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THE RESPONSE OF SPINAL MICROGLIA TO CHEMOTHERAPY-EVOKED PAINFUL PERIPHERAL NEUROPATHIES IS DISTINCT FROM THAT EVOKED BY TRAUMATIC NERVE INJURIES

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Abstract—Painful peripheral neuropathies produced by nerve trauma are accompanied by substantial axonal degeneration and by a response in spinal cord microglia that is characterized by hypertrophy and increased expression of several intracellular and cell-surface markers, including ionizing calcium-binding adapter molecule 1 (Iba1) and Cd11b (a complement receptor 3 antigen recognized by the OX42 antibody). The microglia response has been hypothesized to be essential for the pathogenesis of the neuropathic pain state. In contrast, the painful peripheral neuropathies produced by low doses of cancer chemotherapeutics do not produce degeneration of axons in the peripheral nerve, although they do cause partial degeneration of the sensory axons' distal-most tips, that is the intraepidermal nerve fibers that form the axons' terminal receptor arbors. The question thus arises as to whether the relatively minor and distal axonal injury characterizing the chemotherapy-evoked neuropathies is sufficient to evoke the microglial response that is seen after traumatic nerve injury. We examined the lumbar spinal cord of rats with painful peripheral neuropathies due to the antineoplastic agents, paclitaxel, vincristine, and oxaliplatin, and the anti-retroviral agent, 2′,3′-dideoxycytidine (ddC), and compared them to rats with a complete sciatic nerve transection and the partial sciatic nerve injury produced in the chronic constriction injury model (CCI). As expected, microglia hypertrophy and increased expression of Iba1 were pronounced in the nerve transection and CCI animals. However, there was no microglia hypertrophy or increased Iba1 staining in the animals treated with paclitaxel, vincristine, oxaliplatin, or ddC. These results suggest that the mechanisms that produce neuropathic pain after exposure to chemotherapeutics may be fundamentally different than those operating after nerve trauma.

Key words: 2′3′-dideoxycytidine, microglial hypertrophy, neuropathic pain, oxaliplatin, paclitaxel, vincristine.

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Abbreviations: AOI, area of interest; ATF-3, activating transcription factor 3; CCI, chronic constriction injury; ddC, 2′,3′-dideoxycytidine; DH, spinal cord dorsal horn; DRG, dorsal root ganglion; Iba1, ionizing calcium-binding adapter molecule 1; IENF, intraepidermal nerve fiber; MAPK, mitogen-activated protein kinases; PBS, phosphate buffered saline; PBS-T, PBS with Triton; VFH, von Frey hair; VH, spinal cord ventral horn.

Transection of a peripheral nerve evokes dramatic changes in spinal cord microglia. The changes include an alteration of cell shape (hypertrophy), increased expression of cell surface and intracellular proteins, proliferation of resident microglia, and the in-migration of hematogenous microglia precursors [reviewed in (Inoue and Tsuda, 2009; McMahon and Malcangio, 2009; Milligan and Watkins, 2009)]. These changes are subsumed under the term "activation" following del Rio-Hortega and Penfield's description (1927) of the microglial response to a stab wound in the cerebral cortex. For the response of spinal cord microglia to peripheral nerve injury, activation is generally detected immunocytochemically using antisera directed against the cell surface marker, Cd11b (a complement receptor 3 antigen recognized by the OX42 antibody), or the intracellular marker, ionizing calcium-binding adapter molecule 1 (Iba1); both markers demonstrate increased staining levels and hypertrophy. Following transection of the sciatic nerve, responding spinal microglia are found in the dorsal horn territory where the transected sensory axons terminate, while in the ventral horn the responding microglia are restricted to the lateral motor neuron pools that contain the cells whose axons have been cut (Beggs and Salter, 2007).

Changes in spinal cord microglia after a complete sciatic nerve transection were first reported over 45 years ago [for review see (Gehrmann et al., 1991)], but interest in the phenomenon was greatly increased by reports showing that microglia also responded after partial nerve injuries that evoked neuropathic pain. Such nerve injuries are accompanied by an up-regulation of pro-inflammatory cytokines and chemokines. Microglia are immune cells and they have thus been proposed as a likely source of these substances (Coyle, 1998; DeLeo et al., 1997). Consistent with this idea, microglial inhibitors (e.g., minocycline, propentofylline, and ibudilast (AV411)) block the neuropathic pain state produced by partial nerve injury. These observations have led to the hypothesis that the microglial response is an important, and perhaps a necessary, factor in the pathogenesis of neuropathic pain (Inoue and Tsuda, 2009; McMahon and Malcangio, 2009; Milligan and Watkins, 2009).

We have investigated the painful peripheral neuropathies produced by cytotoxins; specifically, the cancer chemotherapeutic agents, paclitaxel, oxaliplatin, and vincristine, and the anti-retroviral agent, 2′,3′-dideoxycytidine (ddC). With the low-dose protocols that we use, these agents produce neuropathic pain but do not produce degeneration of axons in the peripheral nerve. However, they

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do produce a statistically significant partial degeneration of the intraepidermal nerve fibers (IENF) that form the terminal receptor arbors of the sensory axons that innervate the epidermis (Jin et al., 2008; Siau et al., 2006; Xiao et al., unpublished observations; Xiao et al., 2009).

The experiments reported here investigate the question of whether the relatively minor and very distal degeneration seen in chemotherapy-evoked peripheral neuropathies causes the microglial response that is seen after traumatic nerve injury.

EXPERIMENTAL PROCEDURES

These experiments conformed to the ethics guidelines of the International Association for the Study of Pain (Zimmermann, 1983), the National Institutes of Health (USA), and the Canadian Institutes of Health Research. All experimental protocols were approved by the Animal Care Committee of the Faculty of Medicine, McGill University, in accordance with the regulations of the Canadian Council on Animal Care.

Animals

Adult male Sprague–Dawley rats (200–300 g, Harlan Inc., Indianapolis, IN, USA; Frederick, MD breeding colony) were housed in groups of three to four on sawdust bedding in plastic cages. Artificial lighting was provided on a fixed 12 h light-dark cycle with food and water available *ad libitum*.

Drug administration and surgery

Paclitaxel (Taxol™; Biolyse Pharma Corp.; St. Catherines, ON, Canada) was administered via our standard protocol: the stock solution (in Cremophor/EL) was diluted with saline to a concentration of 2 mg/ml and injected i.p. at 2 mg/kg on four alternate days (D0, D2, D4 and D6) (Flatters and Bennett, 2004; Polomano et al., 2001).

Vincristine sulfate (Novopharm Ltd.; Toronto, ON, Canada) was given by diluting the stock solution with saline to a concentration of 50 μg/ml and injecting i.p. daily at 50 μg/kg for 10 consecutive days (D0–D9) (Siau and Bennett, 2006; Siau et al., 2006).

Oxaliplatin (Eloxatin™; Sanofi-aventis; Laval, QC, Canada) was given according to the protocol of Xiao et al. (unpublished observations): the stock solution was diluted with 5% dextrose in distilled water to a concentration of 2 mg/ml and injected i.p. daily for five consecutive days at 2 mg/kg.

2′,3′-dideoxycytidine (ddC; Sigma-Aldrich; Oakville, ON, Canada) was administered as a single bolus via the tail vein at 50 mg/ml/kg (Joseph et al., 2004; Siau and Bennett, 2006).

For comparisons to the three paclitaxel groups, we used a control group consisting of animals that had received four i.p. vehicle injections (two rats sacrificed on D7 and two on D14). For comparisons to the vincristine, oxaliplatin, and ddC groups, we used another control group consisting of animals that had received five i.p. injections of 5% dextrose in water (1 ml/kg) on five consecutive days and then sacrificed on D35.

Rats with a complete unilateral nerve transection were prepared by cutting the common sciatic nerve at mid-thigh level under isoflurane anesthesia. Rats were prepared with the chronic constriction injury (CCI) as described previously (Bennett and Xie, 1988). The side contralateral to transection or CCI was used as the control comparison. It is known that the contralateral side in such cases may not be normal; however, all published studies of the effects of traumatic nerve injury on microglia activation have used this as the control. All vehicle control, drug, and surgery groups were $n=4$.

Pain assays

The rats were habituated to the testing apparatus on two daily sessions. As per our standard procedure (Flatters and Bennett, 2004), 4 g and 15 g von Frey hairs (VFH) were applied to the plantar hind paw five times on each side for each hair. For each hair, the responses from both sides were summed and expressed as a percent response. Normal rats rarely or never respond to the 4 g VFH, indicating that it is an innocuous stimulus. Increased response frequency to this stimulus is thus indicative of mechano-allodynia. Normal rats respond to the 15 g VFH about 10–20% of the time, suggesting that it is a barely painful stimulus (it evokes a mild stinging pain when applied to our volar wrist). An increase in response frequency to the 15 g VFH is thus indicative of mechano-hyperalgesia.

Paclitaxel-treated rats were tested prior to drug exposure and on D7, D14, or D27. We have shown that paclitaxel-evoked mechano-allodynia and mechano-hyperalgesia begin with a distinct delay after the last injection of paclitaxel. Pain symptoms are absent on D7 (one day after the last paclitaxel injection), first appear 14–18 days after the last injection, and reach peak severity by D27 (Flatters and Bennett, 2004, 2006; Xiao et al., 2009).

Immunocytochemistry

Sciatic nerve transection rats were sacrificed 14 after surgery. CCI rats were sacrificed 8 days after surgery, which is the time of onset of the plateau phase of peak pain severity (Bennett and Xie, 1988). Chemotherapy-treated rats were sacrificed immediately after behavioral testing: paclitaxel-treated rats on D7, D14, or D27; vincristine-treated rats on D16, oxaliplatin-treated rats on D35, and ddC-treated rats on D8. For the vincristine and ddC dosing protocols used here, robust mechano-allodynia and mechano-hyperalgesia are known to be present at the selected time points (Joseph et al., 2004; Siau and Bennett, 2006; Siau et al., 2006). A complete time-course of oxaliplatin-evoked pain with the dosing protocol used here shows that the selected time point is near the beginning of the phase of peak pain severity (Xiao et al., unpublished observation).

The rats were over-dosed with sodium pentobarbital (100 mg/kg; i.p.; Ceva Sante Animale, Libourne, France) and perfused transcardially with a vascular rinse (phosphate buffered saline (PBS) containing 0.05% sodium bicarbonate and 0.1% sodium nitrite) for 1 min; followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The lumbosacral vertebral column was removed and post-fixed overnight, after which the L4/L5 segments of the spinal cord were exposed via laminectomy and identified by tracing the dorsal roots from their respective dorsal root ganglia (DRG). For the animals with CCI and sciatic nerve transection, a notch was made in the ventral side of the spinal cord contralateral to the side of nerve injury. Spinal cords were cryoprotected in 30% sucrose solution at 4 °C overnight and then and stored at −80 °C.

Cryostat sections (30 μm) sections were collected in PBS. Following 1 h incubation in PBS containing 0.2% Triton-X 100 (PBS+T) and 10% normal donkey serum (Jackson ImmunoResearch Laboratories; Mississauga, ON, Canada) at room temperature, sections were incubated for 24 h at 4 °C in PBS+T containing rabbit primary antisera diluted 1:1000 and 5% NDS. The primary antibody was directed against Iba1 (Wako Chemicals; Richmond, VA, USA). After rinsing in PBS+T, sections were incubated in donkey anti-rabbit IgG secondary antibody labeled with Cy3 (Jackson ImmunoReseach) diluted 1:200 for 1.5 h. Negative control sections (no exposure to the primary antisera) were processed concurrently for each rat.