fibrosarcoma cells produced robust mechanical hyperalgesia in INT female animals, while OVX females had significantly less mechanical hyperalgesia and significantly less tumor-induced GFAP and aromatase expression. Since i.t. 17β-estradiol increases tumor pain, we conclude that estrogen may play a critical role in the development of tumor-induced nociception.

EXPERIMENTAL PROCEDURES

Animals

Female INT and OVX C3H animals that are syngeneic to fibrosarcoma 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. The authors certify that all experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised 1996.

Ovariectomy

Ovariectomy (OVX) surgical services were provided by surgeons at the National Cancer Institute (NCI). All OVX animals used were obtained from NCI following surgery and were allowed 7 days to fully recover and acclimate from the surgery prior to beginning any experiments. Serum estradiol samples were obtained and quantified using a commercially available estradiol EIA kit (#582251, Caymen Chemical, Ann Arbor, MI) prior to beginning the experiments to insure successful reduction of circulating estradiol.

Cell culture and preparation for implantation

Fibrosarcoma NCTC 2472 cells were obtained from the American Cell Culture Collection (Manassas, VA) and were maintained in NCTC 135 Medium (Sigma–Aldrich, Munich, Germany). Cells were first washed with 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. 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 plexiglass acrylic 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 a von Frey filament #3.61. This filament produces a force of 0.4 g. 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. A second researcher blinded to the experimental conditions of the animals performed the analysis of the data.

Cold plate hypersensitivity

Separate groups of tumor and saline-injected mice were used to test cold plate hypersensitivity than those used for von Frey testing. Mice were placed on top of a Peltier cold plate (model LHP-1200CPV, TECA Corp., Chicago, IL) in an enclosed container ($20 \times 16 \times 25\ cm^3$) maintained at 0 ± 0.1 °C where the number of licking or rapid shaking behaviors of the hind paw was recorded