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Annular puncture with tumor necrosis factor-alpha injection enhances painful behavior with disc degeneration in vivo

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Abstract

BACKGROUND CONTEXT—Painful intervertebral disc degeneration is extremely common and costly. Effective treatments are lacking because the nature of discogenic pain is complex with limited capacity to distinguish painful conditions from age-related changes in the spine. Hypothesized sources of discogenic pain include chronic inflammation, neurovascular ingrowth, and structural disruption.

PURPOSE—This study aimed to investigate inflammation, pro-neurovascular growth factors, and structural disruption as sources of painful disc degeneration

STUDY DESIGN/SETTING—This study used an in vivo study to address these hypothesized mechanisms with anterior intradiscal injections of tumor necrosis factor-alpha (TNF α), pro-neurovascular growth factors: nerve growth factor and vascular endothelial growth factor (NGF

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and VEGF), and saline with additional sham surgery and naïve controls. Depth of annular puncture was also evaluated for its effects on structural and painful degeneration.

METHODS—Rat lumbar discs were punctured (shallow or deeper puncture) and intradiscally injected with saline, TNF α , or NGF and VEGF. Structural disc degeneration was assessed using X-ray, magnetic resonance imaging (MRI), and histology. The rat painful condition was evaluated using Von Frey hyperalgesia measurements, and substance P immunostaining in dorsal root ganglion (DRG) was performed to determine the source of pain.

RESULTS—Saline injection increased painful responses with degenerative changes in disc height, MRI intensity, and morphologies of disc structure and cell. TNF α and NGF/VEGF accelerated painful behavior, and TNF α -injected animals had increased substance P in DRGs. Deeper punctures led to more severe disc degeneration. Multiple regression analysis showed that the painful behavior was correlated with disc height loss.

CONCLUSIONS—We concluded that rate and severity of structural disc degeneration was associated with the amount of annular disruption and puncture depth. The painful behavior was associated with disc height loss and discal inflammatory state, whereas pro-inflammatory cytokines might play a more important role in the level of pain, which might have resulted from enhanced DRG sensitization. These in vivo painful disc degeneration models with different severities of structural changes may be useful for investigating discogenic pain mechanisms and for screening therapies, although interpretations must note the differences between all surgically induced animal models and the human condition.

Keywords

Animal model; Discogenic pain; Hindpaw mechanical hyperalgesia; Intervertebral disc degeneration; Nerve growth factor; Tumor necrosis factor- α

Introduction

Low back pain is a common and debilitating musculoskeletal disorder associated with intervertebral disc degeneration [1]. Back pain affects 70%–85% of the population at some time in their life [2] and is the leading cause of disability worldwide [3,4]. Sources of painful disc degeneration are complex and multifactorial, and inflammatory, structural, and mechanical causes can all lead to increased nociception. Therefore, improving the understanding of mechanisms underlying back pain and developing relevant models to screen potential therapies are extremely high research priorities.

Neurovascular ingrowth into degenerated discs may play an important role in discogenic pain which often has unknown causes. Nerve fibers and blood vessels are restricted to the outer or middle-third of annulus fibrosus (AF) in normal non-painful discs. However, they extend into inner AF or nucleus pulposus (NP) in painful discs, and the extended nerves are shown to express substance P, a neurotransmitter for transmitting nociceptive information [5]. The increased innervation by sensory nerve fibers was demonstrated to be highly associated with discogenic pain using an in vivo mouse model [6]. Nerve growth factor (NGF) may be critical for nerve growth because the extended nerves expressed trk-A, a

high-affinity NGF receptor [5,7], and vascular growth would be facilitated by growth factor (vascular endothelial growth factor [VEGF]).

Chronic and painful disc degeneration is commonly associated with increased pro-inflammatory conditions. Pro-inflammatory cytokines (eg, tumor necrosis factor- α [TNF α] and interleukin-1 beta) increase in degenerated discs [8–11], and they may result in painful conditions. TNF α can facilitate neurovascular ingrowth via increased production of NGF [12] and VEGF [13,14]. TNF α also facilitates matrix degradation and upregulates substance P, suggesting it may promote structural disruption or sensitize nerves to painful responses [15,16]. Pro-inflammatory cytokines with annular fissures can also result in a permissive environment for neurovascular ingrowth [17]. Together, the literature suggests that TNF α , NGF, and VEGF may all play a critical role for discogenic pain; however, their effects on disc degeneration and pain in vivo have not been investigated.

Rat models have been widely used for studying discogenic pain because the pain associated with disc injury can be assessed with various measurement techniques [18–29]. Most animal studies on disc degeneration and pain have investigated the pathophysiology of disc herniation and radicular pain following posterolateral annular puncture with herniated NP [23,30] or nerve root compression with NP materials from caudal discs [26–28,31]. These studies have provided a group of measurement techniques for pain associated with spine pathology in rats; however, there remains a gap in the investigation of discogenic pain, or disc degeneration-associated pain in the absence of demonstrable herniation and radicular pain.

The development of an in vivo animal discogenic pain model is a high research priority which can provide insights into the pathophysiology of the painful disc degeneration, and may also serve as a screening tool for therapeutic intervention. The rat hindpaw is innervated from L3 to L5 [20] so that decreased withdrawal threshold can be associated with painful disc degeneration and local inflammation in addition to the more commonly observed herniation. Annular puncture with anterior approach without injection was demonstrated to induce reduced hindpaw withdrawal threshold interpreted as painful disc degeneration [32], but the pain was only temporary and recovered within 3 weeks post surgery. It revealed a need for developing an in vivo rat model with more chronic painful disc degeneration. More recently, an anterior puncture with saline injection into three adjacent discs in the lumbar spine demonstrated repeatable disc degeneration and increased pain sensitivity without evidence for herniation [29].

Annular injury is commonly adopted to induce structural disc degeneration in rat lumbar discs, but pain measurements are not common. It is clear that the severity of structural disc degeneration is associated with the size of injury with larger annular injuries inducing more degenerative changes [33,34], which might induce greater segmental motions [35,36]. There is limited information on the depth of annular injury on disc degeneration and discogenic pain, which may be associated with loss of nucleus pressurization. As surgically induced animal models continue to be used, their importance for evaluating the effects of puncture depth as a variable to provide improved interpretations and consistency across research groups becomes evident. Depth of annular defects are relevant when assessing patients,

because painful conditions are most common clinically from complete annular tears (penetrating the full annular depth to expose and depressurize the NP), and less common with incomplete annular tears (penetrating only part of the annular thickness and involving the AF only). However, painful conditions can arise from both complete and incomplete annular tears.

The main purpose of this study was to distinguish between structural and painful disc degeneration using an in vivo rat lumbar spine injection model. To investigate inflammation, pro-neurovascular growth factors, and structural disruption as sources of painful disc degeneration, anterior intradiscal injections included TNF α , pro-neurovascular growth factors: NGF and VEGF, and saline, respectively. Shallow and deep annular punctures with intradiscal phosphate buffered saline (PBS) and TNF α injections were also evaluated to better distinguish roles for structural and inflammatory conditions on discogenic pain. Effects of puncture depth are new in this study and a notable absence from the growing disc puncture model literature. Structural disc degeneration was assessed using X-ray, MRI, and histology. Painful disc degeneration was assessed using Von Frey hyperalgesia measurements and substance P immunostaining in dorsal root ganglion (DRG).

Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee. For aim 1, 30 skeletally mature male Sprague-Dawley rats (4–5 months of age) were used and randomly divided into five groups (six rats per group): naïve, sham, PBS, TNF α , and NGF and VEGF (Table). Lumbar discs of rats in PBS, TNF α , and NGF and VEGF groups were exposed, punctured with shallow needle depth (1.5 mm) to induce an incomplete AF injury, and intradiscally injected with PBS, TNF α , and NGF and VEGF, respectively (Fig. 1). The sham group involved surgery without puncture or injection, whereas the naïve group involved no surgical procedures at all. For aim 2, 12 additional rats were used and divided into two groups (n=6 in each group): PBS_deep and TNF α _deep (Table). Lumbar discs were exposed to deep (3 mm) puncture to induce a complete AF injury and intradiscally injected with PBS or TNF α (Fig. 1). All intradiscal injections used a 26-gauge needle at a depth of 1.5 mm (shallow) or 3 mm (deep) as guided by a needle stopper. A total of 2.5 μ L of PBS, TNF α (0.25 ng in 2.5 μ L) [37], or NGF/VEGF (50 ng/250 ng in 2.5 μ L) were slowly injected into each disc using a calibrated microliter syringe (Hamilton Company, Reno, NV, USA) with doses taken from the literature [38–40]. The volume and method of intradiscal injection were determined from thorough preliminary studies on motion segments of live animals that titrated the injection volume down to 2.5 μ L and demonstrated that there was no fluid leakage or nerve root irritation were visible.

Surgery was performed under sterile conditions and general anesthesia (2% isoflurane in oxygen). A midline incision was made in the abdomen, and the lumbar spine was visualized by dissecting around the major vessels. L3–L4, L4–L5, and L5–L6 discs were identified using the pelvic rim as an anatomic landmark. Discs were punctured at their midline and injected as described. The peritoneum and abdominal wall were closed using 3-0 silk sutures and the skin was closed using 4-0 nylon sutures. The rats were closely monitored to ensure that there were no intraoperative complications. All rats were euthanized 6 weeks post

surgery using carbon dioxide inhalation and analyzed for postmortem MRI and histology of the lumbar spine. Severity of painful disc degeneration was determined using weekly measurements of radiographic disc height and hindpaw mechanical hyperalgesia. The L1 DRGs were immunohistochemically stained for substance P to assess pathophysiology of the painful behavior.

Heights of lumbar discs were measured using lateral radiographs with the animals under general anesthesia. To minimize effects of anesthesia on disc height measurements resulting from muscle relaxation and disc swelling, the rats were initially anesthetized for approximately 10 minutes before taking radiographs [41]. The radiographic film was digitized using a flatbed scanner with backlighting, and the vertebral boundaries were identified to determine the intervertebral space (ie, disc height) using a custom code written in MATLAB (Mathworks, Natick, MA, USA). The disc heights were measured weekly and then compared with those obtained preoperatively.

Hindpaw mechanical hyperalgesia is a measurement of pain sensitivity using a series of calibrated von Frey filaments corresponding to forces between 0.4 and 26.0 g (Stoelting, Wood Dale, IL, USA) [18,19]. After 20 minutes acclimations, calibrated filaments were applied in ascending order from 0.4 g to 26.0 g until a positive response was elicited. The 50% withdrawal threshold was determined using the up-down method [42]. Withdrawal thresholds of left and right hindpaws were assessed and averaged with three trials for each side. This test was performed on days 1, 7, 14, 21, 28, 35, and 42 post surgery, and compared with those presurgically.

Immediately after euthanasia, rat lumbar spines were scanned using a 7-T imager (Bruker BioSpec System, Bruker Corporation, Billerica, MA, USA) with a BGA-S gradient coil. A total of 32 slices with thickness of 500 μ m for each slice was obtained. The midsagittal section with all lumbar discs was identified and examined for disc degeneration and herniation. The gray scale of the NP in both injected discs and self-control discs were quantified. To minimize the variations between animals, the results of injected discs were normalized to self-control discs.

The L4–L6 motion segments were dissected for histologic analyses. The specimens were fixed, decalcified, embedded in paraffin, and sectioned sagittally at 5 μ m intervals. The sections with the needle track were identified, and stained with Safranin-O/fastgreen/hematoxylin for disc morphology and glycosaminoglycan (GAG) content using bright-field microscopy.

Bilateral L1 DRGs were resected following laminectomy, fixed, embedded in paraffin, and sectioned at 5 μ m intervals. Following deparaffinization and rehydration, tissue sections were treated with proteinase K (S3020, Dako North America, Inc, Carpinteria, CA, USA) and protein block (X0909, Dako North America, Inc). Sections were incubated at room temperature for 1 hour with mouse monoclonal primary antibodies against rat substance P (1:300 dilution, ab14184, Abcam, Cambridge, MA, USA) or normal mouse serum (ab7486, Abcam) as negative control. After incubation with anti-mouse IgG peroxidase, secondary antibody (ImmPRESS reagent, Vector Laboratories, Burlingame, CA, USA), and hydrogen

peroxide, the sections were treated with diaminobenzidine-based peroxidase substrate (ImmPACT DAB, Vector Laboratories) to visualize the immunoreactivity. The sections were counterstained with toluidine blue, dehydrated, mounted, and evaluated using bright-field light microscope. The positively stained DRG cells were located, and the percentage of immunopositive cells was determined.

Disc height and withdrawal threshold at each time point were normalized to those obtained presurgically and analyzed using repeated measures analysis of variance (ANOVA) with least significant difference for determining the differences with time compared with presurgery. The differences between groups were evaluated using one-way ANOVA using Tukey's post hoc test. Percentages of immunopositive cells were compared between groups using Kruskal-Wallis with Mann-Whitney tests. A multiple regression analysis was performed to determine the association between paw withdrawal threshold (at week 6), normalized disc height (at week 6), and the percentage of substance P immunopositive DRG cells. Statistical tests were performed using SPSS Statistics version 20 (IBM Corporation, Armonk, NY, USA) with significance level of $p < .05$.

Results

Surgical procedures were well tolerated with no intraoperative complications. There were no obvious effects of surgery or intradiscal injections from mean $\pm$ SD body weight of 487 $\pm$ 52 g, 497 $\pm$ 48 g, 513 $\pm$ 50 g, and 528 $\pm$ 48 g, for presurgery, weeks 2, 4, and 6, respectively, and no differences between groups. "Dropped foot" or foot weakness was observed in four rats (1 PBS, 3 NGF/VEGF animals) that belonged to the initial group of animals after surgery, which may have resulted from muscle or nerve damage when dissecting the surrounding tissue to expose the lumbar spine, and these rats were sacrificed and excluded from this study. For those with dropped foot, they were all from aim 1. The surgical skill improved with experience, and there were no rats in aim 2 with dropped foot following surgery.

Aim 1 investigated the effects of chemical injections. Self-control disc heights (ie, L1–L2 and L2–L3) did not significantly change with time or across groups. The experimental discs (L3–L4, L4–L5, and L5–L6) were averaged for analysis because they had similar patterns. Normalized disc heights of naïve and sham groups remained constant for 6 weeks ($p > .05$ for all time points). However, annular injury with intradiscal injections of PBS, TNF α , or NGF/VEGF induced continuously decreased disc heights ($p < .05$), which were also significantly smaller than naïve and sham groups (Fig. 2). There was no significant difference among the three injection groups.

Withdrawal thresholds of the naïve group remained constant throughout 6 weeks ($p = .662$, Fig. 3). The sham surgery induced a temporarily decreased threshold that recovered 1 week post surgery and showed no significant changes with time ($p = .216$). Withdrawal threshold significantly decreased following PBS injection, but was partially recovered and reached the maximum at day 28 ($p > .05$ compared with presurgery, naïve, and sham groups). However, the thresholds decreased again at days 35 and 42 ($p < .05$ compared with presurgery), and were significantly smaller than the naïve and sham groups, suggesting a failed attempt at recovery. TNF α and NGF/VEGF groups had significantly reduced withdrawal thresholds at

day 1 post surgery that continuously decreased throughout 6 weeks ($p < .05$ compared with presurgery, naïve, and sham). They were also significantly lower than PBS group on days 21 and 28.

Magnetic resonance imaging of self-control and all lumbar discs of naïve and sham groups showed normal signal. Intradiscal injections of PBS, TNF α , or NGF/VEGF reduced MRI signal intensities in one of three injected discs (Fig. 4). The normalized gray scales of the injected discs were 1.16 ± 0.05 , 1.12 ± 0.05 , 0.97 ± 0.14 , 0.98 ± 0.23 , 0.90 ± 0.15 for naïve, sham, PBS, TNF α , or NGF/VEGF groups, respectively, which confirmed that the intensities of the three injection groups were lower than those of naïve and sham groups. No disc herniation was observed in any injected discs.

Naïve and sham discs exhibited normal morphology, including GAG-rich NP with notochordal cells, well-organized AF collagen lamellae, fibroblastic annular cells, and distinct NP-AF boundary (Fig. 5). Annular puncture with injections affected AF morphology with disorganized annular lamellae, and decreased GAG content and annular cells along the needle track. There was no obvious difference among the three injection groups histologically.

Substance P immunoreactivity was mainly located within DRG cell bodies of the neurons. There was a trend toward more DRG substance P in the experimental groups (ie, sham, PBS, TNF α , and NGF/VEGF groups); however, when compared with naïve group, the increase was statistically significant for PBS and TNF α groups only (Fig. 6).

Aim 2 investigated the effects of puncture depth. Deep annular injury with PBS or TNF α injections significantly reduced the disc heights (Fig. 7, Left). When disc height of deep and shallow injection groups were averaged, disc heights of deep injection were significantly smaller than shallow injection groups at all the time points except week 6 (Fig. 7, Right).

Withdrawal threshold significantly decreased after deep annular injury with PBS or TNF α intradiscal injections (Fig. 8, Left). When withdrawal threshold were averaged for PBS and TNF α groups, the thresholds following TNF α injections were significantly lower than PBS injections at all time points except day 1 post surgery (Fig. 8, Right).

Magnetic resonance imaging demonstrated that deep injections with PBS or TNF α induced more degenerative changes and decreased signal intensity in two out of three injected discs (Fig. 4). The normalized gray scales of the injected discs were 0.69 ± 0.15 and 0.64 ± 0.22 for PBS_deep and TNF α _deep groups, respectively, which were lower than those following shallow puncture with PBS, TNF α , and NGF/VEGF injections. No herniation was observed in any injected discs.

In addition to annular disorganization as well as decreased GAG content and annular cells along the needle track, the deep annular injury induced more severe degeneration with decreased GAG content, reduced disc height, more fibrous NP with decreased number of NP cells, and less distinct nucleus boundary (Fig. 5).

Multiple regression analysis showed that the normalized disc height was significantly associated with the paw withdrawal threshold with R^2 of 0.64 ($p < .001$) and non-significantly associated with percentage of substance P positivity R^2 of 0.13 ($p = .076$).

Discussion

Distinguishing painful and non-painful disc degeneration remains a high research priority that can improve understanding of the pathophysiology of degenerative disc disease, and assist in the development of improved clinical treatment options and help inform patient selection criteria for back pain. In vivo disc degeneration models with painful condition were developed based on anterior annular puncture and injection to evaluate how different pro-inflammatory agents and depths of annular puncture affected structural and painful disc degeneration. A rat model was used because it was sufficiently large to induce precise surgically induced disc disruption while also being amenable for painful behavior measurement. The standard diagnostic techniques for discogenic pain in humans (eg, discogram and measures of axial back pain) cannot be applied to rats. However, it is impossible to come up with an animal model that exactly mimics human discogenic pain. The current study provides a relatively close proxy for “discogenic pain,” because it allowed the study of painful behaviors in a small animal without evidence of herniation on chemical and morphological outcomes only. This in vivo animal model also provides us with time and cost savings, and allows us to screen potential therapeutic treatments for discogenic pain with disc degeneration before human clinical studies. In this study, shallow needle puncture and intradiscal injection of PBS induced partial annular tears, moderate disc degeneration, and painful behavior. Shallow injections of $\text{TNF}\alpha$ or NGF/VEGF further accelerated the painful responses without accelerating the rate of structural disc degeneration, suggesting the inflammatory environment was an important driver of pain behavioral changes. Deep needle puncture induced a complete anterior annular tear with more severe and accelerated degenerative changes in disc height, MRI, and morphology. Multiple regression analyses showed a significant association between painful behavior (ie, paw withdrawal threshold) and structural deformity (ie, disc height loss). Overall results, therefore, indicated the severity of disc degeneration was significantly associated with annular injury, whereas the painful behavior was closely associated with both structural deformity and inflammation, as induced by $\text{TNF}\alpha$ injection with no evidence for herniation. These observations suggested these models had important parallels with the human clinical condition.

The current study suggests that the mechanisms for severity of disc degeneration and painful behavior might be different. The deeper puncture induced more disc height loss and MRI intensity reduction when compared with shallow puncture, but there were no obvious differences between PBS, $\text{TNF}\alpha$, and NGF/VEGF groups (with same level of disc puncture). The findings revealed that the level of structural disc degeneration is most strongly related to the amount of disc tissue disruptions due to the puncture rather than the injectate. The concentrations of $\text{TNF}\alpha$ and NGF/VEGF were adopted from previous in vivo or in vitro studies [37–40], and were sufficient to induce observable changes in pain behavior in this study. The deeper annular puncture induced both NP and AF injury with no herniation observed in all injected discs by MRI or histology, although the full AF depth injury could result in some NP exposure in the anterior region. Annular puncture induced matrix

degradation, which might lead to loss of nuclear water content evidenced by reduced MRI signal intensity. Annular puncture also resulted in decreased disc height which might be associated with the loss of nuclear pressure, disorganized annular lamellae, and reduced water content. Consistent with these findings, annular puncture with larger needles previously demonstrated more severe degenerative changes in disc height, MRI intensity, proteoglycan loss, and reduced biomechanical stability [33,35,36,43]. The deep annular puncture may be clinically relevant with progressive annular injury, herniation, and surgical procedures for nucleotomy. These findings suggested that the degenerative changes were highly associated with the size of injury (puncture diameter and depth), which directly related to the amount of disruptions in annular fibers and NP prolapse.

The level of pain behaviors was associated with structural changes and inflammatory state of the disc. Regression analyses showed a significant association between paw withdrawal threshold and disc height loss. Furthermore, when compared with PBS injection, the rats following TNF α injection showed significantly higher painful response (which was nearly identical for shallow or deep annular injection). These findings suggested that the disc inflammatory state might play a more important role in the level of pain. The association between pro-inflammatory cytokines and discogenic pain was previously demonstrated using an intradiscal injection of complete Freund adjuvant, a pro-inflammation mediator, to induce discitis [18]. However, this is the first study to investigate the association between TNF α , a natural pro-inflammatory cytokine thought to be an initiator of disc degeneration, and painful behavior in vivo.

Increased substance P in rat DRGs appeared to be associated with pain sensitization due to an increased pro-inflammatory environment; however, this mechanism needs further investigation. Substance P in the DRG was significantly increased following intradiscal TNF α injection, and also exhibited a non-significant correlation with pain threshold in the multiple regression analysis. Although substance P was increased in the sham group, this was not significantly different from naïve and was largely due to one sham rat that exhibited a notably high substance P level (>1 SD above the mean); this animal had decreased paw withdrawal threshold throughout the 6 weeks of experiment presumably due to an imperfect sham surgery. Substance P was also demonstrated to be upregulated in painful human discs [5], and was also increased in human NP cells when they were cultured with TNF α [16].

All in vivo disc puncture models are acute injuries and differ from the human condition that accumulates over years or decades [44]; however, some parallels with the human condition are worth considering. The intradiscal injection animal models in this study were able to induce pain behavior with relatively longer period of time (ie, 6 weeks) with structural disc degeneration without notable evidence for posterior herniation or evidence for radiculopathy. Discogenic pain in humans can leave some patients with severe back pain and mild disc degeneration, while leaving other patients with severe disc degeneration and little back pain at all [45,46]. Consequently, these models may be useful for assessing treatment strategies that focus on the relief of the painful condition in addition to restoring the degenerated disc, particularly if held out more chronically. The model with complete annular injury (deep puncture) and intradiscal injections of TNF α induced painful condition with severe disc degeneration and may be useful to evaluate the efficiency of more aggressive treatment

strategies, for example, spinal fusion or biological repairing. The model with incomplete annular puncture (shallow) and PBS or TNF α injection induced painful disc degeneration with annular disruption only, and may be suitable to screen more mild treatment strategies, for example, injectable therapeutics and methods for annular repair.

This study was designed to distinguish causes of painful disc degeneration including structural disruption, inflammation, and pro-neurovascular growth factors. Results demonstrated that the rats with degenerated discs showed painful behavior, but the rats with TNF α injection exhibited more severe pain (either shallow or deep injury). The findings suggested that inflammation might play a more important role than disc degeneration for inducing pain in rat. The intradiscal injection of TNF α can induce pain through initiation of a local inflammatory response and sensitization of DRGs with upregulation of substance P. We believe the chronic painful behaviors of the TNF α injection group was predominantly due to intradiscal inflammation because no leakage of any injectate was observed from the disc under physiological spine loading in our preliminary in vitro study. Furthermore, perineural injections to the DRG are known to clear within hours [47] and may not induce the chronic pain behaviors observed in this study. Further study is recommended to evaluate the inflammatory state of the disc, surrounding tissues, and nerve root following TNF α injection to better distinguish specific mechanisms for initiation and progression of painful responses. Injection of TNF α outside of the disc may also be included as another control group to determine the association of regional inflammation and hindpaw hyperalgesia. The NGF/VEGF injection group also exhibited painful behaviors without upregulation of substance P in DRG, and this might also implicate neurovascular ingrowth as an additional source of the observed painful behaviors although it was not investigated here. Altered biomechanics is another potential source of discogenic pain that may result from instability, increased stresses on nerve fibers, or chronic inflammation due to irregular motions and incomplete healing. Although puncture depth was not implicated in increased pain sensitivity in this study, we cannot completely reject altered biomechanics as a cause for pain initiation or progression observed. Previous studies demonstrated that intradiscal stress profile was altered in degenerated disc with decreased NP compressive stress and multiple AF stress contractions [48]. A reduced intradiscal stress might facilitate neurovascular ingrowth and induce discogenic pain.

Conclusions

This study used in vivo rat lumbar spine injection models to evaluate the roles of structural disruption, inflammation, and pro-neurovascular growth factors in leading to painful disc degeneration. The painful behavior was associated with disc height loss and discal inflammatory state. Pro-inflammatory cytokine might play a more important role in the level of pain, which might have resulted from enhanced DRG sensitization. Results therefore support the hypothesis that structural disc degeneration results from the amount of disc tissue disruption whereas painful responses are associated with its inflammatory state. Disc degeneration-associated pain was also induced by intradiscal PBS and NGF/VEGF injections, suggesting that further investigation are warranted to explore localized neurovascular ingrowth and altered biomechanics as pain sources. These in vivo painful disc degeneration models with different severities of disc degeneration can therefore be used to

better understand mechanisms for discogenic pain initiation and chronicity and may also be useful as screening tools to develop interventions in preventing and treating back pain.

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Fig. 1.

Schematic diagram of rat lumbar disc injection procedure. Shallow and deep intradiscal injections were made with an anterior approach. Shallow punctures (ie, 1.5 mm depth) induced an incomplete annular puncture with AF injury only. Deep punctures (ie, 3 mm depth) induced a complete annular puncture with AF tear and NP involvement.

Fig. 2.

All shallow intradiscal injections resulted in similar amounts of disc height loss. (Top) Representative radiographs of rat lumbar spines from naïve, sham and PBS, TNFα, and NGF and VEGF groups. (Bottom) The changes of normalized disc height (ie, L3–L4, L4–L5, and L5–L6 discs, normalized to presurgery) of the five experimental groups, the standard deviations are marked as error bars, and significant differences are indicated. Disc heights of injected discs (ie, PBS, TNFα, and NGF/VEGF groups) decreased continuously and were significantly smaller than those of naïve and sham groups, indicating no effect of injectate material.

Fig. 3.

Shallow intradiscal injections of pro-inflammatory and pro-neurovascular growth factors increased pain sensitivity. The changes of normalized paw withdrawal threshold (normalized to presurgery) of naïve, sham and PBS, TNFα, and NGF/VEGF groups, the standard deviations are marked as error bars, and significant differences are indicated. Thresholds of TNFα and NGF/VEGF groups decreased continuously and were smaller than those of naïve, sham, and PBS groups.

Fig. 4.

Deep intradiscal injections induced greater structural disc degeneration than shallow injections on MRI. Representative T2-weighted MRI images of rat lumbar spines from all experimental groups (for both aim 1 and aim 2). Injected discs showed decreased signal intensity (indicated with red circles). The white arrows highlight discs shown at higher magnification below.

Fig. 5.

Shallow injections induced partial annular tears whereas deep injections induced complete annular tears with NP degeneration. Safranin-O/Fastgreen/hematoxylin staining for rat lumbar discs from all experimental groups (for both aim 1 and aim 2). The NP and AF regions (indicated with blue rectangles) are highlighted and shown at higher magnifications. The black arrows indicate the needle track during annular puncture which did not heal following the 6-week experiment. Normal morphology was observed in discs of naïve and sham groups. Mild degeneration with relatively minor annular disruption from the needle track was found in discs following shallow injections (ie, PBS, TNF, and NGF/VEGF groups). More severe degenerative changes in both AF and NP were shown in discs following deeper injections (ie, PBS_deep and TNF α _deep groups).

Fig. 6.

TNFα injection increased substance P staining in L1 DRGs. (Top) Representative images of rat L1 DRG, with substance P immunohistochemical staining, from naïve, sham and PBS, TNFα, and NGF/VEGF groups. (Bottom) The percentage of substance P immunopositive stained neuron of the five experimental groups, the standard deviations are marked as error bars, and significant differences are indicated. There was a trend toward more DRG substance P in the rats following intradiscal injections of PBS and TNFα ($p < .05$ with naïve).

Fig. 7.

Intradiscal injection depth was an important factor defining the rate and extent of structural disc degeneration. (Top) Changes of normalized disc height of PBS_deep and TNFα_deep groups (compared with naïve, sham, PBS, and TNFα groups), the standard deviations and significant differences are indicated. (Bottom) Disc heights for deep and shallow injections, when averaged across PBS and TNFα groups, exhibited disc heights that were significantly smaller for deep injections than shallow injections.

Fig. 8.

Pro-inflammatory cytokine injection was an important determinant of pain sensitivity. (Top) Mean±SD for changes of normalized paw withdrawal threshold of PBS_deep and TNFα_deep groups (compared with naïve, sham, PBS, and TNFα groups) as normalized to presurgery conditions. Although all PBS and TNFα injections induced enhanced pain sensitivity, TNFα had the greatest effects, and there was no effect of puncture depth. (Bottom) Withdrawal thresholds for PBS and TNFα injection, when averaged across shallow and deep groups, exhibited withdrawal thresholds that were significantly lower for TNFα injections than PBS injections.

Table

The study design and objective of this study for aim 1 and aim 2

Group		No. of animals	Intervention	Time course
Aim 1	Naïve	6	No surgery	6 weeks
	Sham	6	Surgery (expose lumbar discs only)	6 weeks
	PBS	6	Surgery+PBS injection (2.5 ul)	6 weeks
	TNF α	6	Surgery+TNF α (0.25 ng in 2.5 ul) injection	6 weeks
	NGF/VEGF	6	Surgery+NGF and VEGF (50 ng and 250 ng in 2.5 ul) injection	6 weeks
Aim 2	PBS_deep	6	Surgery+PBS injection	6 weeks
	TNF α _deep	6	Surgery+TNF α (0.25 ng in 2.5 ul) injection	6 weeks

Aim 1: To determine the effects of TNF α and NGF/VEGF on disc degeneration and painful behavior in rat, and also to explore the mechanism of the painful behavior observed in the rat.

Outcome measurements: Hindpaw mechanical hyperalgesia, disc height, MRI, IVD morphology, and substance P (DRG).

Aim 2: To determine whether intradiscal injection of TNF α with deeper puncture induced more severe disc degeneration and painful behavior in rat. Outcome measurements: Hindpaw mechanical hyperalgesia, disc height, MRI, and IVD morphology.