Proinflammatory Cytokines Stimulate the Expression of Nerve Growth Factor by Human Intervertebral Disc Cells

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Although chronic low back pain is a major clinical problem that results in physical disability and decreased productivity, its pathophysiology remains poorly understood. Some studies have provided evidence that a common source of low back pain might be the lumbar intervertebral disc (IVD). The IVD is an avascular and aneural tissue, except for the outer third of the annulus fibrosus (AF) in the normal human adult lumbar IVD. However, the presence of nerve fibers extending into the AF and nucleus pulposus (NP) in patients with low back pain, who had been clinically diagnosed by discography, suggested that innervation into the inner part of the disc may be associated with discogenic low back pain. Thus, the mechanism of innervation into the IVD is gaining attention among researchers. Previously published in vivo and in vitro studies have suggested that proteoglycans, particularly aggrecan, might play a role in the suppression of nerve ingrowth into the IVD.

Recent studies have revealed that nerve growth factor (NGF), a neurotrophic factor, promoted nerve ingrowth into the IVD. NGF promotes collateral sprouting of the peripheral sensory nerves and axonal regeneration in the central nervous system. In a different mode of action, NGF can directly modulate the function of nociceptive sensory neurons, resulting in the generation of pathologic pain. The importance of NGF in pain generation was highlighted by a recent histologic study in rat IVD tissues that revealed the majority of disc-innervating dorsal root ganglion (DRG) neurons were NGF-sensitive neurons.

NGF is produced in the peripheral and the central nervous systems. Importantly, NGF can be produced by cells outside the nervous system, such as lymphocytes, mast cells, keratinocytes, and fibroblasts. Recent findings also indicated that adult osteoarthritic articular chondrocytes can produce NGF. In IVD tissues, Freemont et al have shown the expression of NGF mRNA in IVD tissues; however, the localization was confirmed only in microvascular blood vessels, not in disc cells. More recently, Gigante et al have reported that rounded chondrocyte-like cells in the AF were immunohistochemically positive for NGF. One question remains to be answered: which cells produce NGF in IVD tissue?

Much interest has been focused on the role of proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), in the mechanism of disc degeneration. These cytokines stimulate degradation of the extracellular matrix and induce changes in the biochemical

Study Design. In vitro studies of the effects of proinflammatory cytokines on the production of nerve growth factor (NGF) by human intervertebral disc (IVD) cells.

Objective. To determine the constitutive expression and production of NGF and the effect of cytokines on the expression of NGF by human IVD cells.

Summary of the Background Data. NGF may play a role in the collateral sprouting of sensory axons, neural survival, and regulation of nociceptive sensory neurons. NGF is known to be up-regulated by proinflammatory cytokines.

Methods. The presence of NGF protein was analyzed by immunohistochemistry using human IVD cells obtained from cadaveric human spines with known disc disease (MRI Thompson grades 2–4). The effects of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) on NGF production and mRNA expression of NGF by IVD cells were examined. The expression of NGF receptors, TrkA and p75NGFR, was also assessed immunohistochemically.

Results. Cadaveric anulus fibrosus (AF) and nucleus pulposus (NP) cells cultured in vitro in monolayer and in alginate beads positively stained with an anti-NGF antibody. The constitutive production of NGF protein in IVD cells was low (NP) or not detectable (AF). The expression of NGF mRNA was detectable in both cell types. IL-1β and TNF-α up-regulated the NGF mRNA expression and the secretion of NGF protein into the media. TrkA was immunolocalized in AF and NP cells.

Conclusion. Our results demonstrate, for the first time, that human AF and NP cells constitutively express NGF protein and mRNA, and that the proinflammatory cytokines IL-1β and TNF-α stimulate the production of NGF. The precise role of NGF produced by IVD cells in the generation of discogenic pain or on the metabolism of IVD cells, especially under certain physiologic conditions in which cytokines are up-regulated, needs to be clarified in future experimentation.

Key words: nerve growth factor, intervertebral disc, human, cytokine, interleukin-1, tumor necrosis factor, nerve, degeneration, TrkA.
properties of discs, notably a loss of proteoglycans, this change in the proteoglycan content of degenerated discs has been suggested to be a contributing factor in IVD innervation. IL-1β and TNF-α can also considerably up-regulate the expression of NGF and cause inflammatory pain. Based on these reports, we hypothesized that NGF may be induced in the degenerated IVD in which proinflammatory cytokines are up-regulated and play an important role for innervation and generation of discogenic pain. The specific purpose of this experiment was to examine the ability of IVD cells to produce NGF and to examine the effects of proinflammatory cytokines (IL-1β and TNF-α) on the expression of NGF in human IVD cells.

## Materials and Methods

### Cell Preparation and Culture Conditions

Human IVDs were obtained from cadaveric human spines (3 donors, 45–64 years of age; MRE: Thompson Grade 2–4) from a regional organ bank within 24 hours of death. Human IVD cells were separately isolated from both NP and AF tissues, essentially as previously described. Briefly, the outermost layer of the AF (about 0.5 mm) was sharply dissected and discarded to prevent the contamination of cells from ligaments surrounding the IVD. The NP and AF were separated, and cells were isolated from NP and AF tissues by sequential enzyme digestion with 0.4% pronase (EMD Bioscience, La Jolla, CA) for 1 hour and 0.025% collagenase P (Roche Applied Science, Indianapolis, IN) and 0.001% deoxyribonuclease 2 (DNase 2, Sigma-Aldrich, St. Louis, MO) for 16 hours in a 5% CO2/95% air incubator at 37 degrees. The cells isolated from each IVD tissue were cultured in monolayer in 10 cm tissue culture plates or in chamber slides (Lab-Tek II, Nalge Nunc International, Naperville, IL) at 0.2 × 105 cells/cm² or resuspended in 1.2% low-viscosity sterile surgical grade alginate (Keltone LV-(HM), a gift from ISP Alginate Inc., San Diego, CA) solution at 2.0 × 105 cells/mL. The cultures were maintained in complete medium [Dulbecco's modified Eagle medium and Ham's F-12 medium (DMEM/F12: Mediatech, Herndon, VA)] containing 20% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA), 25 μg/mL ascorbic acid (Sigma-Aldrich), 360 μg/mL L-glutamine (Mediatech) and 50 μg/mL gentamicin (Invitrogen, Carlsbad, CA) for 7 days.

### Stimulation of IVD Cells With Cytokines

After 7 days of preculture, the cells in monolayer and in beads were cultured in serum-free medium for 24 hours. The cells were further cultured for 3 days in the presence or absence of recombinant human interleukin-1β (rhIL-1β; Biosource/Invitrogen) at 0.1, 1, or 10 ng/mL, or recombinant human tumor necrosis factor-α (rhTNF-α; Biosource/Invitrogen) at 0.1, 1, 10, or 100 ng/mL in DMEM/F12 containing 0.3% FBS.

### Immunohistochemistry for Cultured IVD Cells

The beads or chamber slides prepared as described above were fixed in methanol for 15 minutes and washed in phosphate-buffered saline at pH 7.4. For immunostaining of the cells cultured in alginate beads, the beads were pretreated with 100 mmol/L BaCl2, which causes irreversible polymerization of the alginate molecules, before fixation in methanol. The samples were first incubated with a blocking solution containing 2% normal goat serum (Vector Laboratories, Burlingame, CA), 1% bovine serum albumin (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich) and 0.05% Tween 20 (Fisher Scientific, Fair Lawn, NJ) for 1 hour at room temperature. Samples were incubated in rabbit anti-NGFβ polyclonal antibody (#sc-548, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The sections were washed with phosphate-buffered saline, secondary antibody [Alexa 488-conjugated anti-rabbit IgG (1:200, Molecular Probes, Eugene, OR)] was applied for 2 hours at room temperature. The cells were also nuclear-stained with propidium iodide (Molecular Probes). The monolayer cells were cover-slipped with Vectashield Mounting Medium (Vector Laboratories). The alginate beads were smashed on the slide, cover-slipped and observed.

In addition to the NGF immunostaining, the monolayer cells were stained for TrkA, which is the high-affinity NGF-receptor, using rabbit anti-TrkA polyclonal antibody (1:100, Santa Cruz Biotechnology, #sc-118), as described above. The low-affinity NGF receptor, p75NGFR, was also stained for using the anti-p75NGFR monoclonal antibody (Clone 8211, 1:50, Chemicon, Temecula, CA) and Alexa 488-conjugated antimouse IgG (1:200, Molecular Probes). To detect nonspecific binding, negative controls with IgG from rabbit serum or isotype-matched IgG (Sigma-Aldrich) were used in place of the primary antibody.

The cells in monolayer culture and the alginate beads were observed with fluorescence microscopy (Eclipse E600, Nikon, Tokyo, Japan) equipped with a digital camera.

### Enzyme-Linked Immunosorbent Assay (ELISA)

In the monolayer cultures, after 3 days treatment with cytokines, NGF concentrations in the cell lysates and in the media were separately assayed by ELISA using the NGF E-max ImmunoAssay System Kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, the ELISA plate was coated with a sheep anti-NGF polyclonal antibody and then blocked. After washing, in order to capture free NGF, the samples were added to the coated plate and incubated. A specific anti-NGF monoclonal antibody was then added to bind to the captured NGF. After washing, the amount of specifically-bound anti-NGF mAb was detected using a species-specific antibody conjugated to horseradish peroxidase (HRP). The plates were incubated with HRP-conjugated anti-rat IgG and developed with tetramethylbenzidine; the absorbance was measured at 450 nm. The detection limit of NGF in this kit was 7.8 pg/mL. All samples from human IVD monolayer cultures were run in triplicate and the resulting quantities were averaged.

### NGF mRNA Detection by Reverse Transcription/Polymerase Chain Reaction (RT-PCR) and Quantitative Analysis by Real-Time RT-PCR

After the 3-day treatment with cytokines, total RNA was isolated from human AF and NP cells in monolayer culture using TRIzol reagent (Invitrogen). Total RNA was reverse-transcribed with the SuperScript First-Strand Synthesis System kit (Invitrogen) using the DNA thermal cycler (Masterecycler Gradient, Eppendorf, Brinkman Instruments, Westbury, NY). Primers for PCR were designed based on the human NGFβ gene from GenBank sequences. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal control. The oligonucleotide primer pairs were as follows: GAPDH: sense, 5'-GAA GAT GGT GAT GGG AIT TC-3'; antisense, 5'-GAA GAT GGT GAT GGG ATT TC-3'; human NGFβ: sense, 5'-ATG GCA TGC TGG ACC CAA-3'; antisense, 5'-TGA AGT TTA GTC CAG TGG GCT-3'. The cDNA produced was amplified with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), essentially as previously described. The cycle consisted of a 1-minute denaturation at 94°C, a 0.5-minute annealing at 58°C (GAPDH) or 59°C
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The expression level of NGFβ was quantified by real-time quantitative PCR using SYBR-Green signal detection (Qiagen) with the primer pairs described above on LightCycler Systems (Roche). The assay was calibrated using GAPDH as the internal control. The cycle consisted of a 1-minute denaturation at 94°C, a 0.5-minute annealing at 59°C (NGFβ) or 58°C (GAPDH), and a 0.5-minute extension at 72°C.

The relative expression of NGF was calculated using the Comparative threshold (Ct) method, as previously described. Briefly, NGF expression was normalized to GAPDH and then to its respective expression levels using the following formula:

\[ \Delta \Delta C_{T(NGF)} = \Delta C_{T(NGF-GAPDH) \text{treatment}} - \Delta C_{T(NGF-GAPDH) \text{control}} \]

Normalized NGF mRNA expression, relative to GAPDH = 2 \(-\Delta \Delta C_{T(NGF)}\)

Figure 1. Immunostaining with anti-NGF antibody in human intervertebral disc (IVD) cells cultured in monolayer. After a 7-day preculture period, annulus fibrosus (AF) and nucleus pulposus (NP) cells were treated with interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α). Under control conditions, both AF and NP cells showed immunoreactivity for NGF. After IL-1β (10 ng/mL) or TNF-α (100 ng/mL) treatment for 3 days, the staining intensity for NGF did not differ between treated cells and control cells; diffuse staining was observed in the cytoplasm, whereas patch-like staining was found in the nuclei. Green: NGF-immunopositive. Red: nuclear staining. Scale bar indicates 20 μm.

Statistical Analysis. The data are expressed as the mean ± SD for 3 independent experiments. The data were subjected to one-way ANOVA using the between-subject factors for the different treatments. The post hoc analyses were performed with the Fisher PLSD test; significant differences are defined at P < 0.05.

Results

Immunolocalization of NGF and TrkA in AF and NP Cells Cultured in Monolayer and in Alginate Beads

Most of the AF and NP cells cultured in monolayer under control conditions showed immunoreactivity for NGF. Diffuse staining was present in the cytoplasm and patch-like staining was found in the nuclei. AF and NP cells treated with IL-1β (10 ng/mL) or TNF-α (100 ng/mL) showed an immunoreactivity similar to cells under control conditions (Figure 1).

To eliminate possible artifacts resulting from dedifferentiation of IVD cells in monolayer culture, immunostaining was performed on cells cultured in alginate beads where IVD cells are shown to maintain their chondrocytic phenotype (Figure 2). NGF immunoreactivity in IVD cells cultured in alginate beads was also con-
Figure 2. Immunostaining with anti-NGF antibody in human intervertebral disc (IVD) cells cultured in alginate beads. As seen in monolayer culture, anulus fibrosus (AF) and nucleus pulposus (NP) cells under control conditions showed positive staining for NGF. Neither cell type, treated with interleukin-1β (IL-1β; 10 ng/mL) nor tumor necrosis factor-α (TNF-α; 100 ng/mL), showed a visually detectable increase in intensity of staining. Green: NGF-immunopositive. Red: nuclear staining. Scale bar indicates 5 μm.

firmed in the cytoplasm and nuclei, as was seen in IVD cells in monolayer culture. IVD cells treated with IL-1β (10 ng/mL) and TNF-α (100 ng/mL) did not differ in staining intensity when compared with IVD cells under control conditions. In all conditions, the localization of NGF did not differ between AF and NP cells.

Under control conditions, AF and NP cells showed immunoreactivity for TrkA in the cytoplasm (Figure 3);
these results are consistent with the report that the anti-TrkA antibody recognizes the intracytoplasmic domain of the receptor. After treatment with IL-1β, the cytoplasmic immunoreactivity for TrkA was similar to that found in IVD cells under control conditions. Immunoreactivity to p75NGFR was not observed under any conditions (data not shown).

**Effect of Proinflammatory Cytokines on NGF Production**

To quantify NGF production by human IVD cells cultured in monolayer (Figure 4), the content of NGF in cell extracts and cultured media was assayed using an NGF ELISA. The content of NGF protein in cell extracts under control conditions was low in the NP (13.5 pg/mL) and under the detectable limit in the AF. In both tissues, neither IL-1β nor TNF-α affected the content of NGF in the cell extracts (NP, no significant difference vs. control; range, 10.7–13.3 pg under given conditions; AF, not detectable). NGF secretion into the medium was not detected under control conditions in either cell types. However, the addition of IL-1β or TNF-α significantly increased the secretion of NGF protein into the medium by both cell types. Values represent the mean ± SD (n = 3). *P < 0.05 versus control. **P < 0.01 versus control.

**Figure 4.** Basal production of nerve growth factor (NGF) protein and the effect of cytokines on human intervertebral disc (IVD) cells cultured in monolayer. After a 7-day preculture period, anulus fibrosus (AF) and nucleus pulposus (NP) cells were treated with interleukin-1β (IL-1β; 0.1, 1, 10 ng/mL) or tumor necrosis factor-α (TNF-α; 0.1, 1, 10, 100 ng/mL) for 3 days. The content of NGF in the medium was quantified using an NGF enzyme-linked immunosorbent assay (ELISA) kit. NGF secretion into the medium was not detected under control conditions in either cell types. However, the addition of IL-1β or TNF-α significantly increased the secretion of NGF protein into the medium by both cell types. Values represent the mean ± SD (n = 3). *P < 0.05 versus control. **P < 0.01 versus control.

**Effect of Cytokines on mRNA Expression of NGF**

To assess the expression of NGF at the mRNA level in human IVD cells, RT-PCR analysis was performed using cDNA isolated from cells in monolayer culture. NGF mRNA was constitutively expressed in control AF and NP cells (Figure 5A). The results from the sequencing of RT-PCR products indicated a complete matching of the sequence to an expected partial strand of human NGF cDNA (222 bp). B, In both AF and NP cells, IL-1β and TNF-α treatment significantly up-regulated NGF mRNA expression.

**Figure 5.** The effect of cytokines on nerve growth factor (NGF) mRNA expression by human intervertebral disc (IVD) cells cultured in monolayer. After a 7-day preculture period, anulus fibrosus (AF) and nucleus pulposus (NP) cells were treated with interleukin-1β (IL-1β; 10 ng/mL) or tumor necrosis factor-α (TNF-α; 100 ng/mL) for 7 days. The expression level of the mRNA was assessed using conventional and real-time reverse transcriptase-polymerase chain reaction (RT-PCR). A, NGF mRNA was constitutively expressed in the control AF and NP cells and was up-regulated by treatment with IL-1β and TNF-α. The results of sequencing of RT-PCR products indicated a complete matching of the sequence to an expected partial strand of human NGF cDNA (222 bp). B, In both AF and NP cells, IL-1β and TNF-α treatment significantly up-regulated NGF mRNA expression.

**Discussion**

The results presented here confirmed, for the first time, the autocrine production of NGF by human NP and AF cells, at both the protein and mRNA level, in vitro. Immunoreactivity for NGF by both cell types was confirmed in monolayer culture and in the alginate bead culture system. A
quantitative analysis of NGF protein by ELISA revealed that the amount of NGF protein in IVD cells under control conditions was not detectable in the AF and was low in the NP. However, IL-1β and TNF-α significantly stimulated the secretion of NGF protein by IVD cells into the medium. The magnitude of the effect of IL-1β on NGF secretion was greater than that of TNF-α in both AF and NP cells. Furthermore, the localization of TrkA, one of the NGF receptors, was confirmed.

The alginate bead 3-dimensional culture was reported to be beneficial in maintaining the phenotype of disc cells. However, recent studies demonstrated that the outer layer of AF cells do not survive well in the alginate bead system, and the monolayer or collagen-based culture system is thus favorable for outer AF cell culture. Therefore, we used 2 different culture systems to confirm our results.

NGF, which had been initially isolated from the submaxillary glands of mice, was shown to play an important role in cells from the nervous, endocrine, and immune systems. Another study reported that NGF accumulated in, or exuded from, inflammatory sites and functioned as an important inflammatory mediator by proliferation and/or activation of lymphocytes, eosinophils, or mast cells. One other important aspect of the role of NGF production was demonstrated by its expression at the site of connective tissue injuries; this indicates that NGF may contribute to tissue repair.

The importance of NGF production by IVD cells may be 2-fold. First, NGF, as a key mediator of sensory and nociceptive nerve physiology during adulthood, was reported to contribute to hyperalgesic phenomena via the high-affinity NGF receptor, TrkA. Nociceptive DRG neurons were shown to include 2 types: NGF-sensitive and glial cell line-derived neurotrophic factor-sensitive neurons. In the IVD, the majority of the disc-innervating DRG neurons was demonstrated to be NGF-sensitive neurons. Therefore, NGF produced by the IVD cells, or by the repair tissue, may sensitize these NGF-sensitive neurons, possibly resulting in discogenic pain. Second, NGF may contribute to nerve ingrowth in the degenerated IVD. Generally, NGF has been reported to be synthesized and released from innervated target tissues and to induce nerve ingrowth into the target tissue. However, a recent study showed that NP tissue inhibits the axonal outgrowth of cultured DRG cells, suggesting that some other factors or matrix components may be counteracting the effect of NGF if NGF acts as a stimulatory factor of axonal growth in the disc tissues. An elucidation of the complex control mechanism of NGF action is worth pursuing.

The stimulatory effect of proinflammatory factors on the production of NGF reported here supports the hypothesis that NGF may contribute to nerve ingrowth and pain generation in degenerative IVDs and herniated discs. Recent studies have suggested that proinflammatory cytokines, such as IL-1β or TNF-α, are expressed by animal IVDs and human IVDs, or herniated IVDs. Under cytokine stimulation, IVDs can produce NGF, as evinced in the present study in which NGF expression and production were observed in both NP and AF cells. However, it should be noted that, in this experiment, IVDs with different grades of degeneration were selected to study the fundamental biology of disc cells. It is possible that the expression level of response to cytokines may be dependent on the grade of disc degeneration; thus, further study using a large number of donor samples will be essential to answer this point.

Our biochemical analysis of NGF production was inconsistent with other immunohistochemical studies that demonstrated the localization of NGF only in microvascular blood vessels and in AF cells. In our study, because most of the NGF produced was secreted into the media and only a minor portion stayed in the cell layer, it is possible that the immunolocalization technique used did not provide information about secreted NGF. Further organ culture studies may prove useful in the detection of NGF under more physiologic conditions.

NGF exerts its biologic activity through 2 receptors; the low-affinity receptor, p75NGFR, or the high-affinity receptor, a transmembrane tyrosine kinase, p140 TrkA. NGF receptors have also been detected on cells other than neural cells, such as human keratinocytes and chondrocytes. Because the participation of NGF has been postulated in the repair of damaged tissues, such as in muscle injury and corneal ulceration, the expression of TrkA in IVD cells may suggest that NGF can affect IVD cells in a paracrine fashion, such as by the induction of NGF/TrkA by positive feedback, the induction of transforming growth factor-β, or by preventing apoptosis.

The results of this paper indicated the presence of a complex cascade in the regulation of NGF. Studies aimed at detecting different cytokines and NGF in the same cadaveric samples, herniated discs, and degenerated discs are ongoing and may help the understanding of the mechanism of innervation and pain generation in degenerative disc diseases.

**Key Points**

- AF and NP cells in monolayer culture and in alginate bead culture positively stained with anti-NGF antibody.
- In both cell types, IL-1β and TNF-α up-regulated the mRNA expression of NGF and the secretion of NGF protein into the media.
- TrkA was immunolocalized in both cell types.

**References**


