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SPINAL ASTROCYTE GAP JUNCTION AND GLUTAMATE TRANSPORTER EXPRESSION CONTRIBUTES TO A RAT MODEL OF BORTEZOMIB-INDUCED PERIPHERAL NEUROPATHY

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Abstract—There is increasing evidence implicating astrocytes in multiple forms of chronic pain, as well as in the specific context of chemotherapy-induced peripheral neuropathy (CIPN). However, it is still unclear what the exact role of astrocytes may be in the context of CIPN. Findings in oxaliplatin and paclitaxel models have displayed altered expression of astrocytic gap junctions and glutamate transporters as means by which astrocytes may contribute to observed behavioral changes. The current study investigated whether these changes were also generalizable to the bortezomib CIPN. Changes in mechanical sensitivity were verified in bortezomib-treated animals, and these changes were prevented by co-treatment with a glial activation inhibitor (minocycline), a gap junction decoupler (carbenoxolone), and by a glutamate transporter upregulator (ceftriaxone). Immunohistochemistry data at day 30 in bortezomib-treated animals showed increases in expression of glial fibrillary acidic protein (GFAP) and connexin 43 but a decrease in GLAST expression. These changes were prevented by co-treatment with minocycline. Follow-up Western blotting data showed a shift in connexin 43 from a non-phosphorylated state to a phosphorylated state, indicating increased trafficking of expressed connexin 43 to the cell membrane. These data suggest that increases in behavioral sensitivity to cutaneous stimuli may be tied to persistent synaptic glutamate resulting from increased calcium flow between spinal astrocytes.

Key words: bortezomib, minocycline, GFAP, connexin 43, GLT-1, GLAST.

INTRODUCTION

Bortezomib is a first-generation proteasome inhibitor chemotherapy drug used primarily for the treatment of multiple myeloma. Among its multiple side effects, bortezomib is known to produce chemotherapy-induced peripheral neuropathy (CIPN) that is often dose-limiting (Argyriou et al., 2012; Ferrier et al., 2013; Argyriou et al., 2014; Miltenburg and Boogerd, 2014). Bortezomib CIPN may extend long after the cessation of treatment, and current treatment options to manage CIPN symptoms are few and limited in their efficacy.

Among other forms of neuropathic pain, there is emerging evidence that spinal astrocytes and microglia may contribute to the phenotype's development and maintenance. However, it is becoming apparent that astrocytes are activated in the apparent absence of microglia in CIPN models. Our own lab has now demonstrated this in paclitaxel, oxaliplatin, and bortezomib models of CIPN (Zhang et al., 2012; Robinson et al., 2014b). However, there is currently limited evidence demonstrating potential mechanism by which astrocytes may contribute to CIPN. Downregulation of glutamate transporters in paclitaxel CIPN (Zhang et al., 2012) and the upregulation of astrocytic gap junctions in oxaliplatin CIPN suggest these also may contribute to bortezomib CIPN (Yoon et al., 2013).

The investigation of glutamate transporter dysfunction as a possible mechanism for CIPN may be justified by observations of behavior and neuronal activity in the spinal dorsal horn. Downregulation of glutamate transporters leads to persistent synaptic glutamate, which is sufficient to potentiate action potentials, drive persistent after-discharges, and promote spontaneous ectopic activity (Matute et al., 2006; Yi and Hazell, 2006; López-Bayghen and Ortega, 2011). Downregulation of glutamate transporters through pharmacological means is sufficient to drive spontaneous nociceptive behaviors (Nakagawa and Kaneko, 2010). Previous work from our lab has demonstrated altered activity in spinal wide dynamic range neurons and downregulation of glutamate transporters in support of such a model in animals treated with vincristine or paclitaxel (Weng et al., 2003; Cata et al., 2006). Work within the bortezomib model has demonstrated behaviors indicating persistent sensation and *in vivo* recordings showing potentiated responses and persistent after-discharges in spinal wide dynamic range neurons (Robinson et al., 2014a). Taken together,

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Abbreviations: CIPN, chemotherapy-induced peripheral neuropathy; GFAP, glial fibrillary acidic protein; HRP, horseradish protein; NP, non-phosphorylated; PBS, phosphate-buffered saline; SEM, standard error of the mean; TBST, Tris-buffered saline with Tween-20.

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it is possible that the changes observed in glutamate transporters in paclitaxel may also carry over to the bortezomib model, as well.

The activity of connexin 43 is another point of interest in CIPN. Connexin 43 is the protein in astrocytes that composes gap junction hemichannels that connect astrocytes together in a functional syncytium (Giaume and Liu, 2012; Theis and Giaume, 2012). The openings that are formed by astrocytic gap junctions are small, allowing only ions and molecules smaller than 1.2 kDa to pass. This is too small for the majority of signaling molecules, but astrocytic gap junctions permit the flow of such messengers as calcium ions and glutamate between cells (Tabernero et al., 2006; Theis and Giaume, 2012; Pannasch and Rouach, 2013). Hemichannel-forming proteins are upregulated in conjunction with astrocyte activation in multiple models of insult or injury, suggesting an increase in intercellular communication in these reactive astrocytes (Homkajorn et al., 2010; Chen et al., 2012, 2014). The potential downstream effects of this increased hemichannel expression are of great interest in CIPN. Increases in intracellular calcium in astrocytes may lead to decreased glutamate uptake or even direct release of glutamate from astrocytes (Malarkey and Parpura, 2008; Devinsky et al., 2013; Hansen and Malcangio, 2013). Thus, increases in connexin 43 potentially indicate a parallel means by which astrocytes decrease glutamate uptake at the tripartite synapse.

The focus of the present study is to assess the activity of the astrocytic glutamate transporters GLT-1 and GLAST and the activity of connexin 43 in bortezomib-treated animals. Data in support of previous findings in other models would explain behaviors and electrophysiological data seen in bortezomib-treated animals. The present work also includes minocycline in treatment groups, since this has been shown to prevent behavioral changes in bortezomib-treated animals. Therefore a direct role for connexins and glutamate transporters in bortezomib-induced peripheral neuropathy may only be established if (1) these proteins are altered in accordance with an observed change of behavior, and (2) if prevention of changes to behavior also prevents changes in these proteins. Carbenoxolone was included in additional treatment groups for behavioral data as a gap junction decoupler (Juszczak and Swiergiel, 2009; Yoon et al., 2013), and ceftriaxone was included as an upregulator of glutamate transporter expression (Lee et al., 2008; Kim et al., 2011). The inclusion of these treatment groups was to establish whether pharmacological strategies to directly counteract possible changes would also prevent the development of behavioral changes. Positive data would indicate that changes to these proteins are sufficient to drive the bortezomib phenotype of CIPN in the animal model.

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. Ninety-four (94) Male Sprague–Dawley rats between 60 and 75 days of age upon beginning treatment (300–350 g) were used for all experiments. Of these rats, all 94 were used for behavioral testing, but 21 of these were used for immunohistochemistry. Another 14 of these were used for Western blotting. 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

Saline, minocycline, and bortezomib were administered by intraperitoneal injection, and volumes were calculated based on body mass to approximate a volume of 0.5 ml. The gap junction decoupler ceftriaxone (Sigma Aldrich, St. Louis, MO, USA) and the glutamate transporter upregulator carbenoxolone (Sigma Aldrich, St. Louis, MO, USA) were administered intrathecally in the space between spinal segments L5 and L6 at a volume of 10 μL. Animals were divided into eight treatment groups: saline alone ($n = 11$), saline + minocycline ($n = 11$), saline + carbenoxolone ($n = 12$), saline + ceftriaxone ($n = 12$), bortezomib alone ($n = 12$), bortezomib + minocycline ($n = 12$), bortezomib + carbenoxolone ($n = 12$), and bortezomib + ceftriaxone ($n = 12$). Bortezomib (Millennium Pharmaceuticals, Cambridge, MA, USA) 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. Equivolume saline was administered to rats not treated with bortezomib. Animals 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. Carbenoxolone (25 μg/day) and ceftriaxone (150 μg/day) were administered on the same schedule as minocycline for cumulative intrathecal doses of 225 and 1350 μg, respectively. Animals not injected with carbenoxolone or ceftriaxone were administered an equivalent volume of saline intrathecally on the same dosing schedule.

Behavior testing

Von Frey filament testing was used to assess mechanical sensitivity over time as previously described (Boyette-Davis et al., 2011; Boyette-Davis and Dougherty, 2011; Robinson et al., 2014b). Briefly, filaments calibrated to a bending force equal to 4, 10, 15 and 26 g were applied 6 times each to the mid-plantar surface of each hindpaw. Animals were allowed a half-hour to habituate to the testing apparatus prior to application of filaments. Testing began with the lowest (4 g) filament and escalated in filament size until a withdrawal threshold was reached. Five to 10 minutes was allowed between filaments in order to minimize responses due to anxiety or sensitization. Filaments were applied with steady force until bending of