SPINE Volume 30, Number 17, pp 1940–1948

Tumor Necrosis Factorα Modulates Matrix Production and Catabolism in Nucleus Pulposus Tissue

Cheryle A. Séguin, MSc,* Robert M. Pilliar, BASc,*† Peter J. Roughley, PhD,‡ and Rita A. Kandel, MD*†

Study Design. This study examines changes in the production of extracellular matrix molecules as well as the induction of tissue degradation in *in vitro* formed nucleus pulposus (NP) tissues following incubation with tumor necrosis factor (TNF)α.

Objective. To characterize the response of NP cells to TNF-α, a proinflammatory cytokine present in herniated NP tissues.

Summary of Background Data. TNF-α is a proinflammatory cytokine expressed by NP cells of degenerate intervertebral discs. It is implicated in the pain associated with disc herniation, although its role in intervertebral disc degeneration remains poorly understood.

Methods. *In vitro* formed NP tissues were treated with TNF-α (up to 50 ng/mL) over 48 hours. Tissues were assessed for histologic appearance, proteoglycan and collagen contents, as well as proteoglycan and collagen synthesis. Reverse transcriptase polymerase chain reaction was used to determine the effect of TNF-α on NP cell gene expression. Proteoglycan degradation was assessed by immunoblot analysis.

Results. At doses of 1–5 ng/mL, TNF-α induced multiple cellular responses, including: decreased expression of both aggrecan and type II collagen genes; decreases in the accumulation and overall synthesis of aggrecan and collagen; increased expression of MMP-1, MMP-3, MMP-13, ADAM-TS4, and ADAM-TS5; and induction of ADAM-TS dependent proteoglycan degradation. Within 48 hours, these cellular responses resulted in NP tissue with only 25% of its original proteoglycan content.

Conclusions. Because low levels of TNF-α, comparable to those present physiologically, induced NP tissue degradation, this suggests that TNF-α may contribute to the degenerative changes that occur in disc disease.

Key words: nucleus pulposus, intervertebral disc, tumor necrosis factor-alpha, matrix metalloproteinases, aggrecanase, proteoglycan, collagen, extracellular matrix degradation. **Spine 2005;30:1940–1948**

Low back pain as a result of intervertebral disc (IVD) degeneration is a common cause of major disability and constitutes one of the leading musculoskeletal disorders responsible for days lost from work. Epidemiologic studies show that chronic low back disability is increasing exponentially, to the degree that a simple backache has a lifetime prevalence of 60% to 80%.[1] The etiology and disease process remain poorly understood, and are largely based on studies that have correlated changes in IVD matrix composition to the grade and stage of IVD degeneration.[2] Alterations in the extracellular matrix of the nucleus pulposus (NP) of degenerate IVDs are known to involve marked decreases in the synthesis and accumulation of proteoglycans, increased accumulation of aggrecan fragments, decreased synthesis and accumulation of type II collagen, increased denaturation of type II collagen, and increased synthesis of type I collagen.[3–6] Recent studies suggest that changes in the extracellular matrix content may be in part be caused by altered patterns of gene expression because the grade of degeneration in human NP correlated with decreases in messenger ribonucleic acid (mRNA) levels for large aggregating and small interstitial proteoglycans, as well as type II collagen.[7,8]

In addition to changes in the production of matrix macromolecules, the activity of zinc-dependent matrix-degrading enzymes, the matrix metalloproteinases (MMP) and the ADAM-TS (a disintegrin and metalloproteinase with thrombospondin motifs), as well as their regulators, the tissue inhibitors of matrix metalloproteinases (TIMP), have been implicated in the tissue resorption that occurs in association with degeneration. The family of MMPs consists of at least 20 structurally similar members, each with substrate specificities that enable MMPs to degrade all components of the extracellular matrix in a concerted manner.[9,10] To maintain the balance between matrix production and turnover, the activities of MMPs are tightly regulated at the level of gene expression, activation of the proenzyme precursors, and specific interactions with the extracellular matrix.[11] TIMPs, endogenous inhibitors that bind the active form of the enzymes, also regulate the local activities of MMPs in tissues.[11,12] ADAM-TS4 and ADAM-TS5 (aggrecanase-1 and aggrecanase-2) are members of the ADAM family and are characterized by their ability to cleave the inter-globulin domain of aggrecan at the Glu^{373}-Ala^{374} bond, a site distinct from the Asn^{341}-Phe^{342} MMPs cleavage site.[13,14] Similar to MMPs, the aggrecanase ac-

From the *BioEngineering of Skeletal Tissues Team, Mount Sinai Hospital, and †Institute of Biomaterials and Biomedical Engineering, University of Toronto, Ontario, and ‡Genetics Unit, Shriners Hospital for Children, McGill University, Montreal, Canada.
Acknowledgment date: August 11, 2004. Revision date: September 29, 2004. Acceptance date: October 11, 2004.
Supported by The Arthritis Society of Canada. Mr. Séguin was supported by a fellowship from the Natural Sciences and Engineering Research Council (NSERC) Canada.
The manuscript submitted does not contain information about medical device(s)/drug(s).
Professional Organization funds were received in support of this work. No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.
Address correspondence and reprint requests to Rita A. Kandel, MD, Department of Laboratory Medicine and Pathobiology, Mount Sinai Hospital, Suite 600, 600 University Avenue, Toronto, Ontario, Canada, M5G 1X5; E-mail: rkandel@mtsinai.on.ca

tivity of ADAM-TS4 and ADAM-TS5 are regulated by TIMPs, namely, TIMP-3.[13]

Studies of human cadaveric IVDs have shown that although MMP expression was absent from the IVDs of infants and young adolescents, expression of MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9 were increased in herniated discs, and correlated with the histologic changes and clinical grade of IVD degeneration.[6,15,16] Aggrecanase-produced aggrecan fragments are increased with disease, their levels correlating with higher degenerative grade IVDs.[6] Although it is clear that MMPs and ADAM-TS regulation by TIMPs is essential for maintaining balanced tissue turnover, the role of TIMPs during disc degeneration remains unknown. While decreased levels of TIMP-1 were found to correlate with the severity of IVD degeneration as determined by magnetic resonance imaging, other studies have reported that TIMP-1 levels increase, while levels of TIMP-2 remain unchanged in herniated discs.[6,16]

In addition to the presence of active MMPs and aggrecanases, NP tissue of herniated IVDs secreted increased levels of nitric oxide, prostaglandin E_2, interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α (TNFα),[17–21] mediators of inflammation known to contribute to extracellular matrix degradation in articular cartilage.[22,23] As a cytokine constituent of herniated NP tissue, recent investigations have described a central role for the proinflammatory cytokine TNFα in the manifestations of sciatica and nerve root damage associated with IVD herniation. Identified as a key pathogenic factor in neuropathic pain states, the liberation of TNFα by herniated NP tissue can initiate a cascade of neuropathologic change, including Schwann cell activation, edema, macrophage recruitment, and impairment of nerve conduction.[19] This response also induced endoneurial TNFα production. The importance of TNFα was shown in an animal model in which topical application of TNFα inhibitor prevented the development of nerve root damage induced by the herniated NP.[24] These findings were reinforced by a pilot study in human beings in which TNFα blockade effectively reduced sciatic pain within 1 hour of infusion.[25]

Although the role of TNFα in cartilage degradation has been well described, the effect of TNFα exposure on NP cells of the IVD and its relative role in the process of IVD degeneration are unknown. Studies aimed at characterizing the causes and cellular mechanisms regulating IVD degeneration have, to date, been limited by the availability of normal disc tissues and the lack of suitable *in vitro* models. NP cells grown in monolayer, on alginate or agarose beads, or in biocompatible scaffolds remain viable, accumulate large aggregating proteoglycans and type II collagen but are not able to form a continuous layer of tissue with extracellular matrix composition or mechanical properties similar to that of native NP tissue.[26–31] Although organ culture of the IVD has been used to study metabolism of the whole tissue, it cannot be maintained at a metabolic level comparable to the *in vivo* tissue and can swell up to 5-fold when placed directly into culture.[32]

We have recently developed a culture system that permits the formation of a continuous layer of NP tissue *in vitro*.[33] This tissue was shown to resemble the native NP, suggesting that it is suitable to use as a model for the study of the processes regulating disc development and NP tissue degeneration. Given that TNFα is produced by NP cells and plays a pivotal role in the pathogenesis of sciatica, as well as the established role of TNFα in inducing degeneration in tissues such as articular cartilage, we examined whether TNFα could alter the synthesis and retention of matrix molecules or patterns of MMPs, ADAM-TS, and TIMPs gene expression in NP cells. Altering the balance between extracellular matrix production and degradation would suggest an important role for TNFα in IVD tissue degeneration.

■ Materials and Methods

Calcium Polyphosphate Substrates. To support NP tissue formation, sintering calcium polyphosphate powder (CPP) generated porous cylindrical substrates (4 mm diameter), as previously described.[34]

***In Vitro* Formation of NP Tissue.** To generate NP-like tissue, cells were isolated from NP obtained from bovine distal caudal spines (6–9 months of age) by sequential enzymatic digestion, as previously described.[33] Tissue from up to 3 animals was combined for each experiment to obtain a sufficient numbers of cells. On average, 2×10^7 cells were obtained from the 6 discs isolated from each caudal spine. NP cells were seeded in Ham F12, supplemented with 25 mmol/L HEPES and 10% fetal bovine serum (FBS), on the upper surface of the CPP substrate (160,000 cells/mm^2), which was surrounded by Tygon tubing (4.3 mm diameter, Thermoplastics Processor Inc., San Jose, CA). Cultures were transferred to Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS 48 hours following seeding, and at day 5 the FBS concentration was increased to 20%. Ascorbic acid (100 μg/mL final concentration) was added to the medium starting at day 7, and the tubing was removed from the NP-CPP composite at day 10. The medium was changed every 2–3 days, and fresh ascorbic acid was added with each change.

Cultures were maintained for 4 weeks to allow for the formation of tissue with properties similar to those of native NP.[33] *In vitro* formed tissues were transferred to DMEM supplemented with 5% FBS 24 hours before addition of TNFα (human recombinant; Sigma, St. Louis, MO). NP tissues were maintained for either 24 or 48 hours in DMEM containing 5% FBS supplemented with various concentrations of TNFα before harvesting.

Histologic Evaluation. The tissues were removed from the CPP surface, fixed in 10% formalin, and paraffin-embedded. 5-μm sections were cut and stained with hematoxylin and eosin or toluidine blue. Three separate sets of cultures were examined.

Deoxyribonucleic acid (DNA) Quantification. The NP tissues were digested by papain (Sigma; 40 μg/mL in 20 mmol/L ammonium acetate, 1 mmol/L ethylenediaminetetraacetic acid

[EDTA], and 2 mmol/L dithiothreitol) for 48 hours at 65°C. The DNA content was determined from aliquots of the papain digest using the HOECHST 33258 dye binding assay (Polysciences, Warrington, PA) and fluorometry (emission wavelength 365 nm, excitation wavelength 458 nm), as previously described.[35] Autofluorescence of NP tissues was corrected for the amount of endogenous fluorescence (in the absence of HOECHST dye). The standard curve was generated using calf thymus DNA (Sigma).

Determination of Proteoglycan and Collagen Contents. The proteoglycan content was determined by measuring the amount of sulfated glycosaminoglycans in the papain-digested samples using the dimethylmethylene blue dye binding assay and spectrophotometry (wavelength 525 nm), as previously described.[36] The standard curve was generated using bovine chondroitin sulfate (Sigma). Collagen content was determined by measuring the hydroxyproline content of the NP tissues. Aliquots of the papain digests were hydrolyzed in 6 N HCl for 18 hours at 110°C, after which the hydroxyproline content was determined using the chloramine-T/Ehrlich's reagent assay and spectrophotometry (wavelength 560 nm), as previously described.[37] The standard curve was generated using L-hydroxyproline (Sigma).

Quantification of Proteoglycan and Collagen Synthesis. To quantify matrix macromolecule synthesis, tissues were incubated in the presence of both $[^{35}S]SO_4$ (6 μCi/construct) to label proteoglycans and [^{3}H]proline (4 μCi/construct) to label collagen during the last 24 hours of TNFα treatment. Radiolabeled tissues were removed from culture and washed 3 times in phosphate-buffered saline to remove unincorporated isotope. Following papain digestion of the tissues, retention of newly synthesized proteoglycan and collagen were determined by quantifying the appropriate radioisotope incorporation using a β-liquid scintillation counter. The proteoglycans in the media were precipitated with 70% ethanol, resuspended in 4 M guanidinium hydrochloride (in 50 mmol/L sodium acetate pH 5.8 containing 0.1 M 6-amino-hexanoic-acid, 50 mmol/L benzamidine HCl, 10 mmol/L EDTA, and 5 mmol/L N-ethylmaleimide), and radioisotope incorporation quantified. Collagen molecules were precipitated from the culture media with 70% ammonium sulfate, resuspended in 10% sodium dodecyl sulfate, and radioisotope incorporation quantified. Total synthesis was determined by combining radioisotope incorporation of both the NP tissue and the culture medium.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was isolated from TNFα-treated tissues by Trizol® (Gibco BRL, Rockville, MD) extraction following disruption of the tissue by mortar and pestle in liquid nitrogen. 0.5 μg of total RNA was reverse transcribed (Superscript First Strand Synthesis System, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Relative gene expression was examined by semiquantitative PCR using Taq Polymerase (Quiagen, Mississauga, ON) and sequence-specific primers in reactions designed to amplify the template sequence of interest within the linear range (Table 1). PCR products were analyzed by electrophoresis on a 1.5% agarose/Tris, Boric Acid, EDTA (TBE) gel containing ethidium bromide and visualized by ultraviolet transillumination. The level of gene expression was determined by densitometry using LabWorks 4.0 Image analysis software (UVP BioImaging Systems, Upland CA) and corrected for 18S rRNA expression.

Quantification of ADAM-TS Message Levels. A competitive PCR that uses an internal RNA standard requiring the same oligonucleotide primers as the authentic message was

Table 1. Gene-Specific Primer Sequences and Amplification Conditions

Gene	Primer Sequence	Product Size (bp)	PCR Conditions
Aggrecan	fwd: 5′-CACTGTTACCGCCACTTCCC-3′ rev: 5′GACATCGTTCCACTCGCCCT-3′	303	95°C 60 s, 60°C 60 s, 72°C 60 s, 26 cycles
Link protein	fwd: 5′-GGTCTGTGCAATATCCCATC-3′ rev: 5′-CCCACTTTAGCAATCTGAGC-3′	231	95°C 60 s, 60°C 60 s, 72°C 60 s, 30 cycles
Type II collagen	fwd: 5′-CCACTGCAAGAACAGCATTG-3′ rev: 5′-CCAGTTCAGGTCTCTTAGAG-3′	463	95°C 60 s, 60°C 60 s, 72°C 60 s, 26 cycles
Type I collagen	fwd: 5′-TGCTGGCCAACTATGCCTCT-3′ rev: 5′-TTGCACAATGCTCTGATC-3′	496	95°C 60 s, 60°C 60 s, 72°C 60 s, 26 cycles
MMP-1	fwd: 5′-AACTCTGGAGCAATGTCACAC-3′ rev: 5′-CCTCATAATCAGCTTGAAGTC-3′	584	95°C 60 s, 55°C 60 s, 72°C 60 s, 30 cycles
MMP-2	fwd: 5′-GCTGAAGGACACCCTGAAGAAGAT-3′ rev: 5′-CCAGATCAGGTGTGTAGCCAATGA-3′	200	95°C 60 s, 55°C 60 s, 72°C 60 s, 28 cycles
MMP-3	fwd: 5′-GTTAGGAGAAAGGACAGTGGTCCTG-3′ rev: 5′-GGCATAGGCATGGGCCAAAACATT-3	402	95°C 60 s, 55°C 60 s, 72°C 60 s, 30 cycles
MMP-13	fwd: 5′-GCTCTGTGATTGACAGGCTT-3′ rev: 5′-GCCAATGAGGTCTACGACAT-3′	1189	95°C 60 s, 60°C 60 s, 72°C 90 s, 28 cycles
ADAM-TS4	fwd: 5′-GCCATTGTGGAGGATGATG-3′ rev: 5′-AGCTGGCACTGGCGGTCA-3′	316	95°C 30 s, 60°C 60 s, 72°C 60 s, 32 cycles
ADAM-TS5	fwd: 5′-AAGCGCTTAATGTCTTCCATC-3′ rev: 5′-TTTTCTTGGTTTTGTCCACACA-3′	471	95°C 60 s, 60°C 60 s, 72°C 60 s, 29 cycles
TIMP-1	fwd: 5′-ATGGCCTCTGGCATCCTGTTG-3′ rev: 5′-AAAGGTGGGAGTGGAAACA-3′	668	95°C 60 s, 60°C 60 s, 72°C 60 s, 28 cycles
TIMP-2	fwd: 5′-GTGGACTCTGGAAAYGACAT-3′ rev: 5′-TCTTCTTCTGGGTGGTGCT-3′	264	95°C 60 s, 55°C 60 s, 72°C 60 s, 28 cycles
TIMP-3	fwd: 5′-CTACACCATCAAGCAGATGAAGATG-3′ rev: 5′-GCTCAGGGGTCTGTGGCATTGAT-3′	457	95°C 60 s, 60°C 60 s, 72°C 60 s, 30 cycles
18S rRNA	fwd: 5′-AAACGGCTACCACATCCAAG-3′ rev: 5′-CCTCCAATGGATCCTCGTTA-3′	150	95°C 60 s, 55°C 60 s, 72°C 60 s, 30 cycles

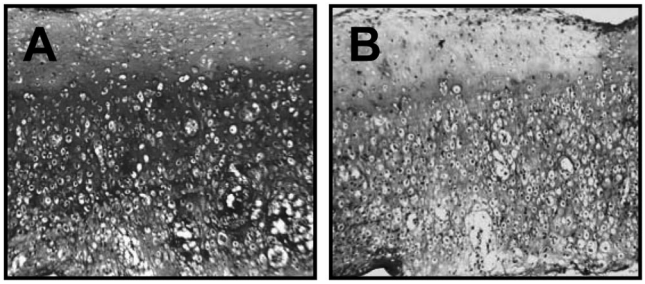


Figure 1. Effect of TNFα treatment on the morphologic appearance of NP tissue. Histologic appearance of untreated *in vitro* formed NP tissue (**A**) and tissue treated for 48 hours with 25 ng/mL TNFα (**B**) (toluidine blue, original magnification ×250).

used to determine the message levels for ADAM-TS4 and ADAM-TS5 in TNFα treated tissues, as previously described.[38] Briefly, templates for the internal standards were designed to contain: the SP6 RNA Pol promoter, the upstream primer sequence corresponding to the message of interest, a bacteriophage lambda sequence, the downstream primer sequence for the message of interest, and a poly-T tail. The length of the lambda sequence was chosen to give a primer separation within the internal standard 60–120 bp larger than the authentic complementary (c)DNA. Message levels in cDNA samples prepared from NP tissues treated for 48 hours with 50 ng/mL TNFα were screened by PCR over a range of internal standard concentrations, using 10-fold dilutions from 10^3 to 10^8 copies. The PCR products were analyzed by electrophoresis, visualized by ultraviolet transillumination, and the ratio of the product intensities derived from the authentic message and internal standard were used to calculate the copy number of the ADAM-TS message in the NP tissues.[38]

Immunoblot Analysis. Matrix proteoglycans were extracted from the NP tissues by incubation in 4 M guanidinium hydrochloride in 50 mmol/L sodium acetate pH 5.8 containing protease inhibitors (0.1 M 6-amino-hexanoic-acid, 50 mmol/L benzamidine HCl, 10 mmol/L EDTA, and 5 mmol/L N-ethylmaleimide) for 48 hours at 4°C. Guanidinium was then removed by dialysis in 25 mmol/L ammonium acetate (1:1000) for 4 days at 4°C, and samples were concentrated by overnight lyophilization. Reconstituted extracts were separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels and electroblotted to nitrocellulose membranes (BioRad, Mississauga, ON). The membranes were then blocked overnight in 3% (wt/vol) bovine serum albumin in tris-buffered saline. Immunodetection of the aggrecan degradation products was performed using a rabbit anti-(aggrecan G1) antibody, prepared as previously described,[39] and an alkaline phosphatase-conjugated goat anti-(rabbit immunoglobulin G) antibody (Promega Corp., Madison, WI). Reactivity was detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Statistical Analysis. Results are expressed as mean ± the standard error of the mean (SEM) from 3 independent experiments. One-way analysis of variance with the Dunnett multiple comparison test was used to compare the untreated NP tissue to TNFα treated samples; as part of this analysis the linear trend of dose responses was also evaluated. P values ≤ 0.05 were considered statistically significant.

■ Results

Histologic Evaluation

Following 4 weeks of culture, a continuous layer of tissue was formed containing the NP cells surrounded by an abundant proteoglycan-rich extracellular matrix (Figure 1A). Within 48 hours, treatment of NP with 25 ng/mL TNFα resulted in decreased toluidine blue staining, suggestive of decreased tissue proteoglycan content (Figure 1B).

Effects of TNFα on Proteoglycan and Collagen Contents of NP Tissue

TNFα induced a dose-dependent decrease in tissue proteoglycan content. There was a significant decrease in glycosaminoglycan content following 24-hour incubation with 5 ng/mL TNFα, which increased to a maximal loss of 55% ± 3% with 50 ng/mL TNFα (Figure 2A). The loss of tissue proteoglycans was more pronounced following 48-hour TNFα treatment, reaching a maximal decrease of 74% ± 2% with 10 ng/mL TNFα. It is interesting that 1 ng/mL TNFα induced a significant loss of tissue proteoglycans within 48 hours. In contrast to the rapid depletion of proteoglycans, TNFα treatment did not significantly alter the collagen content of NP tissues at either time (Figure 2B).

TNFα Induced Changes in Extracellular Matrix Gene Expression

To correlate changes in matrix macromolecule content with changes in gene expression, RNA extracted from TNFα treated tissues underwent RT-PCR using sequence-specific primers (Table 1). Within 24 hours, TNFα induced a dose-dependent decrease in aggrecan

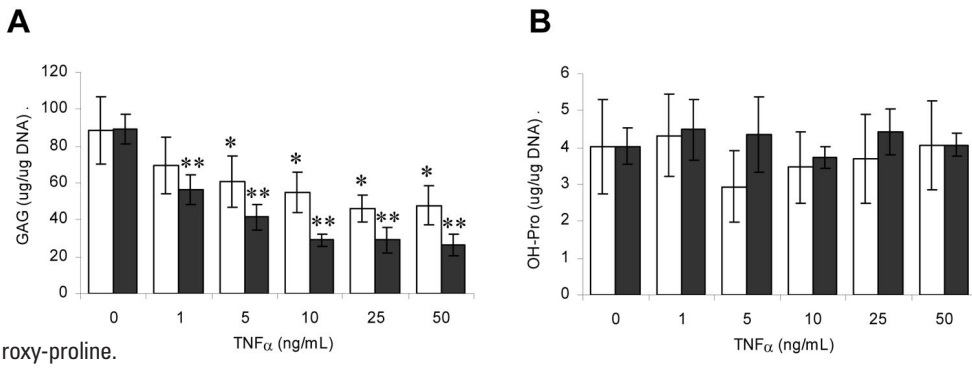
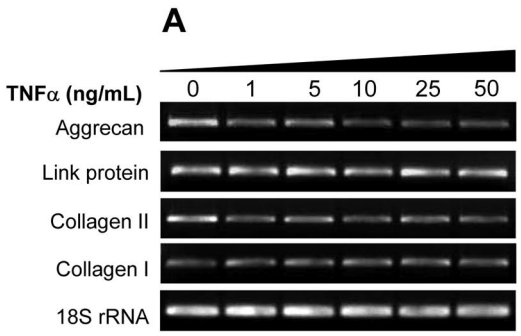


Figure 2. Extracellular matrix content of TNFα treated NP tissue. Proteoglycan (**A**) and collagen (**B**) content of *in vitro* formed NP tissues cultured for 24 (□) or 48 hours (■) with increasing concentrations of TNFα. Results are expressed as mean ± SEM from experiments repeated 3 times. *Values are significantly different from control at 24 hours ($P < 0.05$). **Values are significantly different from control at 48 hours ($P < 0.05$). GAG, glycosaminoglycan; OH-Pro, Hydroxy-proline.



	24 hour					48 hour				
	1 ng/ml	5 ng/ml	10 ng/ml	25 ng/ml	50 ng/ml	1 ng/ml	5 ng/ml	10 ng/ml	25 ng/ml	50 ng/ml
Aggrecan	61± 9	53 ± 6	42 ± 6†	43 ± 14†	35 ± 11†	81 ± 12	62 ± 15	61 ± 5†	59 ± 9†	55 ± 8†
Link prtn	87 ± 7	86 ± 13	73 ± 9	100 ± 10	82 ± 20	94 ± 7	87 ± 9	82 ± 6	89 ± 4	91 ± 16
Collagen II	100 ± 27	100 ± 19	74 ± 14	100 ± 12	82 ± 22	81 ± 17	72 ± 21	76 ± 15	71 ± 16	68 ± 20†
Collagen I	140 ± 28	114 ± 25	125 ± 31	126 ± 42	90 ± 29	103 ± 22	118 ± 25	103 ± 16	107 ± 16	99 ± 26

Figure 3. Effect of TNFα on the expression of extracellular matrix genes. Representative RT-PCR analysis of extracellular matrix gene expression in NP cells treated with increasing concentrations of TNFα for either 24 (**A**) or 48 hours (**B**). Gene expression was semi-quantified by densitometry, as described in the "Materials and Methods," and calculated as percent change from control (corrected for 18S rRNA). The experiment was repeated 3 times, and the results are expressed as mean ± SEM. †Values are significantly different from control at $P < 0.05$. prtn, protein.

gene expression, with a significant decrease observed at 10 ng/mL TNFα (42% ± 6% of control) (Figure 3A). Although unchanged following 24 hours of TNFα treatment, type II collagen gene expression showed a dose-dependent decrease by 48 hours, which was significantly different from controls at 50 ng/mL TNFα (68% ± 20% of control) (Figure 3B). TNFα did not induce any changes in the levels of link protein or type I collagen mRNA at either time (Figure 3).

Effects of TNFα on the Accumulation and Overall Synthesis of Extracellular Matrix Molecules

Patterns of protein synthesis were assessed to determine if TNFα induced changes in gene expression resulted in altered levels of proteoglycan and collagen production. The accumulation of newly synthesized proteoglycan and collagen in NP tissue was quantified in the presence of varying amounts of TNFα up to 50 ng/mL. Twenty-four hour treatment of NP tissues with TNFα induced a dose-dependent decrease in the retention of newly synthesized proteoglycans, with a significant decrease of 28% ± 5% detected at 5 ng/mL TNFα (Figure 4A). The effects of TNFα were more pronounced following 48 hours, resulting in a 45% ± 3% decrease in the retention of newly synthesized proteoglycans at 1 ng/mL TNFα. TNFα induced a similar dose-dependent decrease in the accumulation of newly synthesized collagens. Following

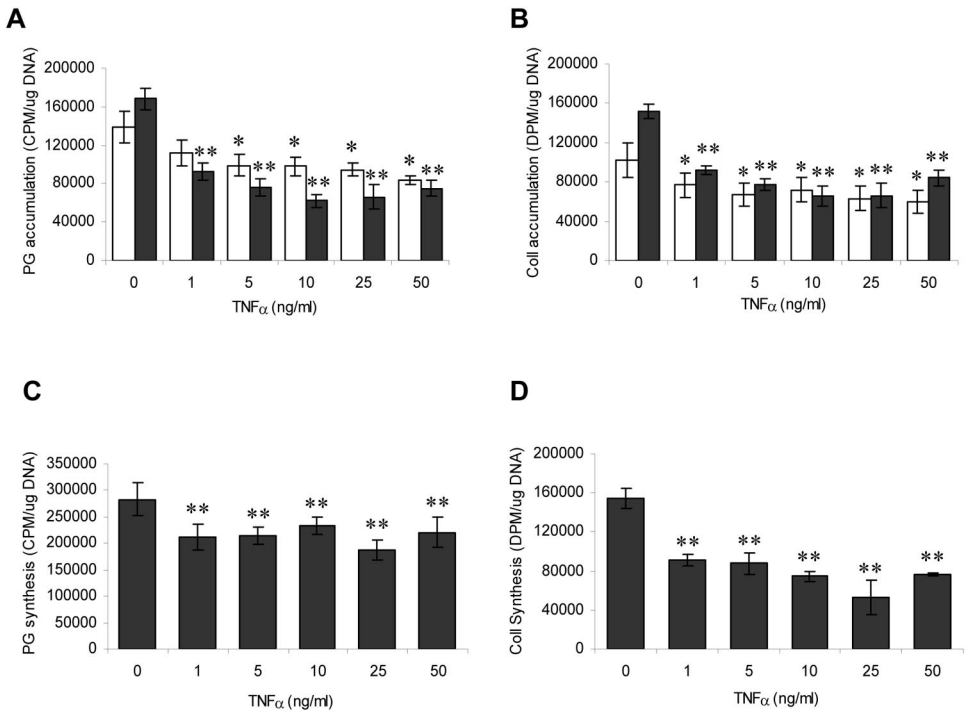


Figure 4. TNFα down-regulates proteoglycan (PG) and collagen (Coll) synthesis. Accumulation of newly synthesized sulfated PGs (**A**) and Colls (**B**) by NP tissues treated for 24 (□) or 48 hours (■) with increasing concentrations of TNFα. Total synthesis of sulfated PGs (**C**) and Colls (**D**) by NP tissues treated with increasing concentrations of TNFα for 48 hours (■). The experiment was repeated 3 times, and the results are expressed as mean ± SEM. *Values are significantly different from control at 24 hours ($P < 0.05$). **Values are significantly different from control at 48 hours ($P < 0.05$).

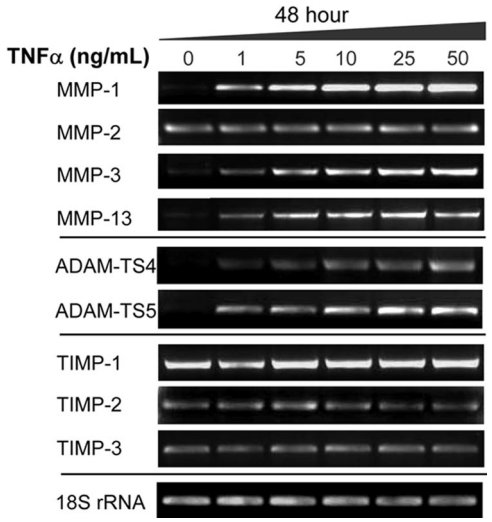


Figure 5. Effect of TNFα on the expression of matrix degrading enzymes and their inhibitors. RT-PCR analysis of gene expression in NP cells treated for 48 hours with increasing concentrations of TNFα. This is a representative gel from an experiment that was repeated 3 times.

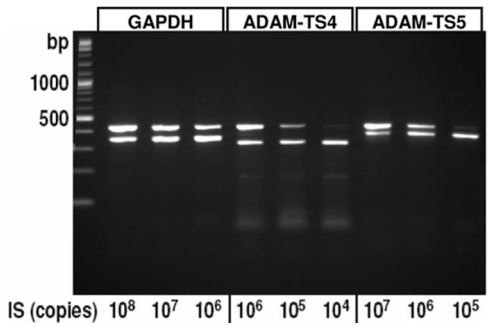


Figure 6. Quantification of TNFα induced aggrecanase gene expression. Quantitative PCR analysis of aggrecanase gene expression in NP cells treated for 48 hours with TNFα (50 ng/mL). cDNA from TNFα treated NP was subjected to PCR amplification for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ADAM-TS4, or ADAM-TS5 in the presence of increasing concentrations of internal standard (IS), as described in the "Materials and Methods."

24 hours, there was a significant decrease in the amount of radiolabeled collagen molecules detectable within TNFα treated NP tissues, which ranged from a 25% ± 4% decrease at 1 ng/mL to 42% ± 3% at 50 ng/mL (Figure 4B). The effects of TNFα were again more pronounced following 48 hours, with a 40% ± 3% decrease in the retention of newly synthesized collagens at 1 ng/mL TNFα.

To ascertain whether the reduced accumulation of newly synthesized proteoglycans and collagens in TNFα treated NP tissues was a result of decreased production, total synthesis was determined following 48-hour TNFα treatment. When compared to untreated tissues, treatment with 1 ng/mL TNFα decreased proteoglycan synthesis by 25% ± 4% (Figure 4C). Although significant, this decrease in proteoglycan synthesis is not as marked as the decreases observed in the retention of newly synthesized proteoglycans in TNFα treated NP tissues (Figure 4A), suggesting that the loss of tissue proteoglycans in response to TNFα may also involve increased proteoglycan catabolism. The 48-hour TNFα treatment caused a significant decrease of 41% ± 0.1% in the synthesis of collagen molecules at 1 ng/mL, with a maximal loss of 67% ± 0.2% at 25 ng/mL (Figure 4D). These results suggest that the decreased retention of newly synthesized collagen molecules in TNFα treated tissues can be accounted for by decreased collagen protein synthesis.

Effect of TNFα on MMP, ADAM-TS, and TIMP Gene Expression

To determine if TNFα treatment was inducing the expression of genes associated with matrix catabolism, the expression of MMPs, aggrecanases, and TIMPs were assessed in NP tissues following 48-hour TNFα treatment (Figure 5). In the absence of cytokine stimulation, NP cells showed constitutive expression of MMP-2, TIMP-1, TIMP-2, and TIMP-3, as well as MMP-1, MMP-3, and MMP-13 at very low levels. Forty-eight-hour TNFα treatment produced a dose-dependent increase in MMP-1, MMP-3, and MMP-13 gene expression, with significant induction at doses as low as 1 ng/mL. TNFα treatment caused a similar dose-dependent induction of aggrecanase gene expression, with significant induction of both ADAM-TS4 and ADAM-TS5 at 1 ng/mL TNFα. TNFα was shown to induce a higher level of ADAM-TS5 gene expression (0.053 ± 0.02) than that of ADAM-TS4 (0.017 ± 0.01) on a per cell basis (relative message levels normalized to glyceraldehyde-3-phosphate dehydrogenase), as determined by competitive PCR (Figure 6). These findings show the preferential induction of ADAM-TS5 gene expression over that of ADAM-TS4 (approximate ratio 4:1). In contrast to the dramatic induction of MMPs and aggrecanase gene expression, the treatment of NP cells with increasing concentrations of TNFα for 48 hours did not alter the expression of TIMP-1, TIMP-2, or TIMP-3.

Mechanism of TNFα Dependent Aggrecan Degradation

To assess the functional activation of proteoglycan-degrading enzymes, TNFα treated NP tissues were examined for the presence of aggrecan degradation. Untreated NP tissue contained low levels of both the MMPs generated fragment (MMP-G1) and the aggrecanase-generated fragment (ADAM-TS-G1) in addition to intact aggrecan (Figure 7A). Treatment of NP tissues with TNFα concentrations higher than 10 ng/mL resulted in the accumulation of the aggrecanase-generated G1 fragment in tissues. No changes in the levels of MMP-generated G1 fragments were observed at all doses of TNFα assayed. In the absence of cytokine stimulation, NP tissue culture medium did not contain detectable levels of either MMPs or aggrecanase-generated fragments (Figure 7B). At concentrations as low as 1 ng/mL TNFα, there was a dramatic accumulation of the aggrecanase-generated G1 fragment in the culture medium. No in-

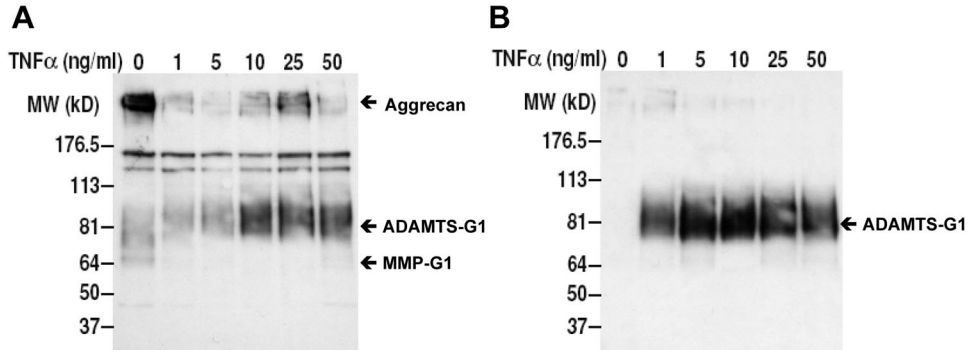


Figure 7. TNFα induces aggrecanase, but not MMP-mediated proteoglycan degradation. Immunoblot analysis of aggrecan degradation products generated in tissues (**A**) and culture medium (**B**) of NP tissues following 48-hour treatment with increasing concentrations of TNFα. Fragments were detected using an antibody reactive with the G1 region of aggrecan, as described in the "Materials and Methods." This is a representative blot from an experiment repeated 3 times. MW, molecular weight.

creased levels of MMP-generated G1 fragments were detected in the culture medium of TNFα treated NP tissues.

■ Discussion

Proinflammatory mediators and cytokines secreted by degenerate IVD tissue are thought to regulate inflammation and tissue resorption. IVD tissue can produce a wide range of inflammatory mediators, including TNFα.[18–20] The current study shows that a brief (48-hour) exposure of NP tissues to low levels of TNFα induces multiple cellular responses, including: decreased expression of both aggrecan and type II collagen genes; decreased accumulation and overall synthesis of aggrecan and collagen; increased expression of MMP-1, MMP-3, MMP-13, ADAM-TS4, and ADAM-TS5; and the induction of aggrecanase dependent proteoglycan degradation. Together, these cellular responses to TNFα result in NP tissue that histologically shows a loss of extracellular matrix and has a maximal loss of 74% of its proteoglycan content. These findings suggest that TNFα could contribute to NP degeneration *in vivo*, however, several factors may influence these findings. First, it is possible that the response of NP cells in the *in vitro* formed tissues to TNFα may be influenced by the tissue culture system used. Alternatively, the presence of the CPP substrate may alter NP cell gene expression. Furthermore, bovine caudal NP cells may differ from human NP cells in their response to inflammatory stimulation, differences that may also reflect physiologic differences between cells in caudal IVD *versus* other locations in the spine.

TNFα production has been detected at levels of approximately 0.4 ng/g in homogenates of surgically removed human lumbar herniated discs.[20] Although recent studies have questioned the origin of TNFα found at the site of IVD degeneration,[40] whether secreted by NP cells in response to proinflammatory stimulus or by macrophages in the surrounding granulation tissue, the current study shows that low levels of TNFα (1 ng/mL), comparable to those present physiologically, increase MMPs and ADAM-Ts gene expression, leading to the depletion of tissue proteoglycans in the early stages *via* aggrecanase mediated degradation. These findings, together with studies showing the prevention of nerve root injury and pain associated with herniated IVD-induced sciatica following anti-TNFα treatment,[24,25,41] show a significant role for TNFα in the pathophysiology of NP degeneration. Furthermore, the up-regulated production of MCP-1 in NP cells following TNFα stimulation has suggested that TNFα may also contribute to the resorption of the herniated disc through the promotion of macrophage infiltration.[42]

Immunohistochemical localization studies in degenerate IVDs have shown the presence of MMPs and, thus, their potential involvement in matrix remodeling and degradation. The current study shows increased gene expression of MMP-1 and MMP-13 following exposure to levels as low as 1 ng/mL TNFα. These findings are in accordance with those of Doita *et al*,[43] who showed increased gene expression and production of MMP-1 in explant cultures of herniated human NP treated for 72 hours with TNFα (50 ng/mL). However, those studies did not differentiate between the latent and active form of the enzymes, and they did not show collagenase-mediated matrix degradation. We observed that although there were changes in collagenase gene expression, the decrease in NP tissue collagen could be accounted for by decreased protein synthesis and, therefore, was not caused by MMP-mediated collagen fibril lysis within 48 hours of TNFα treatment. It appears that NP cells respond similarly to IL-1β because it has been shown that IL-1β induced the synthesis of MMPs, however, the major proportion of the MMPs produced was present in the inactive pro-form, and levels of collagenase activity in NP tissues were unaffected.[44–46] Interestingly, in degenerative diseases such as osteoarthritis and rheumatoid arthritis, proteoglycan degradation precedes collagen catabolism,[47] and it has been shown that the removal of aggrecan is required before collagen degradation occurs in cartilage.[48] Therefore, the cells may require a

more prolonged exposure to TNFα before MMP-mediated collagen degradation is detected in NP tissues.

Our data suggest that MMP-mediated aggrecan catabolism does not contribute to the loss of tissue proteoglycans following short-term exposure to TNFα. Instead proteoglycan degradation appears to be a result of the induction of aggrecanase activity. In chondrocytes, expression of ADAM-TS5 is constitutive and unaltered following exposure of tissues or cells to inflammatory cytokines, whereas expression of ADAM-TS4 is enhanced following TNFα stimulation.[49] Although ADAM-TS4 and ADAM-TS5 show similar substrate specificities, the increased activity level of ADAM-TS4 has led to the suggestion that its expression is more detrimental to the health of cartilage.[47] In contrast to articular chondrocytes, we show that NP cells do not constitutively express either ADAM-TS4 or ADAM-TS5 under our experimental conditions and that expression of the message for both of these enzymes is significantly up-regulated by exposure to TNFα at concentrations as low as 1 ng/mL. Levels of ADAM-TS5 mRNA were significantly higher than those of ADAM-TS4, suggesting that ADAM-TS5 may be the major aggrecanase in the IVD, similar to patterns observed in human articular chondrocytes.[50] These findings may reflect the tissue culture system used to form NP tissues because recent studies have shown that the expression level of many enzymes was significantly higher *in vitro* than *in vivo*. However, in preliminary studies examining gene expression in native bovine NP, we have been unable to show constitutive ADAM-TS expression (data not shown).

It is noteworthy that while the current study shows the induction of MMPs and ADAM-TS gene expression and ADAM-TS enzyme activity in response to TNFα, there were no significant alterations in the expression of TIMP-1, TIMP-2, or TIMP-3, inhibitors that play an important role in the regulation of extracellular matrix degradation. Although conflicting reports exist as to the regulation of TIMPs expression in degenerate IVDs, an imbalance between MMPs and their inhibitors has been proposed to exist in the degenerating disc.[6,16] Because aggrecanase-mediated catabolism appears to play an essential role in the early stages of NP tissue degradation in response to TNFα, the lack of change in TIMP-3 expression may be compounding ADAM-TS mediated degradation. Further study is required to understand the contribution of TIMP-3 to tissue degeneration. It may be that targeting the expression level of this protein may represent a promising avenue toward the prevention of NP tissue degradation.

These studies show the exquisite sensitivity of NP tissues to TNFα stimulation and provide evidence that the ADAM-TS enzymes are responsible for aggrecan cleavage during cytokine-induced IVD degeneration. In addition, although based only on a limited number of genes, this study suggests that TNFα stimulation of NP cells is a good model to study the induction of catabolic processes known to occur during disc degeneration *in vivo*. Delineating the response of NP tissues to cytokine exposure may help us understand the stepwise progression of IVD degeneration or the mechanisms regulating the initiation of NP tissue resorption following herniation.

■ Key Points

- NP tissues show an exquisite sensitivity to TNFα treatment; doses as low as 1 ng/ml dramatically alter patterns of gene expression and protein synthesis.
- Following a brief (48-hour) exposure to TNFα, NP tissues show decreased gene expression and protein synthesis of proteoglycan and collagens.
- Short-term exposure of NP cells to TNFα activates aggrecanase-mediated proteoglycan degradation.

Acknowledgments

The authors thank Mr. Harry Bojarski and Ryding-Regency Meat Packers Ltd., Toronto, Canada, for providing the bovine tissues. They also thank Mona Reid for preparation of histologic sections.

References

1. Waddell G. Low back pain: A twentieth century health care enigma. *Spine* 1996;21:2820–5.
2. Goupille P, Jayson MI, Valat JP, et al. Matrix metalloproteinases: The clue to intervertebral disc degeneration? *Spine* 1998;23:1612–26.
3. Antoniou J, Steffen T, Nelson F, et al. The human lumbar intervertebral disc: Evidence for changes in the biosynthesis and denaturation of the extracellular matrix with growth, maturation, ageing, and degeneration. *J Clin Invest* 1996;98:996–1003.
4. Buckwalter JA. Aging and degeneration of the human intervertebral disc. *Spine* 1995;20:1307–14.
5. Ahsan R, Tajima N, Chosa E, et al. Biochemical and morphological changes in herniated human intervertebral disc. *J Orthop Sci* 2001;6:510–8.
6. Roberts S, Caterson B, Menage J, et al. Matrix metalloproteinases and aggrecanase: Their role in disorders of the human intervertebral disc. *Spine* 2000;25:3005–13.
7. Sive JI, Baird P, Jeziorsk M, et al. Expression of chondrocyte markers by cells of normal and degenerate intervertebral discs. *Mol Pathol* 2002;55:91–7.
8. Cs-Szabo G, Ragasa-San Juan D, Turumella V, et al. Changes in mRNA and protein levels of proteoglycans of the anulus fibrosus and nucleus pulposus during intervertebral disc degeneration. *Spine* 2002;27:2212–9.
9. Nagase H, Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem* 1999; 274:21491–4.
10. Brinckerhoff CE, Matrisian LM. Matrix metalloproteinases: A tail of a frog that became a prince. *Nat Rev Mol Cell Biol* 2002;3:207–14.
11. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. *Circ Res* 2003;92: 827–39.
12. Cawston TF, Billington CF, Cleaver CF, et al. The regulation of MMPs and TIMPs in cartilage turnover. *Ann N Y Acad Sci* 1999;878:120–9.
13. Arner EC, Pratta MA, Trzaskos JM, et al. Generation and characterization of aggrecanase. A soluble, cartilage-derived aggrecan-degrading activity. *J Biol Chem* 1999;274:6594–601.
14. Tortorella MD, Burn TC, Pratta MA, et al. Purification and cloning of aggrecanase-1: A member of the ADAMTS family of proteins. *Science* 1999; 284:1664–6.
15. Weiler C, Nerlich AG, Zipperer J, et al. 2002 SSE Award Competition in Basic Science: Expression of major matrix metalloproteinases is associated with intervertebral disc degradation and resorption. *Eur Spine J* 2002;11: 308–20.
16. Kanemoto M, Hukuda S, Komiya Y, et al. Immunohistochemical study of matrix metalloproteinase-3 and tissue inhibitor of metalloproteinase-1 human in intervertebral discs. *Spine* 1996;21:1–8.

17. Kang JD, Georgescu HI, McIntyre-Larkin L, et al. Herniated lumbar intervertebral discs spontaneously produce matrix metalloproteinases, nitric oxide, interleukin-6, and prostaglandin E2. *Spine* 1996;21:271–7.
18. Olmarker K, Larsson K. Tumor necrosis factor alpha and nucleus-pulposus-induced nerve root injury. *Spine* 1998;23:2538–44.
19. Igarashi T, Kikuchi S, Shubayev V, et al. 2000 Volvo Award winner in basic science studies: Exogenous tumor necrosis factor-alpha mimics nucleus pulposus-induced neuropathology. Molecular, histologic, and behavioral comparisons in rats. *Spine* 2000;25:2975–80.
20. Takahashi H, Suguro T, Okazima Y, et al. Inflammatory cytokines in the herniated disc of the lumbar spine. *Spine* 1996;21:218–24.
21. Rand N, Reichert F, Floman Y, et al. Murine nucleus pulposus-derived cells secrete interleukins-1-beta, -6, and -10 and granulocyte-macrophage colony-stimulating factor in cell culture. *Spine* 1997;22:2598–601.
22. Schuerwegh AJ, Dombrecht EJ, Stevens WJ, et al. Influence of pro-inflammatory (IL-1 alpha, IL-6, TNF-alpha, IFN-gamma) and anti-inflammatory (IL-4) cytokines on chondrocyte function. *Osteoarthritis Cartilage* 2003;11:681–7.
23. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* 1996;14:397–440.
24. Olmarker K, Rydevik B. Selective inhibition of tumor necrosis factor-alpha prevents nucleus pulposus-induced thrombus formation, intraneural edema, and reduction of nerve conduction velocity: Possible implications for future pharmacologic treatment strategies of sciatica. *Spine* 2001;26:863–9.
25. Karppinen J, Korhonen T, Malmivaara A, et al. Tumor necrosis factor-alpha monoclonal antibody, infliximab, used to manage severe sciatica. *Spine* 2003;28:750–3.
26. Alini M, Roughley PJ, Antoniou J, et al. A biological approach to treating disc degeneration: Not for today, but maybe for tomorrow. *Eur Spine J* 2002;11 (suppl 2):S215–20.
27. Baer AE, Wang JY, Kraus VB, et al. Collagen gene expression and mechanical properties of intervertebral disc cell-alginate cultures. *J Orthop Res* 2001;19:2–10.
28. Gruber HE, Stasky AA, Hanley EN Jr. Characterization and phenotypic stability of human disc cells in vitro. *Matrix Biol* 1997;16:285–8.
29. Horner HA, Roberts S, Bielby RC, et al. Cells from different regions of the intervertebral disc: Effect of culture system on matrix expression and cell phenotype. *Spine* 2002;27:1018–28.
30. Maldonado BA, Oegema TR Jr. Initial characterization of the metabolism of intervertebral disc cells encapsulated in microspheres. *J Orthop Res* 1992; 10:677–90.
31. Wang JY, Baer AE, Kraus VB, et al. Intervertebral disc cells exhibit differences in gene expression in alginate and monolayer culture. *Spine* 2001;26: 1747–51.
32. Urban JP, Maroudas A. Swelling of the intervertebral disc in vitro. *Connect Tissue Res* 1981;9:1–10.
33. Seguin CA, Grynpas MD, Pilliar RM, et al. Tissue engineered nucleus pulposus tissue formed on a porous calcium polyphosphate substrate. *Spine* 2004;29:1299–1306.
34. Pilliar RM, Filiaggi MJ, Wells JD, et al. Porous calcium polyphosphate scaffolds for bone substitute applications–In vitro characterization. *Biomaterials* 2001;22:963–72.
35. Kim YJ, Sah RL, Doong JY, et al. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal Biochem* 1988;174:168–76.
36. Goldberg RL, Kolibas LM. An improved method for determining proteoglycans synthesized by chondrocytes in culture. *Connect Tissue Res* 1990;24: 265–75.
37. Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. *Clin Biochem* 1996;29:225–9.
38. Melching LI, Cs-Szabo G, Roughley PJ. Analysis of proteoglycan messages in human articular cartilage by a competitive PCR technique. *Matrix Biol* 1997;16:1–11.
39. Sztrolovics R, Alini M, Roughley PJ, et al. Aggrecan degradation in human intervertebral disc and articular cartilage. *Biochem J* 1997;326:235–41.
40. Burke JG, G Watson RW, Conhyea D, et al. Human nucleus pulposis can respond to a pro-inflammatory stimulus. *Spine* 2003;28:2685–93.
41. Cooper RG, Freemont AJ. TNF-alpha blockade for herniated intervertebral disc-induced sciatica: a way forward at last? *Rheumatology (Oxford)* 2003; 43:119–21.
42. Yoshida M, Nakamura T, Kikuchi T, et l. Expression of monocyte chemoattractant protein-1 in primary cultures of rabbit intervertebral disc cells. *J Orthop Res* 2002;20:1298–304.
43. Doita M, Kanatani T, Ozaki T, et al. Influence of macrophage infiltration of herniated disc tissue on the production of matrix metalloproteinases leading to disc resorption. *Spine* 2001;26:1522–7.
44. Kang JD, Stefanovic-Racic M, McIntyre LA, et al. Toward a biochemical understanding of human intervertebral disc degeneration and herniation. Contributions of nitric oxide, interleukins, prostaglandin E2, and matrix metalloproteinases. *Spine* 1997;22:1065–73.
45. Sakuma M, Fujii N, Takahashi T, et al. Effect of chondroitinase ABC on matrix metalloproteinases and inflammatory mediators produced by intervertebral disc of rabbit in vitro. *Spine* 2002;27:576–80.
46. Shen B, Melrose J, Ghosh P, et al. Induction of matrix metalloproteinase-2 and -3 activity in ovine nucleus pulposus cells grown in three-dimensional agarose gel culture by interleukin-1beta: A potential pathway of disc degeneration. *Eur Spine J* 2003;12:66–75.
47. Tortorella MD, Liu RQ, Burn T, et al. Characterization of human aggrecanase 2 (ADAM-TS5): Substrate specificity studies and comparison with aggrecanase 1 (ADAM-TS4). *Matrix Biol* 2002;21:499–511.
48. Pratta MA, Yao W, Decicco C, et al. Aggrecan protects cartilage collagen from proteolytic cleavage. *J Biol Chem* 2003;278:45539–45.
49. Tortorella MD, Malfait AM, Deccico C, et al. The role of ADAM-TS4 (aggrecanase-1) and ADAM-TS5 (aggrecanase-2) in a model of cartilage degradation. *Osteoarthritis Cartilage* 2001;9:539–52.
50. Bau B, Gebhard PM, Haag J, et al. Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. *Arthritis Rheum* 2002;46:2648–57.