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Detection of bacterial DNA in painful degenerated spinal discs in patients without signs of clinical infection

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Abstract A local inflammatory and potentially painful response, of which the ultimate cause is unknown, has been described in nervous tissues in contact with degenerated disc material in patients with low back and leg pain. With the rationale that a possible cause of such inflammation could be bacterial infection, we utilized PCR (polymerase chain reaction) amplification of the 16S rRNA (ribosomal RNA) gene followed by gene sequencing, to investigate whether bacterial DNA might be detected in the degenerative discs of 10 patients operated for disc herniation or post-discectomy syndrome. One patient with disc hernia harbored DNA homologous to *Bacillus cereus*, and in one

patient suffering from post-discectomy syndrome, *Citrobacter braaki/freundii* DNA was detected. The finding demonstrates that 16S rRNA PCR can be a useful tool in search of bacterial DNA in degenerated discs, which in turn may be indicative of low-grade infection, manifesting itself only as pain rather than as clinical infection.

Keywords Low back pain · Herniated disc · Discitis · Polymerase chain reaction · Bacterial DNA

Introduction

In the spinal motion segment, degenerative changes including disc herniation are associated with pain, but why some persons with disc degeneration experience pain, whereas others do not is not fully understood [22]. Different structures in the degenerative segment, including the disc (annulus, nucleus pulposus and the cartilaginous end plate), may be involved in the pain generating process, which may be of mechanical and/or biochemical origin, including inflammatory responses mediated through proinflammatory cytokines [2, 5, 6, 7, 12, 18, 20, 24, 25, 26, 31].

Proinflammatory cytokine pain mediators like substance P and tumor necrosis factor alpha (TNF-alpha) are released or activated by nervous structures in contact with the nucleus pulposus [2, 23]. Substance P acts as a neuroregulator or neurotransmitter in the conduction of noci-

ceptive stimuli, and is a modulator of neuroimmunoregulation. Its presence has been demonstrated to increase endoneurial vascular permeability and nerve root injury, and it has been proposed to play a role in pain transmission [4, 5, 6, 9]. Increased concentrations of TNF-alpha, and also proinflammatory interleukins 1, 6 and 8 have been demonstrated in patients with chronic pelvic pain syndromes associated with inflammation of the prostate [27]. These substances have also been found to be elevated in patients with viral and bacterial infections [3, 16, 19]. Interestingly, the proinflammatory cytokines interleukin 1 and 6 have been detected at increased concentrations after stimulation of intervertebral murine disc cells with the bacteria *Escherichia coli* [28].

Considering the difficulties in diagnosing even a clinically manifest spondylitis/discitis [35], it should be possible that low virulent “subclinical” infections in the spinal disc could pass undiagnosed, experienced only as back,

leg or neck pain of shorter or longer duration. The pain mechanism could be mediated through the secretion of proinflammatory cytokines induced by immunostimulatory or toxic effects of bacteria or other pathogens. Kahn et al. reported that a low virulent bacteria and skin commensal like *Propionibacterium acnes* could be responsible for an autoimmune response in joints leading to inflammation and pain [17], and Chia et al. found this common bacteria in four cases of discitis following operations for disc herniation and epidural block [8]. Later, Stirling et al. hypothesized that *Propionibacterium acnes* could be responsible for sciatica in patients with disc herniation, and reported a high number of positive cases using serology and culturing [32].

Compared with conventional culturing, the technique of the polymerase chain reaction (PCR) has been shown to be more sensitive. Based on amplification of the 16S rRNA (ribosomal RNA) gene followed by gene sequencing, the technique has the ability to detect many bacteria that are difficult to culture [14, 34]. In a recent study the method was used to identify a wide spectrum of bacterial DNA in inflamed joints from patients with reactive arthritis (ReA), including some not previously associated with ReA [10].

In light of the above we deemed it to be possible that back pain or leg pain in patients with no signs of ongoing clinical infection (discitis) could be associated with the presence of bacteria or other pathogens. Using the PCR technique, we tried to detect the presence of bacteria in painful degenerated discs, both in patients operated for a disc herniation, and in patients operated for other pain conditions associated with disc degeneration.

Material and methods

At the end of 2002, using PCR amplification and sequencing of DNA from a highly informative region of the prokaryotic 16S rRNA gene [34], we examined 10 discs in 10 patients, aged 20–47 years

who had been operated for disc herniation ($n=9$) or post-discectomy syndrome ($n=1$). Five L4-L5 discs and five L5-S1 discs were addressed. Preoperative duration of back/leg pain varied from 2 weeks to more than 24 months. No patient had signs of ongoing clinical infection. Annulus ($n=10$) and nucleus ($n=7$) were sampled under sterile conditions and within 30–60 min stored at -70° . In one patient with post-discectomy syndrome where an interbody fusion was performed, a part from the cartilaginous end plate was also included, giving a total of 18 disc samples. Patient characteristics are given in Table 1.

Sampling procedure

The samples were taken openly (no needle), and a small portion of the annulus fibrosus was sampled ($n=7$), approximately between $2 \times 2 \times 1$ mm and $4 \times 4 \times 2$ mm in size, and a part of the thin posterior ligament could also be a part of this sample. From the nucleus pulposus some of the often quite large protruded, prolapsed, extruded or sequestered part was used ($n=10$), approximately $3 \times 3 \times 5$ – $10 \times 5 \times 5$ mm. The sample sizes were not measured accurately as we concentrated on just getting as much material as needed. We did try to avoid blood contamination and all operations except for the patient who was fused and where DNA from *Citrobacter braachii/freundii* was detected in the cartilaginous end plate (which was found in an "erect" position pointing backward towards the spinal canal from the back corner of the vertebrae), were performed through a microscope, with the use of bipolar diathermy, assuring a very "dry" operation field.

To avoid "mix-up" of the samples, first a part of the annulus was excised and directly put in a sterile dry tube and marked with AF, and with the patient's initials and personal number. The same procedure was used with the nucleus pulposus (NP) and the end-plate samples (CEP). After sampling, all patients received one single i.v. dose of 1 g cloxacillin.

PCR analysis procedure

Preparation of the mastermix, pretreatment of clinical samples, and amplification were performed in three different areas to avoid contamination. In addition the mastermix was UV-irradiated at 312 nm, $11,000 \mu\text{W}/\text{cm}^2$ for 10 min in portions of 40 μl to destroy any contaminating DNA before the DNA from the patient sample was added. To check the mastermix and the method for preparation of patient DNA for contaminating DNA, we used a negative control after at least each fifth sample.

Table 1 Patients operated for disc herniation (Nr 2 fusion): characteristics/results/comments

Nr	Patients, gender/age	Preop leg pain (weeks)	Operated level	Type ^a of herniation	Result 6 weeks leg pain postop	PCR findings	Comments
1	M/34	26	L4-L5	SE	Better	Yes ^c	+ Segmental pain
2	W/47	156	L5-S1	CEP ^b	Good	Yes ^d	+ Fibromyalgia
3	W/35	14	L5-S1	SE	Good/excellent	No	+ Segmental pain
4	M/38	104	L5-S1	SE	Good/excellent	No	
5	M/26	2	L4-L5	SE	Excellent	No	
6	W/26	36	L5-S1	SE	Good	No	
7	M/32	26	L5-S1	SE	Good/excellent	No	
8	W/46	28	L4-L5	SP	Excellent	No	
9	M/20	20	L4-L5	SP	Excellent	No	
10	M/37	4	L4-L5	TE	Good	No	Recurrence of herniated disc

^aSP Subligamentous protrusion, SE subligamentous extrusion, TE transligamentous extrusion, S sequester.

^bFusion after operation for disc herniation 2 years previously. A part of the cartilaginous end plate (CEP) was sampled.

^cDNA from *Bacillus cereus* in nucleus pulposus and in annulus fibrosus.

^dDNA from *Citrobacter freundii* in CEP.

Table 2 Bacterial DNA findings in two patients operated for HD and PDS

Gender, age level	Diagnosis	Bacterial species	Sampled	Sequence identity (%)	Score bits (E-value)	Base pair analyzed
M, 34 L4-L5	HD (SE) contained	<i>Bacillus cereus</i>	NP	NP=100	NP=620 (e-175)	NP=313
			AF	AF=100	AF=529 (e-148)	AF=275
W, 47 L5-S1	PDS (modic sign)	<i>Citrobacter braakii/freundii</i>	NP	-	CEP=1076/1068 ^a (0.0)	CEP=555
			AF	-		
			CEP	CEP=99		

HD Herniated disc.

PDS Post discectomy syndrome.

SE Subligamentous extrusion.

Modic sign type I (edema in the vertebrae adjacent to the disc).

NP Nucleus pulposus.

AF Annulus fibrosus.

CEP Cartilagenous end plate

^aScore bits for *C. braakii* and *C. freundii*, respectively. Further distinction is not possible due to difference of only one nucleotide between the two species in this region.

Approximately 3 mm³ of disc samples were sequentially treated with erythrocyte lysis solution, cell lysis solution and proteinase K, whereafter DNA was extracted by phenol-chloroform and precipitated by ethanol. After pelleting, DNA was suspended in distilled water and subjected to PCR amplification. Primers used were complementary to the conserved regions C1 and C5 [34] of the 16S rRNA gene of *E. coli*, which gives a fragment between bases 41 and 806, irrespective of the species of bacterial DNA (if any) in the specimen. PCR amplification was performed for 40 cycles, and 15 µl aliquots of the PCR products were separated in a 2% ethidium bromide stained agarose gel together with the amplification product of *Staphylococcus aureus* for verification of the product size and a negative control for at least every fifth sample. Positive samples of DNA were cut out, purified and cycle-sequenced using the ABI PRISM Big Dye TM Terminator Cycle Sequencing Ready Reaction kit, version 2.0 (Applied Biosystems, Stockholm, Sweden). DNA sequence search and alignment was performed by the BLAST program (NCBI), providing a Blast result of score and E-value indicating the probability that the match observed was consistent with chance.

Informed consent from patients referred to in the text was obtained.

Results

Three specimens from two patients were positive for bacterial DNA (Tables 1 and 2).

In the patient, male 34 years old, the predominant symptom was leg pain for 6 months, but there was also an element of segmental pain. The preoperative MRI revealed a mostly central herniation between L4 and L5, with a dominant right-sided component (Fig. 1). The patient denied previous infections of the intestines, prostate, or urinary tract. Preoperatively, the CRP was normal (<5). During surgery, a large subligamentous (contained) sequester was removed, and DNA sequences showing identity to *Bacillus cereus* were found in both the nucleus pulposus and the annulus (Table 2). The recovery was slow. After 5 months, an MRI showed a decrease in the preoperative herniation and no specific compromising of any nerve root. A small local "diffuse edema" behind the disc was seen, but this was not assessed as an infectious sign. After 7 months the patient claimed to be "much improved", although somewhat "unstable".

Fig. 1 Preoperative sagittal and axial T2 MRI of the 34-year-old male patient with disc herniation L4-L5 where bacterial DNA was detected

The patient, female 47 years old, had suffered from a post-discectomy syndrome at L5-S1 with predominantly back pain for 2 years. For many years she had had frequent urinary tract infections. She was currently suffering from diffuse muscle and joint pain, which was labeled as fibromyalgia. She was operated for endometriosis, and her uterus had been removed. Preoperative MRI revealed degeneration and slight modic signs type I [21], together with a central disc herniation but no obvious root compression (Fig. 2). CRP preoperatively was normal (<5). An instrumented posterior lumbar interbody fusion was performed. From the side not previously operated on, parts from the nucleus and the annulus were sampled, together with a part from the cartilagenous end plate.

DNA homologous to *Citrobacter braakii/freundii* was found only in the cartilagenous end plate (Table 2). After 3 months, the patient claimed to be 100% improved. After 5 months, the improvement was 70%, and the "usual muscle and joint pain" continued to bother her. The interbody fusion was radiographically solid at that time, with no reaction around the titanium implants. Due to some local tenderness the patient has agreed to device removal.

Fig. 2 Preoperative sagittal and axial T2 MRI of the 47-year-old female patient with disc herniation L5-S1 where bacterial DNA was detected

Discussion

We interpret the present results as true findings of bacterial DNA from *Bacillus cereus*, and *Citrobacter braaki/freund* in the disc samples, based on strict sample collecting and PCR procedure, and due to the presence of only one specific bacterial gene sequence in each sample. Furthermore, in male 34 years, samples from the nucleus and the annulus both demonstrated identical bacterial gene sequences. The finding that no PCR amplicons were found in the remaining 15 samples argues further against contamination. We do not consider possible contamination from blood cells a problem in this study, as bacterial DNA in whole blood in our experience, is difficult to detect using 16S rRNA PCR. For this to occur, the patient has to have as high amounts of bacteria as would regularly give symptoms of sepsis. On the other hand, we cannot exclude that discs of additional patients in the present series also contained bacterial DNA as each structure was only sampled once, and the presence of bacterial DNA in other regions or at other occasions was not a target for detection.

Other researchers have found ordinary skin bacteria, such as *Propionibacterium acnes*, in discs after using serology and conventional bacterial culturing. This bacterial species originates from the skin flora, and the association between such bacteriological findings (as well as the here presented species) and disc pathology and pain should be subjected to larger studies. Interestingly, in spite of the fact that the PCR method used in our study has been effective in detecting *Propionibacterium acnes* in other sample materials, this bacteria was not found in any of the discs, and the natural habitat of the presently detected bacteria was completely different (i.e. gastrointestinal and urogenital regions).

Using PCR both low virulent bacteria identified in the present study, have recently been detected in inflamed joints in patients with reactive arthritis [10], and also in clinically infected joints [30, 33, 36]. For a long time low back pain has been associated with infections in the gastrointestinal and urogenital tracts [1, 11, 29] and *Bacillus cereus* and *Citrobacter braaki/freundii* are common in

these two regions. A regional association between the natural habitats of the two detected bacteria and spinal discs is fully possible.

PCR may be more sensitive than culturing and, furthermore, only a minor fraction of gut bacteria, for example, can be cultured effectively. Therefore 16S rRNA PCR is a better choice for a broad search among microbes. However, it is possible that both the bacteria identified here might have also been detected by culturing, but lacking such information, we cannot exclude a mere presence of bacterial DNA in discs, without productive infection (i.e. an innocent bystander phenomenon as far as a clinical infection was concerned).

The supporting structures of the body, including discs, may constantly be under attack from hematogenously spread bacteria or other microorganisms as a result of incomplete elimination at other locations. It is possible that degeneration of the disc facilitates invasion of pathogenic organisms such as bacteria, virus and fungi [15], and/or hinders their elimination, resulting in a painful inflammatory and toxic immunological response. One reason why some degenerative discs "produce pain" when others do not, could be because of low grade bacterial infection (or indeed other pathogens) in the former. In a recent study, the authors described evidence of complement activation in pathological disc tissue, and an inflammatory response commonly triggered by microbial antigens [13]. While it should be noted that in the present study, we have neither aimed to find nor have we documented the presence of bacterial antigen or inflammatory reactions, this adds an intriguing possibility of an association between the presence of bacteria, inflammation and pain.

The present work reports a pilot study, with no intention of presenting proof that the presence of bacteria in discs causes low back pain, and we therefore did not include a control group, nor did we sample material from other parts of the same patient as controls. We were primarily interested in whether bacterial DNA at all could be detected in discs of patients without a recognizable (with current diagnostic tools) clinical infection, but with low back pain and leg pain. The disc is normally considered as a sterile environment, and detection of bacterial DNA in small amounts of disc tissue in 2 out of 10 patients is therefore surprising. Our findings support the possibility that the presence of bacterial DNA, indicating a low grade bacterial infection in a degenerated disc, may be one of the "hidden factors" sometimes related to back or leg pain of various etiologies.

At this point in time, our and earlier findings should be regarded as hypothesis generating. If, using controlled studies, a correlation between the presence of bacteria in discs and back pain in patients without clinical signs of ongoing discitis could be established, and pathogenic organisms sensitive to antibiotics are involved, this could influence our treatment strategies for different painful degenerative spinal conditions. Such studies are under way.

Conclusions

We consider it of interest that DNA sequences from bacteria previously associated with joint inflammation have

been detected in degenerated painful discs in patients with no signs of ongoing clinical infection.

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