Original Article

Nucleus pulposus cells derived IGF-1 and MCP-1 enhance osteoclastogenesis and vertebrae disruption in lumbar disc herniation

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Abstract: Study design: Chronic strained lumbar disc herniation (LDH) cases were classified into bulging LDH, herniated LDH and prolapse LDH types according to imaging examination, and vertebrae disruptions were evaluated. Cytokines derived from the nucleus pulposus cells were detected, and their effects on osteoclastogenesis, as well as the mechanisms involved, were studied via an in vitro osteoclast differentiation system. Objective: To clarify the mechanisms of lumbar vertebrae resorption induced by lumbar herniation. Summary and background data: Chronic strained lumbar disc herniation induced vertebrae erosion exacerbates quality of patients’ life and clinical outcome. Although nucleus pulposus cells derived cytokines were reported to play an important role in this pathogenesis, the fundamental mechanisms underlying this process are still unclear. Methods: Chronic strained lumbar disc herniation patients were diagnosed with CT scan and T2-weighted magnetic resonance imaging. RNA was extracted from 192 surgical specimens of the herniated lumbar disc and 29 surgical excisions of the lumbar disc from spinal injury patients. The expressions of osteoclastogenesis related cytokines and chemokines were examined using real time PCR. Monocytes were induced into osteoclast with M-CSF and RANKL in vitro, while the IGF-1 and MCP-1 were added into the differentiation procedure in order to evaluate the effects and explore the molecular mechanisms. Results: Vertebral erosion had a positive relationship with lumbar disc herniation severity types. In all of the osteoclastogenesis related cytokines, the IGF-1 and MCP-1 were the most highly expressed in the nucleus pulposus cells. IGF-1 enhances activation of NF-kB signaling directly, but MCP-1 upregulated the expression of RANK, so that enhanced cellular sensitivity to RANKL resulted in increasing osteoclastogenesis and activity. Conclusion: Lumbar herniation induced overexpression of IGF-1 and MCP-1 in nucleus pulposus cells aggravated vertebral erosions. Hence, this study suggests that targeting osteoclastogenesis related cytokines has potential clinical significance in the treatment of lumbar disc herniation patients.

Keywords: Lumbar disc herniation, vertebra disruption, IGF-1, MCP-1, osteoclast

Introduction

Spine-related disorders are among the most frequently encountered clinical condition in medicine. In western countries, low back pain (LBP) alone affects up to 80% of the population in their lifetime, with an annual prevalence of about 15% to 20%. The estimated annual expenditure for the care of low back problems was more than $85 billion in the United States [1, 2]. In China, the LBP prevalence annually is about 8%, however, the overall cost of low back pains may amount to $100 billion due to the huge population base [3]. Low back pain and sciatica caused by lumbar disc herniation (LDH) are the most common causes of activity limitation and visits to physicians [4, 5]. LDH occurs when the nucleus in the center of the disc
presses against the annulus, causing the disc to bulge outward; the most common site is toward the bottom of the spine at L4-L5 or L5-S1 [6]. With further progress, the nucleus herniates completely through the annulus and squeezes out of the disc, placing pressure on the spinal canal or nerve roots [7]. In addition, the nucleus releases chemicals that can irritate the surrounding nerves, causing inflammation and pain [8].

Degeneration of the lumbar disc nucleus and chronic strain are the fundamental and dominant reasons for LDH, although the etiology is multifactorial, which includes environmental, nutritional, lifestyle, and occupational factors [9]. Degenerative changes in the fibrocartilaginous intervertebral disc (IVD) lead to a loss of structural integrity in the surrounding annulus fibrosus (AF), which can lead to herniation of the nucleus pulposus (NP). Biologically, disc cells in the AF and NP actively regulate IVD homeostasis, maintaining balance between anabolic and catabolic processes. This involves modulating their activity through a variety of substances, including enzymes, cytokines, enzyme inhibitors, and growth factors [10]. Anabolic regulators include polypeptide growth factors, such as insulin-like growth factor (IGF), transforming growth factor-β (TGF-β) and the bone morphogenetic proteins (BMPs), while catabolic mediators include various cytokines, enzymes, and aggrecanases [11]. LDH may result from an imbalance between these bio-

logic processes, or the loss of steady-state metabolism maintained in a normal disc [12].

Vertebral body or endplate destruction is often seen in LDH patients in clinic, which changes the adjacent segments of the spine’s mechanical structure, leading to various conditions, including low back pain, spinal stenosis, sciatica, and spinal cord diseases [13]. Previous work has documented the reproducible and reliable radiologic changes with aging in the sand rat lumbar spine, vertebral endplate architecture, and the utility of this model for autologous disc cell implantation. Radiographic and histologic changes in the aging sand rat are very similar to those seen in human disc degeneration [14]. It is hypothesized that experimental discus lesion would initiate not only localized bone remodeling but also increase osteoclast formation in a location remote to the injury site, due to altered spinal biomechanics. It is speculated that these changes in vertebral bone remodeling could be reflected by an increased RANKL expression [15]. The mechanism of vertebral destruction appears to be more complex than what is known, but remains unclear. We studied the effects of NP cells-derived cytokines on osteoclastogenesis and the mechanisms of bone resorption via in vitro osteoclast differentiation system.

Material and methods

Patients

192 patients were recruited between December 2010 to September 2012 from the Peking University People’s Hospital, Dalian University Zhongshan Hospital, Dalian Medical University Second Affiliated Hospital and Tengzhou People’s Central Hospital. All patients underwent a standardized history and physical examination. Inclusion criteria were: recent low back pain (within 3 months), and available magnetic resonance imaging (MRI) demonstrating LDH corresponding to the neurological level and side suggested at the clinical presentation.

### Table 1. Patient characteristics based on different categories

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Bulging</th>
<th>Herniated</th>
<th>Prolapse</th>
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<tr>
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<td>117</td>
<td>39</td>
<td>29</td>
</tr>
<tr>
<td>Age (years)</td>
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<td>22:17</td>
<td>15:14</td>
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<tr>
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<td>5.72±2.08</td>
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<td>31</td>
<td>16</td>
</tr>
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<td>26</td>
<td>88</td>
<td>31</td>
<td>16</td>
</tr>
<tr>
<td>Non-physical labor</td>
<td>10</td>
<td>29</td>
<td>8</td>
<td>13</td>
</tr>
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<tr>
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<td>6</td>
<td>16</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Lumbar spinal stenosis</td>
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<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>14</td>
<td>11</td>
<td>0</td>
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<tr>
<td>None</td>
<td>20</td>
<td>85</td>
<td>25</td>
<td>29</td>
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</tbody>
</table>

*P<0.05; **P<0.01, comparison between the bulging LDH, herniated LDH and prolapse LDH groups.
Exclusion criteria were: known pregnancy; severe active medical or psychiatric comorbidities that would limit study participation; infectious, inflammatory, or neoplastic cause of radiculopathy; significant degenerative or isthmic spondylolisthesis suspected of contributing to symptoms; and prior lumbar spine surgery at the affected level. The normal control group comprised of 29 patients, who suffered from acute vertebral burst fractures caused by violence. There was no history of back pain and lumbar spine MRI showed no pathology or signs of lumbar disc degeneration. All subjects signed the informed consent. The characteristics of the patients involved were summarized in the Table 1.

These patients were divided into three groups based on Computed Tomography, T1- and T2-weighted MRI imaging: the bulging lumbar disc herniation group (Bulging LDH), the herniated lumbar disc herniation group (Herniated LDH) and the prolapse lumbar disc herniation group (Prolapse LDH). The study was approved by the Medical Research Ethics Committee of Dalian Medical University and Peking University.

Quantitative real-time PCR

Nucleus pulposus samples from the patients were procured and rinsed thoroughly by icy 1×PBS immediately after biopsy, removed of annulus fibrosus, cut into the size of 1×1×1
mm, quickly placed in liquid nitrogen and then stored at -80°C until RNA extraction. Total RNA was isolated with the TRIzol reagent (Invitrogen, CA). An aliquot of 1 μg of total RNA was subjected to reverse transcription with SuperScript II RT PCR kit (Invitrogen, CA). 1 μL of the final cDNA was applied to real-time PCR amplification with SYBR Green using the StepOnePlus real-time PCR system (Invitrogen, ABI, CA) and the listed primers (Supplemental Table 1).

**Western blotting**

Cells were harvested and lysed with lysis buffer (Cell Signaling Technology, MA). Cell lysates were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane and immunoblotted with antibodies against phosphorylated or nonphosphorylated NF-κB, p38, ERK, JNK, and AKT. The membrane was stripped and reprobed with anti-β-actin antibody (Sigma-Aldrich, MO) to ensure equal protein loading. Secondary antibodies conjugated to horseradish peroxidase were used for detection, followed by enhanced chemiluminescence (Pierce Biotechnology, IL) and autoradiography.

**Flow cytometry**

After treatment, cultured cells were washed twice with 1×PBS, blocked with human FcR binding inhibitor, then stained with 2 μg of phycoerythrin-conjugated RANK antibody (eBioscience, CA) at RT for 30 minutes, avoiding light, and finally analyzed with a FACS Calibur flow cytometer.

**Differentiation**

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (GE Healthcare) density gradient centrifugation. 1×10^6 PBMCs per well were seeded in 24-well plates and allowed to adhere for two hours in RPMI-1640 (Mediatech Cellgro). Cells were washed three times with fresh medium to remove the non-adherent cells. The adherent PBMC cells were cultured in α-MEM (Gibco) supplemented with 10% fetal bovine serum (HyClone), 100 units/ml penicillin, 100 μg/ml streptomycin, 50 ng/mL human RANKL (R&D Systems), and 25 ng/mL human M-CSF (R&D Systems) (Osteoclast conditioned medium, OCM). OCM was changed every three days. After twelve days of culture, TRAP staining of OCs was performed with Leukocyte Acid Phosphatase kit (Sigma-Aldrich) according to the manufacturer’s instructions.

**TRAP staining and TRACP activity**

The enzymatic marker of osteoclasts, TRAP was stained using a leukocyte acid phosphatase kit (Sigma-Aldrich) following the manufacturer’s instructions. Briefly, differentiated osteoclast cells were fixed with citrate/acetone solution and stained with acetate/naphthol/tartrate solution at 37°C for one hour, and finally the nuclei were stained with hematoxylin at room temperature for 5 minutes. Differentiated cells with characteristics of TRAP staining positive, three or more nuclei and a cell body larger than 100 mm were considered to be osteoclasts. The osteoclast enzyme marker, tartrate-resistant acid phosphatase (TRACP), was detected using a TRACP and ALP Assay kit (Takara, Clontech).

**Statistical analysis**

Experimental values are expressed as mean ± standard error of the mean unless otherwise indicated. The chi-square test was used for categorical variables and the Student’s t-test or One-way ANOVA was used to compare the differences between groups. Statistical significance was analyzed using SPSS 10.0 software and a P value <0.05 was considered statistically significant. All results obtained were from at least three independent experiments.
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Figure 2. Expression profile of osteoclastogenesis-associated cytokines in the herniated disc. Real-time PCR showing relative expressions of OC differentiation-associated cytokines (A) IGF-1 and (B) MCP-1 in lumbar disc nucleus pulposus cells.

Figure 3. Effects of IGF-1 on osteoclast (OC) differentiation from monocytes. A. TRAP staining for mature OCs in monocytes cultured in medium with M-CSF (25 ng/ml) and RANKL (50 ng/mL), in presence or in absence of IGF-1. B. Quantitative analysis of mature OCs generated from cocultures of monocytes with different dosage of IGF-1. Representative results from five independent experiments are shown. C. Real-time PCR showing enhanced expression of OC differentiation-associated proteins CTSK, TRAP, CALCA, and CALCR in monocytes cultured with the addition of RANKL and different dosage of IGF-1, compared with those in monocytes cultured with medium or RANKL only. *P≤0.05; **P≤0.01.

Results

Patient characteristics

192 patients were eligible to participate in this study. In patients enrolled in the current study, the mean age was 57.83±7.25 years (range 47-82 years). The male to female ratio was 105:87. The mean duration of low back pain was 5.29±1.89 years (range 1-10 years). The distribution of type of herniation: bulging LDH in 36 patients; herniated LDH in 117 patients; and prolapse LDH in 39 patients. These patients were divided into three groups accord
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![Image of experimental setup](image)

**Figure 4.** Effects of MCP-1 on osteoclast (OC) differentiation from monocytes. A. TRAP staining for mature OCs in monocytes cultured in medium with M-CSF (25 ng/ml) and RANKL (50 ng/mL), in presence or in absence of MCP-1. B. Quantitative analysis of mature OCs generated from cocultures of monocytes with different dosage of MCP-1. Representative results from five independent experiments are shown. C. Real-time PCR showing enhanced expression of OC differentiation-associated proteins CTSK, TRAP, CALCA, and CALCR in monocytes cultured with the addition of RANKL and different dosage of MCP-1, compared with those in monocytes cultured with medium or RANKL only. *P<0.05; **P<0.01.

To classify the clinical appearance and pathology type and find the relationship between lumbar herniation and bone resorption in lumbar vertebrae, all patients enrolled in this study underwent T1- and T2-weighted magnetic reso-
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Figure 1. Representative images of patients with lumbar disc herniation demonstrated a mass in the posterior epidural space, at the L2-L3 level. The lesion exhibited low intensity on T1-weighted MRI and high and low intensities on T2-weighted MRI. Different types showed different characteristics; for the bulging LDH, both the T1- and T2-weighted MRI images showed the backward shift of the nucleus, but all behind the posterior longitudinal ligament; signals for the disc inferior endplate were even, and intervertebral disc boundaries were clear; on the MRI T2-weighted images, disc intensity signal decreased slightly. In the herniated LDH, the backward shifts of the nuclei were prominent and broke through the posterior longitudinal ligament; disc intensity signal decreased significantly on the MRI T2-weighted images; signals for the disc inferior endplate were heterogeneous, intervertebral disc boundaries were rough, and the vertebra body also became lower. In the prolapse LDH, the backward shift of the nucleus broke through the posterior longitudinal ligament and disconnected with discs, the signals for both side endplates of discs were extremely rough, disc intensity signal decreased to black on the MRI T2-weighted images, and intervertebral spaces became narrow (Figure 1).

With regard to vertebra disruption, CT scans showed that in the bulging LDH groups no obvious disruptions were found in most of the cases. Of all the 36 patients recruited in this
Elevated expression of cytokines in the herniated disc nucleus pulposus cells

Since a positive relationship was found between the bone resorption in vertebrae and herniated lumbar disc, we hypothesized that osteoclast (OC) differentiation or activity may be enhanced during the lumbar disc herniation. In order to analyze various cytokines involved in osteoclastogenesis, we used the real time PCR to detect the mRNA expression profile of cytokines involved in osteoclastogenesis and activity in the nucleus pulposus cells isolated from the surgical samples. According to the previous reports, we examined the mRNA levels of IL-1α, IL-6, RANKL, M-CSF, MCP-1, TGF-β, IGF-1, TNF-α, and PTHrP [16, 17]. Of all these cytokines, there were no significant changes in the mRNA levels of RANKL, M-CSF, or PTHrP, but the IL-1α, IL-6, TGF-β, and TNF-α levels were slightly increased. However, the changes had no significance between groups (data not shown). It is interesting to note that IGF-1 expression increased proportionally with the increasing severity of the lumbar disc herniation, as shown in the Figure 2A. Elevated IGF-1 expression in the bulging group was almost 10 fold higher, the herniated group was 12 fold higher, and the prolapse group was 16 fold higher than the healthy control group, respectively (P<0.01). Although there was no significant difference between the bulging and herniated groups (P>0.05), differences were all significant when compared with the prolapse group, respectively (P>0.05) (Figure 2A). Another cytokine with elevated mRNA expression is MCP-1, which also plays a stimulating role in osteoclastogenesis and activity [18]. We found that MCP-1 mRNA increased over 5 fold in the bulging group, more than 7 fold in the herniated group, and almost 14 fold in the prolapse group compared with the control group, respectively (P<0.001 for all LDH groups vs. control group). Moreover, the differences between groups were all very obvious, particularly the prolapse group (bulging vs. herniated, P<0.05; bulging vs. prolapse, P<0.001; herniated vs. prolapse, P<0.001) (Figure 2B).

IGF-1 and MCP-1 enhanced osteoclastogenesis in vitro

Elevated cytokines from the disc nucleus pulposus cells may enhance osteoclast differentiation and activity around the vertebra bodies. To test the effects of IGF-1 and MCP-1 on osteoclast differentiation, we performed an osteoclast differentiation model using monocytes from the peripheral blood mononuclear cells (PBMC) with 25 ng/ml of RANKL and 50 ng/ml of M-CSF. In line with previous results, with RANKL, monocytes could differentiate into mature OCs (Figure 3A) [19]. In the presence of RANKL, CD14+ monocytes first developed into preOCs in a 7-day culture, and then developed into mature OCs with RANKL after an additional 7 days of culturing. TRAP staining, a specific method to identify the differentiated OCs by showing multinuclear (>3 nucleus per cell) and TRAP+ in the cell cytoplasm for mature OCs, showed enhanced osteoclastogenesis in the presence of IGF-1 at different doses (5 ng/ml and 10 ng/ml) compared to those cultured in a medium without IGF-1 (Figure 3A), because the multinuclear TRAP+ cells are significantly increased with the presence of IGF-1 (P<0.01). Quantitative analysis supported this observation, which showed growing numbers of OCs generated from co-cultures of increasing doses of IGF-1 (Figure 3B). Real-time qPCR revealed enhanced expression of OC differentiation-associated marker genes CALCA, CALCR, CTSK, and TRAP in monocytes cultured with RANKL, and different dosages of IGF-1 compared to those in monocytes cultured with medium or RANKL only (Figure 3C).

Similarly, the enhancing effect of MCP-1 on OC differentiation was also induced in our experiment. TRAP staining for mature OCs showed increased density of multinucleated cells in the presence of MCP-1 at different doses (50 ng/ml and 100 ng/ml) compared to those cultured in medium without MCP-1 (Figure 4A).
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Quantitative analysis of the mature OC cells and real-time PCR for the OC specific marker genes likewise supported this finding, which showed an increased number of OCs generated and enhanced expression of OC differentiation-associated proteins CALCA, CALCR, CTSK, and TRAP genes in monocytes cultured with RANKL and different dosages of MCP-1, compared to those in monocytes cultured with medium or RANKL only (Figure 4B and 4C).

IGF-1 and MCP-1 promotes osteoclast differentiation via different signaling pathways

In addition, we examined signaling pathways involved in the IGF-1 and MCP-1 enhanced OC differentiation respectively. We focused on NF-κB, PI3K/Akt, ERK, JNK, CREB, STAT, and p38 MAPK, because these signaling pathways are important for RANKL-induced OC differentiation [20-22]. When the monocytes were cultured with OCM in presence of M-CSF and RANKL, these signaling pathways were all activated as detected by western blotting (Figure 5A). However, concerning the effects of IGF-1 and MCP-1 on these signaling pathways, mechanism studies showed quite different results. As shown in Figure 5A, the addition of IGF-1 enhanced the phosphorylation status of PI3K, STAT5, Akt, and NF-κB in a dose dependent manner; however, addition of MCP-1 enhanced the activation of PLCγ, ERK, P38, and CREB (Figure 5B). Obviously, IGF-1 enhanced the activation of NF-κB initiated by RANKL, and MCP-1 promoted the activation of CREB to transcript the downstream target genes.

Since RANKL binds to its receptor RANK on the progenitors of OCs and activates OC differentiation-associated signaling pathways, we wondered whether the expression of RANK in monocytes is regulated by the nucleus pulposus cells derived MCP-1, so that the sensitivity of the RANKL should be increased. We treated the monocytes with a different concentration of MCP-1 from 50 ng/ml to 200 ng/ml, and the mRNA and protein levels of RANK were determined by quantitative real-time PCR and flow cytometry, respectively. Our qPCR data showed that the addition of recombinant MCP-1 significantly upregulated mRNA expression of MCP-1 in a dose dependent manner, and with 200 ng/ml of MCP-1 could increase the mRNA level to more than 10 folds (Figure 5C). Moreover, the addition of recombinant MCP-1 significantly upregulated surface protein levels of RANK in monocytes in a dose-dependent manner as detected by flow cytometry (Figure 5D). These results clearly indicate that regulation of RANK by MCP-1 affects RANKL-induced OC differentiation.

Discussion

Our study revealed a new mechanism of vertebra disruption induced by the osteoclastogenesis enhanced cytokines, such as IGF-1 and MCP-1, in the lumbar disc herniation patients. LDH occurs as a result of abnormal pressure on the nucleus of the disc and injures the annulus through abnormal activities, such as repetitive bending, twisting, and lifting [23]. Long-term poor posture when working, or incorrect lifestyle, can place additional stress on the lumbar spine [24]. With aging, discs gradually dry out, lose their strength and resiliency, and easily induce the occurrence of herniation [25]. However, in the current study, we did not find statistically significant differences in the mean age, sex ratio, whether participants were smokers or diabetic, and history of trauma between the early and late groups. The average duration of low back pain from the onset of symptoms to surgery has a close relationship with the severity of LDH; the longer the duration, the worse the LDH. Another contributing factor is employment status. Our analysis shows that physical labor plays an important role in the severity of LDH, because the herniated and prolapsed LDH groups have a higher ratio of patients who are undertaking physical labor than the bulging LDH group, in which the severity of LDH group is much lighter.

Accordingly, we hypothesized that the herniated lumbar disc induced inflammatory cytokines contribute to the vertebra disruption process. The nucleus pulposus cells have been reported to secrete cytokines for LDH induced inflammation [26, 27]. Moreover, some inflammatory cytokines also contribute to OC differentiation and activity, as well as to promote recruitment of OC to bone surface [28]. The maturation of osteoclasts and their participation in bone resorption is regulated in part by hormones and cytokines, such as interleukin (IL-1) [29]. In our study, we examined the mRNA levels of IL-1α, L-6, RANKL, M-CSF, MCP-1, TGF-β, IGF-1, TNF-α and PTHrP that have been reported to improve osteoclast differentiation and activity [16, 17].
We found that most mRNA levels of these inflammatory cytokines, except RANKL, M-CSF, and PTHrP are elevated in LDH patients, and all three groups have higher expression than the healthy control group. Among them, the IGF-1 and MCP-1 showed the highest elevation, and both have a positive relationship with the severity of LDH, because their mRNA expression increase proportionally with increasing severity of the LDH. High levels of IGF-1 and MCP-1 contribute to the recruitment of OC precursor cells to the vertebra surface and activate the OC differentiation.

Insulin-like growth factor (IGF-1) is a major regulator of skeletal growth and remodeling [30]. The role of IGF-1 in regulating osteoclastogenesis is through its promotion of OC differentiation. It is required for maintaining the normal interaction between osteoblasts and osteoclasts, to support osteoclastogenesis through its regulation of RANKL and RANK expression [31]. Studies have shown that IGF-1 acts on bone via both endocrine and autocrine/paracrine pathways [32]. Autocrine/paracrine regulation depends on skeletal IGF-1, produced by osteoblasts and by stromal cells of bone marrow [33]. Recent studies have also shown that MCP-1, a chemokine that plays a critical role in the recruitment and activation of leukocytes during acute inflammation, promotes osteoclast fusion into multinuclear cells. Multinuclear osteoclast formation has been seen to be significantly inhibited, as well as decreased bone resorption and elevated bone mass in MCP-1-deficient mice [34]. Other evidence shows that MCP-1 is related to various pathological conditions such as rheumatoid arthritic bone degradation, or is expressed at the site of tooth eruption and bacterially induced bone loss. It has also been found that increased MCP-1 serum levels in patients with breast, prostate and ovarian cancers correlated with advanced tumor stage and bone resorption. Our results support the observation that the relative expressions of OC differentiation-associated IGF-1 and MCP-1 in lumbar disc nucleus pulposus cells increased with the severity of disc herniation. Quantitative analysis likewise demonstrated that increasing doses of IGF-1 and MCP-1 added to medium significantly enhanced the expression of proteins associated with OC differentiation (CALCA, CALCR, CTSK, and TRAP). While IGF-1 enhanced the activation of NF-kB signaling directly, MCP-1 upregulated the expression of RANK so that enhanced cellular sensitivity to RANKL led to increased osteoclastogenesis and activity. We therefore conclude that lumbar herniation induced overexpression of IGF-1 and MCP-1 in nucleus pulposus cells, which aggravated vertebral erosions. Our results suggest that targeting osteoclastogenesis-related cytokines has potential clinical significance in treatment of lumbar disc herniation patients.

Acknowledgements

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Disclosure of conflict of interest

None.

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### Supplemental Table 1. Primers used for real-time PCR

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<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
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