Elevated IL-1 β and IL-6 levels in lumbar herniated discs in patients with sciatic pain

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Received: 22 March 2012 / Revised: 16 July 2012 / Accepted: 9 September 2012 / Published online: 27 September 2012
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Abstract

Purpose Previous experimental models have shown that proinflammatory cytokines modulate peripheral and central nociception. However, the direct correlation between inflammation and pain in patients remains unclear. Our aim is to correlate the levels of inflammation in the spine with pre- and postoperative pain scores after discectomy.

Methods Paravertebral muscle, annulus fibrosus (AF) and nucleus pulposus (NP) biopsies were intraoperatively collected from ten lumbar disc hernia (LDH) patients suffering from chronic sciatic pain and, as painless controls, five scoliosis patients. IL-1 β and IL-6 expressions in these biopsies were assessed by qPCR and western blot. The

amount of pain, indicated on a 0–10 point visual analogue scale (VAS), was assessed 1 day before surgery and 6 weeks and 1 year after surgery. For analysis purposes, LDH patients were grouped into painful (VAS $\geq$ 3.5) and non-painful (VAS < 3.5). LDH painful patient group showed a onefold increased mRNA expression of IL-1 β in the NP, and IL-6 in the AF and NP ($p < 0.05$ vs. controls).

Results By western blot analysis, both cytokines were clearly visible in all LDH biopsies, but not in controls. However, cytokine expression of the painful patient group did not differ from those of the non-painful patient group. In addition, there was no correlation between VAS scores and either marker.

Conclusions These findings support the idea that LDH is accompanied by a local inflammatory process. Yet, the lack of correlation between IL-1 β or IL-6 expression and the severity pain suggests that these cytokines may not play a leading role in maintaining a pain generating network.

Keywords IL-1 β · IL-6 · Chronic pain · Visual analogue scale · Herniated disc

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Introduction

The primary functions of the intervertebral disc include shock absorption, stabilization, movement and flexibility of the spine. These tasks are possible due to the proper composition and interaction of its two main structures, the hard external ring named annulus fibrosus (AF) and the soft core of the disc named nucleus pulposus (NP). When one of these structures, or both, bulges or migrates out of the normal intervertebral space delimitation, a herniated disc occurs. If a herniated disc develops, the protrusion from the disc could compress one of the nerve roots or the spinal

cord itself. The compression of neural tissue produced by herniated disc material may generate diverse clinical symptoms, among which pain is one of the most common presentations. The symptomatology that results from lumbar disc hernia (LDH), which produces a painful sensation radiating into the leg, is known as sciatica [1, 2]. The modalities to treat sciatic pain may include non-surgical treatment (physiotherapy and medication) and surgery. The general consensus is that conservative therapy should be followed during the first 6–8 weeks from the initiation of symptoms, unless absolute surgical indications are present (increasing muscle weakness, alterations of the urinary bladder function or opioid-resistant pain) [3, 4]. This non-surgical treatment results in recovery in about 80 % of the cases [4]. For the remaining patients the option of disc surgery is considered to be an acceptable treatment alternative, though the outcome after 1 year is comparable to that of non-surgical therapy [1]. This suggests that mechanisms other than pure mechanical compression may underlie the pathophysiology of sciatic pain in LDH.

It is generally recognized that neuroinflammation plays a crucial role in the generation of chronic pain. Numerous in vitro and animal models of pain have demonstrated the contribution of proinflammatory cytokines to peripheral and central nociception [5–10]. Also in human, local and systemically expressed proinflammatory cytokines have been implicated as mediators of pain [11–13]. Among these, TNF- α , IL-1 β and IL-6 are particularly interesting because of their hyperalgesic effects after nerve damage. However, while the contribution of TNF- α to chronic pain has been shown repetitively, data on the roles of IL-1 β and IL-6 in human sciatic pain are scarce. This study assessed if local IL-1 β and IL-6 levels in LDH patients related to presurgical pain and to the outcome of discectomy.

Methods

Patients and pain evaluation

This study was approved by the Ethics Committee at Maastricht University Medical Center. Fifteen patients were prospectively enrolled at the departments of neurosurgery and orthopedics, and included ten LDH patients suffering from leg pain for less than 1 year and five scoliosis patients without painful symptomatology or degenerative disc disease. The LDH patient group had a mean age of 41 years and consisted of five males and five females. The scoliosis patient group had a mean age of 21 years and consisted of three males and two females. LDH patients assessed the intensity of sciatic pain on a 0–10 (0, no pain; 10, worst pain) visual analogue scale (VAS) at 1 day before discectomy, and at 6 weeks and 1 year after surgery. The duration of symptoms before the surgical

procedure in LDH patients included in this study was greater than 3 months and less than 6 months in all cases. General clinical data were recorded prospectively and preoperative MRI was performed in all cases to corroborate the clinical examination findings. All patients in the LDH group ($n = 10/10$) were treated with non-steroidal anti-inflammatory drugs during the period previous to the surgery. Patients were suspended anti-inflammatory medication for 7 days before surgery. All patients underwent elective spinal surgery for the first time. Intraoperatively collected biopsies, obtained during discectomy in LDH patients and during corrective orthopedic surgery in scoliosis patients, were collected from the paravertebral muscle ($n = 15$), the AF ($n = 14$) and the NP ($n = 12$). Immediately upon collection, they were divided into two equal parts (one for qPCR and one for western blot) frozen in liquid nitrogen, and stored at -80°C . LDH patients were divided into two groups according to their postoperative pain scores.

Quantitative polymerase chain reaction

Total RNA extraction was performed using TRIzol (Invitrogen, CA, USA), according to the manufacturer's protocol. RNA purity and concentration were measured by optical density, using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Next, 1 μg RNA was first incubated for 10 min at 70°C to prevent secondary structures and then reverse transcribed using a First Strand cDNA Synthesis Kit (Fermentas International Inc., Canada). Gene expression analysis was performed in optical 96-well plates using a LightCycler 480 Real-Time PCR System. PCR conditions consisted of a 20 μl reaction volume, containing 10 μl SYBR Green I Master (Roche Diagnostics, IN, USA), 0.2 μl forward and reverse primer, 4.8 μl water and 250 ng cDNA in a volume of 5 μl . Amplicons were generated using the following primer sets: IL-1 β -sense (5'-TTGACGGACCC CAAAAGATG-3') and antisense (5'-AGAAGGTGCTC ATGTCCTCA-3'), and IL-6-sense (5'-GTTCTCTGGGAA ATCGTGG-3') and antisense (5'-TGTA CTCCAGGT AGCTATGG-3'). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene to normalize the samples; sense (5'-ACAGTCAGCCGCAT CTTC-3') and antisense (5'-CCAATACGACCAAATC CGTTG-3'). The conditions for the reaction included 50 cycles in a fixed sequence 94°C for 30 s, 63°C for 15 s, and 72°C for 15 s. At the end of the PCR, a general dissociation curve was made and amplicons were resolved on a 2 % agarose gel to assess product specificity.

Western blot analysis

Biopsies were homogenized in lysis buffer containing 0.01 M phosphate buffered saline (PBS), 1 % Igepal,

1 mM EDTA, 1 mM EGTA, 0.1 % Triton and complete protease inhibitor cocktail (Roche Diagnostics; 1 g weight tissue in 9 ml lysis buffer). Next, proteins (20 µg per lane) were separated by 10 % Tris-HCl SDS-polyacrylamide gel electrophoresis and then transferred to a pure nitrocellulose membrane for a period of 60 min at 100 V using a Bio-Rad Laboratories system. After transfer, the membranes were rinsed once with PBS and then blocked for 60 min in 1:2 blocking buffer:PBS (Odyssey blocking buffer; LI-COR Biosciences, NE, USA). Next, membranes were incubated overnight at 4 °C with either 1:500 diluted monoclonal mouse anti-human IL-1 β (clone 5C10, Santa Cruz Biotechnology, CA, USA) and 1:500 diluted monoclonal mouse anti-human β -actin (clone ACTBD11B7, Santa Cruz Biotechnology) or with 1:500 diluted polyclonal rabbit anti-human IL-6 (clone C-20, Santa Cruz Biotechnology) and 1:500 diluted monoclonal mouse anti-human β -actin (clone ACTBD11B7, Santa Cruz Biotechnology). Following primary antibody incubation, membranes were rinsed during three cycles with PBS/PBS + Tween/PBS and then incubated for 60 min at room temperature with their corresponding secondary antibody, i.e., Alexa Fluor 680 donkey anti-mouse IgG or Alexa Fluor 800 goat anti-rabbit IgG (1:10000; Molecular Probes, OR, USA). All antibodies were diluted in 1:2 blocking buffer:PBS. Finally, immunoreactive protein bands were visualized by enhanced chemiluminescence detection using a Odyssey Infrared Imaging System (LI-COR Biosciences), and quantified by calculating the optical density ratio of the 17 kDa IL-1 β or 80 kDa IL-6 band and the 43 kDa β -actin band using a digital imaging analysis system (ImageJ 1.42q NIH, USA).

Statistical analysis

A Kruskal–Wallis test was applied to statistically test differences in protein expression between painful and non-painful LDH patient groups and the scoliosis control group, followed by Dunn post hoc tests. The *p* value was considered significant with an $\alpha < 0.05$. Linear regression analysis was used to measure the correlation between VAS score and IL-1 β or IL-6 protein expression.

Results

VAS scores

Average $\pm$ SD VAS scores were 6.8 ± 1.2 (1 day before surgery), 3.0 ± 1.9 (6 weeks after surgery), and 3.9 ± 2.4 (1 year after surgery). Average $\pm$ SD delta VAS scores compared to presurgical evaluation were 3.8 ± 1.9 (6 weeks after surgery), and 3.0 ± 2.5 (1 year after

surgery). Based on the 1-year follow-up data, the LDH patient group was divided into a painful (VAS ≥ 3.5 ; $n = 5$; two females and three males) and a non-painful (VAS < 3.5 ; $n = 5$; three females and two males) outcome group. This division was performed, since a score of 4 points is described as a disruption in the quality of life.

LDH patients show a localized increase in IL-1 β and IL-6 mRNA expression

IL-1 β and IL-6 gene expression in paravertebral muscle samples from LDH patients were comparable to that from scoliosis controls (Fig. 1a, d). IL-1 β gene expression in AF samples from LDH patients was also comparable to that from scoliosis controls (Fig. 1b). Yet, the IL-6 gene was about 0.8 times more expressed in AF samples from painful LDH patients than in those from controls (Fig. 1e). Also, NP samples of LDH patients with a painful outcome showed a one time higher IL-1 β and IL-6 mRNA expression than controls (Fig. 1c, f). In neither tissue sample did IL-1 β or IL-6 expression levels of the non-painful patient group differ from that of the painful patient group or from controls 1 day before surgery, or 6 weeks and 1 year after surgery.

IL-1 β and IL-6 protein is expressed in LDH samples but not in controls

Immunoblotting showed no detectable IL-1 β or IL-6 in control samples, whereas both proteins were clearly visible in all LDH samples. However, IL-1 β and IL-6 protein expression varied between the different tissue types, with the lowest expression in muscle and the highest in NP samples (Fig. 2). There was no difference in IL-1 β or IL-6 protein level between the painful and the non-painful group in either tissue type 1 day before surgery, or 6 weeks and 1 year after surgery. On the contrary, this same technique showed the presence of both cytokines in all tissue samples from every disc hernia patient. However, certain differences were found between IL-1 β and IL-6 and among tissues. Quantifications of IL-1 β and IL-6 showed no significant differences between painful and non-painful groups of patients in muscle, AF and NP ($p > 0.05$) (Fig. 2). Nevertheless, the protein expression was evidently enhanced in AF for IL-6 and in NP for both cytokines (Fig. 2c, e, f). For IL-6, maximal band intensities of almost twofold over the reference protein were found in AF and NP (Fig. 2e, f).

Correlation between proinflammatory cytokine levels and pain intensity

No correlations were performed for paravertebral muscle samples since no significant differences were found in

Fig. 1 Distribution of individual IL-1 β (a–c) and IL-6 (d–f) mRNA levels in muscle (a, d), annulus fibrosus (b, e) and nucleus pulposus (c, f) samples from LDH patients and scoliosis controls ($n = 5$). Based on 1-year follow-up VAS scores, the LDH patient group was

divided in a painful (VAS ≥ 3.5 ; $n = 5$) and a non-painful (VAS < 3.5 ; $n = 5$) outcome group. Expression levels were normalized to GAPDH as reference gene and are represented as time-fold increase over controls. NS Non-significant, * $p < 0.05$ and ** $p < 0.01$

Fig. 2 Distribution of individual IL-1 β (a–c) and IL-6 (d–f) protein levels in muscle (a, d), annulus fibrosus (b, e) and nucleus pulposus (c, f) samples from LDH patients, and representative western blot for protein expression. Based on 1-year follow-up VAS scores, the LDH patient group was divided in a painful (VAS ≥ 3.5 ; $n = 5$) and a non-painful (VAS < 3.5 ; $n = 5$) outcome group. Protein levels were

normalized to β -actin. Particularly IL-6 was clearly present in annulus fibrosus and nucleus pulposus samples (e, f). There were no significant differences between both patient groups for either cytokine in either tissue type. M muscle, AF annulus fibrosus, NP nucleus pulposus

protein and mRNA levels. Correlation between VAS scores at 1 day before surgery, or 6 weeks and 1 year after surgery, and the proinflammatory cytokines mRNA relative expression levels showed no significant correlations among these parameters in painful and non-painful patients together (Fig. 3). As well, IL-1 β and IL-6 protein levels showed no correlation with the intensity of pain at 1 day before surgery, or 6 weeks and 1 year postoperative assessment (Fig. 4).

Discussion

This study primarily demonstrates the presence of IL-1 β and IL-6 in paravertebral muscle, AF and NP biopsies in LDH patients, but not in control subjects. By detecting proinflammatory cytokines in intervertebral disc biopsies from LDH patients, this study supports the idea that LDH is a condition that is accompanied by a chronic, local inflammation. Yet, the lack of correlation between IL-1 β or

Fig. 3 Within the LDH patient group, VAS scores at 1 year follow-up did not correlate with IL-1 β mRNA levels in annulus fibrosus (**a**, $r = 0.03$), IL-1 β mRNA levels in nucleus pulposus (**b**, $r = 0.05$), IL-6 mRNA levels in annulus fibrosus (**c**, $r = -0.11$) or with IL-6 mRNA levels in nucleus pulposus (**d**, $r = 0.23$)

IL-6 levels and the severity of sciatic pain suggest that these cytokines do not play a role in maintaining a pain generating network. In addition, LDH patients in the painful outcome group reported higher VAS scores after 1 year in comparison to the 6 weeks follow-up. This observation could suggest that the acute clinical improvement could be interpreted as a relief of the mechanical compression created by the LDH, and that the persistent progressive pain could be the consequence of a non-resolved neuroinflammatory process. Nevertheless, according to our findings the inflammatory process related to IL-1 β and IL-6 is apparently unspecific and homogenous between all LDH patients, since no significant differences were found between LDH patients with painful and non-painful outcome.

In recent clinical studies, elevated levels of proinflammatory cytokines (IFN- γ , IL-1 β , IL-2, IL-6 and TNF- α) correlated with elevated pain scores in various types of neurological and rheumatologic disorders [11–20]. In addition, we found that intervertebral TNF- α levels correlate with preoperative pain in LDH patients [21]. In these studies, analysis of inflammatory markers in human samples from peripheral blood, cerebrospinal fluid, periradicular fat tissue, skin punch biopsies, nerve biopsies and lumbar facet joints have demonstrated that both, pro- and anti-inflammatory cytokines play a role in pain control. However, it is difficult

to recognize a shared pathophysiological mechanism because these studies investigated a wide variety of different painful conditions. Another unresolved issue about the role of neuroinflammation in pain disorders is the extent to which local and/or systemic expressed cytokines contribute to the clinical symptoms, or if it is disease specific. For instance, studies of complex regional pain syndrome, painful neuropathies and intervertebral disc degeneration indicate that a local elevation of proinflammatory cytokines is directly related to the amount of pain [11, 13, 22]. Yet studies in patients with neuropathic, nociceptive and mixed types of chronic pain show that elevated peripheral markers related to pain sensation [12, 14, 15, 19]. We are equally in the dark regarding the temporal resolution of neuroinflammatory markers during the transition from an initial precipitating event to chronic pain. There is not enough knowledge to establish clear correlations between the duration of the inflammatory process and the severity that this temporal exposition would have on pain.

The clinical relevance from previous findings and our data still remains to be determined. The lack of a correlation between locally enhanced IL-1 β /IL-6 levels and pain sensation is in line with the observation that some anti-inflammatory drugs perform poorly as treatment for pain and suggests that it may be more efficient to target the pain-specific inflammatory profile. In this study, protein

Fig. 4 Within the LDH patient group, VAS scores at 1 year follow-up did not correlate with IL-1 β protein levels in annulus fibrosus (**a**, $r = 0.02$), IL-1 β protein levels in nucleus pulposus (**b**, $r = -0.08$), IL-6 protein levels in annulus fibrosus (**c**, $r = -0.24$) or with IL-6 protein levels in nucleus pulposus (**d**, $r = -0.31$)

and mRNA levels of both IL-1 β and IL-6 were clearly elevated. This finding was most pronounced for IL-6, which is in line with the observation that this cytokine is expressed persistently in inflamed tissue [23]. Apparently, this long-term exposure does not affect the severity of pain, possibly explained by the capacity of IL-6 to function as an anti-inflammatory cytokine at the same time [24, 25]. Succinctly, IL-6 dual role in both action and time expression could complicate the analysis and therefore the importance of this cytokine in association with pain symptoms. On the other hand, the role of IL-1 β is far more complicated since its contribution to pain has been described as a direct mechanical and thermal hyperalgesic agent on nociceptors, and with the induction of other molecules that are involved in pain sensation, including IL-6 [24].

Conclusion

Intervertebral discs biopsies from LDH patients express proinflammatory cytokines IL-1 β and IL-6. The amount of cytokine is, however, not correlated with the amount of leg pain that is experienced preoperatively or after decompressing surgery. Furthermore, our findings suggest that the acute pain relief obtained by patients could be related to the mechanical decompression, and that the chronic increasing pain could be a consequence of a non-resolved neuroinflammatory process.

Acknowledgments This study was supported by the Netherlands Organization for Scientific Research (NWO-VENI-916.76.042). We would like to acknowledge Prof. J.J. van Overbeeke and Prof. L.W. van Rhijn for their help to include patients in this study.

Conflict of interest None of the authors has any potential conflict of interest.

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