Cytokine Expression in the Epidural Space

A Model of Noncompressive Disc Herniation–Induced Inflammation

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Study Design. Animal study.

Objective. Development of an animal model for the study of biochemical changes that occur in the epidural space after intervertebral disc herniation.

Summary of Background Data. Although strong evidence for an inflammatory component exists, the biochemical processes underlying pain after disc herniation remain unknown.

Methods. Epidural lavage was performed in 48 rats after L5 dorsal root ganglion exposure at baseline and 3, 6, or 24 hours after placement of autologous nucleus pulposus (NP) (N = 15), saline (N = 15), or NP + an interferon-γ antibody (anti-IFN-γ; N = 18) directly onto the dorsal root ganglion. Multiplex assays quantifying interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, tumor necrosis factor α (TNF-α), IFN-γ, and GM-CSF were performed. NP (N = 7) was also analyzed for these cytokines by placing NP into saline and measuring the relative concentration.

Results. Cytokines measured low at baseline (0–100 pg/mL) in all groups. Compared with saline, NP application caused IL-6 elevation, peaking at T = 3 hours, that was prevented by anti-IFN-γ. NP induced elevation of TNF-α, peaking at T = 24 hours and was prevented by anti-TNF-α. IFN-γ was elevated after NP at T = 3 hours and T = 24 hours. IL-1α was similar after saline versus NP. The concentrations of IL-1β and IL-10 were elevated at T = 3 hours, 6 hours, and 24 hours in all groups without between-groups difference. The level of IL-4 peaked at T = 3 hours in the NP group and was different than saline and NP + anti-IFN-γ groups, but the time effect was insignificant. There was no change for GM-CSF. The concentration of cytokines measured in normal NP was less than 2 pg/mL for all cytokines except TNF-α.

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The manuscript submitted does not contain information about medical device(s)/drug(s).

Conclusion. In this model of acute noncompressive disc herniation, NP caused the elevation of epidural IL-6, TNF-α, and IFN-γ—all attenuated by IFN-γ blockade. IL-1β and IL-10 were both significantly elevated by saline alone and their response was not prevented by IFN-γ blockade. This model may prove useful for the study of the biochemical processes by which NP induces inflammation-induced nerve root irritation and radiculopathic pain.

Key words: cytokine, disc herniation, inflammation, epidural, rat, IL-6, TNF-α, interferon-γ.

An association between low back and radiculopathic pain and herniation of nucleus pulposus (HNP) from the intervertebral disc has been recognized for decades, but the specific mechanisms remain unknown. Traditional thought and use of compressive models focused on a mechanism by which mechanical pressure is placed on the dorsal root ganglion (DRG) or nerve roots. However, evidence increasingly supports a mechanism by which neural irritation may result from a combination of compressive and noncompressive etiologies or even in the absence of compression. The observation in human studies that mechanical nerve root stimulation is painful only after prior exposure to nucleus pulposus (NP) supports both this concept and the possibility that the inflammatory milieu resulting from disc herniation without compression may be sufficient to induce symptoms. Furthermore, it has been shown that application of NP to the nerve roots or DRG can rapidly sensitize spinal dorsal horn neuronal responses or primary afferent neurons.

The mediators of neuronal sensitization are under investigation and may include the recruitment of cytokines to the area of inflammation via immunomodulatory cells such as satellite glia or Schwann cells and resident macrophages. As NP has been sequestered from circulation since embryologic formation, it is immunologically naïve, thereby inducing an autoimmune-mediated inflammatory response once herniated. It is likely that multiple modulators are involved in a single or in multiple inflammatory cascades. Evidence of this is provided by the clinical efficacy of broad immunosuppressive agents such as steroids and experimental findings that doxycycline, which can modulate expression of several different cytokines, is more effective at alleviating pain behavior than is blockade of a specific proinflammatory cytokine such as tumor necrosis factor α (TNF-α).
Few studies have evaluated cytokines in the epidural space in humans with neural root irritation thought to result from disc herniation, partially because of difficulty in accessing this potential space in clinical practice.\textsuperscript{22,23} Furthermore, clinical variability, including time to presentation, makes this problem difficult to address in clinical studies. An animal model may therefore prove beneficial for further investigation of the mediators involved in NP-induced inflammation and neural sensitization. Cytokine expression was therefore quantified in the epidural fluid around the fifth lumbar DRG (L5 DRG), using lavage at baseline and at various time points after the application of autologous NP to the L5 DRG. In addition, because interferon-\(\gamma\) (IFN-\(\gamma\)) is a proinflammatory cytokine produced by multiple cell types\textsuperscript{24–27} and can cause the upregulation of various cytokines via the activation of microglia and other cells,\textsuperscript{26–28} the coapplication of a soluble anti-IFN-\(\gamma\) antibody with autologous NP was also investigated.

**MATERIALS AND METHODS**

**Animals**

Forty-eight adult male Sprague-Dawley rats (300–400 g, Charles River Laboratories) were used in this study. The animals were housed in a temperature-controlled room (22 ± 1°C) in a 12-hour light/dark cycle with food and water provided \textit{ad libitum}. The experimental procedures were approved by the Stanford University Institutional Animal Care and Use Committee.

**L5 DRG Exposure**

Rats were anesthetized with isoflurane 3% to 4% in a chamber and then moved to mask anesthesia with 1.5% to 2.5% maintenance isoflurane during surgery. The isoflurane concentration was adjusted so that a strong paw pinch failed to evoke a withdrawal response prior to the incision. An incision over the L5–L6 spinous processes was made and the paraspinous muscles were dissected free from the left L5–L6 lamina. Using a dissection microscope and microrongeurs, the left L5 inferior articular facet was resected, but the superior articular process of L6 was left intact to minimize DRG trauma and bleeding. This procedure allowed visualization of most of the dorsal side of the left L5 DRG and has been previously described.\textsuperscript{4,5} The ligamentum flavum was removed between L5 and L6 on the left side, but the dura was left intact.

**Epidural Lavage of DRG**

Baseline lavage was performed after exposure of the left L5 DRG using a micropipettor to apply 50 \(\mu\)L of phosphate-buffered saline (pH = 7.4) to the DRG. The solution was allowed to sit for 5 seconds prior to aspiration back into the same pipette. The aspirate was placed into a microcentrifuge tube containing a mix of protease inhibitors (Complete, Roche Diagnostics; Indianapolis, IN; 1 tablet per 1.5 mL PBS and then aliquoted into 30 \(\mu\)L per tube) and kept at \(-80°C\) prior to analysis. Multiple time points were analyzed; for 3, 6, and 24 hours postsurgery, animals were randomly assigned to 1 of 3 groups on the basis of the solution applied to the DRG: saline only (\(n = 15\)), NP only (\(n = 15\)), or NP + coapplication of a soluble anti-IFN-\(\gamma\) (\(n = 18\)) (50 \(\mu\)L of 0.1 mg/mL; R&D Systems, MN).

**Coccygeal Disc Exposure**

For animals in the NP or NP + anti-IFN-\(\gamma\) groups, a coccygeal disc at the base of the tail was exposed through a skin incision approximately 10 mm in length and 7 mm in width on the dorsal surface of the tail.\textsuperscript{3} Connective tissue overlying the disc was removed until the outermost surface of the annulus fibrosus was visible. The annulus was incised and the NP was excised with the tip of a #11 scalpel blade and placed onto the exposed L5 DRG immediately. The incision was not taken laterally past the flat surface of the tail, thus avoiding the vasculature, resulting in minimal bleeding. For the animals in the 24-hour experimental group, the spine skin incision was closed with wound clips and the tail skin incision was closed with a 6-0 prolene suture.

**Measurement of Cytokines in Normal NP**

In a separate experiment, autologous NP was extracted from a single coccygeal tail disc of multiple rats (\(n = 7\)), using the same procedure as that described earlier and placed immediately into a microcentrifuge tube with protease inhibitor and frozen for later analysis. The entire NP was always extracted and placed into a constant volume of solution so that the relative concentration measured between samples was of consistent dilution.

**Cytokine Analysis**

The concentrations of 9 cytokines and chemokines (IFN-\(\gamma\), IL-2, IL-4, IL-6, IL-10, GM-CSF, TNF-\(\alpha\), IL-1\(\beta\), and IL-1\(\alpha\)) were quantified in samples using a rat 9-plex inflammatory cytokine panel and the BioPlex 200 System (Bio-Rad, Hercules, CA), following the manufacturer's protocol in a 96-well plate format. This assay uses antibodies linked to polystyrene beads containing different levels of fluorophores and has been validated against standard enzyme-linked immunosorbent assays.\textsuperscript{29}

**Statistical Analysis**

Analysis of variance with post hoc Bonferroni correction for multiple comparisons was used to compare mean cytokine concentrations across the 3 time points compared with baseline and to make comparisons between treatment groups. This analysis was performed using SPSS 9.0 software (SPSS Inc., Chicago, IL). A \(P\) value of less than 0.05 was taken as statistically significant.

**RESULTS**

Data are reported as mean ± standard error of the mean.

**Normal NP**

The concentration of TNF-\(\alpha\) and IL-1\(\alpha\) was measured at 64 ± 13 pg/mL and 2 ± 1 pg/mL, respectively. All other cytokines measured were 0 ± 0 pg/mL in the autologous NP (data not shown).

**REFERENCES**


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Epidural DRG Lavage
IL-6 expression after NP application to the L5 DRG demonstrated a rapid increase over baseline, peaking at 3 hours ($P < 0.001$) and falling back to baseline by 24 hours ($P = 1.00$), respectively, and was significantly greater than after saline application (Figure 1; $P < 0.005$). Soluble anti-IFN-γ coapplication prevented the enhancement of IL-6 when compared with saline; therefore, the saline and NP + anti-IFN-γ mean results were not statistically different ($P = 1.0$).

IL-1β expression after saline, NP, and NP + anti-IFN-γ application increased over baseline with a peak at 6 hours (Figure 2). Although IL-1β levels were statistically significantly elevated at all time points compared with baseline ($P < 0.005$), there was no difference between NP ($P = 1.0$) or NP + anti-IFN-γ ($P = 0.66$) treatment groups compared with saline.

IL-10 expression peaked at 6 hours postsaline application, and the level of IL-10 was significantly elevated at all time points when compared with baseline in all groups (Figure 3; $P < 0.005$). However, there was no significant difference between the saline and either NP ($P = 0.93$) or NP + anti-IFN-γ ($P = 0.68$) groups at any time point.

TNF-α levels peaked at 24 hours after NP application (Figure 4). Significant differences in expression over time were seen between baseline and 3 hours ($P < 0.05$), 6 hours ($P < 0.03$), and 24 hours ($P < 0.001$). NP application caused a statistically significant increase in expression compared with the saline control group ($P < 0.05$). The coapplication of anti-IFN-γ prevented NP-induced elevation of TNF-α levels, resulting in no statistically significant difference between the NP + anti-IFN-γ and saline groups ($P = 0.32$).

The measured concentration of IFN-γ was also significantly greater in the NP group than in the saline group, peaking at 3 hours ($P < 0.05$; Figure 5). Time differences of IFN-γ concentration were seen between baseline, 3 hours, and 24 hours ($P < 0.005$). There was also a significantly lower concentration of IFN-γ after NP + anti-IFN-γ than after NP alone at 24 hours; therefore, there was no difference between the saline and NP + anti-IFN-γ groups at 24 hours ($P = 0.63$).

The concentration of IL-1α was similar after both saline and NP treatments ($P = 1.00$) but was significantly elevated at 24 hours ($P < 0.001$) in the NP + anti-IFN-γ group compared with the saline group ($P < 0.05$; Figure 6). The measured concentrations were not significantly elevated compared with baseline at 3 hours ($P = 1.0$) and 6 hours ($P = 0.11$).

Although the concentrations of IL-2 (Figure 7) and IL-4 (Figure 8) increased over baseline (Figure 7), there was no statistically significant difference between groups over time.
hyperalgesia, whereas anti-inflammatory cytokines can act as negative feedback regulators of these cytokines. Thus, regulation of the balance of pro- and anti-inflammatory mediators could potentially play a role in pain from neural root irritation after disc herniation.

Nucleus Pulposus-Induced Cytokine Expression of IL-6, TNF-α, IFN-γ, and IL-4

The concentrations of IL-6, TNF-α, IFN-γ, and IL-4 were significantly elevated over time after application of NP compared with saline alone. Although there was a marked and
significant elevation of IL-1β, IL-10, and IL-2 over time from baseline levels, there was not a significant difference between the saline and NP application groups, indicating that these cytokines are involved in the inflammatory response to surgery but their expression is not further enhanced by the presence of NP. This could represent either a physiologic ceiling effect of the expression of these cytokines or a divergent inflammatory cascade that varies from that which is mediated by NP.

Interleukin-6 is a proinflammatory cytokine produced by several cell types, including of the mast- and glial-cell lineages, and has been shown to sensitize joint nociceptors and induce hyperalgesia, possibly via the cyclooxygenase-prostaglandin-E2 pathway. In this study, the concentration of IL-6 around the L5 DRG was significantly elevated by the surgery but to a significantly greater extent after NP application. This NP-induced enhancement of IL-6 was prevented by coapplication of a neutralizing soluble IFN-γ antibody, thus providing evidence of an inflammatory pathway that involves IL-6 in an IFN-γ-dependent cascade induced by NP.

An NP-induced upregulation of TNF-α was also observed in this study. TNF-α is a proinflammatory cytokine that plays a critical role in the inflammatory response initiation. It is produced by various cell types, including Schwann cells after nerve injury, neurophilks, and infiltrating and resident macrophages during inflammation. TNF-α may play a role in nociceptor sensitization, allodynia, and hyperalgesia, and a positive correlation between its tissue concentration and neuropathic pain has been observed. In addition, there is prior evidence that NP-induced enhancement of spinal cord nociceptive responses may involve TNF-α. There was a small measurable concentration of TNF-α in the autologous NP. Therefore, the TNF-α measured in the epidural lavage may be the result of an inherent supply both from within the NP and from the production by inflammatory cells locally or recruited to the region in response to NP.

It has been shown that IL-6 and its receptor may be modulated by both TNF-α and IFN-γ, and there is evidence that IL-6 upregulation is a function of the concentrations of TNF-α and IL-1β. TNF-α has been shown to induce the production of IL-1β, IL-6, and IL-8, and there is evidence that IL-1β and IL-6 may act synergistically. These findings may be in agreement with the results of the present study observation that IFN-γ neutralization prevented the NP-induced enhancement of both IL-6 and TNF-α. Although this study did not observe a significant elevation of IL-1β after NP compared with saline, there was a very marked elevation in IL-1β levels over time after the surgery, an observation also consistent with these previous studies demonstrating complex interactions between TNF-α, IFN-γ, IL-6, and IL-1β.

The concentration of IL-1β after NP application was significantly greater than after saline. IFN-γ was undetectable in the autologous NP lavage. Therefore, IFN-γ production may be a mediator of an inflammatory response induced by NP exposure. It has been shown that IFN-γ is produced by multiple cell types, including natural killer cells, macrophages, activated type 1 helper T-cells, astrocytes, and damaged neurons, and is a key proinflammatory cytokine that can sensitize spinal cord dorsal horn neurons, possibly via the activation of microglia.

Interleukin-4 has been shown to inhibit the expression of proinflammatory cytokines IL-1β, TNF-α, and IL-6, as well as the activation of macrophages and microglia, accounting for its antinociceptive properties. The concentration of IL-4, an anti-inflammatory cytokine, was significantly greater in the NP group than in the saline group by the post hoc test did not find a significant time effect, it cannot be concluded with confidence that NP induces the elevation of IL-4 over time compared with saline.

Study Limitations

The epidural or intradiscal concentration of cytokines reported in this study should not be taken as absolute values, because there was some dilution upon lavage and placement of the sample into the microfuge tube containing protease inhibitor. However, because the volumes of lavage and protease inhibitor were known and were consistent across animals, this should not affect relative concentration comparisons. Therefore, the dilution could have reduced the levels of the cytokines (except TNF-α) to undetectable levels in the normal NP samples. This dilution was unavoidable, however, as any quantitative protein concentration assay requires some volume of fluid.

The amount of neutralizing soluble IFN-γ antibody used in this study (50 μL of 0.1 mg/mL) was based on results of in vitro neutralization studies provided by the manufacturer whereby the neutralization dose (ND50) is typically 0.08 to 0.2 μg/mL of antibody in the presence of 2.5 ng/mL of recombinant rat IFN-γ. Because no prior prior in vivo study had been performed, it was unknown how much neutralizing antibody would be required to prevent in vivo IFN-γ activity after saline or NP application.
The study time points of 3, 6, and 24 hours after HNP may seem to be on a much shorter time span than the usual clinical presentation of patients with radiculopathy. However, with the use of standard allometric scaling of physiologic parameters, one can estimate that 3-, 6-, and 24-hour time points in the 0.33-kg rat are equivalent to as many as 7, 14, and 56 days, respectively, in the 75-kg human. The time points used in this study may therefore be clinically relevant to the time course of clinically significant HNP-induced radiculopathy.

Conclusions and Future Studies

This study has provided an in vivo animal model by which to investigate the biochemical changes that occur in the epidural space contiguous with the dorsal root ganglia and nerve root after exposure of NP by disc herniation. Exposure of NP induced a significant elevation of IL-6, TNF-α, and IFN-γ in a manner that is prevented by neutralization of soluble IFN-γ. The surgery alone induced a significant elevation of expression of IL-1β, IL-10, IL-1α, and IL-2, and NP did not cause further enhanced levels. The level of GM-CSF was unchanged over time after surgery. The animal model presented may be useful for further studies investigating not only the epidural but also the neuronal changes within the nerve root and DRG that occur after disc herniation. Finally, this model may enable the evaluation of various inflammatory modulators that may be developed for potential therapeutics. It will be important to determine the correlation of these findings with pain behavior in future studies.

Key Points

- This study describes a new animal model of noncompressive disc herniation.
- NP application to the L5 DRG resulted in rapid and marked elevation of epidural IL-6, TNF-α, IFN-γ, and IL-4.
- IFN-γ blockade prevented NP-induced elevation of IL-6, TNF-α, and IFN-γ.
- There was a marked elevation of IL-1β and IL-10 in response to surgery that was not further enhanced by NP.
- IL-6, TNF-α, and IFN-γ may interact in an important way in the development of neural root irritation caused by exposure of NP from a disc herniation.

References


AUTHOR QUERIES

TITLE: Cytokine Expression in the Epidural Space: A Model of Noncompressive Disc Herniation–Induced Inflammation
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[AQ1]: Shouldn't the funding statement be changed to “Other funds were received to support this work.”?
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[AQ4]: Please provide the manufacturer's location (city/state names).
[AQ5]: Please define “PBS,” if deemed necessary.
[AQ6]: Please check whether the manufacturer's name is OK as typeset.
[AQ7]: Please provide the volume number in references “9 and 12.”
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