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Original Article

Sequential Changes of CX3CR1 in Dorsal Root Ganglion in a Rat Model of Lumbar Disc Herniation

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Objective To investigate the pain-related behaviors and the changes of CX3CR1 expression in the dorsal root ganglion (DRG) in a rat model of lumbar disc herniation.

Method A total of 90 male Sprague-Dawley rats were used. A laminectomy was performed to expose left L5 nerve roots and corresponding DRG. Autologous nucleus puplosus was implanted on the left L5 nerve root proximal to the DRG without mechanical compression. Sham operation was also done with the same procedure as mentioned above. Thermal hyperalgesia and mechanical allodynia were assessed at 1, 5, 10, 20 and 30 days after surgery. Real time PCR and immunohistochemistry after behavioral test were performed.

Results In the lumbar disc herniation rats, significant reduction of thermal withdrawal latency indicating thermal hyperalgesia was shown on the ipsilateral hindpaw on postoperative day 1 ($p<0.01$) and peaked on day 10 ($p<0.05$) and maintained throughout day 30 ($p<0.05$). The reduction of mechanical allodynia threshold, indicating mechanical allodynia, was observed on the ipsilateral hindpaw on postoperative day 1 ($p<0.01$) and continued throughout day 30 ($p<0.01$). Real time PCR showed the decrease in mRNA expression of CX3CR1 in the ipsilateral DRG on day 1 ($p<0.05$) and the significant increase on day 20 ($p<0.05$). The immunoreactivity for CX3CR1 was also increased in ipsilateral DRG on day 10 and 20.

Conclusion These data suggest that lumbar disc herniation induces thermal hyperalgesia and mechanical allodynia and upregulates the expression of CX3CR1 in dorsal root ganglion. Expression of CX3CR1 might be associated with subacute neuropathic pain after intervertebral disc herniation.

Key Words Dorsal root ganglion, CX3CR1, Chemokine, Disc herniation

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INTRODUCTION

Radiculopathy after lumbar disc herniation (LDH), which is one of various kinds of peripheral nerve injury, can induce neuropathic pain such as radicular pain of lower limb, hyperalgesia, mechanical allodynia as well as mild motor weakness, hypoesthesia. Even though without mechanical compression, herniated disc induces chemical inflammatory response, injury of nerve root,[1-5] and change of nerve conduction velocity and histology of nerve root[6,7] through various cytokines and inflammatory mediators such as interleukin-1, 6, 8 (IL-1, 6, 8), tumor necrosis factor-α (TNF-α). Neuroglial cells is known to be important for occurrence and maintenance of pain, and to control nerve cells physiologically and pathologically. Astrocytes and microglias in spinal dorsal horn are activated in a state of nerve injury, and activated glial cells secrete various neural excitation factor and facilitates pain.[8-10] Interestingly, initiation of pain may relate to microglia and maintenance to astrocyte.[11]

Peripheral nerve injury induces the releasing of various inflammatory mediators such as prostaglandins, histamines, bradykinins, cytokines, and chemokines from immune cells. Chemokines have been reported as biologically and pathologically functional, and activated by intracellular signal transmitter after combining to G-protein receptor.[12,13] Chemokines released from immune cells can influence to the migration of astrocyte migration, and microglial activation in spinal cord. They also induce neuropathic pain through facilitation of immune cell migration.[9,14,15]

Fractalkine (CX3CL1), which is activated by only one receptor, CX3CR1, is the only chemokine belonging to CX3C group of four chemokine groups (CXC, CC, C, and CX3C). It sticks to natural killer cell, mononuclear cell or acts as chemotactic agent. It is known that CX3CR1 is expressed mainly from microglia in spinal cord. Activated neuron release fractalkine on its surface, which combined to CX3CR1 on microglia. And CX3CR1 is thought to be important signal transmitter between neuron and glial cells.[10,12] In the previous studies, injected extrinsic fractalkine induced allodynia and hyperalgesia by combining to CX3CR1, and antagonist of fractalkine reduced allodynia and hyperalgesia in neuropathic pain animal model. Fractalkine released from neuron activated microglia in spinal dorsal horn, and aggravated pain in peripheral injury model.[10,16] Direct relationship between DRG and chemokine were reported that chemokine receptors was expressed in neuron in DRG, that intracellular calcium concentration was increased after chemokine injection, and that most chemokine sensitive neurons had relationship to pain.[14,17] In previous study, herniated lumbar disc model using autologous nucleus pulposus graft was rare, and also chemokine and CX3CR1 expression in pain model usually was studied with spinal dorsal horn, not with DRG, and there is no study of temporal changes of CX3CR1 expression in DRG. Therefore, we investigated the temporal change of neuropathic pain and CX3CR1 expression in lumbar disc herniation model using autologous nucleus pulposus graft.

MATERIALS AND METHODS

Animals

Ninety male Sprague-Dawley rats (200-250 g, 6-7 weeks old) were used in this study (n=10 for pain behavioral test, n=9 for real-time PCR for each time course, n=16 for immunohistochemistry). All animal experiments were conducted in accordance to the guidelines of the Institutional Animal Care and Use Committee at the Yeungnam University, South Korea.

Lumbar disc herniation model

Rats were anesthetized by Zoletil (Virbac Laboratories, Carros, France, 50 mg/kg, i.p.). Additional doses were used as required to maintain anesthesia throughout the experiment. A midline dorsal incision was made over the lumbar spine, multifidus muscles were separated along the L4-S1 spinous processes, and left L5 nerve roots and DRG were exposed through laminectomy. Nucleus pulposus, harvested from the tail disc between the second and third coccygeal vertebrae (Co2-3), was implanted next to the nerve root just proximal to the DRG.[18] Animals were received kanamycin (Komipharm, Shihung, Korea, 1 mg/kg, i.m.) for 2 days to prevent infection. Surgery in control rats was identical, except for the implantation of nucleus pulposus.

Pain behavior

Rats were tested for thermal and mechanical sensitivity of the plantar surface of hindpaw 2 days before surgery, and 1, 5, 10, 20 and 30 days after surgery by an investigator blinded to the experimental group and protocol of each rat (experimental group n=10, control

group n=10). We tested for thermal hyperalgesia with Hargreaves' method[19] by measuring the withdrawal response to a painful heat stimulus; rats were placed in a Plexiglas box on top of a glass platform for 30 min to habituate, and 300 watt halogen bulb was put to a glass, and the latency to withdrawal from a thermal stimulus was measured using the radiant heat with a Model 336 Paw/Tail Stimulator Analgesia Meter (Model 336 combination unit, IITC/life Science Instruments, Woodland Hill, USA) set at 2% idle light intensity and 50% working light intensity. The stimulus was turned off manually upon the hindpaw withdrawal or automatically if the 20 sec cut-off time was reached. Each rat received 5 trials of each hindpaw, 30 seconds apart, with 3 min between trials, and the results were averaged for analysis. Data were expressed as the latency to withdrawal in seconds.

We tested for mechanical allodynia by measuring the withdrawal response to a mechanical stimulation of the hindpaw with von Frey filaments (North Coast Medical, Inc., USA) that have been calibrated for the force in grams required to elicit a withdrawal response: rats were placed in a clear plastic cage with a metal mesh floor, adapted to the testing environment for 30 minutes, and the plantar surface of each hindpaw was stimulated with von Frey filaments (North Coast Medical, Inc., USA) of increasing or decreasing thickness, beginning with 0.1 g probe for 6-8 seconds, until a filament consistently gives withdrawal responses to 5 out of 10 stimuli.[20] Fifty percent probability thresholds of mechanical paw withdrawal were calculated.

Real time PCR (polymerase chain reaction)

Total RNA was isolated from lumbar spinal cord and DRG tissue, corresponding to L5 root on 1, 5, 10, 20 and 30 days after surgery (n=9 at each time point, total n=54). Total RNA was isolated from each sample with Trizol reagent (Invitrogen Corporation, Carlsbad, USA) and purity was checked with spectrophotometer. Reverse transcription of 1 μg aliquots of total mRNA was carried out at 45°C for 10 minutes, at 37°C for 120 minutes using a cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, USA). Real-time PCR assays were performed using a 7500 Real Time PCR system (Applied Biosystems, Carlsbad, USA). Primers and the TaqMan probe were designed using Probe Finder software (Universal Probe Library (UPL), Roche, Switzerland). To amplify CX3CR1 and hypoxanthine guanine phosphoribosyl-transferase (HPRT) transcripts the following primers were used: sense primer 5′-GTG GGA CTG GGT GAG TGG-3′ and antisense primer 5′-GAG GTA GGC ATG GTG AGG TC-3′ for CX3CR1 and sense primer 5′-GGT CCA TTC CTA TGA CTG TAG ATT TT-3′ and antisense primer 5′-CAA TCA AGA CGT TCT TTC CAG TT-3′ for HPRT. The HPRT gene was used as an internal control to adjust for differences between samples. The mastermix consisted of 250 nM of UPL probe, 700 nM of each primer (sense and antisense), 4 μm of 5X TaqMan master and 2 μl of cDNA. All PCR reactions were run in duplicate. After pre-incubation at 95oC for 10 minutes, PCR was performed using 50 amplification cycles of denaturation at 95°C for 10 seconds and annealing at 60°C for 60 seconds.

Immunohistochemistry

To assess the expression of CX3CR1, we immunostained the sections of DRG of LDH model on 1, 10, 20 and 30 days after surgery (n=4 at each time point). Rats were anesthetized with Zoletil (Virbac Laboratories, Carros, France, 50 mg/kg, i.p.) and perfused with heparinized saline followed by 500 ml of 4% paraformaldehyde in phosphate buffer (PB) (in 0.1 M, pH 7.4). The DRG on both sides were removed, postfixed for 2 hrs in the fixative used for perfusion, and cryoprotected in 30% sucrose (in 0.1 M PB, pH 7.4). Forty micrometer-thick transverse sections were cut on a cryostat, blocked with 10% normal donkey serum (NDS, Jackson Immunolabs, West Grove, USA) in phosphate-buffered saline (PBS, 0.01 M, pH 7.2) for 10 min, and incubated overnight with primary antibodies. To investigate CX3CR1 expression rabbit anti-CX3CR1 (1 : 500, Torrey Pines Biolab, La Jolla, USA) was used. The next day, sections were rinsed and incubated in 2% NDS for 10 min and then incubated with an appropriate combination of donkey-antirabbit secondary antibodies conjugated to Cy3 (1 : 200, Jackson Immunolabs, West Grove, USA) in PBS for 3 hours. After several rinses, sections were mounted on slides, coverslipped with Vectashield (Vector laboratories, Burlingame, USA), and observed on a Leica DMR microscope (Leica, Solms, Germany). Images were acquired with a CCD camera (F-view II, SIS, Muenster, Germany) attached to the microscope, and contrast and brightness were adjusted with Photoshop (7.0, Adobe Systems Inc, San Jose, USA).

Statistical analysis

Data expressed as mean±standard deviation. Behavio-

ral data were analyzed with the Wilcoxon signed rank test and Mann Whitney U test, and temporal expression data of mRNA were analyzed with paired t-test and independent t-test using SPSS/PC v. 15.0 and significance was set at $p<0.05$.

RESULTS

Pain behavior

Thermal withdrawal latency significantly decreased on 1 day ($p<0.05$), 10 days ($p<0.01$), 20 days ($p<0.05$), 30 days ($p<0.05$) after surgery in ipsilateral side and 1 day ($p<0.05$), 10 days ($p<0.01$) after surgery in contralateral side, respectively. In both sides, the biggest drop of latency occurred on 10 days after surgery, and then latency gradually increased (Fig. 1).

A mechanical withdrawal threshold significantly decreased on 1 day ($p<0.01$), 5 days ($p<0.01$), 10 days ($p<0.01$), 20 days ($p<0.01$), 30 days ($p<0.01$) after surgery in ipsilateral side and 20 days ($p<0.01$), 30 days ($p<0.05$) after surgery in contralateral side, respectively (Fig. 2).

Real time PCR

The expression of mRNA for CX3CR1 was significantly reduced on 1 day ($p<0.05$) after surgery compared to sham control, and then significantly increased on 20 days ($p<0.05$) after surgery, and then reduced again in ipsilateral side. In contralateral side the expression of mRNA for CX3CR1 was significantly reduced on 1 day ($p<0.05$) after surgery alone, but showed relatively

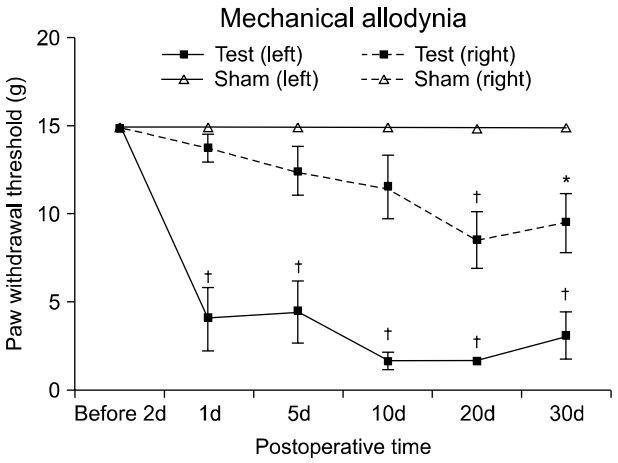

Fig. 2. Comparison of mechanical allodynia. Hindpaw withdrawal thresholds in response to a von Frey filament of the experimental (lumbar disc herniation) and sham groups. A significant reduction in mechanical withdrawal threshold was seen during 1 day through 30 days in the left hindpaw after surgery in experimental group. *$p<0.05$, †$p<0.01$, Values are mean±SE (n=10). Test: lumbar disc herniation, Left: Ipsilateral, Right: Contralateral, D: Day(s).

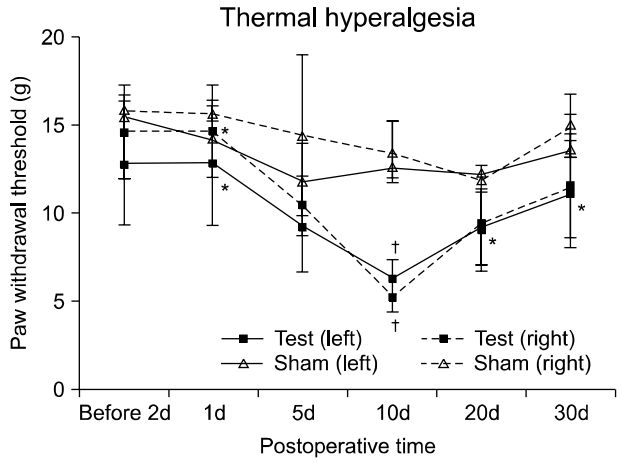

Fig. 1. Comparison of thermal hyperalgesia. Hindpaw withdrawal latencies in response to a radiant heat of the experimental (lumbar disc herniation) and sham groups. Responses to thermal stimulation of the hindpaw reduced during 1 day through 30 days in the left hindpaw after surgery in experimental group. *$p<0.05$, †$p<0.01$, Values are mean±SD (n=10). Test: lumbar disc herniation, Left: Ipsilateral, Right: Contralateral, D: Day(s).

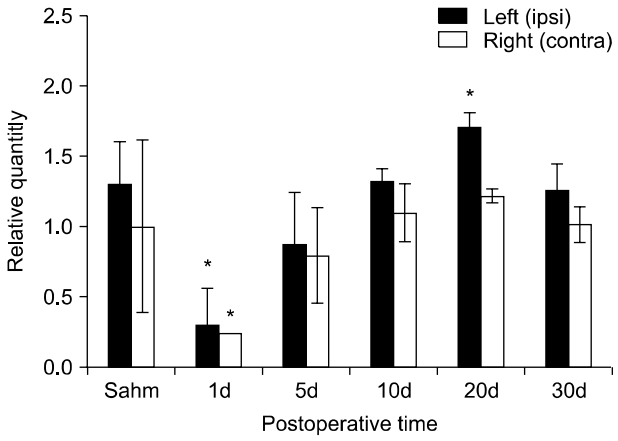

Fig. 3. CX3CR1 mRNA expression of ipsilateral and contralateral dorsal root ganglion in the experimental and sham groups. The expression amount of mRNA for CX3CR1 in the ipsilateral DRG of experimental group was increased at 10 days after surgery. *$p<0.05$ indicate values significantly different from contralateral 24h sham value. Values are mean±SD (n=9). Ipsi: Ipsilateral, Contra: Contralateral, D: Day(s).

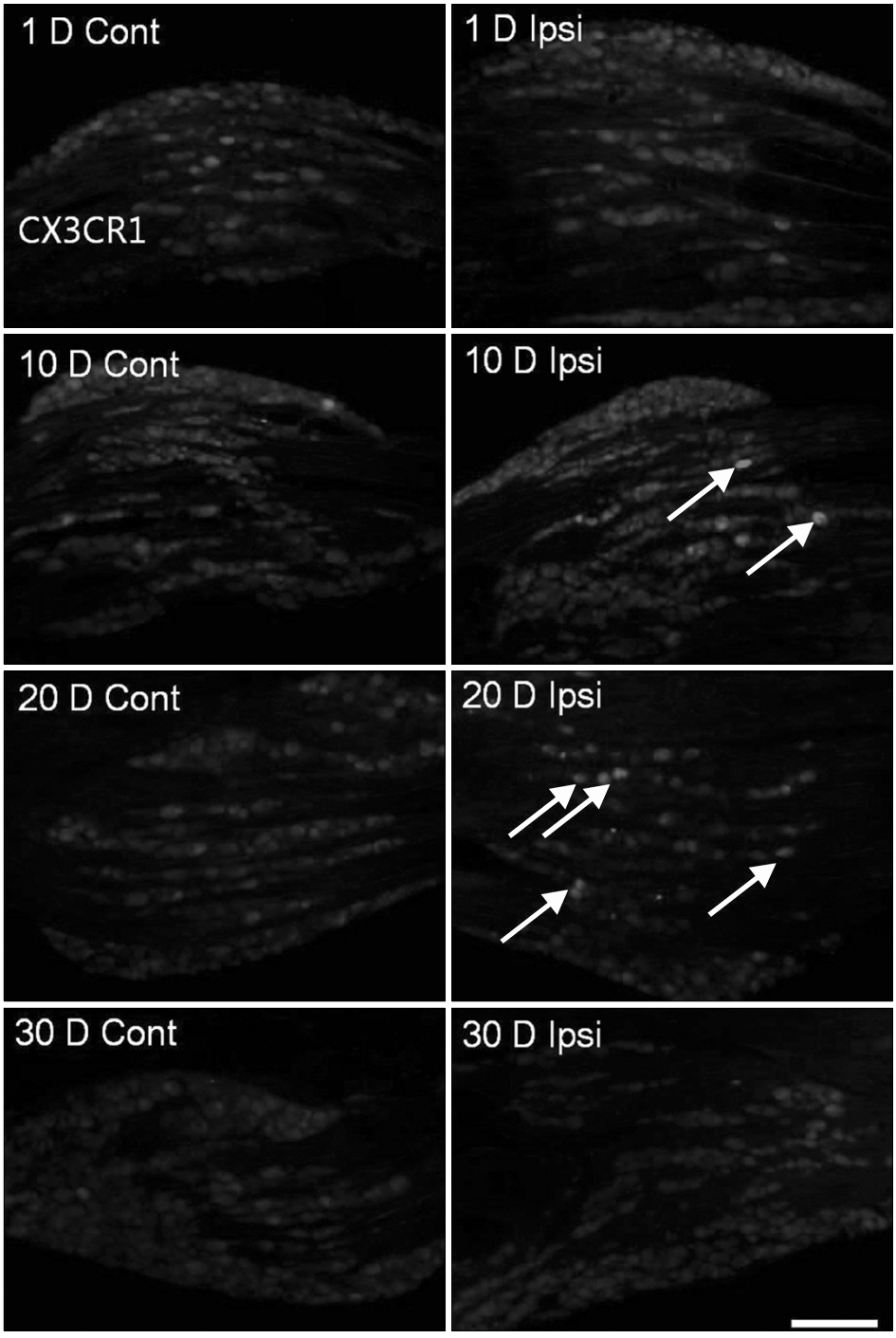

Fig. 4. These immunohistochemistric findings show expression of CX3CR1 in dorsal root ganglion 1, 10, 20 and 30 days after surgery, low power view. Gradual increase of immunoreactivity for CX3CR1 was noted especially in ipsilateral dorsal root ganglia (arrow). Scale bar= 200 μm

similar tendency. Temporal change of mRNA expression was not significant between both sides (Fig. 3).

Immunohistochemistry

The expression of CX3CR1 increased gradually on 10 days, 20 days, not on 30 days after surgery in ipsilateral DRG (Fig. 4).

DISCUSSION

In this study, we investigated the pain behavior of lumbar disc herniation rat model using autologous nucleus pulposus graft to L5 nerve root for 30 days and temporal change of CX3CR1 expression in DRG.

Mechanical withdrawal threshold in ipsilateral side

reduced significantly on 1 day after surgery, and maximally decreased on 10 days after surgery, and then persisted to 30 days after surgery, while it reduced significantly from 20 days after surgery, and persisted to 30 days after surgery in contralateral side. Thermal withdrawal latency in ipsilateral side reduced significantly on 1 day after surgery, and maximally decreased on 10 days after surgery, and persisted to 30 days after surgery, while, significant reduction on only 1 day and 10 days after surgery in contralateral side. In contralateral side, pain was later and weaker than ipsilateral side.

The previous studies reported that mechanical allodynia and hyperalgesia initiated on 1 day after surgery and reduced within 3 weeks after surgery in the autologous nucleus pulposus graft model.[7,21,22] In this study, the pain initiated on 1 day after surgery like other studies, but sustained to 30 days after surgery without decrement. It is assumed because nucleus pulposus was grafted at proximal site of DRG similar to clinical case unlike distal site of DRG in other studies.

Our data showed contralateral as well as ipsilateral hyperalgesia as like other studies.[23-25] Contralateral hyperalgesia, that is, mirror-image neuropathic pain is known to be similar quality of pain, but less intensity and shorter duration. The mechanisms of mirror-image neuropathic pain are speculated as humoral mechanism associated with intravascular circulation of factors from injured nerve, bilateral projection of nerve to target and transmedian sprouting, neuronal communication between two dorsal horns, connection through commissural interneuron,[24] non-neuronal mechanism by wide gap junctional connectivity of spinal glial cells,[26] and mechanism of glial cells and inflammatory cytokines.[25]

The expression of CX3CR1 was reduced significantly on 1 day after surgery, and then gradually increased to 20 days after surgery on both sides. On 20 days after surgery the expression increased significantly in ipsilateral side alone, and reduced on 30 days after surgery.

In immunohistochemistry, the expression of CX3CR1 in ipsilateral DRG increased on 10 days, sustained to 20 days, but not increased on 30 days after surgery. Though maximal increment was a little later compared to the maximal painful time, we could suppose the relationship between pain and CX3CR1 expression because of their similar tendency of increment. So proinflammatory cytokine such as TNF-α, interleukin-1 (IL-1) mediates acute inflammatory pain, and CX3CR1 acts as chemoattractant factor, which could amplify inflammation or intensify neuropathic pain in subacute phase. This could be supposed through Ahn's study,[1] which reported the increment of IL-8, a kind of chemokine in severe subacute radicular pain patients, not TNF-α, IL-1. In the previous studies in spinal dorsal horn, CX3CR1 expression increased from acute phase with microglia in dorsal horn.[13,16] However, this study about DRG showed initial decrement, and next gradual increment to 20 days after surgery, and decrement again against dorsal horn studies. In clinical situation, most patients with radicular pain tend to go to hospital in subacute phase, and CX3CR1 expression persists to 20 days after surgery, so CX3CR1 would be worth considering as important therapeutic target. Especially, transforaminal epidural steroid injection could wet entire nerve root containing DRG, which could suppress CX3CR1 and could reduce radicular pain, and antagonist of CX3CR1 could be helpful to suppress radicular pain. But, we could not confirm the effect of CX3CR1 antagonist on neuropathic pain of autologous nucleus grafted rat model and direct relationship between CX3CR1 and neuropathic pain. Also we could not research the expression of C3XCR1 in dorsal horn and comparison of C3XCR1 expression between dorsal horn and DRG. Further studies about above limitation are necessary to clear pathomechanism of radicular pain, and to treat pain.

CONCLUSION

In lumbar disc rat model using autologous nucleus pulposus graft, herniation neuropathic pain sustained for long time, the temporal expression of CX3CR1 in DRG could be related to neuropathic pain in subacute phase.

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