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ASTROCYTES, BUT NOT MICROGLIA, ARE ACTIVATED IN OXALIPLATIN AND BORTEZOMIB-INDUCED PERIPHERAL NEUROPATHY IN THE RAT

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Abstract—Spinal microglia are widely recognized as activated by and contributing to the generation and maintenance of inflammatory and nerve injury related chronic pain; whereas the role of spinal astrocytes has received much less attention, despite being the first glial cells identified as activated following peripheral nerve injury. Recently it was suggested that microglia do not appear to play a significant role in chemotherapy-induced peripheral neuropathy (CIPN), but in contrast astrocytes appear to have a key role. In spite of the generalizability of astrocyte recruitment across chemotherapy drugs, its correlation to the onset of the behavioral CIPN phenotype has not been determined. The astroglial and microglial markers glial fibrillary acidic protein (GFAP) and OX-42 were imaged here to examine glial reactivity in multiple models of CIPN over time and to contrast this response to that produced in the spinal nerve ligation (SNL) model. Microglia were strongly activated following SNL, but not activated at any of the time points observed following chemotherapy treatments. Astrocytes were activated following both oxaliplatin and bortezomib treatment in a manner that paralleled chemotherapy-evoked behavioral changes. Both the behavioral phenotype and activation of astrocytes were prevented by co-administration of minocycline hydrochloride in both CIPN models, suggesting a common mechanism.

Key words: astrocytes, microglia, oxaliplatin, bortezomib, minocycline.

INTRODUCTION

The precise molecular pathways that govern the induction and maintenance of neuropathic pain phenotypes are not fully understood. However, several lines of evidence indicate that an interaction between sensitized spinal neurons and activated spinal glial cells mediated by the localized release of pro-inflammatory cytokines plays a critical role in this complex process (Sivilotti and Woolf, 1994; Ji and Suter, 2007; Miller et al., 2009; Graeber, 2010). Microglia in particular have been implicated as playing an important role across multiple models of chronic pain via immune or inflammatory activity (Ji and Suter, 2007; Graeber, 2010). Microglia are not distinct in this regard, however, as it has also been known that astrocytes are activated following nerve injury for over 20 years (Garrison et al., 1991). Identification of a common mechanism for glial involvement in multiple models of pain may be important for understanding how such models are developed or maintained.

Chemotherapy-induced peripheral neuropathy (CIPN) is a chronic disorder characterized by numbness, tingling, burning sensations, lack of sensation, or other dysthesias in the extremities (Dougherty et al., 2007). However, the exact symptoms and time to their development in CIPN vary from one drug to another (Cata et al., 2006a,b; Cavaletti and Marmiroli, 2010). To better understand the pathophysiology of CIPN, and to identify new possible treatments that translate well between chemotherapeutics, it is necessary to first identify common mechanisms that contribute to or distinguish CIPN as a type of chronic pain. A recent paper highlighted one such feature in CIPN, a lack of microglial reactivity in CIPN models that is otherwise present in overt nerve injury models (Zheng et al., 2011; Zhang et al., 2012). This finding was surprising in the context of other glial research in chronic pain, which suggested a major role for microglia in chronic pain as a whole. On the other hand, astrocytes have also been shown to be involved in and sufficient for the development and maintenance of some types of chronic pain (Hald, 2009; Gao and Ji, 2010). The involvement of astrocytes in CIPN was previously shown in our lab in the absence of microglial activation using a paclitaxel model (Zhang et al., 2012). The present study tests the generalizability of this observation to oxaliplatin and bortezomib-induced CIPN models under the hypothesis that astrocytes contribute a common activity in CIPN as a whole.

Minocycline hydrochloride has been shown to prevent the development of behavioral indicators of pain in multiple models, presumably through the inhibition of microglia (Hua et al., 2005; Ledeboer et al., 2005; Guasti et al., 2009). However, minocycline has prevented the development of CIPN symptoms in spite of the lack of any sign of microglial activation (Cata et al., 2008; Boyette-Davis and Dougherty, 2011; Boyette-Davis

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Abbreviations: CIPN, chemotherapy-induced peripheral neuropathy; GFAP, glial fibrillary acidic protein; PBS, phosphate-buffered saline; SEM, standard error of the mean; SNL, spinal nerve ligation.

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et al., 2011; Zheng et al., 2011). This would suggest that it is not, as many believe, a selective inhibitor of microglia, but may act through global anti-inflammatory mechanisms. This kind of activity would certainly inhibit microglial activation, but could also prevent inflammatory mechanisms within astrocytes. Accordingly, an important follow-up to investigating astrocyte activity was to examine whether minocycline abrogated potential up-regulation of astrocytes in bortezomib- and oxaliplatin-related CIPN. Abrogation of both astrocyte up-regulation and changes to behavioral phenotype by this single agent would suggest a correlation between the two. Abrogation of mechanical sensitivity by treatment with minocycline alongside oxaliplatin has already been shown (Boyette-Davis and Dougherty, 2011), but effects of minocycline on mechanical sensitivity in bortezomib have not yet been shown. Thus, whereas the first goal of the present study was to establish a glial activation profile in bortezomib compared to oxaliplatin, the second goal was to establish whether any observed changes in mechanical sensitivity and glial activation in either model are similarly blocked by minocycline.

EXPERIMENTAL PROCEDURES

Animals

All procedures were reviewed and approved by the M.D. Anderson Institutional Animal Care and Use Committee and were in accordance with the guidelines established by the NIH and the International Association for the Study of Pain. 111 Male Sprague–Dawley rats between 60 and 75 days of age upon beginning of treatment (300–350 g) were used for all experiments. Rats were housed in a facility with a 12-h light/dark cycle and were given food and water *ad libitum.* All efforts were taken at each stage of the experiments to limit the numbers of animals used and any discomfort to which they might be exposed.

Drugs

All drugs were administered by intraperitoneal injection in a volume of 0.5 ml. Oxaliplatin (Tocris Bioscience) was administered in dextrose vehicle at a dose of 2 mg/kg on days 1, 3, 5, and 7 of experimentation for a cumulative dose of 8 mg/kg as previously described (Boyette-Davis and Dougherty, 2011). Bortezomib (Millennium Pharmaceuticals) was administered in saline vehicle at a dose of 0.15 mg/kg on days 1, 3, 5, and 7 of experimentation for a cumulative dose of 0.60 mg/kg. Groups treated with minocycline hydrochloride (Sigma Aldrich, St. Louis, MO, USA) were injected daily with 25.0 mg/kg minocycline in saline vehicle beginning at day 0 and continuing daily through day 8 (one day past chemotherapy treatment) of experimentation for a cumulative dose of 225 mg/kg. Control groups were injected with an equivalent volume of appropriate vehicles (saline for bortezomib or dextrose for oxaliplatin).

Surgery

As a positive control for activation of both astrocytes and microglia, six rats received spinal nerve ligation (SNL) surgery (Kim and Chung, 1992). The rats were anesthetized using inhaled isoflurane (3–4%) to an adequate depth, verified by loss of nociceptive and blink reflexes. The L5 spinal nerve was exposed immediately distal to the dorsal root ganglion and then ligated with 6–0 silk suture. The wound was then closed in layers using vicryl suture and the skin closed with wound clips. The animal was monitored during recovery until it resumed normal activity. Another six rats received sham surgery, in which the L5 nerve was exposed, but not ligated.

Behavior testing

Sensitivity to mechanical stimuli was assessed in all animals using von Frey filaments (Boyette-Davis and Dougherty, 2011; Boyette-Davis et al., 2011). Filaments calibrated to 4-, 10-, 15-, and 26-g bending force were applied 6 times each to the mid-plantar surface of each hindpaw in order to determine the filament corresponding to the animal's response threshold. Application of filaments began following a half-hour habituation period with the lowest (4 g) filament. This was followed by other filaments of increasing bending force until a withdrawal threshold was obtained. Rats were allowed a resting period of 5–10 min between filaments in order to minimize the possibility of responses due to anxiety during testing. Filaments were applied with steady force until bending of the filament was observed and held for approximately 1second. A response was evaluated as a rapid withdrawal of the paw. The threshold for sensitivity to mechanical stimuli was recorded as the bending force of the filament for which at least 50% of applications elicited a response. The mean of this threshold was reported for each treatment group at each time point. Error was reported as standard error of the mean (SEM), and significance was tested at critical time points (those in which the errors of the two groups did not overlap or in which there was minimal overlap of errors) using Mann–Whitney tests.

The persistence of sensation was also measured based on von Frey responses that evoked exaggerated behaviors such as prolonged lifting, shaking, or licking of the paw. Out of the responses used to determine the 50% withdrawal threshold, the number of these that evoked an exaggerated response was recorded and expressed as percent of total responses. Error was reported as SEM, and significance was tested in bortezomib and bortezomib + minocycline groups versus saline-treated controls using Mann–Whitney tests.

Tissue collection

At the conclusion of the behavioral testing, animals with confirmed CIPN or SNL mechanical hyperresponsiveness were overdosed with sodium pentobarbital (150 mg), then perfused intracardially with room temperature heparinized saline, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer. Spinal cords were removed and post-fixed in 4% paraformaldehyde at 4 °C overnight, then moved to 15% sucrose the following day. Tissue was then moved after 24 h to 30% sucrose for a minimum of 48 h as a cryoprotectant. The lumbar enlargement was mounted