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A role for TNFα in intervertebral disc degeneration: A non-recoverable catabolic shift

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ABSTRACT

This study examines the effect of TNFα on whole bovine intervertebral discs in organ culture and its association with changes characteristic of intervertebral disc degeneration (IDD) in order to inform future treatments to mitigate the chronic inflammatory state commonly found with painful IDD. Pro-inflammatory cytokines such as TNFα contribute to disc pathology and are implicated in the catabolic phenotype associated with painful IDD. Whole bovine discs were cultured to examine cellular (anabolic/catabolic gene expression, cell viability and senescence using β-galactosidase) and structural (histology and aggrecan degradation) changes in response to TNFα treatment. Control or TNFα cultures were assessed at 7 and 21 days; the 21 day group also included a recovery group with 7 days TNFα followed by 14 days in basal media. TNFα induced catabolic and anti-anabolic shifts in the nucleus pulposus (NP) and annulus fibrosus (AF) at 7 days and this persisted until 21 days however cell viability was not affected. Data indicates that TNFα increased aggrecan degradation products and suggests increased β-galactosidase staining at 21 days without any recovery. TNFα treatment of whole bovine discs for 7 days induced changes similar to the degeneration processes that occur in human IDD: aggrecan degradation, increased catabolism, pro-inflammatory cytokines and nerve growth factor expression. TNFα significantly reduced anabolism in cultured IVDs and a possible mechanism may be associated with cell senescence. Results therefore suggest that successful treatments must promote anabolism and cell proliferation in addition to limiting inflammation.

1. Introduction

Pro-inflammatory cytokine expression increases with age and severity of intervertebral disc (IVD) degeneration (IDD) [1]. Pro-inflammatory cytokines can cause structural deterioration via increased catabolism in IVD cells and influence pain-related factors with up-regulation of substance P, NGF and VEGF [2–4], highlighting a role for inflammation in the pathogenesis of painful human IDD. While pro-inflammatory mediators appear to be involved in this pathology it remains unclear if a single inflammatory insult, for example resulting from a single injury, is sufficient to initiate an inflammatory cascade in a healthy IVD. It is also unclear whether pro-inflammatory mediators influence other biologic characteristics associated with IDD such as cellular senescence.

The pro-inflammatory cytokines principally associated with the progression of IDD are tumor necrosis factor-α (TNFα) and interleukin 1-β (IL-lβ) although others have also been implicated [2,4–8]. TNFα is expressed by IVD cells and has been suggested to be involved in the early onset of IDD through initiating an inflammatory cascade [9] although it remains unclear if a healthy IVD can recover following exposure to TNFα.

Along with the increases in TNFα expression that correlate with degenerative grade, a parallel increase in cellular senescence has been observed with the IDD [10–12]. In cartilage a relationship between pro-inflammatory cytokines and senescence has also been demonstrated where IL-1β was shown to induce premature senescence in osteoarthritic chondrocytes [13]. This suggests that increases in TNFα may also be related to the cellular senescence observed in IVD degeneration.

The aim of this study is to investigate a potential role of TNFα in the processes associated with degeneration of the IVD. We hypothesized that healthy bovine IVDs can recover from TNFα exposure and that TNFα exposure would cause a significant catabolic shift and increase the amount of senescent cells observed in the IVD. Bovine IVD organ culture models are widely utilized large animal

Abbreviations: IDD, intervertebral disc degeneration; TNFα, tumor necrosis factor alpha.

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models with similar size and composition as human IVDs, and these models allow multiple measurements of matrix accumulation and breakdown while maintaining *in-situ* cell matrix connectivity [14–16]. IVD organ culture also enables development of cytokine-matrix interactions that are known to strongly influence cellular responses in bone and cartilage tissues [17,18]. Organ culture models have substantial control over boundary conditions enabling investigation of the isolated effects of an individual cytokine in the IVD independent of systemic immune responses. Bovine caudal IVD explant cultures were used to examine responses at the cellular and structural levels at various time points with emphasis on cell viability, cellular senescence, gene expression for matrix structural proteins, enzymes, cytokines and symptom-modifying factors, along with assessment of matrix degradation through histology and western blot.

2. Methods and materials

Caudal IVDs were harvested from skeletally mature bovine tails obtained from a local abattoir (Green Village Packing Co., NJ) and endplates were removed to promote maximal transport and cell viability. After isolation, IVD height, diameter, and weight were recorded. Isolated IVDs were assigned to Control (cultured in control media consisting of high glucose DMEM, 10% FBS, 50 μg/mL ascorbic acid, 1% penicillin/streptomycin and 0.5% fungizone) or TNFα (control media + 200 ng/mL human recombinant TNFα; Invitrogen Cat #PHC3016) groups and cultured for 7 days ($N = 7$/group). Additional cultures for 21 days ($N = 9$/group) consisted of three groups; a Control, TNFα and a Recovery group which involved 7 days with TNFα exposure followed by 14 days of recovery in control media. All IVDs were cultured at 37 °C & 5% CO_2, and loaded under 0.2 MPa static compression. Media was continuously circulated and changed and collected every 3–4 days. Following culture, IVD height, diameter and wet weight were recorded. IVD tissue was divided into four sagittal sections (~4 mm wide) using a custom dissection tool, and each section was used to assess tissue viability, qRT-PCR, histology, and Western blot analysis of aggrecan degradation respectively.

Tissue viability was assessed at 7 and 21 days via a double staining technique using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) to stain viable cells, and 4′,6-diamidino-2-phenylindole (DAPI, Roche Diagnostics, Germany) to stain cell nuclei, with processing and quantification as previously described [16].

Real-time qRT-PCR was performed on 7 days and 21 days specimens. Tissue from the AF and NP regions were separated from the inner AF which was discarded and RNA extracted using the Qiagen RNEasy Kit (Qiagen, USA). Gene expression of housekeeping (18S), anabolic (Aggrecan, Collagen I, and Collagen II), catabolic (MMP-3, MMP-13, and ADAMTS-5) and pro-inflammatory genes (TNFα, IL-1β, IL-6) were assessed using SYBR green bovine specific primers [16]. Gene expression for angiogenic/pain-related genes (VEGF (Bt03213283_m1), NGF (Bt03817604_s1), Substance-P (Bt03259156_m1)) and 18S (Hs03928985_g1) were also assessed via TAQMAN assays on demand (Applied Biosystems, Bedford MA). Gene expression levels were analyzed using the $\Delta\Delta C_T$ method and normalized to 18S and time-matched controls, as described [19].

IVD structure and composition were evaluated with Western blot, water content and histology. Western blot used an antibody specific to the G1 region of aggrecan to assess aggrecan degradation in AF and NP regions [20]. GAG loss to the culture media was analyzed using the dimethylmethylene blue (DMB) dye binding assay, and normalized to initial IVD wet weight [21]. AF and NP water content were calculated from wet and dry weights. Sagittal histology sections 10 μm thick were stained with picosirius red and alcian blue [16] as a further measure of IVD structural integrity and composition. Histological sections were scored by three blinded reviewers using a semi-quantitative histological grading scheme modified from that described by Sive et al. [22] (Supplemental Fig. A). Briefly the grading system assessed three histological characteristics; loss of demarcation between NP and AF (0–3), loss of proteoglycans from the NP (0–3) and the presence & extent of fissures (0–3), with a cumulative score range from 0 (healthy) to 9 (degenerated).

Histology samples from the 21 day timepoint were also processed for senescence associated β-galactosidase (SA-β-gal) immunohistochemistry ($N = 3$/group) which is a common technique for evaluating senescence in the IVD [10]. Sections (10 μm) were deparaffinized, a primary anti-rabbit β-galactosidase antibody (ab4761, Abcam, Cambridge, MA) and a goat anti-rabbit HRP polymer secondary antibody (ab94710, Abcam Cambridge, MA) were used with omission of primary antibody as a negative control. Samples were then counter stained with Toluidine Blue. Twenty images representative of the entire IVD were captured from the each disc at 40x magnification. The percent of positive β-galactosidase stained cells were calculated in each IVD.

Student's t-test compared the $\Delta\Delta C_T$ and water content values for the 7 day results (Control vs. TNFα). One-way ANOVA with a Tukey post hoc test compared the $\Delta\Delta C_T$'s, water content, viability, β-galactosidase quantification and GAG present in the media (DMB) for the 21 day results (Control, TNFα, and Recovery). One-way ANOVA with a Tukey post hoc test compared the semi-quantitative histological grading between four Groups (Control, 7 day TNFα, 21 day TNFα, Recovery). To assess whether exposure to TNFα increased β-galactosidase staining a one-tailed t-test compared Control to pooled TNFα groups (21 day TNFα + Recovery) was used. Statistical analyses were performed using GraphPad Prism 3 (La Jolla, Ca) with $p < 0.05$ significant. Variance is given as standard deviations (SD) throughout.

3. Results

All cultures remained viable throughout culture period with no differences between groups in all regions (AF, IAF, NP) and time points (Supplemental Fig. B). Viability in AF was 80.3 ± 11.4%, 78.2 ± 11.3%, 80.1 ± 8.9% for control, 21 day TNFα, and Recovery groups, respectively. The viability in IAF was 75.6 ± 19.1%, 80.2 ± 10.4%, 77.4 ± 13.2% for Control, 21 day TNFα, and Recovery groups, respectively and in the NP was 61.9 ± 20.4%, 81.8 ± 10.6%, 82.0 ± 12.4% for Control, 21 day TNFα, and Recovery groups, respectively.

In the AF, 7 days of TNFα exposure significantly down-regulated gene expression of aggrecan (−5.7 fold), and significantly up-regulated MMP-3 (3.9 fold), MMP-13 (3.1 fold), IL-6 (2.9 fold), and NGF (3.2 fold) (Fig. 1). After 21 days of TNFα exposure AF gene expression was significantly up-regulated for MMP-3 (10.8 fold) only, and this was not up-regulated for the recovery group. In the NP, 7 days of TNFα exposure significantly down-regulated aggrecan (−18.3 fold), collagen I (−23.1 fold), collagen II (−15.7 fold), and MMP-3 (−14.7 fold) and significantly up-regulated IL-1β (45.0 fold), and IL-6 (56.4 fold). After 21 days, NP gene expression was significantly down–regulated in TNFα and Recovery groups for collagen I (−15.8 fold, −69.4 fold), collagen II (−17.3 fold, −23.5 fold), and ADAMTS-5 (−14.2 fold, −67.3 fold), respectively, while MMP-3 (−35.5 fold) was significantly down-regulated only in the Recovery group. TNFα mRNA was not consistently expressed in any samples.

TNFα induced substantial matrix breakdown. Histological staining demonstrated a reduction in the intensity of alcian blue staining in the NP of TNFα treated samples at 7 and 21 days, suggesting

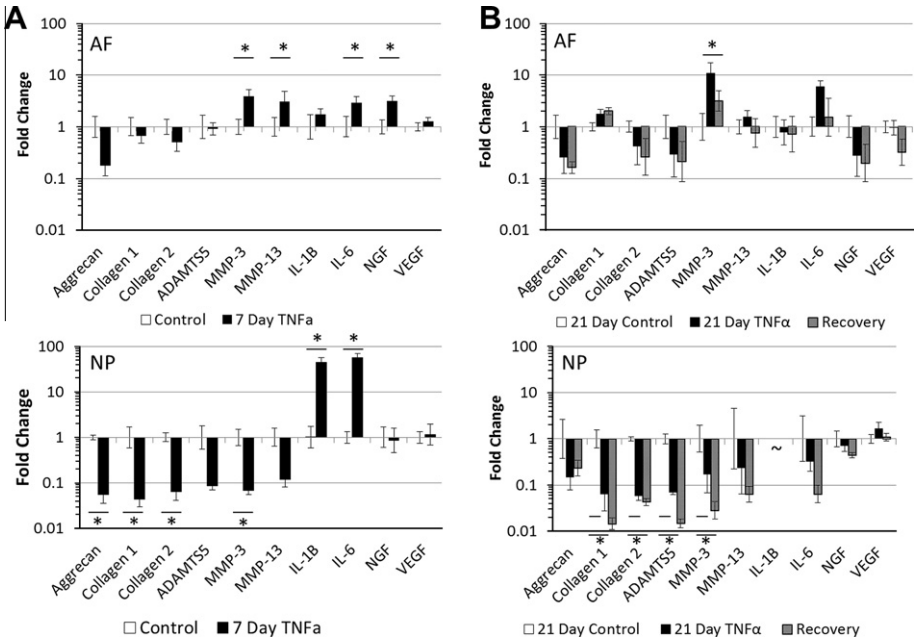

Fig. 1. Gene expression results from the annulus fibrosus (AF) and nucleus pulposus (NP) normalized to 18S and time-matched controls, after (A) 7 days and (B) 21 days in culture. In general, there was a large catabolic shift with down-regulation of anabolic gene expression and up-regulation of some catabolic genes and pro-inflammatory cytokines for TNFα and Recovery groups in both AF and NP regions and for both time points. There were no differences between TNFα and Recovery groups. Significant differences from the value one are noted with ∗ and bars represent differences between groups, $p < 0.05$.

a loss/degradation of proteoglycans relative to Control (Fig. 2). Semi-quantitative analysis demonstrated a significant increase in degenerative grade in all groups treated with TNFα compared to control (Supplemental Fig. C). The histological score was significantly worsened for all TNFα groups with values of 1.4 ± 0.5, 5.8 ± 0.8 ($p < 0.01$), 4.1 ± 2 ($p < 0.05$), and 5.2 ± .7 ($p < 0.01$) for Control, 7 day TNFα, 21 days TNFα, and Recovery groups, respectively. Western blot analysis confirmed an increase in degraded aggrecan products in the NP of TNFα treated IVDs at both 7 and 21 days (Fig. 3A) and a trend of increased GAG in the culture medium was observed at 21 days (ANOVA, $p = 0.073$) with values of 33.2 ± 5.7, 43.4 ± 17.8, and 52.2 ± 22.0 μg GAG/(mg wet weight) for Control, 21 days TNFα, and Recovery groups, respectively (Fig. 3B). Interestingly, at 21 days no differences were observed

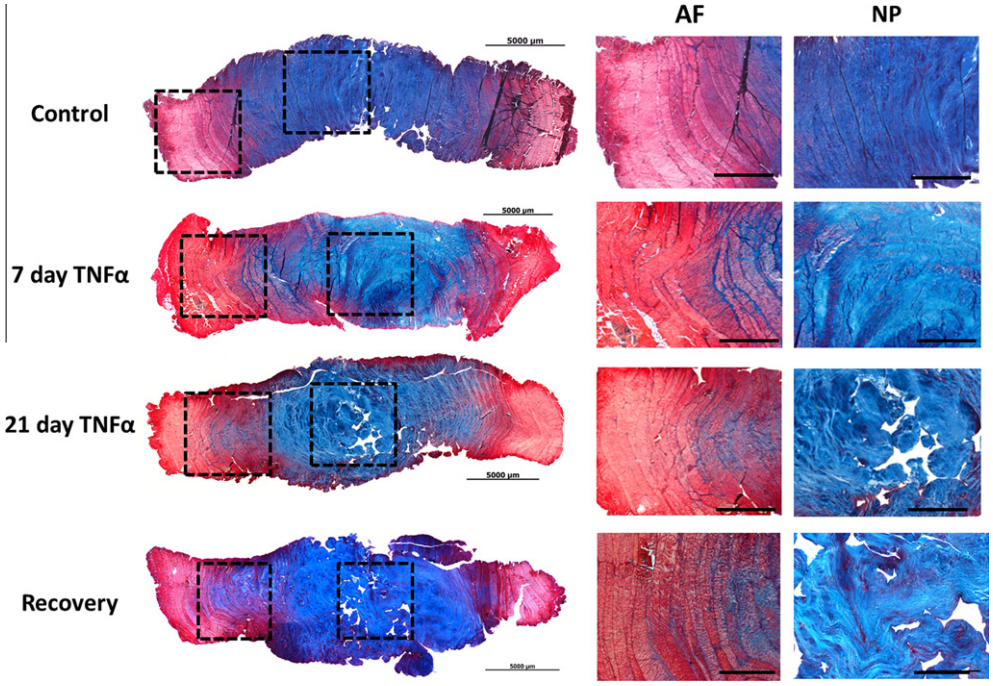

Fig. 2. Structural histology for day 7 and day 21 experimental groups; no differences were observed between 7 and 21 day Control groups. Tissue stained with Picosiruis red Alcian blue. All images were taken at 2.5x and scale bars represents 1000 μm. Notable morphological changes in TNFa and Recovery groups included loss of proteoglycan staining in the nucleus pulposus, loss of demarcation between AF & NP, and AF encroachment into the NP region, particularly for the 21 day groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

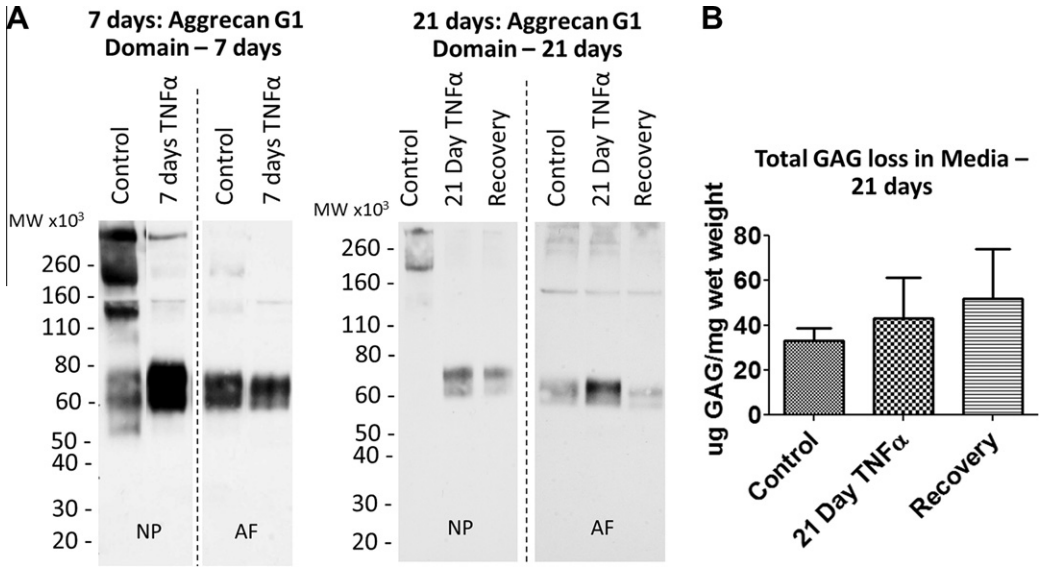

Fig. 3. (A) Western blot for the aggrecan G1 domain in the NP and AF at 7 days and 21 days. There was substantially more degraded aggrecan in the NP of TNFα and Recovery groups at day 7 and 21 with minimal differences in degraded aggrecan observed in the AF region. (B) Total GAG loss to cell culture media at 21 days with μg GAG normalized to mg tissue wet weight.

in the aggrecan degradation products between the 21 day TNFα and Recovery groups. There were no significant changes in water content or disc height between any samples at any time point.

There was an increased trend ($p = 0.108$) of positively SA-β-gal stained cells in pooled TNFα treated IVDs (44.1 ± 29.4%) compared to Control (19.0 ± 15.3%) (Fig. 4A and B). A similar observation was made between Control (19.0 ± 15.3%), 21 day TNFα (33.8 ± 30.0%) and Recovery groups (54.5 ± 30.6%) with $p = 0.32$.

4. Discussion

This study assessed the role of TNFα in contributing to pathologic processes associated with IDD using a large animal IVD organ culture system. TNFα treatment for 7 days induced many of the structural and compositional degenerative changes known to be important in human IDD including large catabolic and anti-anabolic shifts on the gene level, up-regulation of genes predictive of painful conditions, rapid accumulation of degraded aggrecan fragments and structural alterations including AF encroachment into the NP. These degradation patterns were also observed following culture with TNFα for 21 days. However when TNFα was present for the first 7 days of culture and then removed for the last 14 days, there was no apparent recovery suggesting that IVDs cannot recover from TNFα exposure. Interestingly, TNFα was not a source of lost IVD cellularity but suggested an increase in the amount senescent cells which may provide a rationale for the lack of recovery and highlights a potential role for TNFα in the senescence associated with IDD. TNFα exposure also stimulated mRNA production of IL-1β, and IL-6 supporting and strengthening its role as an initiator and regulator of multiple pro-inflammatory cytokines in intact IVDs.

TNFα exposure induced a dramatic catabolic shift with proteoglycan degradation at 7 and 21 days that was not reversed following TNFα removal. Western blot indicated aggrecan degradation was primarily due to ADAMTS activity (two bands between 60–80 kDa MW) and not MMP activity (~50 kDa MW) as suggested by the size of the degraded fragments. TNFα up-regulates ADAMTS-5 expression in NP cells through modulation of syndecan-4 via the NF-κB pathway [8]. This pathway is likely involved our model and is supported by our finding of degradation resulting primarily from ADAMTS and not MMP activity. Another potential mechanism that could be involved in the catabolic shift observed

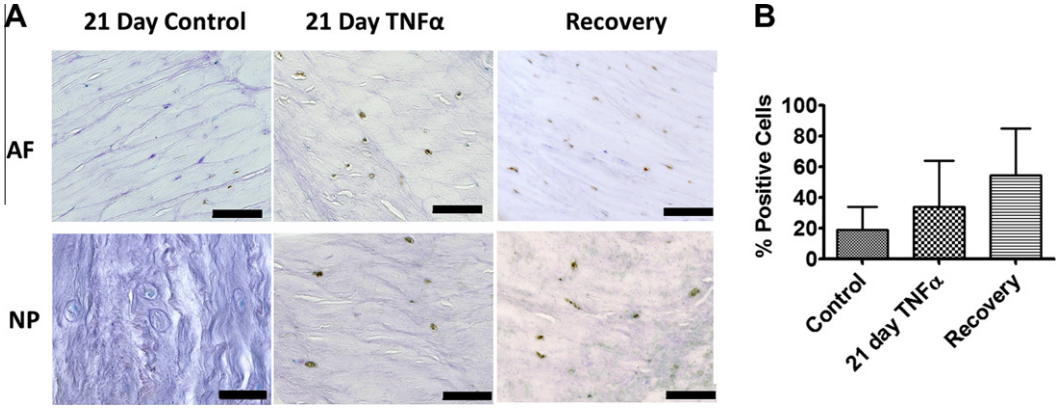

Fig. 4. (A) Immunohistochemistry for senescence associated β-galactosidase (SA-β-gal), demonstrating an increase in staining in the 3 week TNFα and Recovery groups relative to Control. Pictures were taken at 40× and scale bar represents 50 μm. Annulus fibrosus (AF) and nucleus pulposus (NP). (B) The percentage of SA-β-gal stained cells in 21 Day TNFα and Recovery groups compared to Control.

in this model is through TNFα inducing PHD3 expression that promotes NF-kB mediated catabolism [23] .

Comparing the protein changes with gene expression in the NP suggests the majority of the aggrecan degradation may have occurred early in the culture period since there was already substantial aggrecan degradation and a decrease in ADAMTS-5 gene expression in the NP by day 7. The different responses in catabolic gene expression between the AF and NP observed at both 7 and 21 days were notable and may be explained by differences in the kinetic response between NP and AF cells based on their heterogeneity, distinct cell lineages and diverse tissue functions. However, the large amounts of aggrecan degradation in the NP suggest these regional differences are not related to insufficient dosage. While it is possible that ADAMTS-4 or other aggrecanases were expressed and activated, we focused on ADAMTS-5 in this study because it has more potent degradative potential [8,24].

TNFα exposure led to an increased trend in SA-β-gal staining which may have contributed to the continued IVD degradation and inability of the IVD to recover following removal of TNFα. Increases in cellular senescence have been observed in degenerated IVDs and micro-environmental stress-induced cellular senescence may be a potential mechanism [11,25,26]. The literature suggests possible upstream signaling pathways that could lead to pro-inflammatory cytokine induced senescence. TNFα treatment of NP cells increases Wnt/β-catenin signaling [24] and activity of this signaling pathway has also been shown to enhance cellular senescence in IVD cells [27]. Also, treatment of osteoarthritic chondrocytes with IL-1β induced pre-mature senescence mediated by caveolin-1, and IDD is known to be associated with increased caveolin-1 expression, pro-inflammatory cytokine expression and cellular senescence [10,11,26]. Our findings together with the literature suggest a possible mechanism by which excessive pro-inflammatory cytokine may increase cellular senescence in the IVD.

The dramatic catabolic shift in matrix synthesis at both the gene and protein levels induced by TNFα is likely also to be influenced by increases in other pro-inflammatory cytokines, and we measured significant increases in IL-1β and IL-6 gene expression, particularly at 7 days. These results support the hypothesis that TNFα can initiate matrix breakdown and an inflammatory cascade [9]. Cell culture studies have shown TNFα exposure can increase IL-1β mRNA [4,6,9] and in situ zymography demonstrated IL-1β has a greater ability to increase catabolic enzyme activity than TNFα [28]. Results also reinforce the concept that pro-inflammatory cytokines likely play a role in painful IDD since we observed significantly increased NGF mRNA in the AF at 7 days. The literature further demonstrates that IL-1β and IL-6 can up-regulate nerve promoting factors such as NGF and BDNF and are correlated with painful disc degeneration [3,5,29].

The dose of TNFα used in this study was chosen to induce rapid catabolic changes in the IVD, and to mimic those changes observed following decades of pro-inflammatory cytokine exposure that occurs during human degeneration. Cytokines such as a TNFα are known to interact and bind proteoglycans in the matrix [30] thereby decreasing the 'effective concentration' that cells experience. Concentrations of TNFα of 100 ng/mL have also been used in IVD and cartilage explant culture studies [8,31–34]. Because of the large size and relatively low cellularity of bovine IVDs, a pilot study of 100, 200 and 400 ng was conducted and we chose the dose of 200 ng since it consistently induced catabolism whilst maintaining cell viability. A recent study by Ponnappan et al. also explored the role of pro-inflammatory cytokines in disc degeneration using a cocktail of 100 ng/mL TNFα & 10 ng/mL IL-1β in a rat IVD organ culture model over 10 days [34]. They observed a similar suppression of collagen II and aggrecan mRNA providing confirmation that similar effects occur in small and large animal organ culture systems.

In conclusion, TNFα exposure in a bovine organ culture model induced several changes characteristic of the human IDD including a dramatic increase in degraded aggrecan, an anti-anabolic phenotype, up-regulation of catabolic and pain-related genes and changes suggestive of cellular senescence. We demonstrated for the first time that TNFα treatment causes an unrecoverable shift in catabolic and anti-anabolic gene expression and matrix protein degradation. Our data suggested that this lack of recovery may be correlated with increased cell senescence induced by TNFα exposure. This lack of recovery following removal of TNFα therefore supports the concept that future therapeutic interventions are required to stimulate anabolic metabolism and cell proliferation in the IVD following TNFα exposure since long-term catabolic effects can persist long after the cytokine is cleared. This organ culture model system which maintains cell and matrix connectivity can be used in future studies to explore how anti-inflammatory and pro-anabolic interventions can be optimized for large animal IVDs in chronic pro-inflammatory states.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.034.

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