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Transforming growth factor β receptor induction in herniated intervertebral disc tissue: an immunohistochemical study

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Abstract Transforming growth factor β (TGF-β) is a potent inducer of angiogenesis and fibrogenesis. There is presently little information about the pathophysiological function of TGF-β in herniated disc tissue. In order to analyze the cellular role and activation of TGF-β after disc herniation we immunostained frozen material from 38 disc herniation operations and from eight macroscopically normal discs from organ donors. Polyclonal TGF-β-I, TGF-β-II and TGF-β receptor type II antibodies were used with the avidin biotin complex (ABC-) immunoperoxidase method. All the herniated discs were TGF-β immunopositive. Such immunoreactivity was mainly associated with disc cells. In a few samples, capillaries were also TGF-β immunopositive. Immunopositivity was similarly observed in the control discs. To analyze possible differences between the two groups, we calculated the ratio of immunopositive disc cells. For all three antibodies, a statistically significantly (Mann-Whitney test, P=0.0001) higher number of disc cells showed immunopositivity in the herniated discs. The increase in TGF-β receptor immunopositivity suggested induction of TGF-β receptors in herniated discs. Our results support an active regulatory role for TGF-β in disc cell metabolism.

Keywords Herniated disc · TGF-β

Introduction

Both fibrotic and angiogenic reactions take place in disc herniations [7, 8, 29]. Herniated disc tissue has a tendency to decrease in size with time [14, 22], and it has proteolytic activity [17].

The TGF-β superfamily is composed of several growth factors [4]. TGF-β has been found in almost all cells studied [1, 16, 20]. It regulates cellular growth and stimulates extracellular matrix protein incorporation and collagen, hyaluronic acid, and fibronectin production [9, 19]. Subcutaneous injection of TGF-β in mice results in rapid induction of fibrosis and angiogenesis [20]. In cartilage, TGF-β stimulates production of tissue inhibitor of metalloproteinases (TIMP) [28], thus participating in the control process of connective tissue degradation. It also participates in inflammatory responses during articular inflammation [13, 18]. Furthermore, TGF-β has an effect on chondrogenesis and osteogenesis [6, 10].

TGF-βs are secreted from cells in the latent form, which does not bind to TGF-β receptors. The latency-associated peptide is removed by enzymatic degradation by proteases, resulting in activation of TGF-β [2, 5, 15, 23].

Table 1 Description of 38 disc samples from discectomy operations and eight normal controls (*DHT* disc herniation tissue, *DNT* normal disc control tissue, *S* sequester, *E* extrusion, *P* protrusion)

Tissue type	Sex	Prolapse type	Duration of radicular pain Mean (range)	Age Mean (range)
DHT (*n*=38)	22M/16F	S=12; E=24; P=2	6.9 mths (3wks–36 mths)	41.6 (20–74)
DNT (*n*=8, 5 patients)	4M/1F	–	–	36.2 (13–53)

The actions of TGF-β are mediated via binding to cell surface receptors [27].

Since TGF-β has been demonstrated to be a potent activator of disc cells in cell cultures [24] and in vivo [12], in the present study we wanted to analyze its role in herniated disc tissue: specifically, whether the number of TGF-β immunopositive cells is raised by herniation and whether the TGF-β receptor on disc cells is induced.

Materials and methods

Herniated disc material was obtained from 38 discectomy operations. As a normal control we used tissue from eight discs that had been obtained from a disc tissue bank (–70°C) of five organ donors (Table 1). None of the donors had a history of low back pain. After removal, tissue material was immediately frozen to –70°C in the operating theatre, and 8-µm-thick cryostat sections were fixed in ice-cold acetone. For some specimens we performed Zamboni prefixation to detect a possible difference in immunoreaction between prefixed and section-fixed samples. All immunoreactions were detected using an avidin biotin complex- (ABC-) peroxidase staining kit (Vectastain Elite, Vector Laboratories, Burlingame, Calif.). All tissue sections were counterstained by hematoxylin and eosin. Thus, all cells, including those showing no immunoreactivity, could be visualized and counted.

Table 2 Ratio of immunopositive (TGF-β-I, TGF-β-II and TGF-β receptor type II) to total disc cells in herniated disc tissue and control disc samples: values for the total sum of immunopositive disc cells to all disc cells in each group and the means, standard deviations (SD) and 95% confidence intervals (CI) for each group are given. Immunopositive disc cells were counted from eight normal discs and ten herniated discs and their number was then compared with the corresponding total cell number. For counting, five random microscopic fields from each tissue sample were used

Type of studied tissue	Ratio of immunopositive to total disc cells		
	TGF-β-I	TGF-β-II	TGF-β receptor type II
DHT (*n*=10)			
Total sum	550/858	437/787	520/864
Mean	0.64	0.56	0.60
SD	0.09	0.15	0.08
95% CI	0.58–0.69	0.45–0.64	0.54–0.64
DNT (*n*=8)			
Total sum	271/772	100/506	149/489
Mean	0.32	0.16	0.28
SD	0.10	0.11	0.12
95% CI	0.27–0.38	0.10–0.22	0.22–0.34
Group difference	$P<0.0001$	$P<0.0001$	$P<0.0001$

Antibodies

Polyclonal anti-human TGF-β-I, TGF-β-II and TGF-β receptor type II antibodies raised in rabbits were used (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), all at the dilution 1:50. There is no cross-reactivity between these three different antibodies. We chose antibodies to TGF-β-I and -II, in particular, since these are the most common members of the TGF-β superfamily. An antibody to TGF-β receptor type II was chosen, since this receptor is located in the cellular membrane, and it detects all members of the TGF-β superfamily. As a positive control for the immunostaining reaction, we used rheumatoid arthritic synovia tissue. Three sections were stained with each antibody from every specimen.

Immunohistochemical quantitation

All herniated and control disc samples studied consisted of nucleus pulposus tissue only. The immunohistochemical analyses were done in blind review by two observers. Positive staining for all three TGF-β antibodies (TGF-β-I, TGF-β-II, and TGF-β receptor type II) was quantified as the ratio of positive disc cells per cross-sectional area to all disc cells. We took five random microscopic fields at the magnification ×250 from all control discs and from ten herniated discs. The mean of the cell counts obtained by the two observers was used.

We counted 50 fields from herniated disc samples and 40 fields from all control samples, and standard deviations were found to be small (Table 2). Furthermore, when comparing groups, 95% confidence intervals did not overlap at all. Thus, we considered our counting material sufficient for statistical analysis.

For immunostaining control, sections were treated omitting the primary antibody in the staining sequence. For all antibodies (TGF-β-I, -II and receptor type II) preincubation with the corresponding antigen (1:10) was done.

Statistical analysis

Statistical analysis was done using the SOLO statistical software program (BMDP, Los Angeles, Calif.). Groups were compared using the nonparametric Mann-Whitney test. Level of statistical significance was set at $P<0.05$.

Results

The disc samples studied are described in detail in Table 1. Herniated disc samples were classified by the operating surgeon as previously described [25]. In control discs, no signs of autolysis were observed, i.e., all disc cells looked intact, and they all showed a normal morphology macroscopically.

All discs studied, both disc herniations and controls, showed TGF-β-I, -II and receptor type II immunopositivity. The immunoreaction for TGF-βs and TGF-β receptor was mainly disc cell associated, located in the cytoplasm

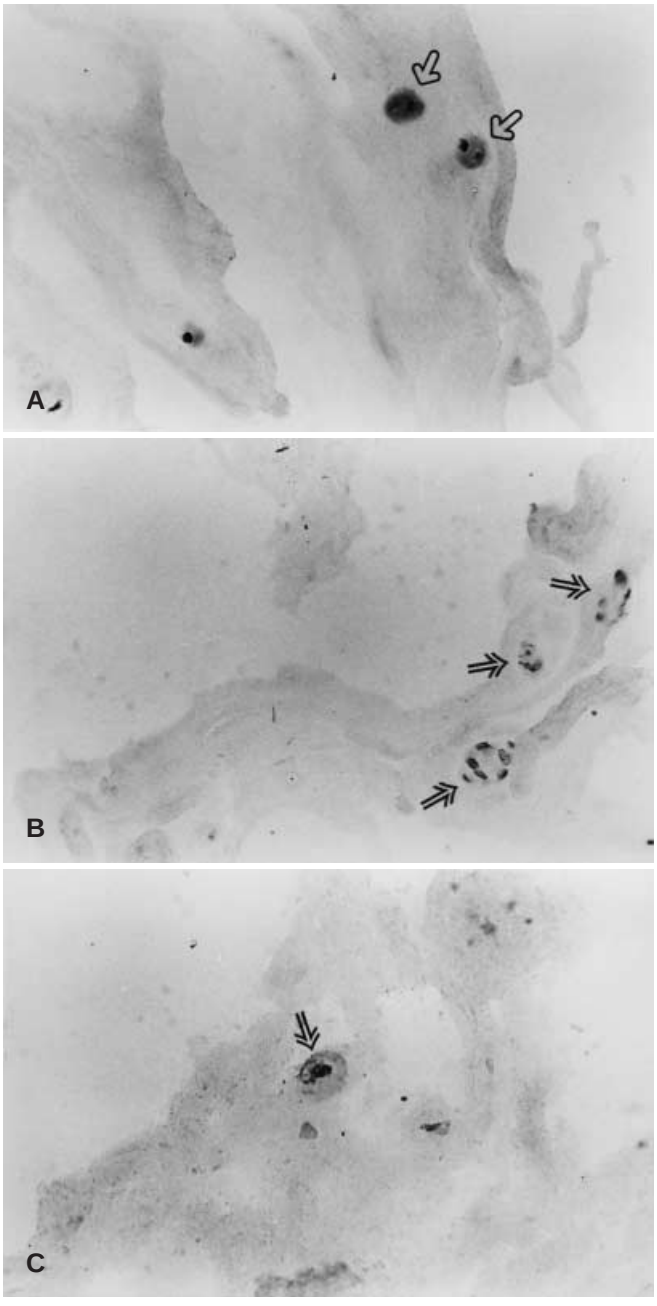

Fig. 1 **A** Transforming growth factor (TGF-)β-I immunopositive disc cells (*open arrows*) in nucleus pulposus of rapidly frozen herniated disc tissue from a 43-year-old male patient. Note the intense cytoplasmic immunoreaction around counterstained nuclei. The operation level of the sequestrated disc was L5-S1 [avidin biotin complex (ABC-) peroxidase immunostaining (Vectastain); hematoxylin counterstaining; original magnification ×241]. **B** TGF-β receptor type II immunopositivity in nucleus pulposus disc cell groups (*open arrows*) in rapidly frozen herniated intervertebral disc from a 40-year-old male patient. The operation level of the disc protrusion was L4-L5 (immunostaining as in **A**; original magnification ×241). **C** TGF-β-II immunopositivity (*open arrow*) in cytoplasm/cell membrane of disc cell in nucleus pulposus. Rapidly frozen herniated intervertebral disc from the same patient as in **B** (immunostaining as in **A**; original magnification ×241)

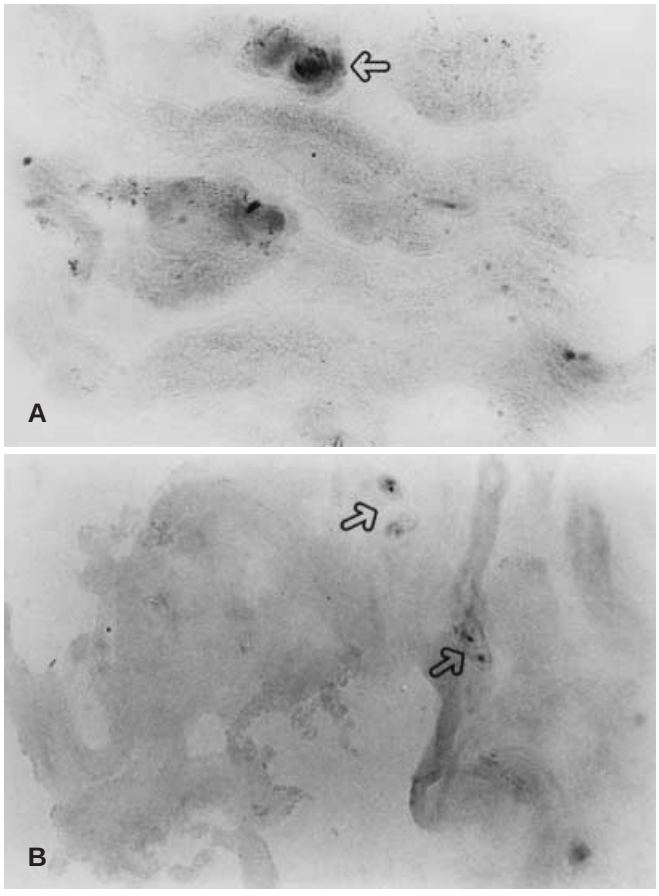

Fig. 2 **A** TGF-β-II immunopositive nucleus pulposus disc cell (*open arrow*) in a 43-year-old female organ donor (control disc). The disc level was L2-L3 (immunostaining as in Fig. 1A; original magnification ×241). **B** Immunostaining control. Antigen preabsorption for TGF-β-II. Note the pale nuclei of nucleus pulposus disc cells (*open arrows*) (immunostaining as in Fig. 1A, original magnification ×241)

of the cell (Fig. 1, Fig. 2 A). In some samples, we also noted blood vessel associated immunoreactivity. For TGF-β-I and TGF-β-II, immunoreactivity in disc cells was noted in all 38 disc herniation samples, whereas only one such sample lacked disc cell associated immunoreactivity for TGF-β receptor type II.

We did not see any difference in immunoreaction between different types of herniated tissue samples, i.e., sequesters, extrusions, and protrusions.

Blood vessel immunoreactivity was observed in 22/38 (58%)(TGF-β-I), 14/38 (37%)(TGF-β-II) and 14/38 (37%) (TGF-β receptor type II) disc herniations, respectively.

Sections stained omitting the primary antibody against corresponding antigen did not show any immunoreactivity. There was no immunoreaction after preincubation with the corresponding antigen for any of the three antibodies employed (Fig. 2B). The rheumatoid arthritis synovia sections were immunopositive for all the antibodies.

Comparison between prefixed and section-fixed samples did not show differences in immunoreactivity.

Control discs showed fewer TGF-β and TGF-β receptor immunopositive disc cells than the disc herniations (Table 2). When comparing the two groups by the Mann-Whitney test, significant differences ($P<0.0001$) were observed for all the three antibodies. A significant correlation (Spearman's $\rho=0.89$, $P<0.002$) between TGF-β-I immunopositivity and TGF-β receptor type II immunopositivity in herniated disc samples was also noted. The correlation between TGF-β-II and TGF-β receptor type II remained at a non-significant level ($\rho=0.45$, $P>0.05$).

Discussion

TGF-β stimulates extracellular matrix component formation and regulates cellular growth and extracellular proteolysis [3, 9, 11, 19, 21]. It participates in angiogenesis and in the early phase of inflammation [13, 18, 21]. Such proteolytic activity has also been observed in herniated disc tissue [17]. Thus TGF-β may be important in mechanisms of disc tissue ageing and repair, which are presently incompletely understood. New blood vessel formation (i.e., neovascularization), inflammation and the regulation of cellular growth and extracellular matrix (e.g. proteoglycan) formation and breakdown are all major processes in tissue healing and/or degradation.

TGF-β may participate in the regulation of extracellular proteolysis [11, 17, 28] in disc herniation tissue. This extracellular proteolysis and the suggested possible role in the regulation of matrix production may be important for the mechanisms of disappearance of prolapsed disc material with time [14, 22].

Yasuma and co-workers have recently noted angiogenesis to be a sign of ageing in disc tissue [29]. Whereas normally blood vessels are known to be sparse in disc tissue, in disc herniation tissue, the expression of growth factors, e.g., fibroblast growth factor (FGF) [25] and TGF-β, in small capillaries is suggestive of an ongoing active neovascularization process. Furthermore, such a neovascularization process is supported by the expression of platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) [26].

Conclusions

The marked statistical difference between immunoreactivity for TGF-β and its receptor type II in herniated intervertebral disc tissue as compared with control discs (Table 2) shows that this particular growth factor is activated. Furthermore, a statistical difference in TGF-β receptor type II immunoreactivity suggests receptor induction in disc herniation tissue, as compared with control disc tissue. We surmise that the increased immunopositivity in herniated disc is due to local production and that the increased immunopositivity for the TGF-β receptor will provide an increased number of binding sites for TGF-β, increasing the action of TGF-β on disc cells.

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