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Comparative immunohistochemical study of group II (synovial-type) and group IV (cytosolic) phospholipases A₂ in disc prolapse tissue

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Abstract Phospholipase A₂ (PLA₂) has been suggested to be present in herniated disc tissue and it could possibly be involved in sciatica/discogenic back pain mechanisms. In the present study the occurrence of two different phospholipase A₂ enzymes, (1) low molecular weight (14 kDa) group II synovial-type (sPLA₂) and (2) high molecular weight (85 kDa) group IV cytosolic (cPLA₂), were compared. Fifty-three disc prolapses obtained at disc operations were analyzed by immunohistochemistry, using anti-human monoclonal antibodies to sPLA₂ and cPLA₂, respectively. Only cell-associated (disc cells, hyaline cartilage chondrocytes) sPLA₂ and cPLA₂ immunoreactivity could be observed. The results showed that sPLA₂ was more common (25/53, 47%) than cPLA₂ (13/53, 25%). sPLA₂ and cPLA₂ were simultaneously present in 13 of 53 samples (25%). However, both PLA₂ enzymes were predominantly present in hyaline cartilage cells (sPLA₂: 16/53, cPLA₂: 5/53), being less commonly observed in disc cells (sPLA₂: 6/53, cPLA₂: 3/53). In addition, three samples for

sPLA₂ and two samples for cPLA₂ exhibited immunoreactivity in cartilage and disc cells simultaneously. sPLA₂ was observed in no other locations, but in 3 of 53 samples cPLA₂ was observed more diffusely in areas of granulation tissue, possibly in macrophages. No gender- or age-related dependence for either type of PLA₂ enzyme immunoreactivity could be observed. Neither did their occurrence relate to clinical data such as straight leg raising or neurological deficit. The results do not support a major role for either of the two disc-cell-associated PLA₂s in disc pathophysiology. For both enzymes, the major pool appears to reside in cartilage tissue cells, presumably in dislodged end-plate fragments. Disc cells are apparently unlikely candidates for major PLA₂ storage.

Key words Disc prolapse · Inflammation · Phospholipases A₂

Introduction

The PLA₂ isoforms include the secretory (also called synovial-type) group II extracellular 14 kD isoform (sPLA₂) and the group IV cytosolic 85–110 kD isoforms (cPLA₂) [6, 21, 22].

It has been suggested that both types of PLA₂ (sPLA₂ and cPLA₂) are implicated in inflammation. These enzymes catalyze the release of arachidonic acid, which is then converted, for instance, to prostaglandins by the cyclo-oxygenases (COX-1 and COX-2). Thus arachidonic acid mobilization seems to be dependent on both types of PLA₂ [6, 16, 19, 21, 22].

PLA2 enzymes may also be involved in cell proliferation [6]. sPLA2 could play a role in cellular defence against infection, because this enzyme activity is bactericidal against *Escherichia coli* [6].

It has been shown that sPLA2 and cPLA2 can be activated by cytokines or mitogens [15, 16] and it was also demonstrated that dexamethasone pretreatment significantly reduced cPLA2 level as well as prostaglandin E2 release in studies of human epithelial cells [16].

The secretory phospholipase (sPLA2) is assumed to be active only in the extracellular space in the presence of millimolar concentration of Ca^{2+} , whereas cytosolic phospholipase (cPLA2) is the enzyme responsible for intracellular-signal-mediated release of arachidonic acid, which is regulated by intracellular Ca^{2+} concentration and phosphorylation [2, 19, 21, 22].

Elevated levels of sPLA2 enzyme are detectable in the serum of patients with septic shock or acute pancreatitis and inflammatory exudates, e.g., synovial fluid of rheumatic joints. High activity of this enzyme is further associated with asthma, psoriasis, and arthritis [2, 6, 15].

In the rat adjuvant arthritis animal model, Burgermeister et al. [2] suggested that an inhibitor of cPLA2 had therapeutic effect in chronic inflammation, whereas inhibitors of sPLA2 had no beneficial effect.

There are presently conflicting views regarding a possible clinical importance of phospholipases A₂ in disc tissue pathophysiology, sciatica, and discogenic pain in particular. So far only sPLA2 has been investigated in intervertebral disc tissue samples [7, 10, 12, 20]. Some investigators suggested that herniated lumbar disc, especially in the case of sequestration, has a high PLA2 activity (sPLA2), which could be of pathophysiological importance in sciatica [17, 20]. On the other hand, in other studies, e.g., by Grönblad et al. [10], neither herniated nor degenerated disc tissue samples demonstrated a higher sPLA2 activity than control disc tissue samples. On the contrary, sPLA2 activity was higher in the control samples, i.e., non-herniated and non-degenerated disc samples, than in herniated disc tissue samples.

There is no doubt that PLA2 enzymes are involved in inflammatory diseases [5, 14], in addition to their more basic role in phospholipid metabolism of all cells [5]. Their role in intervertebral disc tissue and disc tissue pathology is, however, presently less clear. It was recently suggested [12] that sPLA2 enzyme may be an important regulator substance of ordinary metabolism in intervertebral disc tissue, possibly maintaining cellular homeostasis. Different results in prior studies have also depended on different assays used for assessing PLA2 enzyme activity [10, 12, 20].

In the present study two different PLA2 enzymes, sPLA2 and cPLA2, were localized, in parallel, using monoclonal antibodies to sPLA2 and cPLA2, respectively.

Materials and methods

Patients

Samples of herniated disc tissues were collected from 53 patients at disc surgery for radicular pain. As classified by the operating surgeon, 25 of 53 were sequestered disc fragments, 25 of 53 were disc extrusions, and 3 of 53 were disc protrusions [23]. Thirty of the patients were men, 23 women. Their median age was 44 (range 25–75) years. Preoperatively they had root pain symptoms for (median) 4 months (range 0.25–24 months). Further clinical data for individual patients are shown in Tables 1 and 2 together with the immunostaining results.

Treatment of tissue

Prior to freezing, all tissue samples were briefly rinsed in saline solution. They were then frozen in liquid N₂ and kept at –70 °C until analysis by immunohistochemistry.

Antibodies

Two different antibodies to PLA2 were used:

1. A monoclonal antibody (IgG₁ isotype) against sPLA2 (14 kDa; Boehringer Mannheim, Mannheim). According to the manufacturer manual this antibody reacts specifically with human sPLA2, but does not cross-react with pancreatic sPLA2 or cPLA2. It recognizes in Western blot a single band of 14 kD.
2. A monoclonal antibody (IgG_{2b} isotype) against cPLA2 (85 kDa; Santa Cruz Biotechnology, Santa Cruz, Calif.). According to the manufacturer manual (Santa Cruz Biotechnology) the present cPLA2 antibody reacts specifically with cPLA2 of mouse, rat, and human origin by Western blotting, immunoprecipitation, and immunohistochemistry, and is non-cross-reactive with other phospholipases.

Non-specific immunoglobulin isotypes IgG₁ and IgG_{2b} were used as immunostaining controls for the respective antibodies.

Immunohistochemistry

The tissue specimens were sectioned at 8 µm using a Reichert-Jung (Cambridge Instruments, Nussloch, Germany) cryostat. For each antibody, three random sections with a 200-µm interval were stained identically, in parallel, with antibody to sPLA2 and cPLA2. Prior to immunostaining the sections were prefixed for 10 min at –20 °C in acetone. For localization of the respective PLA2 enzymes the alkaline phosphatase anti-alkaline phosphatase method was used as has been described [9]. For the respective antibodies the dilutions 1:200 (sPLA2) and 1:1000 (cPLA2) were used. Cells were either characterized as showing immunostaining “+” or lacking it altogether “–”. Cells showing immunostaining were not quantitated due to their low numbers.

Group comparisons were performed by the two-sided chi-square test. $P < 0.05$ was considered statistically significant.

Results

The results are summarized in Tables 1 and 2. As can be seen from the Tables, sPLA2 was more commonly observed (25/53, 47%) than cPLA2 (13/53, 25%). All of the samples exhibiting cPLA2 immunoreactivity simultane-

Table 1 Clinical patient data (arranged in age order) and immunohistochemical localization of group II (sPLA2) and group IV (cPLA2) phospholipases A₂ in 53 disc prolapses (*DurP* duration of radicular pain symptoms prior to disc operation, *SLR* straight leg raising in degrees, *n.r.* not reported, *Ext* extrusion, *Seq* sequester, *Pro* protrusion, *dc* in disc cells, *cart* in cartilage cells, *other* in cells other than disc cells or cartilage)

Patient (sex/age)	DurP (mo)	SLR ^a	Level	Type ^b	PLA2 immunoreactivity	
					sPLA2	cPLA2
1. M/25	3.5	−/50	L5-S1	Seq	−	−
2. F/26	0.25	n.r.	L4-L5	Seq	+ (cart)	+ (cart)
3. F/27	6	60/30	L5-S1	Pro	+ (cart)	−
4. M/27	9	60/−	L5-S1	Ext	−	−
5. M/28	7	−/−	L3-L4	Ext	−	−
6. M/29	1.5	50/30	L5-S1	Ext	−	−
7. F/30	6	45/−	L5-S1	Pro	−	−
8. M/31	2	30/30	L4-L5	Seq	+ (cart)	−
9. M/32	6	−/45	L4-L5	Ext	−	−
10. F/33	2	45/60	L5-S1	Seq	−	−
11. M/33	5	60/40	L4-L5	Ext	−	−
12. F/35	5	−/50	L5-S1	Ext	+ (cart)	−
13. F/36	6	−/−	L5-S1	Ext	−	−
14. M/36	3	45/−	L5-S1	Ext	+ (dc)	+ (dc, cart)
15. F/37	11	−/−	L5-S1	Ext	+ (cart)	+ (cart)
16. M/37	4	40/−	L5-S1	Ext	−	−
17. F/38	0.75	60/−	L3-L4	Seq	−	−
18. F/39	3.5	40/−	L5-S1	Seq	+ (cart)	+ (dc)
19. M/39	2.5	n.r.	L3-L4	Ext	+ (cart)	−
20. M/40	10	−/45	L5-S1	Seq	−	−
21. M/41	4	−/−	L5-S1	Seq	+ (dc)	−
22. F/41	6	40/50	L5-S1	Seq	+ (dc, cart)	+ (dc, cart)
23. F/41	6	−/−	L4-L5	Seq	+ (cart)	+ (other)
24. M/41	8	−/−	L3-L4	Ext	−	−
25. M/42	12	−/60	L3-L4	Ext	+ (cart)	−
26. M/43	5	30/−	L5-S1	Seq	−	−
27. M/44	0.25	40/40	L4-L5	Ext	−	−
28. F/44	2.5	60/−	L4-L5	Seq	+ (cart)	+ (cart)
29. F/44	0.5	−/40	L5-S1	Seq	+ (cart)	−
30. F/44	6	−/−	L1-L2	Seq	−	−
31. M/45	12	−/−	L4-L5	Ext	+ (dc)	+ (dc)
32. F/45	6	−/60	L5-S1	Ext	−	−
33. M/45	4	40/−	L5-S1	Ext	−	−
34. F/46	6	−/−	L5-S1	Ext	+ (dc)	−
35. F/46	4	60/40	L5-S1	Ext	−	−
36. M/47	4	60/−	L4-L5	Ext	+ (cart)	−
37. M/47	2	−/30	L5-S1	Seq	+ (cart, dc)	−
38. M/47	24	20/−	L5-S1	Seq	+ (dc)	+ (other)
39. F/47	9	30/30	L4-L5	Ext	+ (cart)	−
40. M/51	4	40/20	L4-L5	Seq	+ (cart)	−
41. F/51	3	50/−	L5-S1	Ext	−	−
42. F/51	2	45/−	L4-L5	Seq	−	−
43. F/52	2.5	40/60	L4-L5	Seq	+ (cart)	+ (other)
44. F/53	5	60/−	L4-L5	Seq	−	−
45. M/53	7	−/−	L3-L4	Seq	−	−
46. M/54	6	60/40	L3-L4	Ext	−	−
47. M/56	1	30/30	L4-L5	Seq	−	−
48. M/58	2.5	−/60	L4-L5	Ext	−	−
49. M/59	0.75	30/−	L4-L5	Seq	+ (cart)	+ (cart)
50. M/59	3	−/30	L4-L5	Seq	−	−
51. F/64	4	60/−	L4-L5	Pro	−	−
52. M/68	3	−/−	L4-L5	Seq	+ (dc)	+ (dc)
53. M/75	3	−/−	L3-L4	Ext	+ (dc, cart)	+ (cart)

^a − = negative test ($\geq 70^\circ$), −/− = bilaterally negative test

^b According to classification of Tolonen et al. [23]

Table 2 Imaging and clinical data of patients, in the same order as in Table 1. (Mye Myelography)

Imaging ^a	SLR	Motor deficit ^b	Reflexes ^c		Sensory deficit ^b	PLA2 immunoreactivity	
			Knee	Ankle		s PLA2	cPLA2
1. CT	–/50	–	+/+	+/-	–	–	–
2. CT	n.r.	+	-/-	-/-	+	+ (cart)	+ (cart)
3. CT, mye.	60/30	–	-/-	-/-	–	+ (cart)	–
4. CT	60/-	–	-/-	+/-	–	–	–
5. CT	-/-	–	+/+	+/+	–	–	–
6. CT	50/30	–	+/+	+/-	–	–	–
7. CT	45/-	–	+/+	+/+	–	–	–
8. CT	30/30	+	+/+	-/-	–	+ (cart)	–
9. CT	-/45	–	+/+	-/-	–	–	–
10. CT	45/60	–	+/+	-/+	+	–	–
11. CT	60/40	–	+/+	+/+	–	–	–
12. CT	–/50	+	-/-	+/+	–	+ (cart)	–
13. Mye.	-/-	–	+/+	-/+	–	–	–
14. CT	45/-	–	+/+	+/+	–	+ (dc)	+ (dc, cart)
15. CT	-/-	–	+/+	-/-	–	+ (cart)	+ (cart)
16. CT	40/-	–	+/+	+/+	+	–	–
17. CT, mye.	60/-	–	+/+	+/+	+	–	–
18. CT	40/-	–	+/+	+/-	–	+ (cart)	+ (dc)
19. MRI	n.r.	–	-/+	+/+	–	+ (cart)	–
20. CT	–/45	+	+/-	+/+	–	–	–
21. CT	-/-	–	+/+	+/-	–	+ (dc)	–
22. CT	40/50	–	+/+	+/-	–	+ (dc, cart)	+ (dc, cart)
23. CT	-/-	+	+/+	+/+	–	+ (cart)	+ (other)
24. CT, mye.	-/-	–	+/+	+/-	–	–	–
25. CT, mye.	–/60	–	+/-	+/+	–	+ (cart)	–
26. CT	30/-	–	+/+	+/-	–	–	–
27. Mye.	40/40	+	+/+	-/-	+	–	–
28. CT	60/-	–	+/+	+/-	–	+ (cart)	+ (cart)
29. CT	–/40	+	+/+	-/-	+	+ (cart)	–
30. MRI	-/-	–	+/+	+/+	–	–	–
31. CT, mye.	-/-	–	+/+	+/+	+	+ (dc)	+ (dc)
32. CT	–/60	–	+/+	+/+	–	–	–
33. CT	40/-	–	+/+	-/+	–	–	–
34. CT	-/-	–	+/+	+/+	–	+ (dc)	–
35. CT	60/40	–	+/+	+/+	–	–	–
36. CT	60/-	+	+/+	+/-	–	+ (cart)	–
37. MRI	–/30	–	+/+	-/-	–	+ (dc, cart)	–
38. CT	20/-	–	+/+	+/-	–	+ (dc)	+ (other)
39. Mye.	30/30	+	+/+	+/-	+	+ (cart)	–
40. CT	40/20	–	+/+	+/+	–	+ (cart)	–
41. CT	50/-	–	+/+	+/-	–	–	–
42. CT	45/-	–	+/+	-/-	–	–	–
43. CT	40/60	+	+/+	+/+	–	+ (cart)	+ (other)
44. MRI	60/-	–	+/+	-/+	–	–	–
45. CT, MRI	-/-	+	-/-	-/-	–	–	–
46. MRI	60/40	+	+/-	-/-	–	–	–
47. CT	30/30	+	+/+	+/+	–	–	–
48. CT	–/60	+	+/+	+/+	–	–	–
49. CT	30/-	–	+/+	+/+	+	+ (cart)	+ (cart)
50. CT	–/30	–	+/+	+/+	–	–	–
51. Mye.	-/-	–	+/+	-/-	–	–	–
52. Mye.	-/-	+	+/+	+/+	+	+ (dc)	+ (dc)
53. Mye.	-/-	+	+/+	+/+	+	+ (dc, cart)	+ (cart)

^aImaging modality that identified nerve root compression by herniated disc

^b+ indicates presence of deficit, – indicates no abnormality in this respect

^c+ indicates a normal ankle or knee jerk, – indicates absence of reflexes

Fig. 1 Low-magnification view showing sPLA2 immunoreactivity in chondrocytes of end-plate hyaline cartilage to the left (C) and in disc cells to the right (arrows). Alkaline phosphatase anti-alkaline phosphatase immunostaining, counterstaining with hematoxylin (original magnification $\times 110$)

Fig. 3 Immunoreactivity in disc herniation tissue disc cells (arrows) to sPLA2. Alkaline phosphatase anti-alkaline phosphatase immunostaining, hematoxylin counterstaining (original magnification $\times 280$)

Fig. 2A, B Immunoreactivity in disc herniation tissue cartilage cells (C) to phospholipase A₂ (PLA₂). A sPLA₂, B cPLA₂. Alkaline phosphatase anti-alkaline phosphatase immunostaining, counterstaining with hematoxylin (original magnification $\times 280$)

ously exhibited immunoreactivity to sPLA₂. The observed immunoreactivity was in most samples, and for both sPLA₂ and cPLA₂, located in cartilage tissue cells (Figs. 1, 2). The ratio of immunoreactivity in cartilage tissue cells to disc cells (Fig. 3) was 16:6 for sPLA₂ and 5:3 for cPLA₂. In addition, three samples for sPLA₂ (Fig. 1) and two samples for cPLA₂ contained immunoreactivity in cartilage and disc cells simultaneously. Immunoreactivity to sPLA₂ was not observed in any other location, whereas there were three samples where cPLA₂ was observed in areas of granulation tissue, possibly in macrophages (not shown). Cartilage tissue was present in 27 of the 53 disc samples studied (51%) and immunoreactivity to either sPLA₂ or cPLA₂ was observed in cartilage cells in 20 of 53 disc samples (38%) (Table 1). Thus, when cartilage tissue was present, it was in 74% of cases immunoreactive for PLA₂.

Of the 23 female patients, 12 (52%) exhibited immunoreactivity to sPLA₂, whereas 13 of 30 males (43%) did so. There was thus no clear dependence on gender ($\chi^2 = 0.41$, $P < 0.60$). For cPLA₂ immunoreactivity, 7 of 23 female patients (30%) and 6 of 30 male patients (20%) showed such immunoreactivity. There was thus no gender-associated difference in immunoreactivity ($\chi^2 = 0.77$, $P < 0.40$).

As can be seen from Table 1, no clear age dependence for either PLA₂ enzyme immunoreactivity could be observed either.

When results for PLA₂ immunoreactivity were compared between those having (16/53) and those lacking (37/53) a motor deficit (Table 2), no relationship could be observed for either sPLA₂ ($\chi^2 = 2.16$, $P < 0.20$) or cPLA₂ ($\chi^2 = 0.56$, $P < 0.50$).

When results were assessed in relation to straight leg raising (SLR) (Table 1), 39 of 53 patients were SLR positive. Of these, 16 of 39 (41%) exhibited sPLA₂ and 7 of

39 (18%) did so for cPLA2. In patients with bilaterally negative SLR, 7 of 12 (58%) were immunopositive for sPLA2 and 5 of 12 (42%) for cPLA2. For two patients SLR was not reported. These results did not suggest a higher prevalence of sPLA2 or cPLA2 immunoreactivity with positive SLR.

Discussion

Phospholipase A₂ is widely known as a potent inflammatory enzyme [6, 16, 19, 21, 22]. In previous studies high levels of phospholipase A₂ activity in lumbar disc herniations were measured using biochemical assessment [17, 20]; however, PLA2 was not localized. In addition the authors [17, 20] did not clarify whether the studied disc tissue samples contained fragments of end-plate or not. It may be of relevance for such measurement of PLA2 enzyme activity that herniated disc tissue is a heterogeneous tissue, consisting of various proportions of nucleus pulposus, annulus fibrosus, and end-plate [1, 11, 13]. Such measurable PLA2 enzyme could originate in either end-plate cartilage cells or disc cells.

With the immunohistochemical method used in the present study, both sPLA2 and cPLA2 were observed in a subgroup of the disc prolapse samples studied. sPLA2 was more commonly observed, but similar to cPLA2 it was mostly present in cartilage tissue cells, not in disc cells. Such hyaline cartilage may, for instance, have represented end-plate fragments that had dislodged with the disc herniation. A prior detailed study of disc herniation tissues [13] demonstrated end-plate cartilage in 49% of the disc herniations studied. Other studies have also reported cartilaginous end-plate in disc herniation samples. According to a study by Brock et al. [1], 44% of disc sequesters consisted mainly of end-plate material. In the study on cervical disc herniation tissues by Kokubun et al. [11], all the surgical specimens analyzed had evidence of cartilaginous end-plate fragments.

In the present study hyaline cartilage tissue, perhaps originating from the end-plate, was observed in 51% of the samples, and most of these cartilages (74%) exhibited PLA2 immunoreactivity. Thus, when PLA2 enzyme activity is measured biochemically in disc prolapses, the presence or absence of end-plate material, which seems to be often present in disc prolapses, should be reported. Otherwise, seemingly high PLA2 enzyme activity may be due to mainly end-plate material in the measurement sample.

The immunohistochemical results of the present study are similar to those previously reported, when a different (polyclonal) antibody to sPLA2 was used, employing avidin-biotin-complex (ABC) immunostaining [10]. Somewhat different results from the present were, however, obtained by Miyahara and co-workers [12], who observed sPLA2 immunoreactivity "in all of the chondrocytes ex-

amined in both annulus fibrosus and nucleus pulposus". The method used in their study was, however, somewhat different and as far as the authors know, the effect of such differences in tissue treatment and fixation on PLA2 immunoreactivity has not been studied in greater detail. However, in the present study immunostaining of cartilage was clearly visible with both of the PLA2 antibodies used, supporting the validity of the method and making it unlikely that specific immunostaining had been underreported.

It has been suggested that very high PLA2 activity in pathological disc tissues may explain some of the non-mechanical influences in radiculopathy [7, 20], but more recent studies [10, 12] did not suggest higher levels of PLA2 activity in herniated discs in comparison with control disc tissues that were analyzed in parallel, employing identical assays. Miyahara et al. [12] did, however, report higher PLA2 levels in disc tissues than in normal terminal ileal mucosa and in inflamed colonic mucosa. In another recent study [10], PLA2 enzyme activity in disc tissues was only seldom as high as that measured with the identical assay in inflamed synovial fluid from patients with arthritis. There is thus not presently convincing evidence that PLA2 is a major contributor to disc tissue pathology or a key chemical substance in low back pain syndromes, even if results from various studies differ somewhat.

sPLA2 is known to exist in both an extracellular form, e.g., in synovial fluid and in blood serum, and in a cell-associated form [6, 19]. It was suggested that sPLA2 is synthesized as an active enzyme which is stored in secretion granules and is exocytosed after activation of cells by cytokines (interleukin 1 β , interleukin-6, tumor necrosis factor- α) or lipopolysaccharide [2, 6, 21]. In the present study sPLA2 was located only in cartilage or disc cells, not in extracellular matrix. An explanation for this observation may be that either cartilage and/or disc cells were not activated or that the amount of extracellular sPLA2 was not large enough to be detected by our immunocytochemical technique.

The role of sPLA2, and possibly also of intracellular cPLA2, as was observed in the present study, in disc tissues may be more related to normal metabolism of disc cells and disc tissue homeostasis [12, 18] than to disc tissue pathology. When released from the intracellular compartments of cartilage cells or perhaps disc cells, either PLA2 enzyme could have deleterious effects, depending on their concentrations at the target sites. It is not presently known, however, whether the storage pool present in disc cells, and demonstrable by immunohistochemistry, will suffice to produce, for example, nerve root damage. There are, however, recent studies that have shown such pathological effects on nerve root function by exogenously applied PLA2 enzyme in animal models [4]. Whether such effects are also present in vivo in humans, following the extrusion of intervertebral disc tissue, is, however, not known at present. Since PLA2 is a key en-

zyme in the inflammatory cascade [5, 14], its exact role, and more broadly the role of inflammation and various inflammatory mediators, in disc tissue pathology and low back pain syndromes should be determined by further studies.

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