Inflammatory Cytokines Induce Notch Signaling in Nucleus Pulposus Cells
Implications in Intervertebral Disc Degeneration

Hua Wang1,2, Ye Tian1,3, Jianru Wang1, Kate L.E Phillips4, Abbie L.A. Binch4, Sara Dunn4, Alison Cross4, Neil Chiverton5, Zhaomin Zheng2, Irving M. Shapiro1, Christine L. Le Maitre4, Makarand V. Risbud1*

1 Department of Orthopaedic Surgery and Graduate Program in Cell and Developmental Biology, Thomas Jefferson University, Philadelphia, PA, U.S.A.
2 Department of Orthopaedics, First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, P.R. China.
3 Department of Orthopaedic Surgery, Changzheng Hospital, Second Military Medical University, Shanghai, P.R. China
4Biomedical Research Centre, Sheffield Hallam University, UK
5Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield, UK

Address correspondence to: Makarand V. Risbud, Ph.D., Department of Orthopaedic Surgery, 1015 Walnut Street, Suite 501 Curtis Bldg., Thomas Jefferson University, Philadelphia, PA 19107, U.S.A.
Fax: 215-955-9159; E-Mail: makarand.risbud@jefferson.edu

Background: The regulation of Notch signaling under inflammatory conditions in the nucleus pulposus is unknown.

Result: Expression of select Notch pathway genes including Notch2 and Notch signaling is regulated by IL-1β and TNF-α.

Conclusion: Inflammatory cytokines promote Notch signaling in disc.

Significance: Notch signaling may play a role in pathogenesis of disc disease.

The objective of the study was to investigate how inflammatory cytokines, IL-1β and TNF-α control Notch signaling activity in nucleus pulposus (NP) cells. An increase in expression of selective Notch receptors (Notch1, 2), ligand (Jagged 2) and target genes (Hes1, Hey1 and Hey2) was observed in NP cells following cytokine treatment. A concomitant increase in Notch signaling as evidenced by induction in activity of target gene Hes1 and Hey1 promoters and reporter 12xCSL was seen. Moreover, treatment increased activity of a 2 kb Notch2 promoter. Treatment of cells with NF-κB and MAPK inhibitors abolished the inductive effect of cytokines on Notch2 promoter and its expression. Gain and loss-of-function studies confirmed the inductive effect of p65 on Notch2 promoter activity. In contrast, p50 blocked the cytokine induction of promoter activity. Supporting the promoter studies, lentiviral delivery of sh-p65, and sh-IKKβ significantly decreased cytokine dependent change in Notch2 expression. Interestingly, MAPK signaling showed an isoform specific control of Notch2 promoter; p38α/β2/δ, ERK1 and ERK2 contributed to cytokine dependent induction while p38γ played no role. Analysis of human NP tissues showed that Notch 1, 2 and Hey2 expression correlated with each other. Moreover, expression of Notch2 and IL-1β as well as the number of cells immunopositive for Notch2 significantly increased in histologically degenerate discs compared to non-degenerate discs. Taken together these results explain for the observed dysregulated expression of Notch genes in degenerative disc disease. Thus controlling IL-1β and TNF-α activities during disc disease may restore Notch signaling and nucleus pulposus cell function.

INTRODUCTION

Intervertebral disc (IVD) degeneration and related disorders have been recognized as the major cause of low back pain (1) affecting about 80% of the population during their life time with high economic cost (2). One of the hallmarks of the disease state is increased production of degradative enzymes coupled with decreased synthesis of the extracellular matrix (3,4). These catabolic processes are thought to be mediated by a number of cytokines, including IL-1β and TNF-α (5,6). While, IL-1β has been shown to be directly involved in the decrease in matrix synthesis and increased matrix degradation (7), TNF-α has been linked to disc cell apoptosis, herniation and nerve irritation (8). Our previous studies demonstrated that there was an increase in expression of both IL-1β and TNF-α mRNA in human degenerate nucleus pulposus (9, 10).
It has been postulated that with aging and degeneration NP cells undergo phenotypic transformation and are replaced by fibroblastic cells (11) that secrete atypical fibrous extracellular matrix. This suggests that changes in cellular microenvironment and presence of catabolic mediators likely promote abnormal proliferation and differentiation. That cytokine-mediated shift in nucleus pulposus cell function is supported by other studies (7, 9, 12, 13). Moreover, similar to chondrocytes, our own investigations have shown that disc cell proliferation and differentiation is dependent on the Notch signaling pathway (14-16). This is a highly conserved pathway (17) regulated by interactions between neighboring cells, four cell surface Notch receptors (in mammals, Notch1–4) are activated by ligands, the Delta-like (Dll1,3,4) and Jagged (Jag1,2) on neighboring cells. Upon activation, the intracellular domain of Notch (NICD) is cleaved by the Presenilin proteases (Psen1/2) of the γ-secretase complex. It translocates to the nucleus to associate with RBP-κκ (also known as CSL) and Mastermind-like (Maml) proteins to activate the transcription of target genes, including Hes and Hey (18). Our previous work has shown that all components of the Notch signaling pathway, namely receptors, ligands and target genes, are expressed in intervertebral disc cells (16). Moreover, our studies suggest that Notch signaling is critical for the maintenance of NP cell proliferation in the hypoxic niche of the disc (16).

The purpose of this study was to evaluate whether inflammatory cytokines IL-1β and TNF-α that are associated with disc degeneration promote the activity of Notch signaling proteins in NP cells. It was found that cytokine activation of Notch signaling was dependent on both NF-κB and MAPK pathways.

**EXPERIMENTAL PROCEDURES**

**Reagents and Plasmids**

Plasmids were kindly provided by Drs. Urban Lendahl, Karolinska Institute, Stockholm, Sweden (Notch responsive reporters: 12xCSL-Luc and Hes1-Luc) (19), Diane Hayward, Johns Hopkins University (Notch responsive 4xwtCBF1-Luc and 4xmtCBF1-Luc reporters containing 4 copies of the wild-type or mutant CBF1 binding elements) (20), Jonas Ungerbäck, Linköpings University, Sweden (Human Notch2 promoter: N2PR -2327/-99 and N2PR -246/-99, in pGGL3) (21), Jiahui Han, Scripps Research Institute, La Jolla (p38αAF, p38β2AF, p38δAF, and p38γAF) and Melanie Cobb, University of Texas Southwestern Medical Center, Dallas (ERK-1K71R and ERK-2K52R). Expression plasmids for sh-p65, and sh-IKKβ in lentiviral FSVsi vector that co-expresses YFP was gift from Dr. Andree Yeremian, University of Lleida, Spain, Hey1 reporter was from Manfred Gessler. Plasmids pCMX-1kB (catalog #12330), RelA/p65 (#20012), p50 (#20018), RelB (#20017) and cRel (#20013) developed by Dr. Didier Trono were obtained from Addgene. pRL-TK (Promega) containing the Renilla reniformis luciferase gene was used as an internal transfection control (22). Fibroblasts derived from p65 null and wild type mice were a kind gift from Dr. Denis Guttridge, University of Ohio, Columbus. Notch1, Notch2, Notch3, p65, IKKβ antibodies were from Cell Signaling, Hey2 from Peprotech, β-tubulin from Developmental Studies Hybridoma Bank and GAPDH were from Novus Biologicals. TNF-α and IL-1β were purchased from Peprotech, NJ. Real time PCR pre designed primer/probe mixes were purchased from Applied Biosystems, Warrington, UK. Notch 2 antibody for immunohistochemical localization was purchased from Abcam, Cambridge, UK.

**Isolation of nucleus pulposus cells and treatments**

Rat and human nucleus pulposus cells from 7 patients (Pfirrmann grades 2-5, mean age: 46.71 year (range 36-64 y), 4M:3F) were isolated using a method previously described by Risbud et al. (22). Nucleus pulposus cells were maintained in Dulbeccos Modified Eagles Medium (DMEM) and 10% fetal bovine serum (FBS) supplemented with antibiotics. To investigate effect of cytokines, cells were treated with IL-1β (10 ng/ml) and TNF-α (25-50 ng/ml) for 24 h.

**Human tissue collection and grading**

Human lumbar IVD tissue was obtained either at surgery or post-mortem (PM) examination with informed consent of the patient or relatives (Sheffield Research Ethics Committee #09/H1308/70). Six PM IVDs were recovered from 2 donors. They consisted of intact IVDs within the complete motion segment from which the IVDs were removed. Sixty two surgical IVD tissue samples were obtained from patients undergoing micro-discectomy procedures for the treatment of low back pain and root pain as caused by prolapse of the IVD. NP tissue was divided into two, one half was fixed in 10% neutral buffered formalin and processed for histological and immunohistochemical examination. The remaining tissue was used for...
RNA isolation. H&E stained sections were used to score the degree of morphological degeneration, sections were scored numerically between 0 and 3 for four features of degeneration: 1) the presence of cell clusters; 2) the presence and extent of fissures; 3) the loss of demarcation between NP and AF regions and 4) the loss of haematoxophilia (indicating reduced proteoglycan content) from the NP. The scores from each category were then summed to provide histological grades between 0-12. Where scores of 0 to 3 are considered as representing histologically non-degenerate tissue samples and scores ≥4 represent degenerate tissue samples (7). Gene expression study samples were classified as non-degenerate (≤ 3.9), mid-grade degenerate (4-6.9) and moderate/serve degenerate (≥7) based on histological examination. Grading was performed independently by two researchers and grades averaged.

**Quantitative Real-Time RT-PCR**

Human tissues were processed as described previously. Extracted RNA was subjected to treatment with DNase (Qiagen, Crawley, UK) and purified using Qiagen MinElute Cleanup kit prior to cDNA synthesis using MMLV Reverse Transcriptase (Bioline, London, UK) and random hexamers. Real-time PCR analysis was performed using pre-designed, FAM-labeled Taqman® Gene Expression Assays (Applied Biosystems). A total of 49 IVDs were used for this component of the study. Twelve histologically non-degenerate discs (mean histological grade: 2.66 (range: 1-3.5); mean age: 36.7 year (range: 20-49); 11M:1F). Nineteen histologically mid-grade degenerate discs (mean histological grade: 5.53 (range: 4-6.9); mean age: 44.7 year (range: 25-73); 11M:8F). Eighteen moderate/serve degenerate discs (mean histological grade: 7.87 (range 7-9); mean age: 37.8 year (range: 28-52); 5M:14F). For cultured rat and human NP cells, 1-2 µg of total DNA free-RNA was used to synthesize cDNA using SuperScript III cDNA synthesis kit (Invitrogen). Reactions were set up in triplicate in 96 well plate using cDNA and appropriate PCR Master Mix (Applied Biosystems). PCR reactions were performed in a StepOnePlus real-time PCR system (Applied Biosystems) according to the manufacturer’s instructions. HPRT, β-actin was used to normalize the mRNA expression for in vitro experiments while GAPDH and 18s used to normalize mRNA in human samples. For human tissue analysis an average of housekeeping gene Ct value was used to normalize data using 2^ΔΔCt. Gene primers used in analysis of cultured cells were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

**Protein extraction and Western blotting**

Cells were placed on ice immediately following treatment. They were lifted, washed with ice-cold PBS and harvested in mammalian protein extraction reagent (MPER) buffer (Pierce). All the wash buffers and MPER buffer included 1X protease inhibitor cocktail (Roche), NaF (5 mM) and Na3VO4 (200 µM). Total cell proteins were resolved on 8-12% SDS-polyacrylamide gels and transferred by electroblotting to PVDF membranes (Bio-Rad, CA). The membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% tween 20) and incubated overnight at 4°C in 3% non-fat dry milk in TBST with the antibodies against Notch1 (1:2000), Notch2 (1:5000), Notch3 (1:1000), Hey2 (1:1000), p65 (1:1000), IKKβ (1:1000) or β-tubulin (1:1000). Immunolabeling was detected using the ECL reagent (Amersham Biosciences).

**Transfections and dual luciferase assay**

Rat NP cells were transferred to 48-well plates (2 × 10³ cells/well) one day before transfection. To measure the effect of cytokine treatments on Notch signaling, cells were transfected with 250 ng of reporter plasmids with 250 ng pRL-TK plasmid. To investigate the effect of NF-kB and MAPK on Notch2, cells were cotransfected with 50-150 ng of p65 or p50 or p65 with p50 or DN-p38α/β2/γ/δ, or DN-ERK1/-ERK2 with or without appropriate backbone vector(s) and 175 ng N2PR-W1 reporter and 175 ng pRL-TK plasmid. In some experiments, cells were treated with inhibitors SM7368 (10 µM), SB203580 (10 µM), PD98059 (50 µM) or SP60025 (10 µM) (Calbiochem) 1h prior to treatment with cytokines. Lipofectamine 2000 (Invitrogen) was used as a transfection reagent. For each transfection, plasmids were premixed with the transfection reagent. 24 h after transfection, some cells were treated with IL-1β or TNF-α. 48 h after transfection, the cells were harvested and a Dual-Luciferase™ reporter assay system (Promega) was used for sequential measurements of firefly and Renilla luciferase activities. Quantification of luciferase activities and calculation of relative ratios were carried out using a luminometer (TD-20/20, Turner Designs, CA). At least three independent transfections were performed, and all analyses were carried out in triplicate.

**Lentiviral particle production and viral transduction**

HEK 293T cells were seeded in 10 cm plates (1.3 x 10⁶ cells/plate) in DMEM with 10% heat-inactivated FBS two days before transfection.
Cells were transfected with 2.5 µg of sh-control, or sh-p65 or sh-IKKβ plasmids along with 1.875 µg psPAX2 and 0.625 µg pMD2.G. After 16 h, transfection media was removed and replaced with DMEM with 5% heat-inactivated FBS and penicillin/streptomycin. Lentiviral particles were harvested at 48 and 60 h post-transfection. Human NP cells (Grade 2) were plated in DMEM with 5% heat-inactivated FBS one day before transduction. Cells in 10 cm plates were transduced with 5 ml of conditioned media containing viral particles along with 6 µg/ml polybrene. After 24 h, conditioned media was removed and replaced with DMEM with 5% heat-inactivated FBS. Cells were harvested for mRNA and protein extraction 5 days after viral transduction.

Site-directed mutagenesis
N2PR-W1 reporter plasmid was used to mutate either the NF-κB site a (AGGATTCC to ATGATCTCTC) or the NF-κB site b (GGGAAAAACCC to GTGATACCAC) or both. Mutants were generated using QuickChange II XL site-directed mutagenesis kit (Stratagene, Wilmington, DE, USA), using forward and reverse primer pairs containing the desired mutation, following the manufacturer’s instructions. The mutations were verified by sequencing.

Immunohistochemical analysis
Immunohistochemistry was used to confirm and localize production of Notch 2 in 31 IVDs; 4 PM and 27 surgical samples. Five histologically non-degenerate discs (mean histological grade: 2.86 (range: 2-3.5); mean age: 37.2 year (range: 26-45); 4M:1F). Thirteen histologically mid-grade degenerate discs (mean histological grade: 5.31 (range: 4-6); mean age: 48.66 year (range: 25-82); 7M:6F). Ten moderate/severe degenerate discs (mean histological grade: 7.86 (range 7-9); mean age: 47.5 year (range: 35-82); 2M:8F). 4 µm tissue sections were dewaxed, rehydrated, endogenous peroxidases quenched and following heat antigen retrieval, blocked in goat serum. Sections were incubated overnight at 4°C with rabbit polyclonal antibodies against human Notch 2 (1:200). Pre-immune rabbit IgG (Abcam) was used as a negative control. After washing, sections were incubated with biotinylated goat anti-rabbit antiserum (1:400 dilution; Abcam) and binding detected by the formation of streptavidin-biotin complex (Vector Laboratories, Peterborough, UK) with 3,3′-diaminobenzidine tetrahydrochloride solution (Sigma-Aldrich). Sections were counterstained with Mayers Haematoxylin (Leica Microsystems, Milton Keynes, UK), dehydrated, cleared and mounted in Pertex (Leica Microsystems). Sections were visualized images captured using an Olympus BX60 microscope and QCapture Pro v8.0 software (MediaCybernetics, Marlow, UK). Random fields of view were assessed for immunopositive and immunonegative cells until a total of 200 NP cells were counted in each section and the number of immuno-positive cells expressed as a percentage of the total count.

Statistical analysis
All measurements were performed in triplicate. Results are presented as the mean ± SEM. Differences between groups were assessed by analysis of variance. P values less than 0.05 were considered statistically significant. RT-PCR expression data of human tissues was found to be non-parametric in distribution and so the Kruskal-Wallis test combined with post-hoc analysis by Conover-Inman test was used to determine significance between 2-ΔCT values and percentage immunopositivity between study groups. In addition, Spearman’s rank correlation was deployed to determine correlations between gene expression for different targets.

RESULTS

IL-1β and TNF-α regulation of Notch signaling in rat nucleus pulpos cells
To test the premise that cytokines concerned with disc degeneration control Notch signaling, rat NP cells were treated with IL-1β or TNF-α and expression of Notch signaling components was analyzed. Cytokine treatment induces expression of Notch1 (Fig. 1A) and Notch2 (Fig. 1B) but not Notch3 mRNA (not shown). Notch1 mRNA expression exhibits a delayed increase compared to Notch2 which is induced as early as 8 h following treatment (Fig. 1B). Similarly, mRNA expression of Notch ligand, Jagged1 showed an induction (Fig. 1C), while Jagged2 showed no change in expression is seen for Jagged1 and Dll4 (not shown). In contrast, expression of Dll1 is suppressed by the cytokines (not shown). Moreover, there is increased expression of Notch target genes, Hey1, Hey2, Hes1 following treatment with both the cytokines (Fig. 1D).

To further examine the activation of Notch signaling after cytokine treatment, Notch1, Notch2, Notch3 and Hey2 protein expression was evaluated by Western blot analysis. Figure 1E shows that cytokine treatment increases the levels of cleaved/active Notch1 and Notch2, but not of Notch3 in rat NP cells. Increase in Hey2 level is also seen (Fig. 1E). Densitometric analysis confirmed that formation of cleaved Notch1 (Fig. 1F) and Notch2 (Fig. 1G) but not that of cleaved Notch3
NF-κB signaling controls Notch2 promoter activity in NP cells

To explore the role of NF-κB in the transcriptional regulation of Notch2 expression, we used JASPAR core database to identify putative NF-κB binding motifs in the Notch2 promoter (23). Sequence analysis reveals two putative binding sites; location in the promoter, sequence and relative score were: -253/-244 (GGGGTTTCCC, 0.94) and -1764/-1755 (AGGATTTCCTC, 0.87) (Fig. 2D). To confirm that Notch2 promoter activity is responsive to NF-κB signaling, we first performed a loss of function study. When rat NP cells were co-transfected with DN-NFκB (IkBαM) plasmid, cytokine dependent induction of promoter activity is completely inhibited (Fig. 2F, G). To determine if p65/RelA subunit of NF-κB controlled Notch2 promoter activity and if this was cell type specific, we measured promoter activity in p65 wild type and null mouse fibroblasts. Only in the wild type cells is the promoter activity cytokine inducible (Fig. 2H).

To elucidate role of NF-κB in controlling promoter activity in rat NP cells, we measured N2PR-W1 activity following over-expression of NF-κB subunits p65/RelA or p50 or RelB or c-Rel. Co-transfection with p65 results in a dose dependent increase in N2PR-W1 activity (Fig. 3A). Interestingly, co-transfection of 100 ng each of p50 with p65 blocked the inductive effect of p65 on Notch-2 promoter (Fig. 3B), this suppression was evident even when p50 dose was reduced to 50 ng in presence of 100 ng of p65 (Fig. 3C). Noteworthy, p50 alone has no effect on the promoter activity. Furthermore, p50 suppresses the inductive effect of IL-1β (Fig. 3D) as well as TNF-α (Fig. 3E) on N2PR-W1 promoter activity. Co-transfection with RelB elicits a small but significant up-regulation in Notch2 promoter activity, only at a dose of 100 ng, while addition of c-Rel has no effect at any given concentrations (Fig. 3F).

NF-κB interaction with the Notch2 promoter controls its activity in NP cells

Next, we evaluated the role of conserved NF-κB binding sites in controlling Notch2 promoter activity (Fig. 3G). For this purpose using N2PR-W1 fragment as a template, we generated three mutant reporters that contained mutation in either one (M1, M2) or both (M3) NF-κB binding sites (Fig 3G) and examined the effect of IL-1β and TNF-α on their activity in rat NP cells. In contrast to the wild-type promoter, the two single site mutants are less responsive to cytokine treatment (Fig. 3H). Noteworthy, there is a complete lack of induction in activity of double mutant by TNF-α or IL-1β (Fig. 3H).

NF-κB signaling controls IL-1β and TNF-α dependent Notch2 expression in NP cells

To further elucidate the role of the NF-κB signaling pathway on the regulation of Notch2 expression, we silenced the expression of individual NF-κB signaling components and transduced human NP cells with lentivirus co-expressing YFP and p65-shRNA or IKKβ-shRNA. Figure 4A shows that there is a robust YFP expression in the virally transduced cells, indicating a high level of transduction efficiency and transgene expression. There is a significant decrease in the expression of p65 and IKKβ mRNA (Fig. 4B) and protein (Fig. 4C, D) in cells transduced with sh-p65 and sh-IKKβ respectively when compared to cells transduced with control ShRNA. Importantly, suppression of NF-κB signaling components significantly decreases the inductive effect of IL-1β on the expression of Notch2 mRNA (Fig. 4E) and the level of cleaved Notch2 protein (Fig. 4F, G).

TNF-α and IL-1β promote Notch2 expression and signaling through MAPK and NF-κB pathways
We determined if in addition to the NF-κB pathway, MAPK signaling controls cytokine dependent induction of Notch2 expression and activity. For these studies, rat NP cells were treated with pathway specific inhibitors prior to the exposure of IL-1β or TNF-α. When induced with the cytokines, pretreatment with inhibitors results in a significant suppression in Notch2 promoter activity (Fig. 5A, B) and mRNA expression (Fig. 5C, D). Similarly, Western blot and densitometric analysis showed a pronounced suppression in cytokine dependent increase in cleaved Notch2 protein in the presence of all the pathway specific inhibitors (Fig. 5E-G). In addition to Notch2, we evaluated activity of Notch responsive reporters: 12xCSL-Luc and 4xwtCBF1 to NF-κB and MAPK inhibitors in rat NP cells. Results show that cytokine mediated induction in activity of 12xCSL-Luc (Fig. 6A, B) and 4xwtCBF1-Luc (Fig. 6C, D) is completely blocked by NF-κB and MAPK pathway inhibitors.

MAPK control Notch2 promoter activity in isoform specific manner in NP cells

We investigated if MAPK regulates Notch2 expression in an isoform specific manner. Rat NP cells were transfected with dominant-negative (DN)-p38α/β2/γ/δ, or DN-ERK1 or DN-ERK2 expression plasmids and Notch2 promoter activity was measured in the presence of IL-1β or TNF-α. Cytokine dependent induction in Notch2 promoter activity is significantly inhibited by co-transfection with DN-P38α (Fig. 7A) or DN-p38β2 (Fig. 7B) or DN-p38γ (Fig. 7C). In contrast, another p38 isoform, p38γ (Fig. 7D) had little or no effect on the induction of promoter activity by IL-1β and TNF-α respectively. Co-transfection with DN-ERK1 (Fig. 7E) or DN-ERK2 (Fig. 7F) also inhibited cytokine induction of Notch2 promoter activity.

Notch expression correlates to elevated cytokine levels in degenerate human intervertebral discs

An increase in Notch2 mRNA level is seen in mid-grade degenerate discs (graded 4-6) (Fig. 8A) (P=0.04). Increased expression of Notch 1 (Fig. 8B) and Hey 2 (Fig. 8C) within the mid-grade degenerate discs (graded 4-6) were also seen although they failed to reach statistical significance (Notch 1: P=0.19; Hey 2: P=0.35). Noteworthy, expression of Notch 1 and Notch 2 (Fig. 8D) (P<0.0001), Notch 1 and Hey 2 (Fig. 8E) (P=0.0012) and Notch 2 and Hey 2 (Fig. 8F) (P=0.0034) significantly correlated with each other. Interestingly, this disease group also displayed highest levels of IL-1β (Fig. 8G) (P=0.018), this trend failed to reach significance for TNF-α (Fig. 8H) (P=0.38). Again IL-1β and TNF-α showed a positive correlation with each other (P=0.0001) (not shown). Notch2 expression is further investigated at the protein level. Notch2 is localized to the membrane and cytosol of NP cells (Fig. 9A) and a significant increase in number of cells displaying Notch2 immunopositivity was seen in the degenerate discs (graded 4-6) compared to non-degenerate (P=0.002) or high grade degenerate discs (P=0.018) (Fig. 9B). In addition to direct expression analysis from human tissues, we treated NP cells that were isolated from 6 degenerate human tissue samples with TNF-α for 24 hour. Fig. 9C shows that cells exhibit TNF-α dependent increase in levels of both cleaved-Notch1 and Notch2. Notch2 shows a more consistent response to the treatment, although patient to patient variation was clearly evident (Fig. 9D).

DISCUSSION

The experiments described in this investigation demonstrated for the first time that expression of specific Notch pathway genes, concerned with regulation of cell proliferation, differentiation and fate determination was up-regulated by the inflammatory cytokines IL-1β and TNF-α in NP cells. Another observation was that the cytokine-mediated expression and activation of the Notch pathway was dependent on MAPK signaling. Importantly, in terms of clinical relevance, analysis of human tissues indicated that the Notch1/2 and Hey2 were expressed by degenerate discs, and that expression levels of Notch2 were higher in mid-grade degenerate discs, which also displayed highest expression levels for the inflammatory cytokines. These results for the first time provide a mechanistic link between the high levels of inflammatory cytokines and the dysregulated expression of Notch signaling components observed during disc disease (16). To test the premise that Notch signaling is modulated by the inflammatory conditions, we first investigated the impact of cytokine exposure on expression of Notch signaling proteins. The marked increase in mRNA and protein expression of receptors Notch1 and 2, ligand Jagged2 and target genes Hes1, Hey1 and Hey2 in the NP cells indicated that the cytokines implicated in degeneration affect Notch signaling. Cytokine-dependent induction at both the mRNA and protein level suggested that the response was primarily transcriptional.
Noteworthy, this induction was specific only to a select group of Notch receptors and ligands; in contrast to Jagged2, Dll1 showed a decrease in expression. The physiological significance of the selectivity of this response is currently under investigation.

In addition to the effects of cytokines on Notch receptors and ligands, we noted that there was a cytokine-dependent increase in Notch target genes Hes1, Hey1 and Hey2 and a robust increase in Hey2 protein was also apparent. The cytokines increased the activity of synthetic reporters 12xCSL, 4xwtCBF1 and also Notch target gene promoters Hes1 and Hey1. Moreover, when a CBF1 reporter with a mutation in CSL binding site was used (4xmutCBF1) cytokine dependent inducibility was abolished. Taken together these studies indicated that the cytokines increase expression of Notch pathway genes results in activation of Notch signaling. These results are consistent with previous observations that showed increased Notch signaling in stem cells and other cell types in response to cytokines (24-27).

In osteoarthritic cartilage, a condition linked to degenerate discs, a robust decrease in Hes1, Hey1 and Hey2 expression was noted. This decrease was specific to osteoarthritic cartilage compared to normal cartilage, indicating a potential role of Notch signaling in the degenerative process. This observation was consistent with previous studies that showed a decrease in Notch signaling in osteoarthritic cartilage (24-27).

In addition, the expression of syndecan-4, one of the target genes of Notch signaling, was decreased in osteoarthritic cartilage. This decrease was specific to osteoarthritic cartilage compared to normal cartilage, indicating a potential role of Notch signaling in the degenerative process. This observation was consistent with previous studies that showed a decrease in Notch signaling in osteoarthritic cartilage (24-27).

In conclusion, the results of this study suggest that cytokines play a role in regulating Notch signaling in osteoarthritic cartilage. This regulation is likely to be mediated through the activation of Notch targets such as Hes1, Hey1 and Hey2. The decrease in Notch signaling in osteoarthritic cartilage may contribute to the progression of the disease. Future studies are needed to further elucidate the mechanisms underlying this regulation and the potential therapeutic implications of targeting Notch signaling in osteoarthritic cartilage.
positive and negative fashion, NF-κB dependent signaling and inflammatory cytokine production in many cell types and in disease contexts (37-41). However, as function of notch signaling is context and cell type specific if such a feedback between Notch and NF-κB or cytokines exists in nucleus pulposus cells remains to be seen.

Relevant to the discussion of increased expression and activity of Notch proteins by cytokines, in human and animal models of disc degeneration increased levels of both IL-1β and TNF-α have been reported (7, 42), likewise, elevated levels of several Notch pathway genes in degenerate discal tissues has been shown (16). In concert with these previous studies, (7, 16, 42), our studies confirmed that Notch2 expression is elevated in the degenerate human disc tissue and that staining is localized to NP cells. Importantly, expression of Notch2 mRNA and protein expression was highest in the mid grade degenerate discs, which also displayed the highest levels of cytokines IL-1β and TNF-α. Results of the present study clearly demonstrate it is likely that increased level of cytokines alter Notch expression and activity during degeneration. Further support to this notion is provided by in vitro studies using human NP cells isolated from degenerate tissues; cells evidenced preservation of sensitivity of Notch/2 expression to cytokine exposure. Likewise, TNF-α and IL-1β treatment did not suppress NP cell proliferation, inhibition is seen when Notch activity is suppressed (not shown). One possible explanation for the increase in Notch activation is that it serves as a protective mechanism and is a part of a compensatory response that maintains resident NP cell proliferation necessary to replace the lost or nonfunctional cells. On the other hand, Notch signaling has been shown to form a positive feedback with NF-κB and cytokines under inflammatory conditions (37-39). Thus depending on the exact role it plays during pathogenesis of the disease, whether targeting Notch signaling pathway has important therapeutic implications remains to be seen.

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FIGURE LEGENDS

**Fig. 1.** Cytokine dependent expression of Notch receptors, ligands and target genes in rat nucleus pulposus (NP) cells. Real-time RT-PCR analysis showed selective induction in expression of Notch1 (*A*), Notch2 (*B*) and Jagged2 (*C*) by NP cells treated with IL-1β and TNF-α. *D* Expression of Notch target genes Hey1, Hey2 and Hes1 was induced by IL-1β and TNF-α treatment of NP cells. Represented as fold change relative to corresponding untreated control (considered as 1). *E* Western blot analysis shows that when treated with IL-1β or TNF-α NP cells increase levels of cleaved-Notch1, cleaved-Notch2, and Hey2 but not of cleaved-Notch3. Densitometric analysis of multiple blots from experiment (*E*) above showed that levels of cleaved- F) Notch1, G) cleaved-Notch2 and H) Hey2. Values shown are mean ± SE, of 3 independent experiments; *p*<0.05.

**Fig. 2.** Inflammatory cytokine promote Notch signaling in rat NP cells. Activity of Notch responsive reporters A) 12xCSL B) 4xwtCBF1Luc and target gene promoters C) Hey1 and Hes1 was induced by IL-1β and TNF-α. In contrast, activity of reporter 4xmtCBF1Luc that contained mutation in RBPjκ site was unaffected by cytokines. D) Schematic of different length human Notch2 promoter constructs used in the study, putative NF-κB elements are shown; numbers are relative to the translational start site (ATG). *E* NP cells were transfected with different length Notch2 promoter fragments (N2PR-W1 and N2PR-W2), and the induction in luciferase activity was measured following IL-1β and TNF-α treatment. Unlike small increase in N2PR-W2 activity, a significant induction in N2PR-W1 reporter activity was seen. *F, G) Co-transfection of DN-NF-κB completely blocked F) IL-1β and G) TNF-α dependent induction in Nocth2 (N2PR-W1) promoter activity. *H* p65 WT and p65 Null MEFs were transfected with Notch2 reporter and treated with IL-1β or TNF-α. Only wild type cells evidenced an
increase in Notch2 reporter activity. Values shown are mean ± SE, of 3 independent experiments; *p<0.05.

Fig. 3. NF-κB regulation of Notch2 expression. A) Rat NP cells were transfected with 25-150 ng of p65 plasmid and Notch2 promoter activity measured. There was a dose-dependent increase in Notch2 promoter activity. B, C) NP cells were co-transfected with p65 and/or p50 and promoter activity was measured. Transfection with p65, but not p50, resulted in increased promoter activity. Together, p50 significantly blocked p65-mediated induction in Notch2 promoter activity with suppression evident even at 50 ng dose. D, E) Co-transfection with p50 completely abolished both D) IL-1β and E) TNF-α mediated induction in Notch2 promoter activity. F) In contrast to p65, RelB and cRel had small or no effect on Notch2 promoter activity. G) Schematic of constructs N2PR-M1, N2PR-M2 and N2PR-M3 containing mutation in either one or both NF-κB sites generated by site directed mutagenesis. H) NP cells were transfected with either wild type (N2PR-W1) or NF-κB mutant reporter plasmids, and the induction of luciferase activity was determined following IL-1β and TNF-α treatment. Compared to wild type reporter, treatment caused a much smaller induction in activity of single site mutants. Activity of double mutant reporter was unresponsive to the treatment. Values shown are mean ± SE, of 3 independent experiments; *p<0.05.

Fig. 4. A) Immunofluorescence detection of YFP in human NP cells (Grade 2) transduced with lentivirus co-expressing YFP and either shp65 or shlkkβ show high transduction efficiency. Mag. X20. B) Real-time RT-PCR and C, D) Western blot and corresponding densitometric analysis of cells transduced with LV-shC, LV-shp65, and LV-shlkkβ. Expression of p65 and Ikκβ was significantly suppressed by the respective shRNAs compared to cells transduced with a lentivirus expressing control-shRNA. E) Real-time RT-PCR and F, G) Western blot and densitometric analysis of Notch2 expression in cells infected with LV-shC and LV-shp65 and LV-shlkkβ following IL-1β treatment. Note that the IL-1β dependent induction in Notch2 mRNA (E) and protein (F, G) is significantly blocked by suppression of components of the NF-κB pathway. Values shown are mean ± SE, of 3 independent experiments; *p<0.05.

Fig. 5. Cytokines modulate Notch2 expression through NF-κB/MAPK signaling in rat NP cells. A, B) Activity of Notch2 promoter following IL-1β (A) and TNF-α (B) treatment for 24 h with or without NF-κB inhibitor: SM7368 (SM) or JNK inhibitor: SP60025 (SP) or p38 inhibitor: SB203580 (SB) or ERK inhibitor: PD98059 (PD). A significant blocking of cytokine dependent induction in Notch2 promoter activity is seen by all inhibitors. C, D) Real-time RT-PCR analysis of Notch2 expression shows significant blocking of cytokine dependent induction in Notch2 mRNA expression by the inhibitors. E, F) Western blot (E) and corresponding densitometric analysis shows that IL-1β (F) and TNF-α (G) dependent increase in cleaved-Notch2 level in NP cells was blocked by signaling inhibitors. Values shown are mean ± SE, of 3 independent experiments; *p<0.05.

Fig. 6. Cytokines modulate Notch signaling activity through NF-κB/MAPK pathways in rat NP cells. Activity of Notch responsive reporters 12xCSL (A, B) and 4xCBF1 (C, D) following IL-1β and TNF-α treatment for 24 h with or without inhibitors: SM7368 (SM), SP60025 (SP), SB203580 (SB), or PD98059 (PD). Increased activation of both the reporters by IL-1β (A, C) or TNF-α (B, D) was suppressed by NF-κB and MAPK inhibitors. Values shown are mean ± SE, of 3 independent experiments; *p<0.05.

Fig. 7. MAPK regulation of cytokine–mediated induction of Notch2 promoter activity in rat NP cells. NP cells were cotransfected with Notch2 promoter construct along with either (A) DN-p38α or (B) DN-p38β2 or (C) DN-p38δ or (D) DN-p38γ or (E) DN-ERK1 or (F) DN-ERK2 or empty backbone vector pcDNA3. Transfected cells were treated with IL-1β or TNF-α and reporter activity was measured 24 h after the treatment. Co-transfection with DN-p38α, DN-p38β2, DN-p38δ, DN-ERK1 and DN-ERK2 resulted in nearly complete suppression of cytokine dependent induction of the Notch2 promoter activity. However, cotransfection of DN-p38γ did not block IL-1β mediated induction of the reporter activity, there was a small inhibition in TNF-α treatment group. Values shown are mean ± SEM, of 3 independent experiments; *p<0.05.
**Fig. 8.** mRNA expression of Notch2 and inflammatory cytokines IL-1β and TNF-α in 49 human disc samples. A) Notch2 mRNA expression was highest in mid-grade degenerate discs (grades 4-6) which was significant compared to non-degenerate discs (p=0.04). B) Notch1 mRNA expression was highest in mid-grade degenerate discs (grades 4-6) although this failed to reach significance (p=0.19). C) Hey2 mRNA expression was highest in mid-grade degenerate discs (grades 4-6) although this failed to reach significance (p=0.35). D) A positive correlation in mRNA levels between Notch1 and Notch2 was observed (p<0.0001). E) A positive correlation in mRNA levels between Notch1 and Hey2 was observed (p=0.0012). F) A positive correlation in mRNA levels between Notch2 and Hey2 was noted (p=0.034). G) IL-1β mRNA expression was significantly increased in mid-grade degenerate discs compared to non-degenerate discs (p=0.018). H) TNF-α mRNA expression was increased in mid-grade degenerate discs although this failed to reach significance (p=0.38).

**Fig. 9.** A) Immunohistochemical analysis demonstrated that Notch2 was localized to NP cells within human discs, whilst IgG controls were negative for staining, B) The number of cells which displayed immunopositivity for Notch2 was significantly higher in discs histologically graded 4-6, compared to non-degenerate (≤3) (p=0.002) and high grade degenerate discs (≥7) (p=0.018). C) NP cells isolated from 6 patient tissue samples (S) with different Pfirrmann grades were treated with TNF-α and Notch1 and Notch2 expression analyzed by Western blot. Spinal level is indicated at the bottom of blot. D) Densitometric analysis of Western blot. Notch expression levels are normalized to β-tubulin and represented as fold change relative to corresponding untreated control (considered as 1). As expected, sample to sample variation was seen, although Notch2 levels were higher in 5/6 treated samples, while Notch1 expression in 4/6 samples was increased by TNF-α.
Fig. 1

A. Relative Notch1 mRNA to Ctr (normalized to Hprt)

B. Relative Notch2 mRNA to Ctr (normalized to Hprt)

C. Relative Jag2 mRNA to Ctr (normalized to Hprt)

D. Fold change in mRNA to Ctr (normalized to Hprt)

E. Western Blot of Notch1, Notch2, Notch3, Hey2, and β-Tubulin

F. Densitometry of Notch1 Protein Level to Ctr

G. Densitometry of Notch2 Protein Level to Ctr

H. Densitometry of Hey2 Protein Level to Ctr
Fig. 3

A

Fold (Luciferase Activity)

0 0.5 1.0 1.5 2.0 2.5 3.0

- 25 50 100 150 p50 (ng)

* +

B

Fold (Luciferase Activity)

0 0.5 1.0 1.5 2.0 2.5 3.0

- + + + + p65 p50

* +

C

Fold (Luciferase Activity)

0 0.5 1.0 1.5 2.0 2.5 3.0

- + + + + p50 (ng)

* +

D

Fold (Luciferase Activity)

0 0.5 1.0 1.5 2.0 2.5 3.0

- + + + + IL-1β p50 (ng)

* +

E

Fold (Luciferase Activity)

0 0.5 1.0 1.5 2.0 2.5 3.0

- + + + + TNF-α p50 (ng)

* +

F

Fold (Luciferase Activity)

0 0.5 1.0 1.5 2.0 2.5 3.0

0 100 200 RelB (ng)

0 100 200 cRel (ng)

ns

G

NF-κB

-1764 -1755

ATG

-253 -244

N2PR-W1

LUC

N2PR-M1

LUC

N2PR-M2

LUC

N2PR-M3

LUC

-2327 -256 -99

H

Fold (Luciferase Activity)

0 0.5 1.0 1.5 2.0 2.5 3.0

Cr IL-1 TNF Cr IL-1 TNF Cr IL-1 TNF Cr IL-1 TNF Cr IL-1 TNF

W1 M1 M2 M3

ns
Fig. 4

A) Western blots of p65 and β-Tubulin from LV-shC and LV-shp65 infected cells.

B) Densitometry of p65 and β-actin.

C) Western blots of IKKβ and β-Tubulin from LV-shC and LV-shp65 infected cells.

D) Densitometry of IKKβ and β-actin.

E) Relative Notch2 mRNA to Ctl (normalized to β-actin) in LV-shC, LV-shp65, LV-shIKKβ infected cells with or without IL-1β.

F) Western blots of Notch2 and β-Tubulin from LV-shC, LV-shp65, LV-shIKKβ infected cells with or without IL-1β.

G) Densitometry of Notch2 and β-Tubulin.
Fig. 6

(A) 12xCSL (Luciferase Activity)

(B) 12xCSL (Luciferase Activity)

(C) 4xCBF1 (Luciferase Activity)

(D) 4xCBF1 (Luciferase Activity)
Fig. 7

(A) Fold (Luciferase Activity) vs. IL-1β and TNF-α with different doses of DN-p38α (ng).

(B) Fold (Luciferase Activity) vs. IL-1β and TNF-α with different doses of DN-p38β (ng).

(C) Fold (Luciferase Activity) vs. IL-1β and TNF-α with different doses of DN-p38δ (ng).

(D) Fold (Luciferase Activity) vs. IL-1β and TNF-α with different doses of DN-p38γ (ng).

(E) Fold (Luciferase Activity) vs. IL-1β and TNF-α with different doses of DN-ERK1 (ng).

(F) Fold (Luciferase Activity) vs. IL-1β and TNF-α with different doses of DN-ERK2 (ng).

* indicates significant difference.
Fig. 9

A. Notch2

Cell clusters

IgG Control

Single cells

B. Expression of Notch 2 Protein

% Immunopositive cells

Histological Grade of degeneration

C.

Pfirrmann-Grade 3

|   | S1 | S2 | S3 |
|---|----|----|----|
| kDa |    |    |    |
| 120 | -  | +  | -  |
| 55  | -  | +  | +  |
| 110 | -  | -  | +  |
| 55  | -  | -  | +  |

L5/S1, C4/C5, L4/L5

Grade 4

|   | S4 | S5 | S6 |
|---|----|----|----|
| kDa |    |    |    |
| TNF-α | Notch1 | β-Tub |
| Notch2 | β-Tub |

D.

Relative to respective untreated Ctrl (normalized to β-Tubulin)

L5/S1, C6/C7, L4/L5

S1, S2, S3, S4, S5, S6
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