Lumbar posterolateral fusion inhibits sensory nerve ingrowth into punctured lumbar intervertebral discs and upregulation of CGRP immunoreactive DRG neuron innervating punctured discs in rats

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Abstract Degeneration of lumbar intervertebral discs is thought to be a cause of low back pain. Studies have found that a cause of discogenic low back pain is intervertebral disc inflammation and axonal growth of afferent fibers innervating the disc. Lumbar spine fusion for chronic discogenic low back pain is considered an effective procedure. However, no study has investigated the mechanism of pain relief. We did this by applying Fluoro-Gold (FG) to the ventral aspect of the L4–L5 intervertebral discs of 40 rats. We exposed the nucleus pulposus to the annulus fibrosus in a disc punctured model. Rats were divided into 4 groups. Group A: Punctured intervertebral disc with sham posterolateral fusion (PLF) ($n = 10$), Group B: Punctured intervertebral disc with PLF ($n = 15$), Group C: Normal intervertebral disc (no puncture) with PLF ($n = 10$), and Group D: Normal disc (no disc puncture) with sham PLF ($n = 5$). Four weeks after surgery, bilateral L1–L5 dorsal root ganglia (DRGs) were stained with growth-associated protein 43 (GAP43), a marker of axonal growth, and calcitonin gene-related peptide (CGRP), a neuropeptide marker of pain. Bone union was evaluated using X-ray imaging. Of the FG-labeled neurons, the proportions of GAP43- and CGRP-immunoreactive (IR) neurons in Group A were significantly higher than in Group D ($P < 0.05$). The proportions of GAP43- and CGRP-IR neurons in bone union rats in Group B were significantly lower than in nonunion rats in Group B and in the rats in Group A ($P < 0.05$). No

significant differences in GAP43- and CGRP-IR neurons were observed between bone union and nonunion rats in Group C and the rats in Group D ($P > 0.05$). PLF is strongly related to the downregulation of GAP43 and CGRP expression. Therefore, PLF may suppress the increase of inflammatory neuropeptides and the process of axonal growth. Moreover, these results may explain, in part, the mechanism of pain relief following lumbar spinal fusion for chronic discogenic low back pain in humans.

Keywords Low back pain · Posterolateral fusion · Intervertebral disc · Immunohistochemistry · Nerve ingrowth

Introduction

A majority of people suffer from low back pain and are obliged to disrupt normal daily activities, including work and recreation, at some point during their life [1, 9]. The most common cause of chronic low back pain is thought to originate in the intervertebral discs [10, 24]. Moreover, intervertebral disc degeneration is associated directly with chronic low back pain, as approximately 40% of patients report persistent symptoms of unknown origin [34]. The normal intervertebral disc has been shown to be aneural except in the outermost part of the annulus fibrosus [5, 21]. According to several histological studies, in response to disc degeneration and lamellar disruption, neovascularization with unmyelinated nerve fibers and ingrowth of Schwann cells occur [8, 12, 17].

Recently, it was reported that in humans, inflammatory mediators such as interleukin-1, nerve growth factor and tumor necrosis factor were expressed in degenerated intervertebral discs [7, 39]. Furthermore, in humans,

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intervertebral disc inflammation promotes axonal growth of calcitonin gene-related peptide (CGRP; a marker of pain neuropeptide)-immunoreactive (IR) fibers into the inner layers of the disc, and the presence of CGRP-IR fibers in the disc indicates that the disc can be a source of pain sensation [2, 23, 30]. On the other hand, it was reported that growth-associated protein 43 (GAP43; a marker of axonal growth)-IR fibers were detected in human painful intervertebral discs and inhibition of nerve fibers ingrowth into the discs could benefit patients with chronic low back pain [3, 12, 35, 36].

Traditionally, surgical treatment for patients with unremitting discogenic low back pain has been lumbar spine fusion, which is the most important part of reconstructive surgery [15]. The results of lumbar spine fusion for chronic discogenic low back pain are variable and have been controversial in terms of efficacy and type of fusion [15, 37]. Reported outcomes for lumbar spine fusion in patients with chronic discogenic low back pain have varied substantially [22]. However, outcome studies that included posterolateral fusion (PLF) have shown good clinical results [31]. These surgeries were carried out under the premise that pain sensation is caused by the movement of a painful or unstable motion segment and that fusion of this segment would prevent future pain and disability [19]. Nevertheless, consideration of lumbar spine fusion for patients with chronic discogenic low back pain remains controversial, because the pathomechanism of chronic discogenic low back pain and the mechanism of pain relief are still unknown.

The purpose of the current study was to examine changes in dorsal root ganglion (DRG) neurons innervating punctured intervertebral discs after PLF in rats using two markers, which are GAP43 and CGRP.

Materials and methods

Animals

Forty male Sprague-Dawley rats (200–250 g) were used. The rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally) and treated aseptically in all surgical procedures. The protocols for animal procedures followed the 1996 revision of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and received approval from our Local Ethical Committee of our institution.

FG application and rat lumbar disc puncture model

This procedure was performed as described previously [16, 29]. Briefly, a midline abdominal incision was made in each rat, and the left iliopsoas muscle was dissected to

expose the ventral aspect of the L4–L5 intervertebral disc. A puncture approximately 3 mm deep was made on the middle of the ventral surface of the disc with a 22-gauge needle, and two crystals of Fluoro-Gold (FG; Fluorochrome, Denver, CO, USA) were placed into the puncture site to detect DRG neurons innervating the intervertebral discs. The application site was sealed immediately with cyanoacrylate to prevent leakage of FG.

Rat posterolateral fusion

One week after FG application, under sterile conditions, the posterolateral lumbar spine was exposed through a midline skin incision followed by two paramedian fascial incisions on either side of L4–L6. The dorsal paraspinal muscles were reflected by blunt dissection to expose the lamina and transverse processes of L4–L5. Through the same skin incision, separate fascial incisions were made along the bilateral posterosuperior iliac crests. Following exposure of the inner and outer tables, cortico-cancellous bone was harvested and pulverized. Bone grafts were placed spanning the L4–L5 laminae and transverse processes on both sides.

Experimental groups

The rats were divided into the following four groups. Group A: Disc puncture model with sham PLF ($n = 10$), Group B: Disc puncture model with PLF ($n = 15$), Group C: Normal disc (no disc puncture) with PLF ($n = 10$), and Group D: Normal disc (no disc puncture) with sham PLF ($n = 5$). Rats in the normal disc groups (C and D) underwent the same first surgical procedure as the rats in the disc puncture model groups (A and B), including exposure of the ventral aspect of their L4–L5 intervertebral discs, except that (a) their discs were not punctured and (b) two crystals of FG were applied onto the surface of their L4–L5 intervertebral discs in Group C and D and the application site sealed immediately with cyanoacrylate to prevent leakage of FG.

One week after FG application, rats in the sham PLF groups (A and D) underwent the same second surgical procedure as the rats in the PLF groups (B and C), except that they did not undergo bone grafting. Thus, the sham PLF rats still underwent bone extirpation from their iliac crests: their lamina and transverse processes were exposed and cortico-cancellous bone was harvested from the bilateral iliac crests following the same procedure as in the rats undergoing PLF.

Evaluation of posterolateral fusion

Four weeks after PLF surgery, a single posterolateral radiograph was taken to evaluate bone union. Fusion was

defined as radio-dense bone bridging the L4–L5 transverse processes bilaterally. Furthermore, after sacrifice of the animals, the lumbar spines were explanted. We evaluated these specimens by manual palpation in a blind fashion. Specimens were considered fused if no motion was detected between the L4 and L5 vertebrae with direct manipulation. The use of manual palpation as a measure of fusion has been correlated previously with rigorous biomechanical examination [4].

Histology

Four weeks after PLF surgery, disc and adjacent vertebrae were harvested, fixed in formalin, decalcified in formic acid, dehydrated and embedded in paraffin. Five-micro sections were taken by cutting through the nucleus pulposus parallel to the direction of the puncture. The sections were stained with hematoxylin and eosin.

Double-staining immunohistochemistry

Four weeks after PLF surgery, the rats were perfused transcardially with 0.9% saline followed by 500 ml of 4% paraformaldehyde in phosphate buffer (0.1 mol/l, pH 7.4). We have reported previously that the rat L4–L5 disc is innervated by DRG neurons primarily from the L1–L5 levels [31]. Therefore, the bilateral DRGs from the L1–L5 levels were resected and examined with the aim of characterizing the DRG neurons innervating the L4–L5 disc. The specimens were immersed in the same fixative solution overnight at 4°C. After storage in 0.01 M phosphate-buffered saline (PBS) containing 20% sucrose for 20 h at 4°C, each DRG was sectioned at a 10- μ m thickness on a cryostat and mounted on poly-L-lysine-coated slides. Specimens were treated for 120 min in blocking solution (0.01 M PBS containing 0.3% Triton X-100 and 3% skim milk) at room temperature.

They were processed for GAP43 and CGRP immunohistochemistry with a mouse antibody to GAP43 (1:1000; Chemicon International, Temecula, CA, USA) and a rabbit antibody to CGRP (1:1000; Chemicon International, Temecula, CA, USA) for 24 h at 4°C, followed by incubation with goat anti-mouse Alexa 488 fluorescent antibody conjugate (for GAP43-IR 1:600) and goat anti-rabbit Alexa 594 fluorescent antibody conjugate (for CGRP-IR 1:600; Molecular Probes Inc., Eugene, OR, USA). As a negative control, 12 sections from each group were incubated in buffer with omission of the primary antibody. After each step, the sections were rinsed three times with 0.01 M PBS.

Microscope observation

The sections were observed with a fluorescence microscope (Nikon Corp., Tokyo, Japan). Only FG-labeled DRG

neurons with a clearly visible nucleus or cell body were counted, using the same method that previous reports have described [2, 16]. The number of FG-labeled neurons, FG-labeled GAP43-IR neurons and CGRP-IR neurons in the DRGs were counted by an independent observer who had not performed surgery or immunohistochemistry and who was blinded to the group from which the specimen was obtained. Twenty serial sections of DRGs from each rat were counted. DRG neurons were counted at 400 $\times$ magnification using a counting grid. The numbers of FG-labeled neurons, FG-labeled GAP43-IR neurons and CGRP-IR neurons per 0.0225 mm² were counted and averaged for each animal.

Statistical analysis

The percentages of FG-labeled neurons with either GAP43- or CGRP-IR in the DRG from L1–L5 were calculated for each rat. The differences among the groups were compared using analysis of variance. Statistical analysis of the values was carried out using a nonpaired *t* test. Differences were considered to be statistically significant at *P* < 0.05.

Results

Evaluation of posterolateral fusion

Posterolateral fusions were obtained in 9 rats in Group B and 7 rats in Group C. The mean union ratio was 64%. Figure 1 shows an example of PLF in a union rat.

Histology

Architectural changes, such as collagen layer disorganization, and cellular shape changes were observed in the Group A and among the nonunion rats in Group B, while no architectural changes were observed in Group D (Fig. 2). Mild changes were observed between the union rats in Group B (Fig. 2).

Expression of GAP43-IR and CGRP-IR DRG neurons innervating the lumbar discs

FG applied to the lumbar intervertebral disc was transported to the L1–L5 DRG neurons innervating the disc. In Fig. 3, i-1, ii-1, and iii-1 show DRG neurons innervating the intervertebral discs. The number of FG-labeled DRG neurons were counted (Table 1). A total of 343 FG-labeled neurons in the 10 rats in Group A and 440 FG-labeled neurons in the 15 rats in Group B were observed. Likewise, a total of 319 FG-labeled neurons in the 10 rats in Group C and 169 FG-labeled neurons in the 5 rats in Group D were observed. The distribution of the percentage of FG-labeled

Fig. 1 Representative posteroanterior radiograph of rat spine illustrating the presence of posterolateral fusion. Bilateral fusion masses were found between the L4 and L5 transverse processes. This plain radiograph was taken 4 weeks after the first surgery

neurons at each level from L1 to L5 was not significantly different among the four groups. The averages of these distributions in the four groups were (in descending order): L5 (35.6%), L3 (28.3%), L2 (24.4%), L1 (6.1%) and L4 (5.7%).

Fig. 2 Representative sagittal sections of intervertebral discs, stained with hematoxylin and eosin (original magnification $\times 100$). These sections were taken 4 weeks after PLF surgery. No architectural changes were observed in the sections from Group D while architectural and cellular changes, including collagen layer disorganization and cellular shape changes, were observed in the sections from Group A and from the nonunion rats in Group B

In Fig. 3, i-2, ii-2, and iii-2 show GAP43-IR neurons in FG-labeled DRG neurons and i-3, ii-3, and iii-3 show CGRP-IR neurons in FG-labeled DRG neurons. Each FG-labeled GAP43- and CGRP-IR DRG neuron was counted and analyzed (Table 1). The number of both GAP43- and CGRP-IR FG-labeled neurons in each group was also analyzed (Table 1). Of the 343 FG-labeled neurons in Group A, 86 (25.1%) were positive for both GAP43- and CGRP-IR, corresponding to 80.4% of the GAP43-IR FG-labeled neurons ($n = 107$) and 42.8% of the CGRP-IR FG-labeled neurons ($n = 201$). In the union rats in Group B, 14.2% were positive for both GAP43- and CGRP-IR, corresponding to 61.2% of GAP43-IR F-G-labeled neurons ($n = 67$) and 37.6% of CGRP-IR F-G-labeled neurons ($n = 109$); in the nonunion rats in Group B, 24.5% were positive for both CGRP- and GAP43-IR, corresponding to 71.2% of the GAP43-IR FG-labeled neurons ($n = 52$) and 40.7% of the CGRP-IR FG-labeled neurons ($n = 91$) (Table 1). Intergroup comparisons of the numbers of both GAP43- and CGRP-IR FG-labeled neurons in each group are illustrated in Fig. 4.

Discussion

The goal of the current study was to investigate changes in DRG neurons innervating degenerated intervertebral discs after PLF. Investigation of discogenic pain involves study of three crucial factors: intervertebral disc innervation, inflammation and hypermobility [20].

Fig. 3 *i*, *ii*, and *iii* are from the same section respectively. The *arrowheads* show FG-labeled DRG neurons (*i*-, *ii*-, *iii*-1). The *arrows* indicate GAP43-IR (*i*-, *ii*-, *iii*-2) and CGRP-IR DRG neurons (*i*-, *ii*-, *iii*-3). These *arrows* show both GAP43- and CGRP-IR in FG-labeled DRG neurons. All examples were harvested from animals 4 weeks after surgery. *i*: Group A, *ii*: Nonunion rat in Group B, *iii*: Union rat in Group B. 1: FG-labeled DRG neurons, 2: GAP43-IR DRG neurons, 3: CGRP-IR DRG neurons

Table 1 Numbers of immunoreactive DRG neurons

The name of Group (the number of rats)	FG-labeled neurons	FG-labeled CGRP-IR neurons (%)	FG-labeled GAP43-IR neurons (%)	FG-labeled Double stain neurons (%)
Group A (10)	343	201 (58.6)**	107 (31.2)**	86 (25.1)**
Group B union (9)	289	109 (37.7)*	67 (23.2)**	41 (14.2)*
Group B nonunion (6)	151	91 (60.3)*	52 (34.4)**	37 (24.5)**
Group C union (7)	235	69 (29.4)	32 (13.6)	20 (8.51)
Group C nonunion (3)	84	22 (26.2)	12 (14.3)	7 (8.33)
Group D (5)	169	46 (27.2)	27 (16.0)	16 (9.47)

Statistically significant differences between the Groups (* $P < 0.05$, ** $P < 0.01$). All analyses were performed between all Groups and Group D

Innervation

Intervertebral disc pain presupposes disc innervation [12]. The normal intervertebral disc has been shown to be aneural except in the outermost part of the annulus fibrosus both in humans and rats [5, 21]. Therefore, the intervertebral disc is theoretically considered to be an insensible tissue. It has been reported that one of the causes of discogenic low back pain is an extensive innervation into the inner annulus fibrosus of the disc [12]. A series of detailed studies has revealed that the ventral portion of the rat L5–L6 intervertebral discs is innervated predominantly by the L1 and L2 DRG, [26] whereas the dorsal portion of the L5–L6 disc is innervated extensively by the L1–L6 DRG [29]. Although that series of studies focused mainly on

innervation of the L5–L6 intervertebral disc, the L4–L5 disc was selected in the current study to allow more distance from the iliac bones. In this study, similar nonsegmental multilevel innervation patterns were observed.

Inflammation, hypermobility, and disc degeneration

To create a degenerative intervertebral disc model, the discs were punctured. Puncture incision models have been widely used with various animals, also including rats [14, 16, 25, 33, 38]. The morphologic evidence of puncture-related intervertebral disc degeneration reported in previous studies is similar to most of the human age-related degenerative disc disease features [6]. Following disc puncture, disc inflammation is induced [16, 20]. Intervertebral disc injury

Fig. 4 Numbers of both GAP43- and CGRP-IR FG-labeled neurons in each group. Significant differences between Group A and D were observed, but no significant differences were observed between the rats in Group A and the nonunion rats in Group B ($P > 0.05$). The percentages of labeled neurons were significantly lower among the bone union rats in Group B than among the rats in Group A, whereas no significant differences were observed between the nonunion rats in Group B and the rats in Group A ($*P < 0.05$). No significant differences in GAP43- and CGRP-43-IR neurons were observed between the union and nonunion rats in Group C and the rats in Group D ($*P > 0.05$)

in animals initiates the production of proinflammatory molecules. The postinflammatory response expands with proinflammatory mediators, such as tumor necrosis factor alpha, interleukin-1, -6 and nerve growth factor [28].

These changes in intervertebral disc organization alter the constraints placed on adjacent vertebrae, leading to lumbar hypermobility [20]. Therefore, these three decisive factors interact in ways that enhance and perpetuate the risk of disc degeneration and discogenic pain. Because of these three factors, PLF was performed to reduce hypermobility and disc degeneration. In the current study, histological findings indicate that the nucleus pulposus in punctured intervertebral discs was replaced by annulus fibrosus-like cartilage cells, but that this phenomenon was suppressed in union rats after PLF. These results suggest that intervertebral disc puncture promotes a degenerative response, and that PLF partially inhibits this puncture-induced disc degeneration.

Neuropeptide markers of pain in punctured discs and the effect of PLF

In this study, we showed that FG-labeled neurons innervating the L4–L5 intervertebral discs were co-labeled with GAP43 and CGRP. Of the FG-labeled neurons in Group A, the percentages of both GAP43- and CGRP-IR neurons were significantly higher than those in Group D ($P < 0.05$). These results suggest that DRG neurons innervating the lumbar intervertebral discs undergo ingrowth of their axons

into these discs and express painful inflammatory neuropeptides. On the other hand, of the FG-labeled neurons among the bone union rats in Group B, the percentages of GAP43- and CGRP-IR neurons were significantly lower than the corresponding percentages among the rats in Group A ($P < 0.05$). No significant difference in the percentages of GAP43- and CGRP-IR neurons in the FG-labeled neurons were observed between the rats in Group A and the nonunion rats in Group B. These results indicate that PLF for punctured intervertebral disc is strongly related to the downregulation of neuronal expression of GAP43 and CGRP. Therefore, it is possible that PLF for punctured intervertebral disc suppresses the upregulation of inflammatory neuropeptides and axonal growth. From clinical studies, PLF has been reported to reduce discogenic low back pain effectively [13, 18].

In the current study, we evaluated PLF performed 1 week after disc puncture. This situation is not similar to conditions of human disc degeneration; however, there were some limitations in the animal study. We evaluated disc degeneration 4 weeks after surgery as shown in Fig. 2. However, we did not show disc degeneration at the time PLF was performed. In this regard, this model was a punctured disc model, and not a disc degeneration model at the time of PLF surgery. In sensory nerves innervating intervertebral discs, proinflammatory mediators induced axonal ingrowth and upregulation of neuropeptide markers of pain within 10 days after disc puncture [16], so we performed PLF 1 week after surgery to examine changes in the characteristics of sensory nerves. However, further study is needed to strengthen our current hypothesis using examination of true disc degeneration in animals and humans.

On the other hand, significant differences in the proportions of GAP43- and CGRP-IR FG-labeled neurons and disc degeneration were observed between the union rats in Group B and the rats in Group D ($P < 0.05$). This finding indicates that PLF does not completely control the progression of intervertebral disc degeneration, the upregulation of inflammatory neuropeptides, or axonal growth. PLF prevents hypermobility because of disc degeneration; however, it does not treat the intervertebral disc. This may be a limitation of PLF compared with anterior interbody fusion for disc degeneration and pain relief. Interbody fusion is thought to be the best method to reduce inflammation, innervation, and hypermobility compared with PLF. On the other hand, one randomized controlled trial examining the method of surgery for discogenic LBP has found no significant differences among them (2). Two hundred twenty-two patients were blindly randomized into three surgical groups. The patients had therapy-resistant chronic LBP that had persisted for at least 2 years and radiologic evidence of disc degeneration (spondylosis) at

L4–L5, L5–S1, or both. Three groups consisting of PLF, PLF + PS, PLF + PS + anterior interbody fusion, and PLF + PS + posterior interbody fusion were compared. All surgical techniques were found to reduce pain and decrease disability substantially, but no significant differences were found among the groups. Further study is needed to examine changes in sensory nerve fibers innervating disc in the interbody fusion model compared with those in the PLF model.

In the current study, we found downregulation of the expression of GAP43 and CGRP in neurons innervating punctured intervertebral discs following PLF in conjunction with no significant differences in the percentages of GAP43- and CGRP-IR neurons between bone union and nonunion rats in Group C and those in Group D ($P > 0.05$). These results indicate that PLF did not affect the sensory nerves in non-punctured discs but that PLF suppressed neuropeptides and inhibited nerve ingrowth only into punctured discs. Our results thus suggest that PLF may be able to ameliorate activated sensory nerve fibers innervating punctured discs.

Major limitations of rat studies are that because rats are quadrupeds, they are different from humans regarding mechanical loading of intervertebral discs and bone union. In humans, reported discogenic low back pain is typically mechanical and intolerant to axial activities, such as standing and sitting. Accordingly, the results of this study may not be applicable to humans. Nevertheless, it was reported that the similarities between rat and human compression and torsion mechanical properties, when normalized for geometry, validate the use of the rat intervertebral disc as a mechanical model of the human disc [11, 27]. It has been previously demonstrated that rat lumbar discs are the closest representation of the human lumbar intervertebral disc geometry with regard to the parameters of disc height, anteroposterior width of the entire disc, and nucleus pulposus area [11]. Furthermore, it has been demonstrated that the averages of compression and torsion stiffness, normalized by geometry, compared well with human motion segment stiffness [27]. These reported studies concluded that comparison of experimental animal models to the human lumbar disc is valid to aid both interpretation and planning for experimental disc research. Therefore, the results of this study were thought to approximate the human condition at least to some degree.

A further limitation is that a difference in bone union between animals and human is observed. In the current study, we observed 64% bone union 4 weeks after PLF surgery. The PLF model has been used previously in some animals. Boden et al. [4] showed that radiographic bone union, biomechanical strength, stiffness of the fusions, and histological increase of woven bone were achieved

4 weeks after surgery in rabbits. Rao et al. showed that radiographs of the mice spine at 4 weeks after PLF revealed no fusion mass in decorticated controls (no graft material). However, an obvious, and dense fusion mass was seen in the PLF group (harvested from the iliac crest) [32]. So we evaluated the rats at 4 weeks after surgery in the current study. However, more solid fusion may decrease the inflammation in punctured discs, so long-term experiments are needed to clarify the pathomechanism.

In conclusion, PLF for punctured intervertebral disc inhibits architectural changes and is related to downregulation of the expression of GAP43 and CGRP in neurons. These effects of PLF, in turn, may positively impact innervation, inflammation and hypermobility. These results may explain, in part, pain relief following lumbar spine fusion surgeries, including other procedures for chronic discogenic low back pain in humans.

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