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Spinal astrocyte gap junctions contribute to oxaliplatin-induced mechanical hypersensitivity

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Abstract

Spinal glial cells contribute to the development of many types of inflammatory and neuropathic pain. Here the contribution of spinal astrocytes and astrocyte gap junctions to oxaliplatin-induced mechanical hypersensitivity was explored. The expression of glial fibrillary acidic protein (GFAP) in spinal dorsal horn was significantly increased at day 7 but recovered at day 14 after oxaliplatin treatment, suggesting a transient activation of spinal astrocytes by chemotherapy. Astrocyte-specific gap junction protein connexin 43 was significantly increased in dorsal horn at both day 7 and day 14 following chemotherapy but neuronal (connexin 36) and oligodendrocyte (connexin 32) gap junction proteins did not show any change. Blockade of astrocyte gap junction with carbenoxolone prevented oxaliplatin-induced mechanical hypersensitivity in a dose-dependent manner and the increase of spinal GFAP expression, but had no effect once the mechanical hypersensitivity induced by oxaliplatin had fully developed. These results suggest that oxaliplatin chemotherapy induces the activation of spinal astrocytes and this is accompanied by increased expression of astrocyte-astrocyte gap junction connections via connexin 43. These alterations in spinal astrocytes appear to contribute to the induction but not the maintenance of oxaliplatin-induced mechanical hypersensitivity. Combined these results suggest that targeting spinal astrocyte/astrocyte-specific gap junction could be a new therapeutic strategy to prevent oxaliplatin-induced neuropathy.

Introduction

Peripheral neuropathy is one of the most common side effects following chemotherapy and causes prominent sensory disability and persistent pain even after chemotherapy has discontinued^{19,29,41,55}. Oxaliplatin is the third-generation platinum-based compound used as the primary therapy for metastatic colorectal cancer and other malignancies such as lung, breast, and ovarian cancers^{20,23,44,50}. Oxaliplatin induces prominent neuropathic pain which is characterized by pronounced cold and mechanical hypersensitivity and spontaneous pain^{4,6,52}. Several types of neuroprotective compounds including thiols, neurotrophic factors, anticonvulsants, and antioxidants have been tested in preventing oxaliplatin-induced

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neuropathy, but no definite effects have been found in clinical studies³. Thus, it still remains a high priority to identify safe and effective approaches to prevent or ameliorate oxaliplatin-induced painful neuropathy.

Spinal astrocytes have been shown to contribute to the development of chronic pain in various conditions including surgery, inflammation and nerve injury^{9,22,26,28,39}. Astrocytes are typically interconnected by gap junctions to form the organization of a functional syncytium¹². Gap junctions are formed by the linking of two hemi-channels one in each of two opposing cells, with each hemichannel composed of a hexamer of gap junction proteins called connexins, the most common of which in astrocyte hemichannels is connexin 43³⁵. Gap junctions appear to play an important role in the expression of various types of inflammatory and neuropathic pain. Spinal astrocyte gap junction protein is increased following chronic constriction of sciatic nerve⁵⁷; and blockade of gap junctions reduces peripheral nerve injury-induced central sensitization in medullary dorsal horn^{13,49}. We have previously shown that paclitaxel-induced painful neuropathy is associated with the activation of spinal astrocytes⁶¹. It is not known however whether spinal astrocytes are also activated in oxaliplatin-induced neuropathy and whether astrocyte involvement in chemotherapy-induced neuropathy may engage a gap-junction mechanism. In the present study, we examined the activation of spinal astrocytes and the expression of the astrocyte-specific gap junction protein as well as gap junction proteins in neurons and oligodendrocytes in a rat model of oxaliplatin-induced peripheral neuropathy. In addition, we tested the effect of carbenoxolone, a gap junction decoupler, on mechanical hypersensitivity and spinal astrocyte activation during and after oxaliplatin treatment.

Materials and Methods

Animals

The experiments were performed using 200 male Sprague-Dawley rats (330–380 g; Harlan Laboratories, Indianapolis, IN, USA) housed in colony cages with free access to food and water and maintained in temperature- and light-controlled rooms (23 ± 2°C, 12/12-hour light/dark cycle with lights on at 07:00) for at least 1 week prior to the study. One hundred and thirty (130) animals were used in the Western and immunohistochemistry studies (4–5 per group) and 70 animals were used in the behavioral pharmacology studies (6–8 per group). The specific allotment of animals to each experiment is detailed below. The experimental protocols for animal usage were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center and conformed to National Institutes of Health guidelines (NIH publication No. 86-23, revised 1985).

Drug administration

Oxaliplatin (Tocris Bioscience) was prepared by diluting to 1mg/ml in saline (0.9%) from a stock solution (5 mg/ml in 5% dextrose) and injected intraperitoneally at a dosage of 2 mg/kg every other day for a total of four injections (Days 1, 3, 5, and 7)⁷. Control animals received an equivalent volume of vehicle, which consisted of 5% dextrose and saline in the same final concentration as the oxaliplatin solution.

Carbenoxolone (CBX, Sigma-Aldrich Corp, USA) was diluted in saline. For assessment of its preventive effect on oxaliplatin-induced neuropathic pain, CBX (10 µl per injection) was injected intrathecally by lumbar puncture at sacral level, starting 24 h prior to the first dose of oxaliplatin (day 0) and continued once daily for the consecutive 7 days for a total of eight injections (Days 0–7). On days when both oxaliplatin and CBX were to be administered (Days 1, 3, 5, and 7), CBX was given 30 minutes prior to oxaliplatin. For assessment of the

effect of CBX on established oxaliplatin-induced neuropathic pain, CBX was administered once daily on days 14–21 after chemotherapy. Doses of carbenoxolone ranged from 1 to 25 µg per animal per day. Control animals received 10 µl saline in the same fashion.

Behavioral assessments

Mechanical withdrawal threshold was determined for all rats using an ascending series of von Frey filaments as previously described^{10,59}. Rats were placed in a clear Plexiglas compartment (25.4 × 25.4 × 10.16 cm; 10 × 10 × 4 in.) on an elevated metal mesh grid and allowed to acclimate for 10–20 minutes before testing. Each monofilament was applied six times in ascending order to the mid-plantar region of each hind paw of rat. The monofilament that produced a paw withdrawal, flinch, or lick in three of the six applications was defined as the 50% paw withdrawal threshold. This test was administered periodically starting on day 0 and ending on day 28. Behavioral experiments were conducted in a quiet behavioral testing room. The behavioral investigator was blinded to the treatment of animals during the experiments.

Western blot

Spinal cord samples were collected from vehicle- (with normal behavioral phenotype) and oxaliplatin-treated rats (with confirmed phenotype of CIPN) at day 7 or day 14 following treatment. Animals were deeply anesthetized with intraperitoneal injection of pentobarbital (90 mg/kg body weight; Lundbeck, Deerfield, IL, USA) and spinal dorsal horn of both sides from L4-L6 segment was dissected and quickly frozen in liquid nitrogen and stored at –80°C until further processing. The tissues were then homogenized by sonication in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂ ethylene-diaminetetra-acetic acid, 1 mM ethylene glycol tetra-acetic acid, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, mixed with protease and phosphatase inhibitor cocktails (Sigma). The protein concentration of the extraction was determined by Lowry protein assay (Bio-Rad, Hercules, CA, USA). Samples (30 µg protein in total) were then heated at 95°C for 5 minutes, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride (PVDF) membrane using a Transblot SD apparatus (Bio-Rad).

After being washed with twice-buffered saline with Tween-20 (TBST, 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20), the blots were blocked with 5% skim milk in TBST at room temperature for 30 minutes and incubated at 4°C overnight with primary antibody, followed by an incubation with horseradish peroxidase-conjugated appropriate secondary antibody at room temperature for one hour. The target protein with proper size was detected and visualized by an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, UK). Primary antibodies included rabbit anti-Cx43 (1:1000; Invitrogen), rabbit anti-Cx32 rabbit (1:1000; Millipore) rabbit anti-Cx36 rabbit (1:300; Invitrogen) and mouse anti-GFAP (1:5000, Cell Signaling Technology). The loading control of each sample was quantified by the level of β-actin (mouse, 1:10000, Sigma). Negative controls included omission of the primary antibody (data not shown), whereas positive controls were isolation of single bands at the published expected molecular weight.

Immunohistochemistry

Spinal cord immunohistochemistry was performed as previously described⁶¹. Rats were deeply anesthetized by pentobarbital (90.0 mg/kg, i.p.) and perfused transcardially with warm 0.9% saline followed by cold 4% paraformaldehyde. Spinal cord segment of L3–6 was removed, post-fixed in 4% paraformaldehyde at 4°C overnight and then cryoprotected in 30% sucrose at 4°C for at least two nights. Serial transverse sections (30 µm) were cut

from L4–5 spinal cord using a cryostat (Leica) and collected in PBS. Free-floating sections were then sequentially incubated with blocking solution (5% normal donkey serum plus 0.3% Triton X-100 in PBS) at room temperature for one hour and primary antibodies against Cx43 (1:1000) and GFAP (1:1000) in diluents (1% normal donkey serum plus 0.3% Triton X-100 in PBS) at 4°C overnight. After five washes with PBS, the sections were incubated with Cy3- and FITC-conjugated secondary antibodies (1:500, Jackson ImmunoResearch) at 4°C overnight. After washing, the tissue slices were mounted onto slides and viewed using an Eclipse E600 fluorescence microscope (Nikon). Negative controls included omission of the primary antibody (data not shown).

Statistical analysis

Results are expressed as means $\pm$ SEM. Data analysis and statistical comparisons were performed using GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA). Differences between two groups were assessed using Student's *t*-test. For multiple comparisons, one-way ANOVA followed by post hoc Dunnett test or two-way ANOVA followed by post hoc Bonferroni analysis was performed. Differences with $p < 0.05$ were considered significant.

Results

Oxaliplatin induced increased expression of GFAP in spinal dorsal horn

Astrocyte reactivity to chemotherapy was assessed by the expression of GFAP at days 7 and 14 following oxaliplatin treatment. A significant increase in GFAP expression was observed by western blot analysis at day 7 but not at day 14 in oxaliplatin-treated animals when compared to vehicle-treated animals ($n=4$ in each group) (Fig. 1A). Immunochemical analysis confirmed an increase of GFAP in spinal dorsal horn at day 7 following chemotherapy (Fig. 1B), but GFAP staining intensity at day 14 was comparable to baseline levels ($n=4$ in each group). Astrocytes in spinal dorsal horn at day 7 showed hypertrophied cell bodies with thickened and elongated processes after chemotherapy (Fig. 1B) but resumed a more quiescent morphology by day 14.

Oxaliplatin increased the expression of astrocyte-specific gap junction protein Cx43 in spinal dorsal horn

Several cell-specific subtypes of connexins (Cx) have been found⁵⁶ and connexin 43 (Cx43) is the primary component of intercellular gap junction connections in astrocytes^{5,12,16,17}. The expression of Cx43 in spinal dorsal horn following chemotherapy was assessed by western blotting. Oxaliplatin-treated animals showed a significant increase in the expression of Cx43 in spinal dorsal horn at both day 7 and day 14 after treatment by western blot analysis when compared to vehicle-treated animals ($n=5$ in each group) (Fig. 2A, B). This increase in expression was shown by immunohistochemistry to be widespread throughout the dorsal horn (Fig. 2C). In contrast, the expression level of neither Cx32, a neuron-specific gap junction protein, nor Cx36, a oligodendrocyte-specific gap junction protein, was changed at days 7 or 14 following oxaliplatin treatment ($n=4$ in each group) (Fig. 3). Double immunofluorescent staining further confirmed that CX43 was co-localized with the astrocyte marker GFAP in both spinal dorsal horn (Fig. 4BI) and white matter (Fig. 4BII).

Spinal application of carbenoxolone prevents oxaliplatin-induced mechanical hypersensitivity

To test the involvement of spinal astrocyte gap junctions in the development of oxaliplatin-induced mechanical hypersensitivity, carbenoxolone was delivered intrathecally in animals

co-treated with chemotherapy. The effect of carbenoxolone on the induction of oxaliplatin-induced mechanical hypersensitivity was first tested when animals were treated with carbenoxolone prior to and during chemotherapy (pre-emptive treatment). As shown in Figure 5A, oxaliplatin induced mechanical hypersensitivity in animals co-treated with saline (i.t.) as evidenced by a significant decrease in 50% threshold starting at day 10 following chemotherapy. The development of mechanical hypersensitivity following oxaliplatin treatment observed here is consistent with our previous study⁷. In contrast, rats treated with carbenoxolone during chemotherapy did not develop mechanical hypersensitivity as there was no decrease in 50% threshold (Fig. 5A). Carbenoxolone or vehicle alone did not cause any change in mechanical sensitivity (Fig. 5A). In addition, the preventive effect of carbenoxolone on oxaliplatin-induced mechanical hypersensitivity showed a dose-dependent effect with maximal effect achieved at 25 µg per injection (Fig. 5B, C).

To test the effect of carbenoxolone on established mechanical hypersensitivity following chemotherapy, carbenoxolone was applied on day 14 following the oxaliplatin treatment when animals consistently showed prominent mechanical hypersensitivity and continued once daily for 8 days (Days 14–24). As shown in Figure 5D, carbenoxolone (25 µg) showed no effect on fully developed mechanical hypersensitivity induced by oxaliplatin.

Spinal application of carbenoxolone prevents oxaliplatin-induced increase of spinal GFAP expression

To test whether blocking astrocyte gap junction connections could change the activation of spinal astrocytes induced by oxaliplatin treatment, the expression of spinal GFAP was examined in animals co-treated with carbenoxolone in the pre-emptive manner (n=6–8 per group, see Figure 6). Consistent with the data shown in Figure 1, oxaliplatin induced a marked increase in GFAP expression at day 7 but not day 14 following chemotherapy (Fig. 6). The application of carbenoxolone (25 µg) at the dose shown to prevent the development of oxaliplatin-related mechanical hypersensitivity also prevented the increase in GFAP expression normally induced by oxaliplatin treatment. The baseline level of GFAP expression was not affected by the administration of carbenoxolone or vehicle alone (Figure 6).

Discussion

Here we show that oxaliplatin induced a prominent activation of spinal astrocytes evidenced by the increased expression of GFAP. Oxaliplatin also induced a significant increase in the expression of astrocyte-specific gap junction protein CX43 in spinal cord, suggesting an enhancement in function of spinal astrocytes through either gap junction connections or hemichannels. Spinal application of carbenoxolone to block gap junction connections between astrocytes prevented the oxaliplatin-induced mechanical hypersensitivity and activation of astrocytes but had no effect once the oxaliplatin-induced hypersensitivity had fully developed.

The involvement of astrocytes in central nervous system in the pathogenesis of chronic pain has been demonstrated in many studies^{34,36,54}. Peripheral nerve injury induced the activation of astrocytes in spinal dorsal horn and trigeminal nucleus^{15,40,51}. Astrocytes can respond to neurotransmitters released from presynaptic terminals of neurons through a variety of receptors such as *N*-methyl-D-aspartate receptor³¹ and purinergic receptors⁵³ on their membrane. Activated astrocytes can modulate neuronal activities by releasing various signaling molecules including cytokines, nitric oxide synthase, and glutamate^{14,15,24,25,46}. Astrocytes can also downregulate the expression of glial glutamate transporters which will lead to the sensitization of postsynaptic neurons through the impaired clearance of glutamate in inter-synaptic space^{33,37,38,60}. Different from the persistent activation of spinal astrocytes

by paclitaxel⁶¹, the activation of spinal astrocytes induced by oxaliplatin is transient with complete recovery 14 days after the treatment. This may suggest different roles of spinal astrocytes in neuropathy induced by different chemotherapeutic drugs.

We found a persistent increase in the expression of astrocyte-specific gap junction protein Cx43 in spinal cord following oxaliplatin treatment whereas the expression levels of neuronal gap junction protein Cx32 and oligodendrocyte gap junction protein Cx36 were not affected. Adjacent astrocytes can be connected with each other through intercellular gap junctions, which provide a pathway for direct exchanges of ions and small molecules including cAMP, ATP, and IP3 to coordinate activities between individual cells^{8,21}. Plasticity of astrocyte gap junctions has been demonstrated by several studies in different conditions of chronic pain^{1,11,30}. The astrocyte-specific gap junction protein Cx43 has been shown to be involved in mediating ionic and metabolite exchanges between the processes of neighboring astrocytes and supporting intercellular communication via propagating Ca²⁺ waves between cells^{2,47}. Astrocytes can also release glia-transmitters through hemichannels^{18,27,35}. Thus our finding that the expression of spinal Cx43 was increased after oxaliplatin treatment suggests enhanced functional status of spinal astrocytes through forming new gap junction connections or hemichannels induced by chemotherapy. The increased expression of Cx43 in spinal cord was also reported following the chronic constriction injury of sciatic nerve⁵⁷. A potential limitation however, is that we did not rule out that part of the increased expression of Cx43 following chemotherapy may have in fact been as a consequence of de novo expression in neurons or oligodendrocytes.

Carbenoxolone is a disodium salt of 3'-*O*-hydrogen succinate of glycyrrhetic acid that has been widely used to disrupt intercellular communication via gap junctions^{42,49,58}. The effects of carbenoxolone shown in the present study further support an active role of spinal astrocyte gap junctions in promoting oxaliplatin-induced neuropathic pain. Daily intrathecal treatment with carbenoxolone in a pre-emptive manner dose-dependently prevented the development of mechanical hypersensitivity. However, administration of carbenoxolone had no effect on mechanical hypersensitivity once this behavior has been fully developed following the treatment of oxaliplatin. This observation suggests that spinal astrocytes gap junction connections play a key role in the induction but not the maintenance of oxaliplatin-induced neuropathy, though the underlying mechanism is still not clear. Another interesting finding is that the pre-emptive treatment of carbenoxolone prevented the activation of spinal astrocytes as shown by the expression of GFAP in oxaliplatin-treated animals, suggesting the activation of spinal astrocytes induced by oxaliplatin is dependent on the intact functioning of glial gap junctions. The similar effect of carbenoxolone on pain behavior has been observed in other models of chronic pain. Intrathecal or intracisternal carbenoxolone significantly suppresses formalin-induced hyperalgesia^{32,43}. Intrathecal injection of carbenoxolone also significantly reduced the development of mechanical allodynia in a rat model of thrombus-induced ischemic pain⁴⁸. Prolonged treatment with carbenoxolone has been associated with hypokalemic paralysis⁴⁵, yet animals in this study showed no sign of motor involvement.

In summary, our data has shown that oxaliplatin markedly activated spinal astrocytes and increased the expression of astrocyte-specific gap junction protein Cx43 in spinal cord. The preemptive blockade of astrocyte gap junction connections in spinal cord prevented the oxaliplatin-induced mechanical hypersensitivity and activation of spinal astrocytes. In contrast, carbenoxolone treatment had no effect on the fully developed mechanical hypersensitivity induced by oxaliplatin. These results suggest that spinal astrocyte gap junctions contribute to the induction of the mechanical hypersensitivity and the activation of spinal astrocytes but not the maintenance of oxaliplatin-induced neuropathy. Though the methodology for implementation at this juncture remains unclear, manipulations directed at

spinal astrocyte gap junctions may ultimately provide a new therapeutic strategy to prevent oxaliplatin-induced neuropathy.

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Abbreviations

Cx43	connexin 43
GFAP	glial fibrillary acidic protein
CBX	carbenoxolone

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Perspective

Spinal astrocytes but not microglia were recently shown to be recruited in paclitaxel related chemoneuropathy. Here spinal astrocyte gap junctions are shown to play an important role in the induction of oxaliplatin neuropathy.

Figure 1. Oxaliplatin (OXAL) treatment but not vehicle induced an increase in the expression of GFAP in spinal cord. (A) Western blot data shows the increase of GFAP at day 7 but not day 14 following the initiation of chemotherapy (N=4 for each group). * $p < 0.05$ oxaliplatin vs. vehicle group, one-way ANOVA. (B) Representative images of GFAP immunochemical staining in spinal dorsal horn at day 7 (center) and day 14 (right) following oxaliplatin in comparison to vehicle treatment (left). Magnified images of astrocytes in spinal dorsal horn (bottom row) show hypertrophy in the oxaliplatin group. Scale bar: 200 μ m (upper row) and 20 μ m (bottom row).

Figure 2.

Oxaliplatin (OXAL) treatment increased the expression of CX43 in spinal cord. (A) Western blotting data showed increased expression of Cx43 at both day 7 (A) and day 14 (B) following systemic oxaliplatin treatment (n = 5 for each group). Immunohistochemistry images show astrocyte-specific protein Cx43 in spinal dorsal horn after oxaliplatin treatment (C). Oxaliplatin induced a markedly increase in the expression of Cx43 throughout the spinal cord compared to vehicle at day 7 (C) and this persisted through day 14 (not shown).

*p < 0.05 oxaliplatin vs. vehicle group, one-way ANOVA.

Figure 3.

Oxaliplatin (OXAL) treatment did not change the expression of Cx32 or Cx36 in spinal cord. The expression of Cx32 (A) and Cx36 (B) did not show any change at either day 7 or day 14 following chemotherapy (N=4 for each group).

Figure 4. Double immunohistochemistry shows extensive co-localization of Cx43 (red) and GFAP (green), confirming that Cx43 is an astrocyte-specific gap junction protein. Boxed areas are displayed in detail with magnified images separately. Scale bar: 200 μ m (A, B top row) and 20 μ m (BI and BII).

Figure 5.

The effect of spinal application of carbenoxolone (CBX) on oxaliplatin-induced mechanical hypersensitivity. (A) Intrathecal carbenoxolone (25 μ g) was administered once daily for days 0–7 (solid line) while oxaliplatin was administered once every other day (arrows). Carbenoxolone but not saline treatment prevented oxaliplatin-induced decrease of 50% paw withdrawal threshold (PWT) (Two-way ANOVA). Vehicle or carbenoxolone alone did not cause any change of paw withdrawal threshold. (B) The effect of carbenoxolone on oxaliplatin-induced mechanical hypersensitivity was dose dependent when carbenoxolone was injected at 1, 5 and 25 μ g (Two-way ANOVA). (C) The analysis of area under curve showed a significant difference of carbenoxolone with 1 and 25 μ g (One-way ANOVA). (D) Daily CBX treatment (25 μ g, from days 14 to 21) did not change the fully developed

mechanical hypersensitivity following oxaliplatin treatment (two-way ANOVA). * $p < 0.05$, ** $p < 0.01$.

Figure 6.

Pre-emptive treatment of carbenoxolone (days 0–7) prevented the increased expression of GFAP in dorsal spinal cord induced by oxaliplatin (OXAL). (A) Carbenoxolone prevented the increase of GFAP in spinal dorsal horn at day 7 following oxaliplatin treatment. (B) At day 14, no significant differences were observed between the treatment groups. * $p < 0.01$, one-way ANOVA.