Original Article

Fractalkine receptor chemokine (CX3CR1) influences on cervical and lumbar disc herniation

In-Soo Oh, Dong-Whan Suh, Sung-Ryeoll Park, Kee-Yong Ha[1]

Abstract

Background: Herniation of nuclear or disc material along with, inflammatory chemokines such as prostaglandin E2, interleukin-6, matrix metalloproteinase and nitric oxide has definite correlation, possibly they are over produced. CX3CL1 and its receptor (CX3CR1) are part of chemokine system involved in leukocyte recruitment and adhesion in chronic inflammatory disease, but its role in spinal herniated nucleus pulposus (HNP) is unknown. We evaluated the expression of CX3CL1 and CX3CR1 in patients with disc herniation to clarify the role
of CX3CL1 and CX3CR1 in the disc degeneration and to compare between cervical and lumbar HNP.

Materials and Methods: The mRNA concentrations of CX3CL1/CX3CR1 chemokine were analyzed in the surgically obtained disc specimens from C-HNP (n = 13) and L-HNP (n = 13) by real-time polymerase chain reaction (PCR). The localization of CX3CL1/CX3CR1 chemokine in the disc of C-HNP and L-HNP patients was determined using immunohistochemical study. Blood samples from patients with C-HNP and L-HNP patients were stained for CX3CR1 with flow cytometric analysis.

Results: The CX3CL1 positive cell ratio in the discs was observed in both groups by immunohistochemical study. CX3CR1 was strongly expressed on endothelial cells in C-spine disc, but sparely expressed in L-spine disc. There was greater CX3CR1 mRNA expression in C-HNP patients than in L-HNP patients as quantified by reversal transcription-PCR (P = 0.010). CX3CR1 positive cell frequencies and CX3CR1 expression levels were increased in CD4 (+) T-cells and natural killer (NK) cells from patients with C-HNP (P = 0.210 and P = 0.040).

Conclusions: This study identified that increases in CX3CL1 and CX3CR1-expressing cells are significantly related to pathomechanism of HNP for the first time. Especially, CD4 (+) T-cells and NK cells expressing CX3CR1 may play an important role in developing C-HNP.

Key words: Lumbar disc, fractalkine receptor, herniated nucleus pulposus
MeSH terms: Lumbar, cervical, disc, herniated, receptors, chemokine

Introduction

Herniated nucleus pulposus (HNP) is a multifactorial disease of the complex nature. It's relation to disc degeneration was well known in the literature; however, recent studies propose that this condition is more genetic, innate and degenerative rather than extrinsic. The pathophysiology of intervertebral disc disease derives its explanation from a notion that disc disease actually is a degenerative process or a course of senescence. As such, progressive annular degeneration follows physiologic change of nucleus and frank herniation of nuclear material as a result. Herniation of nuclear or disc material along with inflammatory chemokines such as prostaglandin E2, interleukin-6, matrix metalloproteinase and nitric oxide has definite correlations, possibly they are over produced.[1]

CX3CL1, a chemokine that signals through a single known receptor CX3CR1 is implicated in recruitment and adhesion of both monocytes and T-cells in atherosclerosis, rheumatologic disorders and HIV-1.[2-4] Importantly, CX3CL1 is a dual function molecule. It potently attracts CX3CR1-expressing cells when it is in soluble form and when in the membrane-bound form, mediates firm adhesion of CX3CR1-expressing monocytes/macromphages, natural killer (NK) cells and a subpopulation of T-cells, even without activation of integrins.[4-7] Therefore, when it comes to attraction, migration and activation of C X3CR1-expressing monocytes/macrophages, NK cells and a subpopulation of T-cells, CX3CL1-CX3CR1 axis proves versatility.[4-6]

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A critical role of CX3CL1/CX3CR1 chemokine has been established in chronic inflammatory process in a variety of organs, including liver, renal, lung, and heart.[8-11] A role of CXC3CL1/CX3CR1 has not been explained in other chronic conditions such as HNP, in which relation to increment of inflammatory chemokine is reported, while its involvement well known in rheumatoid arthritis and osteoarthritis (OA).

We evaluated the expression of CX3CL1and CX3CR1 in patients with disc herniation to clarify the role of CX3CL1 and CX3CR1 in the disc degeneration and to compare between cervical and lumbar HNP.

Materials and Methods

This study was approved by the Institutional Review Board (IRB approval No. OC 11SISI0082). Decompressive surgery was performed on 26 patients and surgically obtained disc specimens from C-HNP patients (n = 13) and L-HNP patients (n = 13) were analyzed. Mean (SD) age at the time of surgery was 42.1 (standard deviation (SD) 7.9) years old in the C-HNP group and 38.6 (SD 10.6) years old in the L-HNP group. There was no significant difference (P = 0.786) as a control group. None of these patients received epidural, elective nerve root blocks and revision surgery. Patients with medical comorbidities were excluded. Tissues were stored in a liquid nitrogen tank immediately after harvesting and kept at −70°C until they were used in experiments. Informed consent was obtained from all subjects in a written format before starting the study. Peripheral blood samples were collected from all subjects on admission for flow cytometry study.

Histologic and immunohistochemical studies

Sections of 5 μmm thickness were obtained from paraffin blocks. Sections were placed on glass slides, dried, dewaxed in xylene and rehydrated in graded concentrations of ethanol. Sections were incubated in a 10 mmol/L sodium citrate buffer (pH: 6.0) with a microwave oven to retrieve antigenicity from formalin fixated samples. The sections were treated at 4°C with antihuman CX3CL1 antibody (antigen affinity-purified polyclonal goat IgG, R and D systems), CX3CR1 antibody (antigen affinity-purified polyclonal rabbit IgG, Novus Biologicals) after incubation with normal blocking serum. Sections were counterstained with hematoxylin, incubated with a secondary antibody, treated with an avidin biotin-peroxidase complex, and colored with diaminobenzidine. Results were evaluated using contrast stains with hematoxylin.

Laboratory methods

Quantitative polymerase chain reaction

Quantitative real-time polymerase chain reaction (PCR) analysis of CX3CL1 and CX3CR1 mRNA expression was performed using a Bio-Rad iCycler (Bio-Rad Laboratories, Berkeley, California), and each mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ribosomal RNA expression for each sample. Total RNA 1 μg was used in the reversal transcription (RT) reaction with 0.5 μg of oligodT primer, 10 mmol/L of each of the 4 dNTP, 25 mmol/L of $MgCl_2$, 10 U of RNA inhibitor and 50 U of superscript II reverse transcriptase (all purchased from Invitrogen) according to the manufacturer's instruction. The final solution was used directly for PCR amplification. Each cDNA reaction was diluted, along with a calibrator sample containing the transcript of interest and run in 25 μL of Bio-Rad SYBR Green (Bio-Rad Laboratories, Berkeley, California, USA) reactions using 10 pmol of primers designed by Primer 3.[12] Human gene-specific oligonucleotide sequences were as follows: CX3CR1, forward primer-5'-GTGGTGCTGACAAAGCTTGGAA-3', reverse primer 5'-TCACTGGGTGCCATCGTAAGAA-3', CX3CL1, forward Primer 5'-GGATGCAGCCTCACAGTCCTTAC-3', reverse primer 5'-GGCCTCAGGGTCCAAAGACA -3'. GAPDH, forward primer 5'-GCACCGTCAAGGCTGAGAAC -3', reverse primer 5'TGGTGAAGACGCCAGTGGA -3'. Reactions were processed using one initial denaturation cycle (5 min at 94°C), then 30 cycles of denaturation (30 s at 95°C), annealing (45 s at variable for gene) and amplication (1 min at 72°C) followed by melt curve determination consisting of one denaturation cycle (1 min at 95°C), annealing (one cycle for 1 min at 55°C) and then 80 cycles (5 s each at 55°C-95°C). The GAPDH ribosomal RNA primer pair reaction was run on every sample for template content normalization purpose. A calibrator RT sample containing cDNA of interest was run to yield a standard curve for each primer set and individual primer reaction efficiencies were calculated from this curve using the Bio-Rad iCycler (Bio-Rad Laboratories, Berkeley, California, USA) software. The iCycler software calculated a threshold cycler for each sample; threshold cycles and primer pair efficiencies were used in the comparative computed tomography method to yield differences in each mRNA expression as normalized to the GAPDH housekeeping gene.

Flow cytometry study

CX3CR1 expression levels by peripheral blood mononuclear cells (PBMC) were examined. Heparinized blood samples were collected and placed on ice. The samples were then processed with Ficoll 1077 (sigma, USA) and washed with phosphate buffered saline for twice. Antibody staining with anti human CX3CR1 PE (2A9-1), anti human CD4 FITC (RPA-T4), anti human CD8a PerCP (SK 1), anti human CD16 APC (CB16) from eBioscience (San Diego, California, USA) (San Diego, CA) was performed at 4°C with a predetermined optimal concentration of the test monoclonal Ab for 20 min. After staining with antibody, the product was aliquoted to 1×10^6 ; 5×10^5 cells per tubes.

Cells were washed and analyzed with a Fluorescence-activated cell sorting (FACS) scan flow cytometer. FACS analysis was performed on a FACS Canto (BD Biosciences, San Diego, CA). Positive and negative populations of cells were determined with unreactive isotype matched monoclonal Abs (Beckman Coulter Korea Ltd., Seoul, Korea) as controls for background staining.

Statistical analysis

All statistical analyses of data from both groups were conducted by the SPSS system (SPSS Inc., Version 18.0., Chicago, USA). Mann-Whitney U-test was used to assess the difference in both CX3CL1 and CX3CR1 concentration between C-HNP and L-HNP. All data are expressed as mean ± standard error of the mean. The difference between groups was considered significant when $P < 0.05$.

Results

CX3CL1/CX3CR1 expression by immunohistochemical analysis

We examined the expression of CX3CL1/CX3CR1 in the disc in patients with C-HNP and L-HNP by immunohistochemistry. The ratio of CX3CR1 positive mononuclear cell was higher in disc tissue of cervical HNP group compared with lumbar counterpart. CX3CL1 was expressed on infiltrated mononuclear cells in C-HNP disc tissue while that of L-HNP shows sparsely populated CX3CR1 positive cells [Figures 1 and 2].

CX3CL1/CX3CR1 mRNA expression by reversal transcription-polymerase chain reaction

In quantitative real-time PCR, we also observed higher CX3CL1/CX3CR1 expression in the C-HNP patients when compared with L-HNP patients. An amount of CX3CR1 mRNA expression in the C-HNP patients was relative higher than in the L-HNP patients ($P = 0.021$) [Table 1 and Figures 3 and 4].

CX3CR1 expression on peripheral blood mononuclear cells by flow cytometry

CX3CR1 expression on PBMC from patients with systemic sclerosis was assessed by immunofluorescence staining with flow cytometry. CX3CR1 (+) cell frequencies and CX3CR1 expression levels were increased in CD4 (+) T-cells and NK cells from patients with C-HNP, but this was not observed in patients with L-HNP ($P = 0.210$ and $P = 0.040$) [Figure 5].

Discussion

Nontraumatic disc herniation may develop as a result of degenerative and/or mechanical process. Biochemical

Table 1: The average of statistical result of each group by flow cytometry and RT-PCR

Variables	C-spine	L-spine	*P* value[a]
Relative amount of mRNA by quantitative real-time PCR			
CX3CL1	0.143±0.096	0.122±0.074	0.779
CX3CR1	0.298±0.132	0.160±0.073	**0.010[a]**
CX3CR1 by flow cytometry			
CD4 (+)	6.029±2.694	3.025±1.504	**0.021[a]**
CD8 (+)	25.21±6.421	19.037±2.551	0.720
NK cell	28.85±6.968	17.825±2.891	**0.004[a]**

[a]Statistical significance test was done by Mann-Whitney U-test. *P*<0.05 was significant and shown in bold. NK=Natural killer cell, RT-PCR=Reversal transcription-polymerase chain reaction

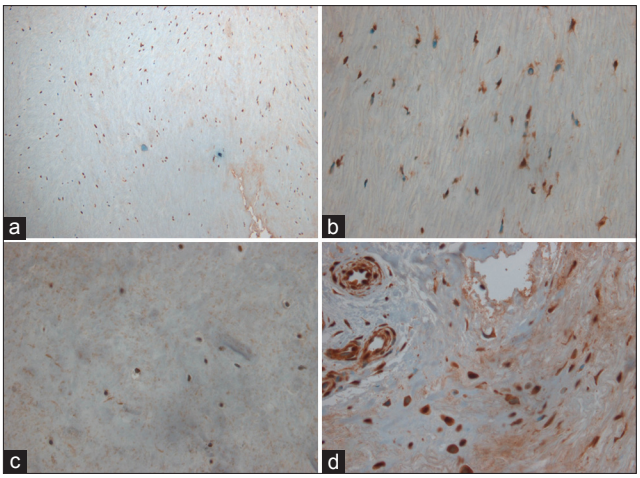

Figure 1: Immunohistochemical stainings for CX3CL1 in C-herniated nucleus pulposus (HNP) and L-HNP patient. Degenerations of the disc and expression of CX3CL1 are markedly noted in the both group. Cells stained red are expressing CX3CL1. These are mononuclear cells, disc cells, endothelial cells, CD4 (+) T-cells and NK cells (counterstain with H and E; (a and b) ×100, (c and d) ×400)

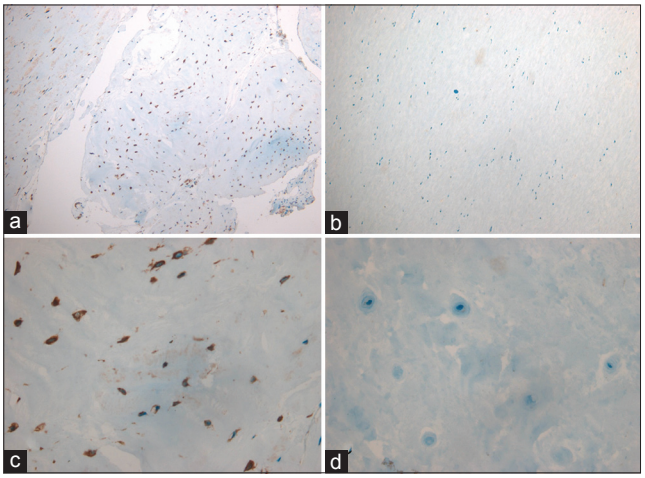

Figure 2: Immunohistochemical stainings for CX3CR1 in C-herniated nucleus pulposus (HNP) and L-HNP patient. Expression of CX3CR1 is markedly noted in the disc of C-HNP patient. (a and c) while nearly sparse expression of CX3CR1 was observed in the L-HNP sample (b and d). Cells stained red are expressing CX3CR1. These are mononuclear cells, disc cells, endothelial cells, CD4 (+) T-cells and NK cells (counterstain with H and E; (a and b) ×100, (c and d) ×400)

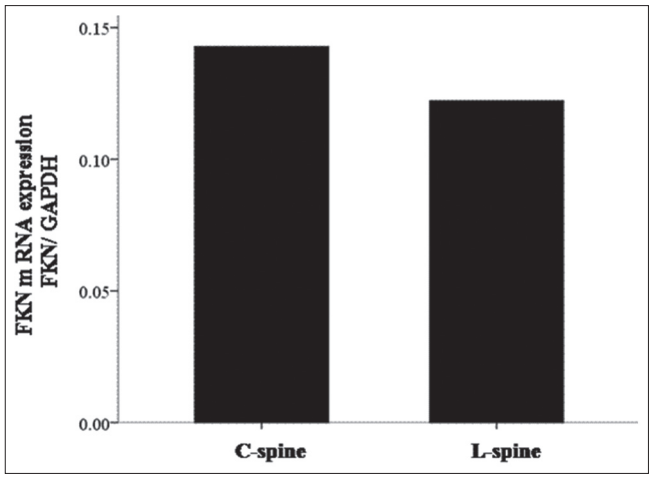

Figure 3: Comparison of relative amount of CX3CL1 mRNA normalized by glyceraldehyde-3-phosphate dehydrogenase between C-herniated nucleus pulposus (HNP) disc and L-HNP disc using quantitative real-time polymerase chain reaction

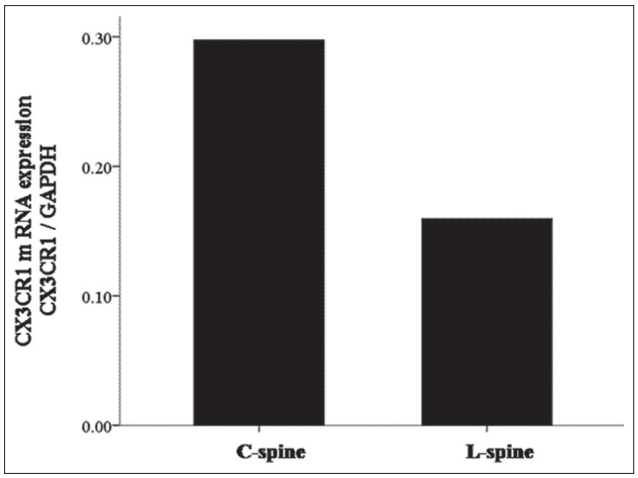

Figure 4: Comparison of relative amount of CX3CR1 mRNA normalized by glyceraldehyde-3-phosphate dehydrogenase between C-herniated nucleus pulposus (HNP) disc and L-HNP disc using quantitative real-time polymerase chain reaction

and structural changes of intervertebral disc occur with aging. Such changes can influence biomechanical response following disc degeneration and vice versa.[13] Narrowing of the intervertebral discs has previously been considered one of the signs of aging of the lumbar spine.[14,15] Recent studies have shown that lumbar discs do not always narrow with age, implying that a process other than aging is in work.[16] Similarly, pathologists regard tears of the annulus as a degenerative change even after it has been shown that radial tear do not always correlate with age.[14,17,18] Furthermore, although all discs are of the same age, those at lower lumbar levels exhibit degenerative changes far more often than in the upper levels.[16] This puts mechanical loading in a place of causative factor when it comes to lumbar disc degeneration. The clinical fact that lumbar HNP presents as ruptured form, while cervical HNP does suggest degenerative role more relevant in pathomechanism of lumbar HNP.

In this study, CX3CL1 expression was increased in the endothelial cells of the regional disc tissues from patients with C-HNP and L-HNP. And, CX3CR1 expression was enhanced on peripheral CD4 (+) T-cells and NK cells in patients with C-HNP compared to patients with L-HNP. Accordingly, the number of mononuclear cells expressing CX3CR1 was increased in the herniated cervical disc tissues. These findings suggest that enhanced CX3CL1/CX3CR1 interaction contributes to the disease process more so in C-HNP. A critical role of CX3CL1/CX3CR1 chemokine in chronic inflammatory process has been investigated in several joint disease such as rheumatoid arthritis (RA), OA, and degenerative disc disease (DDD).[8-11,19] Nanki *et al*.[20] insisted that up-regulated expression of CX3CL1 in the vascular endothelial cells and overexpression of CX3CR1 on peripheral mononuclear cells cooperatively promoted CD4 (+) T-cells and NK cells, leading to initiation of inflammation and cytotoxicity in RA synovium. However, it is still controversial whether there is a correlation between the expression of CX3CL1/CX3CR1 axis and DDD, while several studies show the infiltration of macrophages and other inflammatory cells in herniated disc tissues.[21-23]

In accordance with our statistical results, CX3CL1/CX3CR1 is thought to play an essential role in inflammatory cell migration into degenerative herniated disc tissues. Outside the central nervous system, CX3CR1 is expressed in NK cells, CD8 (+) T-cells, monocytes, dendritic cells, endothelial and epithelial cells. The involvement of CX3CR1 in T-cell migration to inflamed tissue has been concisely studied in RA and inflammatory bowel disease patients, while studies regarding disc degeneration are lacking. We hypothesized that CD4 (+) T-cells and NK cells migrate toward inflamed degenerative disc lesions, in response to fractalkine up-regulation. Indeed, immunohistochemistry and RT-PCR revealed that these cells are present in the disc lesions of cervical spine. CX3CL1 expression was both noted in pathology of cervical and lumbar disc tissue. However, CX3CR1 expression was markedly increased in cervical disc tissue and almost absent in lumbar disc tissue. This observation is an evidence of a difference in contents of herniated disc tissues from both lesions. Overall, our results demonstrate the potential contribution of CX3CR1-expressing CD4 (+) T-cells and NK cells in response to inflammation is the pathogenesis of cervical disc degeneration which may lead to herniation.

However, our study had several limitations. First, sample numbers were limited and it was technically difficult to sample from the same anatomic area in each sample, especially C-HNP patients. We attempted to consistently

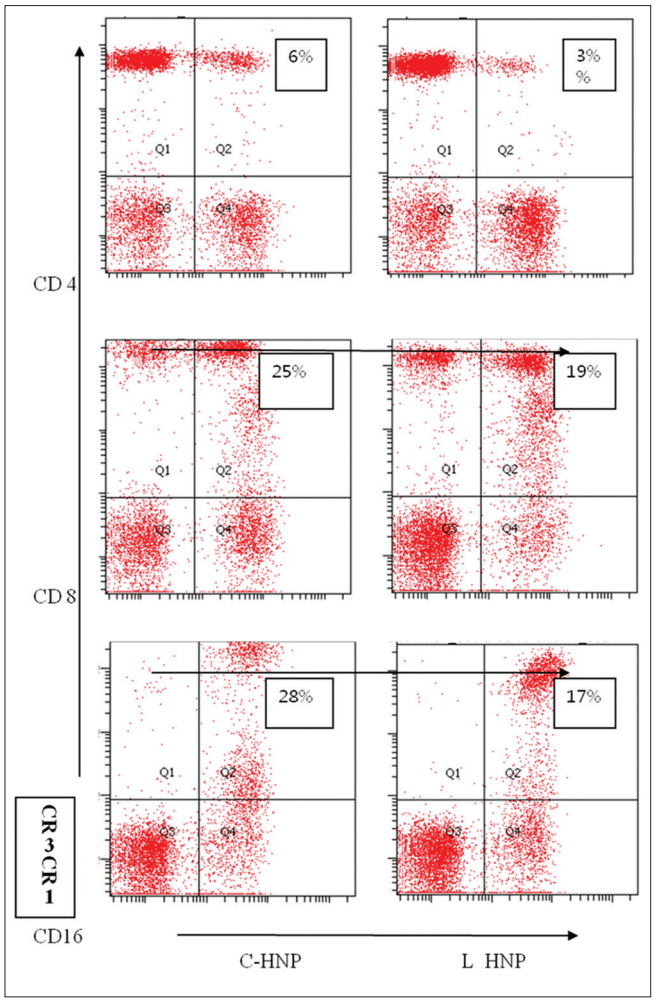

Figure 5: Representative expression of CX3CR1 on CD8⁺ T-cells, CD4⁺ T-cells, and CD16⁺ NK cells in peripheral blood from patients C-herniated nucleus pulposus (HNP) and L-HNP

sample from herniated disc; however, we cannot guarantee that cervical disc obtained is the herniated nucleosus pulposus. In case of C-HNP patient, obtaining sufficient amount of herniated disc tissue was not an easy task because we usually performed anterior cervical approach. Second, laboratory experiment with western blot to verify expression of protein also is limitation of our study. The main reason is the molecular size of the chemokine. Size of human CX3CL1 is smaller than 10 kD, a size that cannot be separated by western blot, thus could not be detected. Third, CX3CR1 up-regulation cannot fully explain the occurrence of C-HNP, though our results strongly support correlation between CX3CR1 activity and C-HNP development since HNP is a multi factorial disease and other risk factors are considered. In the results, CX3CR1 was highly expressed on cells in C-spine disc, but rarely in L-spine disc. There was greater CX3CR1 mRNA expression in C-HNP than in L-HNP, leading to assumption that different degenerative pathomechanism is at work and that C-HNP undergoes more inflammatory processes. More studies should be needed at this point.

CX3CR1 was found to be localized at the site of degeneration in human cervical intervertebral disc tissue. This finding is the first to provide direct evidence for the involvement of CX3CR1 in the pathomechanism of cervical disc degeneration and its herniation.

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