Pain Markers and Epidural Fibrosis Caused by Repeated Spinal Surgery in Sprague–Dawley Rats

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Abstract

Background

Epidural fibrosis is one of the aetiologies of pain following spinal revision surgery. However, roles of epidural fibrosis caused by repeated spinal surgery and pain-related proteins in causing the post spinal surgery syndrome remain unknown. In this study, using a rat spinal surgery epidural fibrosis and adhesion model, we evaluate and investigated the relationship between pain marker and epidural fibrosis caused by repeated spinal surgery in Sprague-Dawley rats.

Methods

Sprague–Dawley rats that underwent repeated spinal surgery were divided into three groups: group A (single laminectomy), group B (two repeated surgeries) and group C (three repeated surgeries). Dural thickness was measured in each experimental group, and immunohistochemical analysis and western blotting of mitogen-activated protein kinases were performed (ERK, p38 and JNK).

Results

Dural thickness was 6.363 ± 1.911 µm in group A, 13.238 ± 2.123 µm in group B and 19.4 ± 2.115 µm in group C. In western blotting, phosphorylated ERK expression was higher in groups B (1.77 fold) and C (2.42 fold) than in group A. Phosphorylated p38 expression was higher in groups B (1.17 fold) and C (1.33 fold) than in group A. Immunohistochemical analysis revealed that phosphorylated ERK and p38 expression gradually increased with the number of repeated surgeries, as evidenced by western blotting.

Conclusions

Repeated spinal surgery may increase dural thickness and expression of phosphorylated ERK and p38 in the spinal dorsal horn, suggesting that pain increases with repeated surgery.

Background

Each year, 1 million people worldwide undergo lumbar disc surgeries for disc herniation and spinal stenosis, making it one of the most common treatments for spinal diseases [1–4]. However, occasionally, despite appropriate decompression, the outcomes of such spinal surgeries are not necessarily correlated with the clinical outcomes. This may be because of the development of epidural fibrosis and adhesions, which is a normal reaction during healing following spinal surgery [5]. However, epidural fibrosis causes pulling, stretching or compression of the associated nerve root or dura mater and can lead to persistent back and leg pain; this is known as the post laminectomy syndrome, failed back syndrome or post spinal surgery syndrome [6, 7]. Epidural fibrosis and adhesion are inevitable following spinal surgery, and
despite advancements in surgical techniques, some patients continue to suffer from recurrent postoperative pain [1]. It is difficult to expect good outcomes even when repeated surgery is performed to eliminate epidural fibrosis and adhesions. In addition, such repeated surgeries may lead to dural tears, nerve root injuries and excessive bleeding [8].

Neuronal injury and post-injury regeneration progress as neural peptides and signal transduction molecules are expressed in the dorsal root ganglion (DRG) and spinal dorsal horn. In this regard, mitogen-activated protein kinases (MAPKs) are attracting much attention [9, 10]. MAPKs are serine/threonine protein kinases that conventionally comprise extracellular signal-regulated kinases 1/2 (ERK1/2), p38 and c-Jun N-terminal (JNK) [11]. MAPKs are activated by diverse extracellular stimuli, such as hormones and growth factors, and they transduce extracellular stimuli to intracellular transcriptional and post-transcriptional responses [9, 12]. Increasing evidence has shown that MAPKs play important roles in the induction and maintenance of chronic pain [13–15]. However, the roles of epidural fibrosis caused by repeated spinal surgery and pain-related proteins expressed in the spinal dorsal horn in causing the post spinal pain syndrome remain unknown.

In the present study, we evaluated the extent of epidural fibrosis by measuring dural thickness following repetitive surgery using a previously established rat model of laminectomy and investigated the association of MAPK expression in the spinal dorsal horn with post spinal surgery syndrome or chronic pain syndrome development.

**Methods**

**Animals**

In this study, a total of 45 male Sprague–Dawley rats (age, 8 weeks) were obtained from Orient Bio Inc company (Korea). All animal experiments were approved by the Institutional Review Board of St. Mary`s Hospital of Catholic University (CMCDJ-AP-2012-017). The animals were randomly divided into three groups; group A (n = 15) underwent a single spinal laminectomy; group B (n = 15) underwent two laminectomies repeated at an interval of 3 weeks; and group C (n = 15) underwent three laminectomies repeated at intervals of 3 and 6 weeks (Table 1).

| Group | Operation time | Operation data                       | Total survival |
|-------|----------------|--------------------------------------|----------------|
| A     | 1              |                                      | 3 weeks        |
| B     | 2              | One more trial after 3 weeks         | 6 weeks        |
| C     | 3              | One more trial after 3 + 3 weeks     | 9 weeks        |

**Surgery**
All animals were intraperitoneally anaesthetised with 40 mg/kg ketamine hydrochloride (Ketamine 50®; YUHAN, Korea) and 5 mg/kg Rompun injection (Rompun®; BAYER, Korea). After shaving the lower back, the surgical site was sterilised with povidone. The sterilised site was then covered with sterile surgical sutures, and the lumbosacral fascia and paraspinous muscles were dissected, followed by L4–6 laminectomy (Fig. 1). During surgery, the animal’s body temperature was maintained using a warm pad. At the end of the experimental period, the animals were euthanized using CO₂ gas. Using knife, the entire laminectomy site was excised according to the spinal cord levels; step by step under a dissecting microscope (Nikon, SMZ800N, Japan); the site was dissected using a micro-rongeur and micro-curette for the removal of muscle and other tissues. After the removal of the surgical site, half of the area was fixed in 10% formalin solution and the remaining half was stored at −80 °C in a cryotube for protein extraction.

**Measurement of dural thickness**

The fixed specimen was axially cut through from the upper L4 to lower L6 levels to isolate the laminectomy site. After decalcification and dehydration, paraffin blocks were prepared, and 4-µm-thick section of the laminectomy site were cut and stained with hematoxylin. Slides were evaluated in a blinded manner by a histologist who analysed dural thickness. Dural thickness was measured at 3 points; the first sample was harvested from the midpoint of the laminectomy defect, the second sample was obtained 2 mm from the right side of the first sample, and the third sample was obtained 2 mm from the left side of the first sample [16]. Mean dural thickness was considered for statistical evaluation.

**Western blotting**

For protein extraction, tissues were placed in RIPA buffer (CBR0002; LPS solution, KOREA) with cOmplete™ EDTA-free protease inhibitor cocktail tablets (0469332001; Sigma-Aldrich, Germany) and shaken in a tissue lyser (TissueLyser II, QIAGEN, Germany) at the rate of 30 times/min for 3 min. The protein content was quantified using BSA, and the extracted and quantified proteins were diluted to 20 µg/µL for western blotting. Proteins were loaded onto 10% sulphate-polyacrylamide gels for electrophoresis (#456–1034; Bio-Rad Laboratories, Inc., USA). After transfer to nitrocellulose membranes and blocking, proteins were incubated with the following primary antibodies overnight at 4 °C: total-ERK1/2 (1:1000; #4348, Cell Signaling Technology, Inc., USA), total-p38 (1:1000; #9212, Cell Signaling Technology, Inc. USA), total-JNK (1:1000; #9258, Cell Signaling Technology, Inc. USA), phosphorylated ERK1/2 (1:2000; #4370, Cell Signaling Technology, Inc. USA), phosphorylated p38 (1:1000; #4511, Cell Signaling Technology, Inc. USA), phosphorylated JNK (1:1000; #9251, Cell Signaling Technology, Inc. USA) and β-actin (1:1000; #5125, Cell Signaling Technology, Inc. USA). After washing three times with TBST, the proteins were incubated with the secondary anti-rabbit IgG HRP-linked antibody (1:5000; #7074, Cell Signaling Technology, Inc. USA) at room temperature for 2 h, and ECL (iNtrON Biotechnology, Inc., Korea) of the solution was determined.

**Immunohistochemical analysis**
Formalin-fixed tissues were sectioned to prepare the slides. The prepared slides were dehydrated in xylene and graded ethanol, immersed in citrate buffer and boiled for 10 min using an electronic rage. Immunostaining was performed following the ABC kit manual. After blocking, the slides were incubated with the following primary antibodies at 4 °C overnight: phosphorylated ERK1/2 (1:400; #4370, Cell Signaling Technology, Inc. USA), phosphorylated p38 (1:1600; #4511, Cell Signaling Technology, Inc. USA) and phosphorylated JNK (1:100; #9251, Cell Signaling Technology, Inc. USA). Subsequently, the slides were incubated with a secondary antibody at room temperature for 1 h. All slides were developed using the ImmPACT™ NonaRED™ peroxidase substrate (Sk-4805; Vector Laboratories Inc., USA), mounted and observed under a microscope. Three fields of sections from each tissue sample were imaged at 200 × to quantify the percent p-ERK-positive, p-p38-positive and p-JNK-positive cells.

**Statistical analysis**

All data are presented as mean and standard deviation. Correlations were analysed using Student’s two-tailed and paired t-tests. Statistical Package for the Social Sciences v.12.0 software (Chicago, IL, USA) was used for data analysis. $P<0.05$ was considered significant.

**Results**

**Epidural fibrosis following repeated laminectomy**

Epidural fibrosis was indirectly analysed by measuring the dural thickness. Thickness was $6.363 \pm 1.911 \mu m$ in group A, $13.238 \pm 2.123 \mu m$ in group B and $19.4 \pm 2.115 \mu m$ in group C (Fig. 2). The thickness in group A was significantly lower than that in groups B and C ($p<0.05$), and the thickness in group B was significantly lower than that in group C ($p<0.05$). Therefore, repetitive laminectomy seemingly increases the dural thickness. In other words, repetitive spinal surgery alone increases epidural fibrosis.

**ERK1/2 protein expression in the spinal cord following repeated laminectomy**

In western blotting, total-ERK expression was similar across groups, but compared with group A, group B showed a 1.04-fold increase and group C showed a 0.89-fold increase in expression.

Phosphorylated ERK expression was in the lowest in group A; group B showed 1.77-fold increased expression compared with group A, and group C showed the highest expression, with a 2.42-fold increased expression compared with group A (Fig. 3A and B). In addition, phosphorylated ERK expression was significantly different between groups A and C and between groups B and group C ($p<0.05$).

Immunohistochemical staining revealed that phosphorylated ERK1/2 was expressed in the spinal dorsal horn in group A with primary surgery, in group B with secondary surgery and in group C with tertiary surgery. Moreover, phosphorylated ERK1/2 expression gradually increased with the number of surgeries,
and expression in group B and C was significantly different from that in group A ($p < 0.05$) (Fig. 6A and B).

These results indicate that phosphorylated ERK1/2, which is considered to be involved in causing neuropathic pain, is overexpressed following repeated laminectomy.

**p38 protein expression in the spinal cord following repeated laminectomy**

In western blotting, total-p38 expression showed a decreasing trend with the number of laminectomies. Group A showed the highest expression, while groups B and C showed 0.81-fold and 0.57-fold decreases compared with group A; expression in groups A and C was significantly different ($p < 0.05$).

Phosphorylated p38 showed an increasing trend with the number of laminectomies. Group A showed the lowest expression, and groups B and C showed a 1.17-fold and 1.33-fold increased expression compared with group A; expression in groups A and C was significantly different ($p < 0.05$).

This decrease in total-p38 expression and increase in phosphorylated p38 expression indicate that epidural fibrosis occurs due to repetitive laminectomy and that phosphorylated p38 is involved in this process (Fig. 4A and B).

Immunohistochemical staining revealed that phosphorylated p38 was expressed in group A with primary surgery, in group B with secondary surgery and in group C with tertiary surgery. Moreover, phosphorylated p38 expression gradually increased with the number of surgeries, as confirmed by the results of western blotting (Fig. 6A). Moreover, compared with group A, groups B and C were showed significantly increased expression ($p < 0.05$) (Fig. 6B).

Therefore, phosphorylated p38, which is considered to be involved in neuropathic pain, is overexpressed following repeated laminectomy.

**JNK protein expression in the spinal cord following repeated laminectomy**

Western blotting results revealed that total-JNK expression was the lowest in group A, while groups B and C showed 1.64-fold and 1.24-fold increases in expression compared with group A, and the expression in groups A and group B was signicantly different ($p < 0.05$). Expression increased in group B but decreased in group C.

Phosphorylated JNK expression was the lowest in in group A, and compared with group A, groups B and C showed 1.62-fold and 1.43-fold increased expression; expression in groups A and B was significantly different ($p < 0.05$) (Fig. 5A and B).

Immunohistochemical staining revealed that phosphorylated JNK expression was the lowest in group A. Compared with group A, groups B and C showed increased expression ($p < 0.05$). However,
phosphorylated JNK expression in the spinal dorsal horn was higher in group B than in group C, as confirmed by the results of western blotting (Fig. 6A and B).

**Discussion**

Epidural fibrosis—a common complication of lumbar disc surgery—causes repeated radicular pain or back pain due to compression of the exposed dura and nerve roots [17]. Recently, some surgeon tries to approach with indirect decompression in revision surgery to avoid these scar formations and incidental durotomy complications [18].

Post-laminectomy epidural fibrosis is well known, and Turkoglu et al. [7] have identified the mechanism of action of etanercept after inducing spinal epidural fibrosis in a rat model post laminectomy. Similarly, Alkalay et al. [19] have demonstrated that a post-laminectomy epidural fibrosis model could be used to prevent epidural fibrosis of bioplastic materials. In addition, Kurt et al. [20] have used a post-laminectomy epidural fibrosis model to compare the effects of waxed paper and Gore-Tex on the prevention of post-laminectomy epidural fibrosis. Therefore, in the present study, we constructed a rat model of post-laminectomy epidural fibrosis. But repeated multiple spinal surgery model is not existing in animal study. As this reason, we evaluated multiple spinal surgery rat model to evaluation pain marker expression and relationship between dural thickness and surgery time.

In animal models of neuropathic pain caused by peripheral nerve injury, neuropathic pain was not completely manifested at an early stage (i.e. 0–3 days post-lesion), but it was well developed at a later stage (i.e. 7–21 days post-lesion) [19]. In addition, in a partial sciatic nerve ligation model, mechanical allodynia was well established in the affected hind paw at 3 weeks post-lesion [9, 21]. Therefore, in this study, the reoperation interval was set as 3 weeks. Furthermore, in our experiments, laminectomy was repeated once, twice or thrice, and it was confirmed that epidural fibrosis progressed as the number of repeated surgeries increased. These findings suggest that repeated spinal surgeries increase dural thickness and which in turn causes neuropathic pain. Therefore, repetitive spinal surgery may increase epidural fibrosis.

Increasing studies on MAPKs have uncovered their roles in the generation of chronic central neuropathic pain due to spinal cord injury [22–24]. In addition, MAPKs such as ERK and p38 have been reported to contribute to dorsal horn hyperexcitability in a peripheral neuropathic pain model [25–28]. Thus, in the present study, we evaluated MAPK expression in the spinal cord following repetitive surgery.

The ERK/MAPK pathway plays an important role in cell proliferation and differentiation. Additionally, the activation of ERK/MAPK signalling contributes to the pain response of the dorsal horn and dorsal root ganglia following inflammation and/or nerve injury [10]. These data suggest that the MAPK family is actively involved in the pain-related processes [9]. Zhuang et al. [28] have suggested that ERK acts on neurons, microglia and astrocytes via spinal nerve ligation as well as contributes to mechanical allodynia in a neuropathic pain model. Likewise, in our study, ERK was expressed following laminectomy, and the
protein expression of phosphorylated ERK gradually increased with the number of repetitions of surgery, suggesting that ERK contributes to pain development due to epidural fibrosis.

Phosphorylated p38/MAPK induction by nerve injury mainly occurs in the spinal dorsal horn and dorsal root ganglia, which has been extensively studied in terms of the initiation and maintenance of neuropathic pain [9]. In addition, the phosphorylated forms of ERK 1/2 and p38 are reportedly upregulated in similar regions of the spinal cord in injured rats, which induced mechanical allodynia [22]. These findings suggest that activated ERK1/2 and p38 regulate changes in nociceptive reactivity in peripheral nerve injury models [29–31]. In our experiments, phosphorylated ERK and p38 were expressed in the spinal dorsal horn. Furthermore, in the present study, the expression of phosphorylated ERK and p38 was upregulated as the number of repeated surgeries increased, suggesting that p38 and ERK contribute to pain development due to epidural fibrosis.

In neuropathic pain, the role of JNK is lesser known than those of ERK and p38, with only few studies having been conducted. Zhuang et al. [15] have reported that JNK acts on the sensory nerves and astrocytes to develop and maintain neuropathic pain. However, Crown et al. [22] have reported that increases in expression of activated forms of ERK1/2 and p38 but not JNK are correlated with the expression of at-level mechanical allodynia following spinal cord injury. In our study, JNK was expressed in the spinal dorsal horn following laminectomy; however, its expression level did not gradually increase with the number of repeated surgeries. Moreover, protein expression level of phosphorylated JNK did not increase with the number of repeated surgeries. Therefore, JNK may not contribute to pain development due to epidural fibrosis.

Repetitive spinal surgery was stimulated by the spinal dorsal horn, resulting in increased ERK1/2 and p38 expression. Thus, neuropathic pain is likely induced by epidural fibrosis, and ERK1/2 and p38 are the potential pain-related factors.

Conclusions

This study was the first to analyse the association between pain markers and epidural fibrosis due to repeated spinal surgery in rats. Repeated spinal surgeries seemingly increase dural thickness, ultimately leading to epidural fibrosis. In addition, repeated spinal surgeries increased expressions of pain markers such as ERK and p38, indicating that pain increased with the repeated surgeries. However, the DRG was not focussed upon in this study because repeated surgery does not define the anatomical region of the DRG. Thus, DRG function should be evaluated and pain behaviour should be tested through further animal studies.

Abbreviations

DRG
dorsal root ganglion; MAPKs: mitogen-activated protein kinases; ERK1/2: extracellular signal-regulated kinases 1/2; JNK: c-Jun N-terminal;

Declarations

Ethics approval and consent to participate: The study was approved by the Ethics committee of St. Mary’s Hospital of Catholic University. All animal experiments were performed with the approval and guidance of the Institutional Review Board (CMCDJ-AP-2012-017).

Consent for publication: All authors have read and approved the publication.

Availability of data and materials: Authors declares that data and materials in the study are available from the corresponding author Y.Y. Kim on reasonable request.

Competing interests: None

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Author contributions: MQ: participated in experimental acquisition and data, analysis of results, and drafting of the manuscript; JK and WH: conducted the analysis of results and statistical analysis; and YK: participated in study design, formation of hypotheses, interpretation of data, and preparation of manuscript.

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Figures
Figure 1

Photograph of laminectomy. S-D rats were anesthetized and subjected to laminectomy at the L4-6 site.
Figure 2

Dural thickness following repeated laminectomy (* p< 0.05 versus group A and # p< 0.05 was used to compare different groups).
Figure 3

(A) Table showing protein bands for Total-ERK, p-ERK, and β-actin across Group A, Group B, and Group C.

(B) Bar graphs comparing Total-ERK/β-actin and p-ERK/β-actin levels across Group A, Group B, and Group C.
Total-ERK and p-ERK proteins expression following repeated laminectomy. (A): Western blotting, and (B): density measurement (* p< 0.05 versus group A and # p< 0.05 was used to compare different groups).
Figure 4
Total-p38 and p-p38 protein expression following repeated laminectomy. (A): Western blotting, and (B): density measurement (* p<0.05 versus group A).
Figure 5

(A) | Group A | Group B | Group C
---|---|---|---
Total-JNK | | | 54kD 46kD
p-JNK | 54kD 46kD |
β-actin | 43kD |

(B)

![Bar charts showing the comparison of Total JNK/β-actin and p-JNK/β-actin between Group A, Group B, and Group C.](chart)

*Significant difference.
Total-JNK and p-JNK protein expression following repeated laminectomy. (A): Western blotting, and (B): density measurement (* p<0.05 versus group A).

Figure 6

Immunohistochemical analysis of phosphorylated MAPK markers in the spinal dorsal horn. (A): images of immunohistochemical analysis of p-ERK, p-p38 and p-JNK and (B): quantitative assessment of the percent immunopositive cells (* p<0.05 versus group A; circle: spinal dorsal horn).

Supplementary Files

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- WesternblottingPainmarkers.pptx
- NC3RsARRIVEGuidelinesChecklist.pdf