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Cytokines and growth factors in the protruded intervertebral disc of the lumbar spine

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Abstract Nerve root irritation induced by factors produced by the intervertebral disc may play a crucial role in the pathophysiology of sciatic pain production. In this study we used immunohistochemistry to investigate the presence of transforming growth factor-β1 (TGF-β1), insulin-like growth factor-1 (IGF-1), interleukin-6 (IL-6), IL-6-receptor (IL-6R) and fibronectin in lumbar disc bioptic specimens from 30 patients with disc herniation (protrusion type). Chondrocytes of herniated discs stained positive for TGF-β1, IGF-1, IL-6 and fibronectin. We demonstrated for the first time the presence of IL-6-R in the chondrocytes of herniated tissue. Specimens from autoptic healthy tissue were used as controls. In these sections no immunoreaction for TGF-β1, IL-6, or IL-6R was found, while they expressed IGF-1 and fibronectin, but in lower quantities than herniated discs. These results demonstrated the production of factors such as TGF-β1, IGF-1, IL-6, IL-6R and fibronectin at the site of lumbar disc herniation.

Keywords Herniated disc · Immunohistochemistry · Transforming growth factor-β1 · Insulin-like growth factor-1 · Interleukin-6

Introduction

The pathogenetic mechanism of low back pain and nerve root damage in lumbar disc herniation is the subject of ongoing debate. In particular, given that the mechanical-ischaemic hypothesis alone cannot explain the cause-effect relationship between disc degeneration and neurological impairment, a biochemical mechanism is likely to be involved. It has recently been demonstrated that the autologous nucleus pulposus can induce histological and functional changes in spinal nerve roots when applied epidurally [17]. Cells of the nucleus pulposus can produce prostaglandin E2, interleukin-1 and interleukin-6 (IL-6) [31], phospholipase A2 [26], and growth factors like fibroblast-like growth factor (FGF) [32] and insulin-like growth factor-1 (IGF-1) [18]. These factors are known to control cell metabolism and to promote inflammatory processes. Moreover, recent studies suggest a critical role for IL-6 and its receptor in the modulation of pain [8]. The mechanisms of action underlying the possible effect of these factors on spinal nerve root structure and function are, however, still unknown. In addition, the fact that nerve roots differ from peripheral nerves in their anatomical, biomechanical, and physiological properties prevents extrapolation from the extensive literature and the numerous experimental models of peripheral nerve compression to the pathophysiology of root injury [5, 23, 25]. The working hypothesis of the present study is that transforming growth factor-β1 (TGF-β1), IGF-1, IL-6 and IL-6-receptor (IL-6R) can be produced at the site of herniation. This hypothesis was immunohistochemically explored by analysing normal and protruded intervertebral disc tissue for these factors.

Materials and methods

Patients and specimens

Intervertebral disc specimens, including the nucleus pulposus with the anulus fibrosus, were obtained from 30 patients undergoing herniectomy and discectomy at the Orthopaedic Department of the Ancona Medical School between April 1998 and February 1999. Patients were 19 men and 11 women, with a mean age of 40.7 years (±6.3 SD). Informed consent to specimen collection was obtained from all patients. Symptom duration before surgery (sciatic pain) was longer then 1 year in all the patients. The levels involved were L4-5 (15 patients), L5-S1 (12 patients), L2-3 (1 patient), and L3-4 (2 patients). Disc herniation was assessed by computed tomography (CT) and/or magnetic resonance imaging (MRI), and confirmed by operative findings. Only protruded intervertebral discs bulging into the spinal canal without a breach of the posterior longitudinal ligament were studied. Autoptic L4-5 disc tissue from seven age-matched (mean age 42.2±5.9 SD) subjects with no history of back pain was collected and used as control. The time interval between death and disc harvesting was less then 24 h. All specimens were en-bloc embedded in paraffin, longitudinally and transversally cut into 5-μm-thick serial sections, and stained with haematoxylin-eosin-alcian blue.

Immunohistochemistry

Sections were processed by the standard avidin-biotin peroxidase complex procedure (Vectastain Elite kit, Vector, Calif, USA). Non-specific binding was blocked with 3% normal goat serum in a phosphate-buffered saline solution pH7.4 (PBS) for 30 min at room temperature. The slides were then incubated with primary antibodies overnight at 4°C. The following antibodies were used: monoclonal anti-TGF-β1 (1:50), polyclonal anti-IGF-1 (1:50) (both from Chemicon, Calif, USA), polyclonal anti-IL-6 (Endogen Inc, Boston, USA) (1:50), monoclonal anti-IL-6R (1:50) (Chemicon), polyclonal anti-fibronectin (1:600) (Sigma, Italy) and monoclonal anti-α-smooth muscle actin (Novocastra Laboratories, UK) (1:100). Rabbit and mouse immunoglobulins at the same dilutions as the primary antibodies were used as controls. Peroxidase activity was revealed by incubation with 0.05% 3,3'diaminobenzidine tetrahydrochloride (Sigma) in PBS containing 0.03% peroxide for 5 min at room temperature; slides were then washed, dehydrated and mounted with Eukitt (O.Kindler GmbH & Co., Germany). Experiments were reproduced in triplicate and slides were examined under a Zeiss Axiophot (Germany) light microscope by two experienced observers blinded to each other and to the harvested tissue. The intensity of the immunostaining was evaluated by considering reactive cells or fibres in 20 random fields per slide (lens: 20×). Staining intensity was evaluated on a grey scale ranging from 0 (white) to 255 (black), considering values less than 30 as background. Images from immunostained sections were captured using a Sony XC77 CCD camera connected to Macintosh IIvX computer (Apple Computer, Cupertino, Calif, USA) by means of a Scion LG3 frame grabber (Scion Corporation, Frederick, Mass, USA); data were analysed using one-way analysis of variance (ANOVA).

Results

All the herniated tissue samples stained positive for alcian blue, which reveals the presence of a cartilaginous matrix rich in proteoglycans. In the nucleus pulposus, chondrocytes were surrounded by a strongly alcian blue-positive pericellular matrix, whereas staining was weaker in the anulus fibrosus. In many areas, a crowding of 5–20 chondrocytes (chondrocyte "cloning") was observed (Fig. 1). Cells were scattered irregularly through the collagen fibres, which exhibited the characteristic fish-bone arrangement [30]. In one case a fragment of the cartilaginous endplate was observed in contact with the outer anulus fibrosus.

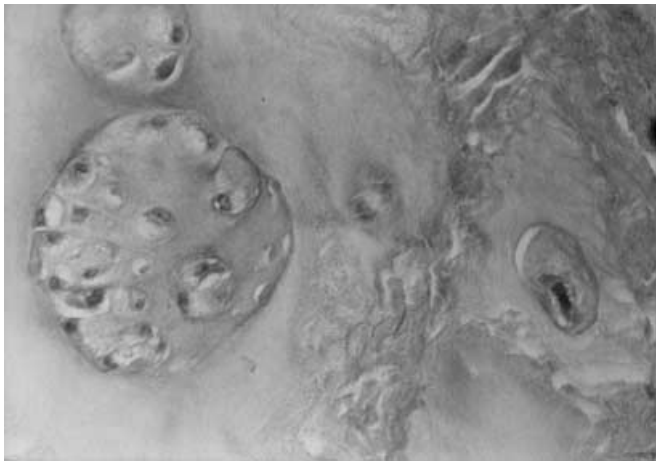

Fig. 1 Chondrocyte crowding in herniated intervertebral disc tissue (haematoxylin-eosin-alcian blue, ×400)

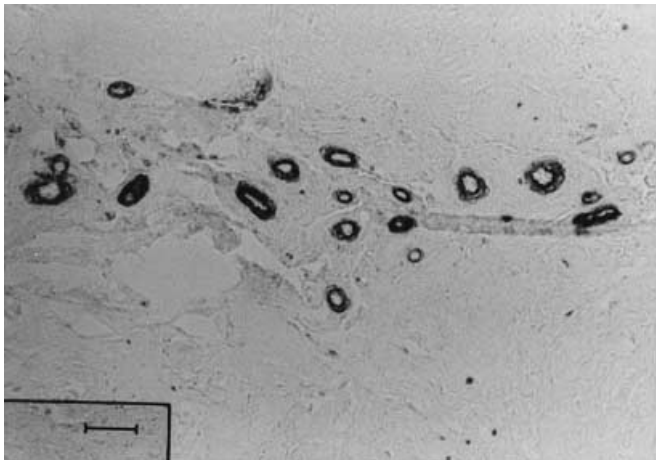

Fig. 2 Immunoreactivity for α-smooth-muscle actin in vessels of herniated disc (*bar* = 50 μm, ×200)

Herniated disc tissues from 24 patients showed the presence of granulation tissue, preferentially localized at the edges of the herniated tissue. This newly formed tissue appeared rich in small, spindle-shaped fibroblasts and in vessels, its presence being revealed by α-smooth muscle actin immunoreactivity (Fig. 2). TGF-β1 was expressed in herniated discs, particularly in chondrocytes (Fig. 3A), in endothelial cells and in the granulation tissue of the surrounding matrix (Fig. 3B).

IGF-1 was present in chondrocytes of both normal and pathological tissue, with a stronger labelling in the latter

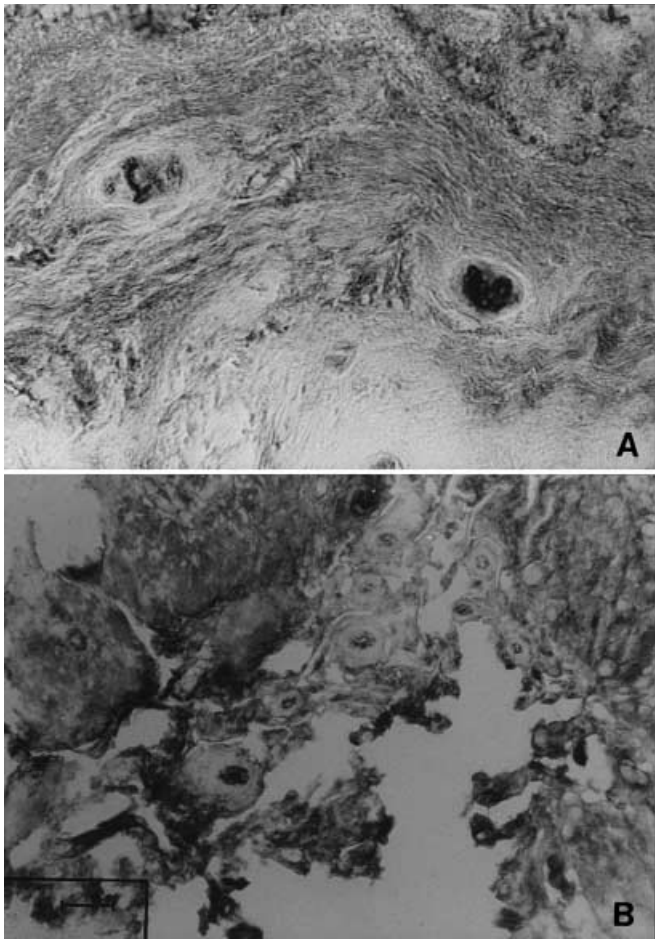

Fig. 3 Transforming growth factor-β1- (TGF-β1)- immunoreactivity in **A** chondrocytes and in the extracellular matrix of herniated tissue (×630), and **B** endothelial cells of neoangiogenetic areas (*bar* = 50 μm, ×200)

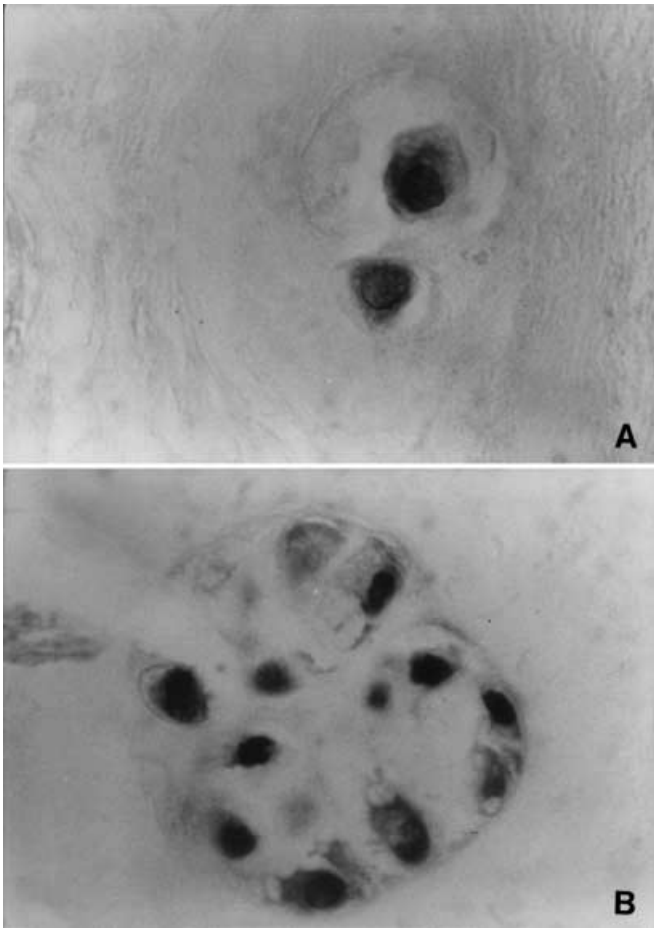

Fig. 5A, B Interleukin-6 (IL-6) immunoreactivity in chondrocytes of herniated tissue. The labelling was observed in **A** sporadically distributed cells (×1000) and **B** in cells forming clusters (×1000)

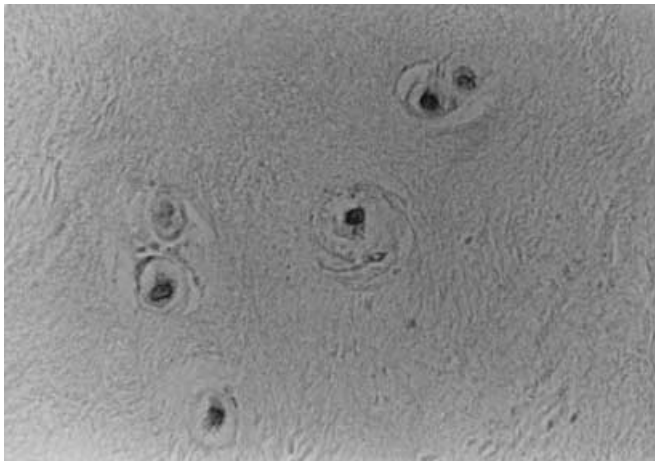

Fig. 4 Insulin-like growth factor-1 (IGF-1) in herniated disc: the labelling was localised in chondrocytes exhibiting an intracellular expression pattern (×630)

(Fig. 4), while no immunoreactivity was found in the granulation tissue. IL-6 and IL-6R immunoreactivity was detected in the cytoplasm of chondrocytes of the protruded intervertebral discs (Fig. 5, Fig. 6). Fibronectin immunoreactivity was stronger in the herniated tissue than in normal tissue, and was observed in the chondrocytes (Fig. 7) and in the granulation tissue areas.

Sections from autoptic disc tissue stained with haematoxylin-eosin and α-smooth muscle actin antibody revealed no edge vascularization. In the normal intervertebral disc, chondrocytes did not show any specific immunoreaction for TGF-β1, IL-6 and IL-6R (Fig. 8), while they expressed low quantities of IGF-1 and fibronectin. Differences detected between herniated and normal tissues were statistically highly significant ($P<0.001$) (Table 1).

No staining was observed with mouse and rabbit immunoglobulins used as controls (Fig. 9).

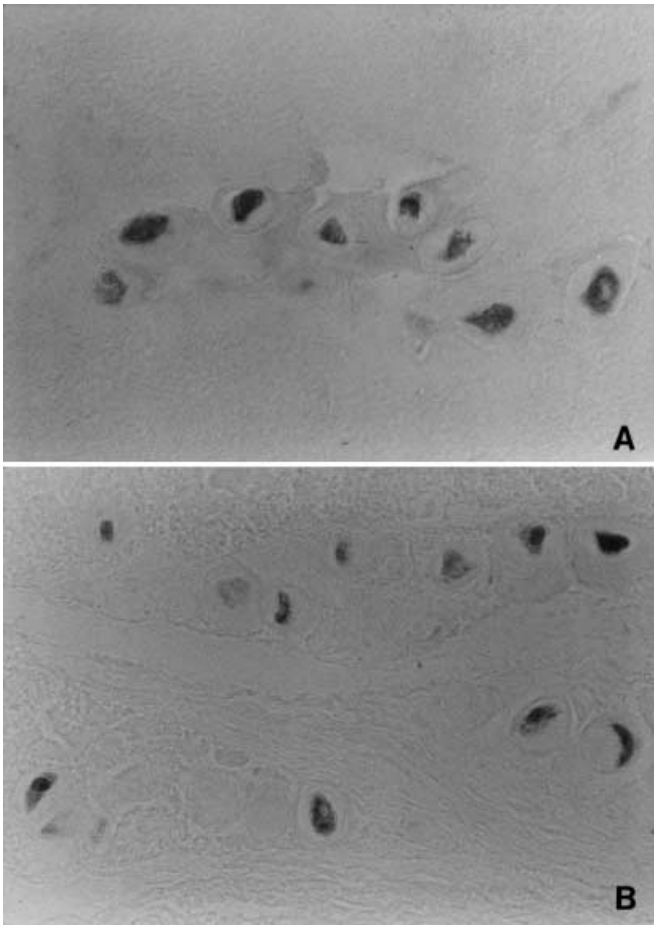

Fig. 6A, B IL-6-receptor (IL-6R) immunoreactivity in chondrocytes of herniated intervertebral disc. The reaction was localised in the cytoplasm (×630)

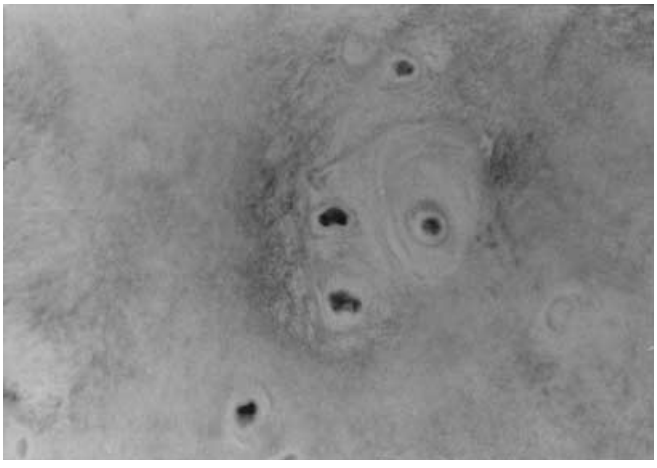

Fig. 7 Fibronectin immunoreactivity was localised in chondrocytes and in neoangiogenetic areas of the herniated disc (×400)

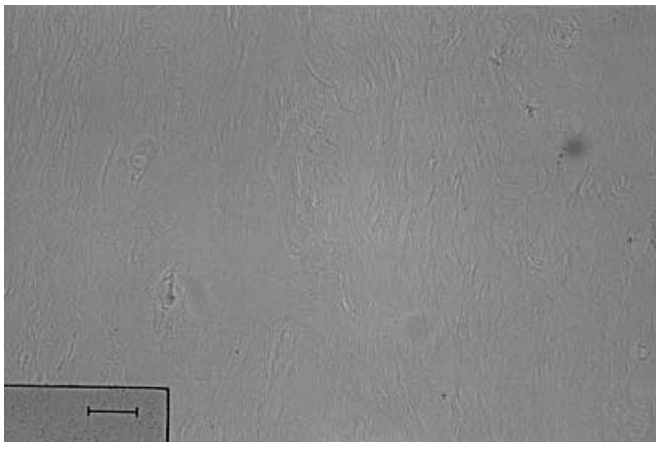

Fig. 8 No immunostaining for IL-6R was observed in the control tissues (*bar* = 50 μm, ×200)

Table 1 Intensity of immunoreactivity in the herniated and normal disc tissues evaluated on a grey scale ranging from 0 (white) to 255 (black), considering values less than 30 as background. Data are presented as the mean ±SD in 20 random fields per 37 slides, one for each patient (*TGF-β* transforming growth factor-β, *IGF-1* Insulin-like growth factor-1, *IL-6* interleukin-6, *IL-6R* IL-6-receptor)

	Herniated disc tissue	Normal disc tissue	Statistical significance
TGF-β	68.3±23.6	18.2± 8.4	$P<0.001$
IGF-1	123.7± 3.7	87.3±15.6	$P<0.001$
IL-6	72.9±17.7	19.5±15.3	$P<0.001$
IL-6R	52.7±10.3	12.4± 3.2	$P<0.001$
Fibronectin	137.3± 7.9	111.5± 5.6	$P<0.001$

Discussion

Mechanical nerve root compression has long been known to be one important pathomechanism of sciatica [13], but a recent report indicates that nucleus pulposus also has marked inflammatogenic properties and can induce nerve root injury [17]. In an animal study, autologous nucleus pulposus, applied without mechanical pressure to normal cauda equina nerve roots in live healthy pigs, produced a significant reduction in electrical conduction times; this result was confirmed by histological findings consisting of fibre atrophy, Schwann cell oedema and axonal vacuolisation [16]. The biochemical mechanisms underlying nerve root damage remain, however, unknown. The present study was undertaken to verify whether TGF-β1, IGF-1, IL-6 and IL-6R are located in the protruded disc tissue, and to clarify the possible inflammatory property of the herniated intervertebral disc. Chitkara [3] demonstrated that one of the determinants of edge neovascularisation is the site of herniation: cervical discs rarely showed evidence of new vessel formation. Our histologi-

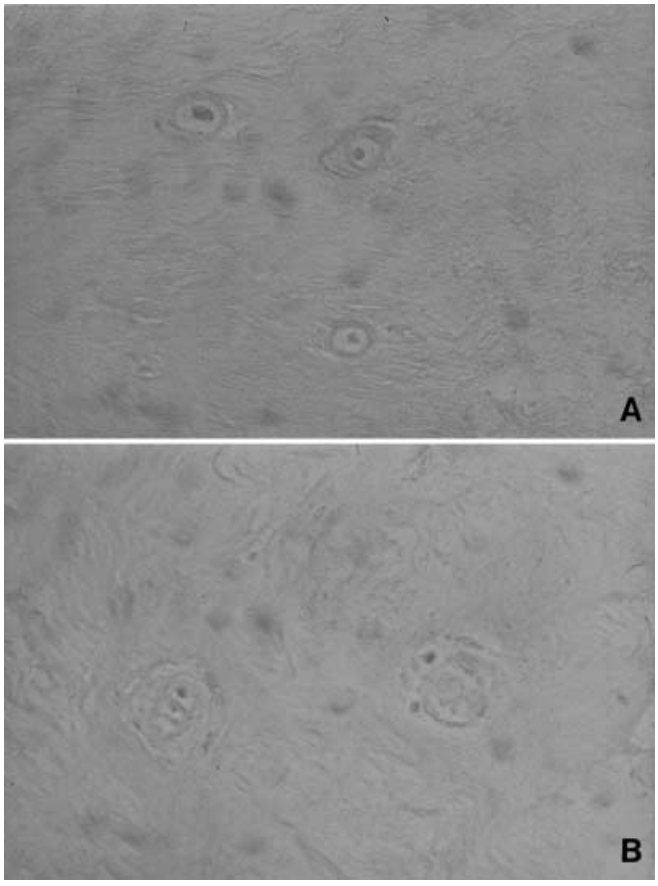

Fig. 9A, B No immunostaining was observed with mouse and rabbit immunoglobulin (×400)

cal results showed the presence of granulation tissue with an edge vascularisation of fibrocartilage fragments in most of the lumbar herniated discs. Another factor determining the presence or the absence of granulation tissue in degenerated discs is the duration of sciatic pain: the longer the radiculopathy the higher the amount of granulation tissue [3]. Our patients, indeed, suffered sciatica for more then 1 year.

Transforming growth factor-β1

Immunohistological analysis showed TGF-β1 to be expressed in the granulation tissue of the herniated discs, especially in chondrocytes and in endothelial cells. TGF-β1 is known to interfere with the reparative process of connective tissue and to be able to induce the formation of inflammatory and granulation tissues [12]. The actions of TGF-β1 in reparative processes are directed to several cell types that are recruited during wound healing: fibroblasts, monocytes and endothelial cells [12]. TGF-β1 enhances the synthesis of collagens and fibronectin and the formation of the extracellular matrix in vivo [22]. TGF-β1 is also a chemoattractant for fibroblasts and monocytes, and may thus stimulate the migration of cells to the injured area [20]. Its relationship to endothelial cells seems to be contradictory, as it inhibits endothelial cell proliferation [7] and migration [2] in vitro, but it has been reported to stimulate microvessel formation in vivo [21]. During the formation of new capillaries, endothelial cells degrade the basal membrane and migrate in the surrounding stroma, forming the tubular arrays characteristic of microvessels [12]. We can hypothesise that in the herniated disc TGF-β1 is produced in response to a tissue damage leading to the formation of granulation tissue.

Insulin-like growth factor-1

We found that the expression of IGF-1 is stronger in chondrocytes of the herniated disc than in controls. IGF-1, which is produced mainly in the liver in response to the action of growth hormone and is transferred into the serum [28], is known to stimulate the growth of bone and cartilage [27]. In particular, it might stimulate proteoglycan synthesis in the nucleus pulposus, as demonstrated by Thompson et al. [33]. More recently, local production of IGF-1 has been demonstrated in the intervertebral disc: the expression of IGF-1 mRNA and IGF-1-receptor in disc tissue was greater in cells of the nucleus pulposus of fetal bovine intervertebral discs than in those of the adult disc [18]. The production of IGF-1 during tissue repair [9] suggests that its presence in the adult herniated intervertebral disc might represent an attempt to repair the matrix.

Fibronectin

Similarly to IGF-1, our data demonstrated that more fibronectin was expressed in the herniated tissue. Fibronectin is a glycoprotein consisting of repeating units of amino acids, which form domains that enable the molecule to interact with a variety of cells through both integrin and non-integrin receptors [24]. It has been demonstrated that fibronectin levels are elevated in the degenerated discs and frequently present as a fragment, suggesting a role in disc degeneration [14]. Particular attention is devoted to a role for adhesive interactions mediated by fibronectin during invasion. Invasion is the movement of cells of one type into the fabric of other contiguous tissues, and is responsible for the migration of cells engaged in wound healing and neoangiogenesis [1]. We believe that fibronectin expression in the herniated disc may promote the formation of neoangiogenetic areas, considering that the intervertebral disc extracellular matrix is sparsely cellular. In fact, it has been demonstrated that fibronectin facilitates invasion into a sparsely cellular extracellular matrix and prevents invasion where the host tissue is densely cellular [1].

Interleukin-6 and interleukin-6 receptor

The expression of IL-6 in herniated discs has previously been reported [10, 11, 31], while IL-6R detection has never been demonstrated before. IL-6 is mainly produced by neurons, glial cells, haematopoietic precursor cells, B cells, T cells, keratinocytes and osteoclasts [19], and is considered an important mediator of acute inflammatory responses. Its action is exerted via a receptor complex consisting of a specific receptor (IL-6R) and a signal-transducing subunit (gp130) [8]. Soluble forms of both receptor components are generated by shedding [19] and the complex of IL-6 and soluble IL-6R modulates the receptor system, acting as an agonist [8]. Recent observations indicate that IL-6 and its soluble receptor have a protective role in the metabolism of cartilage, as indicated by the induction of the tissue inhibitor of metalloproteases (TIMP) [29]. Cohen et al. [4] demonstrated, using northern analysis, that treatment of various cell lines with IL-6 for 6–48 h resulted in a significant induction of vascular endothelial growth factor (VEGF) mRNA. It has been reported that IL-6 also interferes with pain sensation in rats; for instance, intracerebro-ventricular injection of IL-6 induces thermal hyperalgesia [15]. In line with this finding is the observation that intrathecal IL-6 production results in allodynia and thermal hyperalgesia following peripheral nerve injury [6]. The possible effects of IL-6 on nerve root are still unknown. The presence of IL-6 and its receptor suggests that an autocrine/paracrine mechanism of IL-6 biosynthesis may be present in the herniated disc. This finding is interesting, especially for chondrocytes of the intervertebral disc, which is the largest avascular tissue of the human body.

However, this study is preliminary, and further studies are required to elucidate the possible role of these factors in causing radicular pain.

Conclusion

This study provides the first demonstration of the expression of IL-6R in the chondrocytes of herniated tissue, and confirms the presence in the protruded intervertebral disc of TGF-β1, IGF-1, and IL-6 – factors produced in response to tissue damage.

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References

1. Armstrong PB, Armstrong MT (2000) Intercellular invasion and the organizational stability of tissue: a role for fibronectin. Biochim Biophys Acta 1470:9–20
2. Baird A, Durking T (1986) Inhibition of endothelial cell proliferation by type β-transforming growth factor: interactions with acid and basic fibroblast growth factors. Biochem Biophys Res Commun 138:476–482
3. Chitkara YK (1991) Clinicopathologic study of changes in prolapsed intervertebral disks. Arch Pathol Lab Med 115:481–483
4. Cohen T, Nathari D, Cerem LW, Neufeld G, Levi BZ (1996) Interleukin-6 induces the expression of vascular endothelial growth factor. J Biol Chem 271:736–741
5. Creange A, Barlovatz-Meimon G, Gherardi RK (1997) Cytokines and peripheral nerve disorders Eur Cytokine Netw 8:145–151
6. De Leo JA, Colburn RW, Nichols M, Malkotra A (1996) Inetrleukin-6-mediated hyperalgesia/allodinia and increased spinal IL-6 expression in a rat mononeuropathy model. J Interferon Cytokine Res 16:695–700
7. Frater-Schroder M, Muller G, Birchmeier W, Bohlen P (1986) Transforming growth factor-β inhibits endothelial cell proliferation. Biochem Biophys Res Commun 137:295–302
8. Gadient RA, Otten UH (1997) Interleukin-6 (IL-6): a molecule with both beneficial and destructive potentials. Prog Neurobiol 52:379–390
9. Jennische E, Skottner A, Hansson HA (1987) Dynamic changes in insulin-like growth factors I immunoreactivity correlate to repair events in rat ear after freeze-thaw injury. Exp Mol Pathol 47:193–201
10. Kang JD, Giorgescu HI, McIntyre-Larkin L, Stefanovic-Racic M, Donaldson WF, Evans CH (1996) Herniated lumbar intervertebral discs spontaneously produce matrix metalloproteinases, nitric oxide, interleukin-6, and prostaglandin E2. Spine 21:271–277
11. Kang JD, Stefanovic-Racic M, McIntyre-LA, Giorgescu HI, Evans CH (1996) Toward a biochemical understanding of human intervertebral disc degeneration and herniation. Contributions of nitric oxide, interleukins, prostaglandin E2, and matrix metalloproteinases. Spine 22:1065–1073
12. Laiho M, Keski-Oja J (1989) Growth factors in the regulation of pericellular proteolysis: a review. Cancer Res 49: 2533–2553
13. Mixter WJ, Barr JS (1934) Rupture of the intervertebral disc with involvement of the spinal canal. N Engl J Med 211:210–215
14. Oegema TR, Johnson SL, Aguiar DJ, Ogilvie JW (2000) Fibronectin and its fragments increase with degeneration in the human intervertebral disc. Spine 25:2742–2747
15. Oka T, Oka K, Hosoi M, Hori T (1995) Intracerebroventricular injection of interleukin-6 induces thermal hyperalgesia in rats. Brain Res 692:123–128
16. Olmarker K, Rydevik B, Nordborg C (1993) Autologous nucleus pulposus induces neurophysiologic and histologic changes in porcine cauda equina nerve roots. Spine 18:1425–1432
17. Olmarker K, Blomquist J, Stromberg J, Nannmark U, Thomsen P, Rydevik B (1995) Inflammatogenic properties of nucleus pulposus. Spine 20:665–669
18. Osada R, Hiroshi O, Ishihara H, Yudoh K, Sakai K, Matsui H, Tsuji H (1996) Autocrine/paracrine mechanism of insulin-like growth factor-1 secretion, and the effect of insulin-like growth factor-1 on proteoglycan synthesis in bovine intervertebral discs. J Orthop Res 14:690–699

19. Peters M, Meyer zum Buschenfelde KH, Rose-John S (1996) The function of the soluble IL-6 receptor in vivo. Immunol Lett 54:177–184
20. Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH (1987) Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor β. J Exp Med 165:251–256
21. Roberts AB, Sporn MB (1988) Transforming growth factor beta. Adv Cancer Res 51:107–145
22. Roberts AB, Sporn MB, Assoian RK, et al (1986) Transforming growth factor type-β: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 83:4167–4171
23. Rogister B, Delree P, Leprince P, et al (1993) Transforming growth factor beta as a neuronoglial signal during peripheral nervous system response to injury. J Neurosci Res 34:32–43
24. Romberger DJ (1997) Fibronectin. Int J Biochem Cell Biol 29:939–943
25. Rufer M, Flanders K, Unsicker K (1994) Presence and regulation of transforming growth factor beta mRNA and protein in the normal and lesioned rat sciatic nerve. J Neurosci Res 39:412–423
26. Saal JS, Franson RC, Dobrow R, Saal JA, White AH, Goldthwaite N (1990) High levels of inflammatory phospholipase A2 activity in lumbar disc herniations. Spine 15:674–678
27. Schoenle E, Zapf J, Humbel RE, Froesch ER (1982) Insulin-like growth factor-1 stimulates growth in hypophysectomized rats. Nature 296:252–253
28. Schwander JC, Hauri C, Zapf J, Froesch ER (1983) Synthesis and secretion of insulin-like growth factor and its binding protein by the perfused rat liver: dependence on growth hormone status. Endocrinology 113:297–305
29. Silacci P, Dayer JM, Desgeorges A, Peter R, Manueddu C, Guerne PA (1998) Interleukin (IL)-6 and its soluble receptor induce TIMP-1 expression in synoviocytes and chondrocytes, and block IL-1 induced collagenolytic activity. J Biol Chem 273:13625–13629
30. Sobotta J, Hammersen F (1984) Istologia. Atlante di citologia, istologia ed anatomia microscopica, 2nd edn. USES-Edizioni Scientifiche, Florence, p 61
31. Takahashi H, Suguro T, Okazima Y, Motegi M, Okada Y, Kakiuchi T (1996) Inflammatory cytokines in herniated disc of the lumbar spine. Spine 21:218–224
32. Takashi N, Kazuo Y, Simpei M, Masaya T, Keiro O (1995) Distribution of the basic fibroblast growth factor and its receptor gene expression in normal and degenerated rat intervertebral discs. Spine 20:1972–1978
33. Thompson JP, Oegema TR Jr, Bradford DS (1991) Stimulation of mature canine intervertebral disc by growth factors. Spine 16:253–260