

Is the Fibronectin-Aggregan Complex Present in Cervical Disk Disease?

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Objective: To investigate the presence of inflammatory cytokines and the fibronectin-aggregan complex (FAC) in persons undergoing surgical treatment for cervical radiculopathy caused by disk herniation.

Design: Single-center, prospective, consecutive case series.

Setting: A single large academic institution.

Patients: A total of 11 patients with radiculopathic pain and magnetic resonance imaging findings positive for disk herniation elected to undergo single-level cervical discectomy.

Methods or Interventions: Lavage was performed by needle injection and aspiration upon entering the disk space for fluoroscopic localization before discectomy.

Main Outcome Measurements: The lavage fluid was assayed for pH and the FAC, as well as for the cytokines interleukin-6 (IL-6), interferon- γ , monocyte chemotactic protein (MCP), and macrophage inhibitory protein-1 β .

Results: The subjects were 7 women and 4 men with a mean age of 50.6 years (SE 9.7; range, 36-70 years). The mean concentrations (SE; range) in picograms per milliliter were 7.9 (4.4; 0-44) for IL-6, 25.3 (15.5; 0-159) for interferon- γ , 16.1 (11.9; 0-121) for MCP, and 6.1 (2.8; 0-29) for macrophage inhibitory protein-1 β . The optical density of the FAC at 450 nm was 0.151 (0.036; 0.1-0.32), and the pH was 6.68 (0.1; 6.10-7.15). Statistically significant correlations were found between MCP and FAC ($P = .036$) and between FAC and pH ($P = .008$).

Conclusions: Biochemical analysis of injured cervical intervertebral disks reveals the presence of inflammatory markers such as MCP, fragments of structural matrix proteins such as FAC, and a correlation with pH. Further evaluation of the FAC as a potential diagnostic biomarker or therapeutic target is warranted in the cervical spine.

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INTRODUCTION

Although degenerative disk disease has been studied extensively, much remains unknown about its etiology and treatment. Most of the work to date has focused on the lumbar spine. In comparison, the cervical spine has been studied less often, and we have a paucity of knowledge about pathways involving cytokines and degenerative breakdown products in the etiology of cervical spine disease [1-3].

Our group previously demonstrated that specific cytokines and degenerative breakdown products play important roles in the etiology of lumbar intervertebral disk disease of the lumbar spine and painful conditions of synovial joints [4-6]. Investigation of painful meniscus tears has identified a novel complex of cartilage breakdown products associated with knee pain [4,5]. These biomarkers have cross-reactivity with interferon (IFN)- γ , suggesting a role in the pathophysiology of pain-related disease in the musculoskeletal system. Most recently, we showed that the presence of the fibronectin-aggregan complex (FAC) predicts a favorable response to epidural steroid injections in the lumbar spine in patients with radiculopathy from a herniated nucleus pulposus (HNP) [6]. In the current study, we sought to determine whether the same mediators are implicated in the etiology of cervical disk disease.

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MATERIALS AND METHODS

Subjects

Independent institutional review board approval was obtained (Sterling, Inc., Atlanta, GA), and all patients provided informed consent to participate in the study. Patients considered candidates for the surgical treatment of cervical disk disease leading to radiculopathy were included in the study if they were at least 21 years of age and had a history of pain for at least 3 months after not responding to expectant management with nonsteroidal anti-inflammatory drugs (NSAIDs), activity modification, and/or physical therapy. A trial of NSAIDs was prescribed for all the patients in this study, but none of them experienced lasting and/or adequate relief of their symptoms while taking the medications.

The study participants were identified from a consecutive group of patients that met the aforementioned clinical criteria and were offered enrollment in the study. This cohort was drawn between January 2008 and May 2009 from the patients of a single board-certified orthopaedic surgeon trained in spine surgery. Patients were excluded from the study if they had a history of oral or injected corticosteroid use within a 3-month period before surgery, chronic medical conditions associated with metabolic or inflammatory disorders (eg, insulin-dependent diabetes mellitus, severe coronary artery disease, and rheumatic or autoimmune diseases), or the primary complaint of myotomal weakness.

Sample Acquisition, Storage, and Preparation

During surgery, disk lavage was performed at the time of localization, as we have previously described [7]. In brief, the patient was positioned supine, and monitored anesthesia was induced. After exposure through a standard anterior cervical approach, a 22-gauge spinal needle was placed anteriorly into the disk space with the use of C-arm fluoroscopy. One patient had the localizing needle placed in a subjacent, normal level and underwent lavage before the needle was removed; it was subsequently repositioned to the correct surgical level. Lavage was undertaken by injection and aspiration of approximately 1-2 mL of 0.9% normal saline solution without preservative. The lavage fluid was transferred into a sterile polypropylene tube and frozen at -80°C until the time of sample analysis. At the time of analysis, each patient sample was thawed to room temperature, clarified by centrifugation at 5000g, and filtered with the use of $0.45\ \mu\text{m}$ of low-protein binding filter. The collected filtrate was immediately assayed as described in the section "Multiplexed Bead Analysis."

Enzyme-linked Immunosorbent Sandwich Assay Analysis

A heterogeneous, enzyme-linked immunosorbent sandwich assay (ELISA) that was developed and validated with a previous series of patients was used again for our analysis in this study [4]. This assay detects a protein complex of fibronectin and the aggrecan G3 domain. In brief, anti-aggrecan G3 domain antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in phosphate-buffered saline/Tween 20/thimerosal was used to coat a 96-well microplate. The plate was treated with bovine serum albumin in the same buffer overnight at 4°C to block excess binding sites, then washed with 6 cycles of phosphate-buffered saline/Tween 20/thimerosal. The centrifuged and filtered sample was divided at 3 serial dilutions in triplicate into the microplate and incubated for 1 hour to facilitate binding of the complex to the immobilized antibody. After the sample was washed 6 times with the wash buffer, antifibronectin antibody labeled with horseradish peroxidase (US Biological, Swampscott, MA) was added and incubated for 1 hour. After 6 washes, 3,3',5,5'-tetramethylbenzidine substrate was added, and the reaction product was measured by optical density at a 450-nm wavelength. Human fibronectin (BD Biosciences, San Jose, CA) at a concentration of $10\ \mu\text{g}/\text{mL}$ was used as a negative control.

Multiplexed Bead Analysis

A multiplexed bead immunoassay was performed for the analysis of cytokine content. The Bio-Plex system (BioRad, Hercules, CA), a multiplex bead array immunoassay, was used for analysis of the lavaged fluid. This system uses a sandwich-style immunoassay with capture antibodies linked to polystyrene beads and detection antibodies conjugated to fluorophores. It has been validated against standard enzyme-linked immunosorbent assays in human blood [8]. The immunoreactivity (immunofluorescence) of 17 cytokines/chemokines (IFN- γ , interleukin [IL]-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, tumor necrosis factor- α , IL-1 β , monocyte chemoattractant protein [MCP]-1 β , and macrophage inhibitory protein-1 β [MIP-1 β]) was measured with use of the premixed human inflammatory 17-plex panel. Each multiplex assay was performed in duplicate and according to the manufacturer's specifications. Standard curves were included in each run, and sample concentrations were calculated with Bio-Plex Manager software from these fluorescence measurement curves.

Statistical Methods

Data were analyzed by Wilcoxon signed-rank tests. For $N = 11$ samples, the power of Wilcoxon signed-rank tests was 83%, assuming an α value of 0.05 and a very large effect size (Cohen $d = 1.0$).

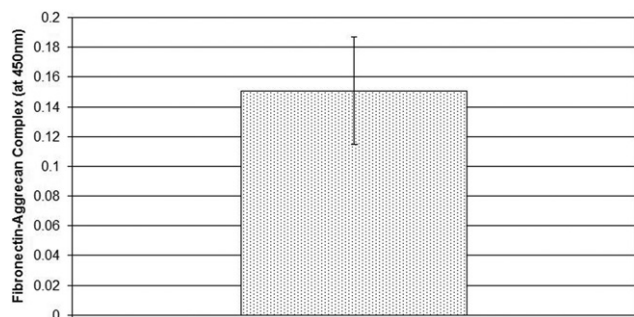


Figure 1. Mean value of the fibronectin-aggregan complex optical density at 450 nm by enzyme-linked immunosorbent sandwich assay for all patients in the series. The error bar represents the standard error of the mean.

RESULTS

The subjects were 7 women and 4 men, with a mean age of 50.6 years (SE 9.7; range, 36-70 years). Twelve disks were lavaged in the 11 patients. The mean concentrations (\pm SE; range) in picograms per milliliter (pg/mL) were as follows: IL-6, 7.9 pg/mL (\pm 4.4; range, 0-44); IFN- γ , 25.3 pg/mL (\pm 15.5; range, 0-159); MCP-1, 16.1 pg/mL (\pm 11.9; range, 0-121); and MIP-1 β , 6.1 pg/mL (\pm 2.8; range, 0-29). The mean optical density of the FAC at 450 nm was 0.151 (\pm 0.036; range, 0.1-0.32), and the mean pH was 6.68 (\pm 0.1; range, 6.10-7.15). Statistically significant correlations were found between MCP-1 and FAC ($P = .036$) and between FAC and pH ($P = .008$) per the Wilcoxon signed-rank test. Figures 1 and 2 summarize our results. Table 1 details the profiles of the inflammatory cytokines in the disk spaces of patients in this study.

DISCUSSION

Degenerative disk disease, particularly of the lumbar spine, has been widely studied. Recently, the relationship between pain and molecular markers in degenerative syndromes has garnered a great deal of interest because of the novel therapeutic implications in bioengineering and pharmacology. Although a complex molecular and cellular cascade of disk degeneration has been elucidated involving inflammatory mediators, structural proteins, and degradation fragments, the exact details are still unknown. We sought to determine whether similar markers of degeneration and inflammation are present in the cervical spine as in the lumbar spine. The results of this pilot study confirm the existence of similar processes at a molecular level. This model may further our understanding of degenerative disk disease in the cervical spine on a molecular level.

Inflammatory cytokines such as MCP and MIP, which were previously identified as being elevated in painful conditions of other joints, also were shown to be elevated in the cervical spine

[9]. Furthermore, the FAC that we previously showed to be correlated to painful meniscal pathology also was elevated in degenerative cervical spine disease in this study, which underscores the potential similarity of physiologic processes in cartilage tissues throughout the musculoskeletal system. This potential similarity may ultimately lead to similar biologic interventions by targeting the common link.

The present study also demonstrates that the FAC previously identified in the epidural space of the lumbar spine in patients with radiculopathy who respond favorably to steroid injections is also present in the disk space in a select group of patients being treated for radiculopathy related to HNP in the cervical spine [6]. However, it is not present in all disks, which suggests that different processes may be occurring in degenerative disk disease of the axial spine, depending on the level involved. Similarly, we noted that FAC was not elevated in one 57-year-old patient who underwent lavage during fluoroscopic localization. This patient had some evidence of degeneration at the level that was asymptomatic. The remainder of the patients had various levels of FAC present in the disk as indicated by lavage.

Regarding the inclusion criteria for the present study, we included patients with painful radiculopathy and specifically excluded patients with weakness. Mechanical neurocompression from HNP causing weakness possibly represents a complementary pathophysiologic pathway from pain caused by inflammation and chemical mediators. Anecdotally, we did not observe the presence of the FAC in our 2 patients who presented with a primary complaint of weakness (not reported).

The relationship between the FAC and pH is under investigation. The intervertebral disk is known to be a tenuous physiologic environment that is poorly vascularized, with low oxygen tension and low pH. Variability in pH may be caused by the physiologic environment or by sample processing. Preliminary laboratory data indicate that the FAC may be sensitive to the pH of the sample, resulting in additional complexity that makes interpretation of the results a challenge.

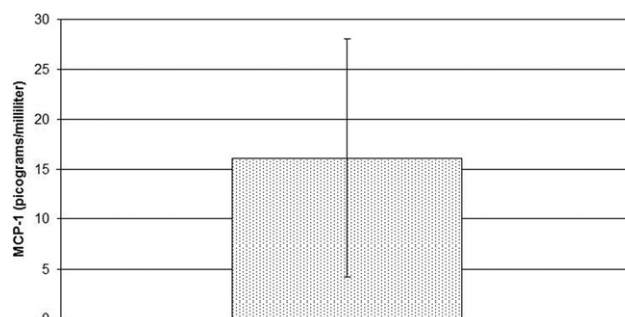


Figure 2. Mean value of monocyte chemoattractant protein-1 (MCP-1) in picograms per milliliter by multiplexed bead immunoassay for all patients in the series. The error bar represents the standard error of the mean.

Table 1. Detailed profile of all patients in the study with involved disk level and corresponding raw levels of inflammatory cytokines present in the disk space

Patient	Gender	Age (y)	Level	Pathology/ Diagnosis	pH	Complex G3 ELISA (450 nm)	Pain/ Correction	Presence of Weakness	IL-6	IFN- γ	MCP	MIP-1 β
1	F	46	C4-5	HNP	7.10	0.10	Yes	Yes	44	159	2	0
2	F	53	C5-6	HNP	7.12	0.20	Yes	Yes	0	39	0	9
3	F	70	C6-7	HNP/spondylosis	6.50	0.09	Yes	Yes	5	0	25	29
4	F	53	C4-5	HNP	6.67	0.32	Yes	Yes	0	0	7	0
5	F	41	C3-4	HNP	6.44	0.12	Yes	No	0	0	0	6
8	F	36	C5-6	HNP	6.58	0.01	Yes	Yes	0	10	2	4
9	F	42	C5-6	HNP	6.7	0.03	Yes	Yes	1	28	4	8
10	M	60	C6-7	HNP/spondylosis	7.15	0.23	Yes	No	1	0	121	0
11	M	57	C5-6	DDD, level aspirated, no diskectomy	5.89	0.00	No	No	0	67	0	4
11	M	57	C6-7	HNP	6.63	0.26	Yes	No	Low volume	Low volume	Low volume	Low volume

ELISA = enzyme-linked immunosorbent sandwich assay; IL = interleukin; IFN = interferon; MCP = monocyte chemotactic protein; MIP = macrophage inhibitory protein; HNP = herniated nucleus pulposus; DDD = degenerative disk disease.

Nonetheless, these data provide the first preliminary clinical evidence that the presence of the FAC is related to pH.

Our study has several notable limitations. The sample size is small, and thus no definitive conclusions can be drawn because of the possibility of false-negative results. An experimental versus observational study design is necessary to determine whether we are merely observing a degenerative phenomenon or whether the molecular profile is correlated to operable disease. Although NSAIDs may have affected our results, none of the patients experienced long-term relief upon use of these medications before they underwent lavage; therefore, our protocol was consistent with all of the patients. Moreover, because all patients were taking NSAIDs before undergoing lavage, use of the drugs may have attenuated the concentration of the complex identified in the disk spaces. Although we did not definitively prove cervical radiculopathy in our patients through electrodiagnostic testing, all patients had clear involvement of a particular nerve root on the basis of history, physical examination, and imaging findings. The sampling method is invasive and mimics diskography, which is known to have several inherent risks associated with its use and may in turn limit diagnostic potential [10]. The amount of fluid recovered can be variable and is largely dependent on the experience level of the surgeon. The procedure may result in only a small volume of aspirate after injection and aspiration of a variable amount of diluents [11]. Diskography also may lead to accelerated adjacent level disk degeneration, which is another important weakness of this technique [12]. These considerations, in conjunction with the biology of the disease process, may result in a wide range of concentrations for biomarkers that are assayed. Thus a sensitive assay with a high dynamic range is required. In the present study, these issues are addressed with the use of a sensitive heterogeneous sandwich ELISA, although the lack of a synthesizable positive control makes absolute quantification impossible. In addition, different values for the limit of detection would yield different estimates of positive versus negative test values.

CONCLUSION

The identification of the FAC in the disk space of patients with axial syndromes represents another step in furthering our understanding of the sequence of events leading to the development of painful states in degenerative disk disease, whether it be in the cervical spine or the lumbar spine. Further elucidation of the physiology in degenerative or painful states may assist us in developing therapeutic solutions to various pathologic conditions of the spine. Additional studies examining neurogenic claudication may better delineate specific roles of these inflammatory markers and further our understanding of different pathologies. However, because consensus has not been reached regarding a gold standard for the diagnosis or treatment of degenerative disk disease, studying it can be a challenge. Therefore we must approach it from many directions to fully define and understand its clinical manifestations. Nevertheless, we believe that this pilot study represents an important contribution to the understanding of the pathophysiology of degenerative disk disease of the cervical spine.

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