CCN2 Suppresses Catabolic Effects of Interleukin-1 β through α5β1 and αVβ3 Integrins in Nucleus Pulposus Cells

Implications in Intervertebral Disc Degeneration

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Background: The relationship between inflammatory cytokines TNF-α and IL-1β and CCN2 in nucleus pulposus cells is unknown.

Results: Cytokines suppress CCN2 expression, whereas CCN2 represses catabolic action of IL-1β.

Conclusion: In nucleus pulposus, cytokines and CCN2 form a negative regulatory circuit.

Significance: CCN2 may play an important role in pathogenesis of intervertebral disc degeneration.

The objective of the study was to examine the regulation of CCN2 by inflammatory cytokines, IL-1β, and TNF-α and to determine whether CCN2 modulates IL-1β-dependent catabolic gene expression in nucleus pulposus (NP) cells. IL-1β and TNF-α suppress CCN2 mRNA and protein expression in an NF-κB-dependent but MAPK-independent manner. The conserved κB sites located at −93/−86 and −546/−537 bp in the CCN2 promoter mediated this suppression. On the other hand, treatment of NP cells with IL-1β in combination with CCN2 suppressed the inductive effect of IL-1β on catabolic genes, including MMP-3, ADAMTS-5, syndecan 4, and prolyl hydroxylase 3. Likewise, silencing of CCN2 in human NP cells resulted in elevated basal expression of several catabolic genes and inflammatory cytokines like IL-6, IL-4, and IL-12 as measured by gene expression and cytokine protein array, respectively. Interestingly, the suppressive effect of CCN2 on IL-1β was independent of modulation of NF-κB signaling. Using disintegrins, echistatin, and VLO4, peptide inhibitors to ανβ3 and α5β1 integrins, we showed that CCN2 binding to both integrins was required for the inhibition of IL-1β-induced catabolic gene expression. It is noteworthy that analysis of human tissues showed a trend of altered expression of these integrins during degeneration. Taken together, these results suggest that CCN2 and inflammatory cytokines form a functional negative feedback loop in NP cells that may be important in the pathogenesis of disc disease.

The intervertebral disc comprises a central proteoglycan-rich nucleus pulposus (NP) that is surrounded circumferentially by a fibrocartilaginous annulus fibrosus. The high concentration of hydrophilic aggrecan molecules in the NP causes a high swelling pressure that is contained by the annulus fibrosus. Together these structures form a unique hydrodynamic system that accommodates applied biomechanical forces on the spine (1, 2).

Disc degeneration, a major cause of lower back pain, is characterized by the loss of water-binding proteoglycans through increased degradation and an overall shift toward a more fibrotic matrix (2). The resulting dehydration causes a reduction in disc height and altered mechanical loading that leads to progressive changes in tissue microenvironment and cell function. In the NP, an increase in the inflammatory cytokines, IL-1β, and TNF-α, during degeneration drives the expression of many degradative matrix metalloproteinases (MMPs) and aggrecanases, such as A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4) and ADAMTS-5 (3–5). Moreover, the inflammatory milieu of degenerate disc causes a phenotypic shift in NP cells, resulting in atypical proliferation, cell death, synthesis of a fibrotic matrix, and secretion of factors such as NGF, BDNF, and VEGF that promote angiogenesis and nerve ingrowth (6–8). Thus, it is clear that during degeneration, the changing microenvironment has profound effects on NP tissue. The modulation of this microenvironment could offer a means to affect cell function to promote tissue regeneration and attenuation of the catabolic processes that contribute to the degenerative state.

CCN2/CTGF, a member of the CCN family of matricellular proteins, is an important constituent of the intervertebral disc microenvironment that is responsive to many growth factors and environmental stimuli (9–11). By promoting cellular adhesion, proliferation, migration, and extracellular matrix synthesis, CCN2 plays a role in a range of processes, including wound healing and inflammation (9, 10, 12–17). The multimodular domain structure of CCN2 allows it to interact with growth factors and matrix molecules, whereas the outside-in signal-

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2 The abbreviations used are: NP, nucleus pulposus; CCN2, connective tissue growth factor; MMP, matrix metalloproteinase; ADAMTS, A disintegrin and metalloproteinase with thrombospondin motifs; ECH, echistatin; PHD, prolyl hydroxylase.
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The effect of inflammatory cytokines, inhibitors of NF-κB (SM7368), p38 (SB203580), JNK (SP60025), or ERK (PD98059) signaling (10 μM each) were used to pretreat cells before addition of inflammatory cytokines.

Real Time RT-PCR Analysis—Total RNA was extracted from NP cells using RNeasy mini columns (Qiagen). Before elution from the column, RNA was treated with RNase-free DNase I (Qiagen). The purified, DNA-free RNA was converted to cDNA using RNA to cDNA EcoDry™ premix (Clontech). Template cDNA and gene-specific primers were added to SYBR green master mix (Applied Biosystems), and mRNA expression was quantified using the Step One Plus real time PCR system (Applied Biosystems). HPRT1 and β-actin were used to normalize gene expression. Melting curves were analyzed to verify the specificity of the RT-PCR and the absence of primer dimer formation. Each sample was analyzed in duplicate and included a template-free control. All the primers used were synthesized by Integrated DNA Technologies, Inc. (Corvalle, IA).

Western Blotting—Cells were placed on ice immediately following treatment and washed with ice-cold Hanks’ buffered salt solution. All the wash buffers and final resuspension buffer included 1× protease inhibitor mixture (Roche Applied Science), NaF (5 mM), and Na3VO4 (200 μM). Conditioned medium was collected following 24 h of treatment and concentrated using centrifugal filter units (10 kDa; EMDMillipore, Billerica, MA). Proteins were resolved on 10% SDS-polyacrylamide gels and transferred by electroblotting to PVDF membranes (Bio-Rad). The membranes were blocked with 5% nonfat 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% nonfat dry milk in TBST with the anti-CCN2 (1:900; Santa Cruz) or anti-MMP3 (1:1000; Abcam) or anti-β-tubulin antibody (1:2000; Developmental Studies Hybridoma Bank). Immunolabeling was detected using the ECL reagent (Amersham Biosciences).

Immunofluorescence Microscopy—Cells were plated in flat-bottomed 96-well plates (4 × 10^3/well) and cultured with IL-1β or TNF-α for 24 h. After incubation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked with PBS containing 5% FBS, and incubated with antibodies against CCN2 (1:200; Santa Cruz) at 4 °C overnight. As a negative control, cells were reacted with isotype IgG under similar conditions. After washing, the cells were incubated with Alexa Fluor 488-conjugated anti-goat secondary antibody (Invitrogen), at a dilution of 1:50 for 45 min at room temperature. Cells were imaged using a laser scanning confocal microscope (Olympus Fluvioview).

Transfections and Dual Luciferase Assay—Cells were transfected to 48-well plates (2 × 10^4 cells/well) 1 day before transfection. To measure the effect of inflammatory cytokines, cells were transfected with 250 ng of CCN2 reporter plasmids and 250 ng of pRL-TK plasmid. For each transfection, plasmids were premixed with the transfection reagent Lipofectamine. The transfected reporter plasmids contained a template-free control. All the primers used were synthesized by Integrated DNA Technologies, Inc. (Corvalle, IA).
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2000 (Invitrogen). The next day, 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, Sunnyvale, CA). At least three independent transfections were performed, and all analyses were carried out in triplicate.

Site-directed Mutagenesis—Site-directed mutagenesis of the human CCN2 promoter was performed according to manufacturer’s protocol using the In-Fusion HD cloning kit (Clontech). Primers used to mutate kBS sites in human CCN2 promoter with underlined are as follows: kBS site at −93/−86 bp, forward, 5′-gaggagtctgaATCTGGTCATGttggtgtgaccctcat-3′; kBS site at −546/−537 bp, 5′-gtaggctctcACTCTCtcaatg-gttttaaagacta-3′. Mutations were verified by sequencing using the Applied Biosystems 3730 DNA sequencer.

Chromatin Immunoprecipitation—Human NP cells were plated in 10-cm plates and treated with or without 10 ng of IL-1β for 24 h. ChIP assay was performed using ChIP-IT® high sensitivity kit (Active Motif, Carlsbad, CA) according to the manufacturer’s recommendations. Briefly, chromatin was sheared by sonication, and input DNA was generated by treating aliquots with RNase, proteinase K, and heat, followed by ethanol precipitation. DNA complexes were immunoprecipitated by incubation with p65 antibody (Cell Signaling) overnight at 4 °C followed by binding to protein G-agarose beads for 3 h at 4 °C. Cross-links were reversed by treatment with proteinase K and heat for 2.5 h, and DNA was purified using DNA purification elution buffer (Active Motif). Real time PCR analysis was performed using ChIP-IT® quantitative PCR analysis kit (Active Motif) using the following primer pairs for putative NF-κB sites within the CCN2 promoter: −93/−86 bp, forward, 5′-GTCAAGATCAAAGTCCTG-3′ and reverse, 5′-AATGATGATGATGATTCCCTGC-3′.

Negative control primers and standard curve primers used were provided with kit. Real time PCR was performed with Power SYBR® Green PCR Master Mix (Applied Biosystems). The Ct values for untreated and IL-1β-treated samples were recorded, and the data were normalized based on primer efficiency, input DNA Ct values, amount of chromatin, and resuspension volume, based on manufacturer’s recommendations.

Lentiviral Particle Production and Viral Transduction—HEK293T cells were seeded in 10-cm plates (1.3 × 10⁶ cells/plate) in DMEM with 10% heat-inactivated FBS 2 days before transfection. The cells were transfected with 2.5 μg of control shRNA (shCtrl-LV), shRNA against p65 (shp65-LV), or shRNA against CCN2 (shCCN2-LV) plasmids along with 1.875 μg of psPAX2 and 0.625 μg of pMD2.G. After 16 h, the transfection medium 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. NP cells were plated in DMEM with 5% heat-inactivated FBS 1 day before transfection. 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, media was removed and replaced with DMEM with 5% heat-inactivated FBS. The cells were harvested for protein extraction 5 days after viral transduction.

Cytokine Protein Array—Human NP cells were plated at 2 × 10⁵ in a 6-well plate and transduced with lentiviral particles encoding shCtr or shCCN2. Five days after transduction, the culture medium was removed and replaced with serum free OPTI-MEM + Gluta Max-I (Invitrogen). Serum-free conditioned medium was collected from cells 24 h after medium replacement and was probed for inflammatory cytokines using the RayBio Human Cytokine Antibody Array 3 (Ray Biotech Inc., Norcross, GA) following the manufacturer’s instructions. Briefly, the membranes were blocked for 1 h before incubation with conditioned medium at 4 °C overnight. Membranes were washed and incubated with biotin-conjugated primary antibody mixture for 2 h. Following washing, membranes were incubated with HRP-conjugated secondary antibody for 2 h. Lastly, membranes were washed, and the chemiluminescence was detected using Digital Imaging System ImageQuant LAS-400 (GE Healthcare).

Statistical Analysis—All measurements were performed in triplicate. The data are presented as means ± S.E. Differences between groups were analyzed by Student’s t test (*, p < 0.05). The correlation between the expressions of integrin subunits from human disc samples was calculated using Spearman’s rank order correlation.

RESULTS

Pro-inflammatory Cytokines, IL-1β and TNF-α, Decrease CCN2 Expression in NP Cells—To examine CCN2 regulation by IL-1β and TNF-α, NP cells were cultured with IL-1β (10 ng/ml) or TNF-α (50 ng/ml), and expression of CCN2 was analyzed using real time RT-PCR. Fig. 1A shows that treatment with inflammatory cytokines results in a significant decrease in CCN2 mRNA expression at 24 h by NP cells. Western blot analysis of cell lysates indicates that CCN2 levels are decreased by both the cytokines in a time-dependent fashion (Fig. 1B). Densitometric analysis of several Western blot experiments shows that TNF-α suppressed levels of CCN2 in cell lysates as early as 4 h, whereas IL-1β treatment results in a significant change by 24 h (Fig. 1C). A pronounced decrease in levels of secreted CCN2 by inflammatory cytokines was also confirmed (Fig. 1D). Additionally, immunofluorescence microscopy of cells cultured with IL-1β and TNF-α for 24 h confirms that there is a decrease in CCN2 staining when compared with untreated control (Fig. 1E). It is worth noting that TGF-β treatment can override CCN2 suppression by either inflammatory cytokine (Fig. 1, F and G), perhaps explaining the elevated expression of both CCN2 and inflammatory cytokines during degeneration (5, 11, 22).

IL-1β and TNF-α Suppress CCN2 Expression through NF-κB Signaling—To investigate the mechanism of CCN2 regulation by IL-1β and TNF-α, we treated NP cells with the inflammatory cytokines with or without specific inhibitors of NF-κB (SM7368) and MAPK: p38 (SB203580), JNK (SP60025), or ERK (PD98059) signaling. Fig. 2 (A and B) shows that only pretreatment with the NF-κB specific inhibitor was able to block the
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FIGURE 1. IL-1β and TNF-α decrease CCN2 expression in NP cells. A, real time RT-PCR analysis of NP cells treated with IL-1β or TNF-α for 4, 8, and 24 h shows that CCN2 is significantly decreased at 24 h. B, Western blot analysis of cells treated with IL-1β or TNF-α for 4, 8, and 24 h shows a decrease in CCN2 protein levels. C, densitometric analysis of several independent experiments as shown in B confirms that CCN2 is significantly decreased by IL-1β or TNF-α treatment. D, densitometric analysis of several Western blots of conditioned media from IL-1β- or TNF-α-treated cells shows significantly decreased levels of secreted CCN2 with IL-1β or TNF-α treatment. E, immunofluorescence microscopy of NP cells treated with IL-1β and TNF-α for 24 h shows that CCN2 protein is decreased with treatment compared with untreated control. TGF-β counteracts suppression of CCN2 by IL-1β and TNF-α. F, Western blot of NP cells treated with IL-1β and TNF-α with and without TGF-β. IL-1β and TNF-α suppress CCN2; however, the addition of TGF-β together with either IL-1β and TNF-α negates the suppressive effects of the inflammatory cytokines on CCN2 expression. G, densitometry analysis of at least three Western blot experiments as shown in F reveals that although IL-1β and TNF-α suppress CCN2, the addition of TGF-β abrogates this suppressive effect. The values shown are means ± S.E. from at least three independent experiments. *p < 0.05. Ctr or ctr, control.

suppression of CCN2 by both the cytokines. Densitometric analysis of several experiments confirms that NF-κB signaling is involved in suppression of CCN2 by IL-1β (Fig. 2C). It was noteworthy that unlike TNF-α, IL-1β treatment in presence of NF-κB inhibitor resulted in robust increases in CCN2 levels compared with the untreated control, suggesting that IL-1β modulation of other signaling pathways may have been involved. Finally, to confirm that the effects of inflammatory cytokines on CCN2 are dependent on NF-κB signaling, we silenced p65/RelA subunit of NF-κB using lentiviral delivery of p65shRNA (shp65-LV) and measured CCN2 expression following treatment with IL-1β and TNF-α. Fig. 2D indicates that there is robust expression of YFP by the virally infected cells, indicating high transduction efficiency. Western blot analysis shows that the inflammatory cytokine treatment is unable to suppress CCN2 levels when p65 is silenced (Fig. 2E). Densitometric analysis of several independent experiments reveals that silencing of p65 was significant (Fig. 2F) and that the knock-
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FIGURE 2. IL-1β and TNF-α suppress CCN2 expression through NF-κB signaling. A and B, Western blot analysis of NP cells treated with IL-1β (A) or TNF-α (B) with or without pretreatment with NF-κB inhibitor (SM7368), p38 (SB203580), JNK (SP60025), or ERK (PD98059) inhibitors used at 10 μM each. Note that suppression of CCN2 by IL-1β or TNF-α treatment is ablated only by pretreatment with the NF-κB inhibitor. C, densitometric analysis of several independent experiments as shown in A above confirms that suppression of CCN2 by IL-1β is abrogated only by treatment with NF-κB inhibitor. D, immunofluorescence image of NP cells transduced with a lentivirus co-expressing shRNA against p65 and YFP (Shp65-LV) demonstrates high transduction efficiency. E, Western blot analysis of NP cells transduced with lentivirally mediated shRNA against a scrambled control sequence (Ctr-LV) or p65 (Shp65-LV) that were treated with IL-1β and TNF-α. CCN2 is suppressed by IL-1β or TNF-α, but with p65 knockdown this effect is abrogated. F, densitometric analysis of several independent experiments shown in D shows that p65 was significantly knocked down when cells were transduced with Shp65-LV. F and G, densitometric analysis of several independent experiments shows that the suppression of CCN2 protein levels by IL-1β (G) or TNF-α (H) is rescued by knockdown of p65. The values shown are means ± S.E. from at least three independent experiments. *, p < 0.05. Ctr, control.

ECHR AGR ECHS ECR ECHR ECHR ECHR ECHR ECHR

down of p65 renders IL-1β and TNF-α ineffective in suppressing CCN2 (Fig. 2, G and H).

We then examined the response of the human CCN2 promoter to inflammatory cytokine treatment. There are two conserved NF-κB binding sites at −93/−86 and −546/−537 bp on the human CCN2 promoter (24, 25). We used the Multiz alignment in the UCSC Genome Browser to compare conservation of these sites between species. Fig. 3A shows that these κB sites, especially the site at −93/−86 bp, are highly conserved between many species. To determine the importance of these sites in CCN2 regulation by inflammatory cytokines, we mutated both of the sites individually or in combination. Fig. 3B shows a schematic of the CCN2 promoter constructs used for the transfection experiments. Treatment with inflammatory cytokines decreases only the wild type CCN2 promoter activity, whereas the mutation of either of the NF-κB binding sites abrogates this suppression (Fig. 3C). Furthermore, ChIP experiments confirmed that there is binding of p65 to κB sites at −93/−86 bp and −546/−537 bp on the human CCN2 promoter in the presence or
absence of IL-1β. There was no enhancement of p65 binding in the presence of cytokine.

CCN2 Suppresses IL-1β-mediated Induction of Several Catabolic Genes—To determine whether CCN2 modulates the effects of IL-1β, NP cells were cultured with IL-1β (10 ng/ml), CCN2 (100 ng/ml), or both IL-1β and CCN2 together. Real time RT-PCR analysis was performed to analyze the expression of catabolic genes, including MMP-3, ADAMTS-5, syndecan 4, and prolyl hydroxylase 3 (PHD3) (Fig. 4, A–D). As expected, IL-1β treatment significantly induces the expression of these catabolic genes, whereas CCN2 treatment alone does not influence basal expression (Fig. 4, A–D). Interestingly, when CCN2 is added with IL-1β, expression of all the measured catabolic genes is significantly decreased compared with expression levels induced by IL-1β alone (Fig. 4, A–D). Western blot and corresponding densitometric analysis of secreted proteins from several independent experiments confirms that the CCN2 exerts an inhibitory effect on IL-1β-dependent induction of MMP-3 (Fig. 4, E and F).

Silencing of CCN2 Results in the Induction of an Inflammatory Phenotype by Human NP Cells—We silenced CCN2 expression in human NP cells using lentivirus-mediated shRNA against CCN2 (shCCN2-LV) and measured catabolic gene expression. Real time RT-PCR (Fig. 5A) and Western blot (Fig. 5B) analysis shows that CCN2 expression is successfully silenced in the shCCN2-LV group compared with cells transduced with control shRNA (Ctr-LV). Fig. 5 (C–G) reveals that silencing of CCN2 increases the transcript levels of several

**FIGURE 3.** Suppression of CCN2 promoter activity by IL-1β and TNF-α requires p65 binding to NF-κB binding sites. A, Multiz alignment of two NF-κB binding sites at −93/86 and −546/−537 bp on human CCN2 promoter shows relatively high conservation between vertebrate species. B, schematic of WT and mutant (MT1, MT2, or MT1,2) human CCN2 promoter constructs with NF-κB binding sites located at −93/86 bp and −546/−537 bp. C, NP cells were transfected with WT and mutant (MT1, MT2, or MT1,2) human CCN2 promoter constructs and treated with IL-1β or TNF-α. Only the WT promoter shows a decrease in promoter activity in response to IL-1β and TNF-α treatment, whereas the mutant promoter constructs show no significant change (ns). D, ChIP analysis shows binding of p65 to both the x8 sites within CCN2 promoter in presence or absence of IL-1β. Negative primers show little or no amplification, indicating the high specificity of the ChIP assay. The asterisk indicates significant increase in binding/enrichment over negative control (Neg. Ctr). The values shown are means ± S.E. from three independent experiments. *, p < 0.05.
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![Graphs](image)

**FIGURE 4.** CCN2 treatment decreases the IL-1β-mediated induction of several catabolic genes. A, real-time RT-PCR analysis of NP cells treated with IL-1β (10 ng/ml), CCN2 (10 ng/ml), or both IL-1β and CCN2 together. A–D, IL-1β causes the induction in catabolic genes MMP-3 (A), ADAMTS-5 (B), syndecan 4 (SDC4) (C), and PHD3 (D), whereas CCN2 alone has no significant effect. When the cells are treated with both CCN2 and IL-1β, CCN2 suppressed IL-1β-dependent induction of catabolic genes compared with IL-1β alone. E, Western blot analysis of conditioned media from NP cells treated with IL-1β, CCN2, or both IL-1β and CCN2 together shows that the level of secreted MMP-3 is induced by IL-1β treatment and unchanged by CCN2 treatment. Compared with MMP3 levels induced by IL-1β alone, MMP-3 levels are decreased when cells are treated with CCN2 and IL-1β together. F, densitometric analysis of at least three independent experiments as in E shows a statistically significant increase MMP-3 protein levels with IL-1β treatment that is significantly decreased when cells are treated with both IL-1β and CCN2 together. The values shown are means ± S.E. from at least three independent experiments. *, *p < 0.05. ctr, control.
on catabolic gene expression (supplemental Fig. S1, A–D). Western blot analysis of MMP-3 secreted in the conditioned medium confirms that inhibition of α5β1 integrin blocks the ability of CCN2 to suppress IL-1β action (Fig. 6, F and G).

Expression Analysis of β1, α5, and αV Integrin Subunits in Human Degenerative Disc Samples—Analysis of human degenerative disc samples shows a trend of altered expression of β1, α5, and αV integrin subunits in severely degenerated disc samples (grades 4 and 5) compared with moderately degenerated discs (grades 2 and 3) (Fig. 7, A–C). However, the trends were not statistically significant because of a large patient to patient variation in human tissue samples and a limited number of grade 2 samples available for analysis. It is noteworthy that correlation between β1 and α5 (p = 0.0038) (Fig. 7D), β1 and αV (p = 0.0038) (Fig. 7E), and αV and α5 (p = 2E-07) (Fig. 7F) integrins revealed a strong positive correlation between the expression of these integrin subunits.
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**FIGURE 6.** Anti-catabolic effect of CCN2 requires binding to integrin receptors. A, NP cells were transfected with a prototypic reporter responsive to NF-κB activity (NRE-luc) and treated with CCN2, IL-1β, or both together. IL-1β treatment alone induces NF-κB activity, whereas CCN2 has no significant effect. When CCN2 is added in combination with IL-1β, there is no significant difference in reporter activity compared with IL-1β alone. B–E, real time RT-PCR analysis of aggrecan (ACAN) gene expression with treatment of CCN2 with and without inhibitors of integrin binding, ECH, which inhibits αvβ3, or VLO4, which inhibits α5β1. CCN2 treatment alone increases aggrecan expression, whereas pretreatment with either inhibitor ablates this effect. Note that inhibitors alone have no significant effect on ACAN expression. Real time RT-PCR analysis following treatment of NP cells with IL-1β or IL-1β and CCN2 together, with or without ECH or VLO4. Treating cells with CCN2 in combination with IL-1β causes a significant decrease in the expression of MMP3 (C), MMP13 (D), and PHD3 (E) compared with IL-1β alone. Interestingly, pretreatment with ECH or VLO4 before IL-1β and CCN2 treatment not only abrogates the suppressive effect of CCN2 on IL-1β but also results in further induction in catabolic gene expression. F, Western blot analysis of conditioned media of NP cells treated with IL-1β, CCN2, or both together, with and without ECH or VLO4 shows that IL-1β induction of secreted MMP3 levels are decreased when cells are treated with CCN2 together with IL-1β. Integrin αvβ3 or α5β1 inhibition by ECH or VLO4 abolishes the decrease in MMP3 by the addition of CCN2. G, densitometric analysis of at least three independent experiments as shown in F reveals that the increase in MMP3 by IL-1β is significantly decreased by CCN2. When cells are pretreated with ECH or VLO4, not only is this effect abolished, but also MMP3 levels are significantly elevated over IL-1β treatment alone. The values shown are means ± S.E. from at least three independent experiments. *, p < 0.05. ctr, control.
DISCUSSION

The results of this study demonstrate for the first time that in NP cells, IL-1β and TNF-α suppress CCN2 expression through NF-κB signaling. We also showed that CCN2 suppresses several IL-1β-induced catabolic genes and that this effect requires binding of CCN2 to integrin receptors αvβ3 and α5β1 on the cell surface. Taken together, these studies suggest the presence of a functional loop between CCN2, the cognate integrin receptors, and inflammatory cytokines in the NP. Disruption of any of these components would be expected to influence the progression of degeneration in the intervertebral disc.

We first examined the regulation of CCN2 expression by inflammatory cytokines, IL-1β, and TNF-α. Our results clearly showed that both IL-1β and TNF-α suppressed CCN2 in an NF-κB-dependent manner and that inhibition required p65 binding to κB sites in the CCN2 promoter. Interestingly, our ChIP analysis shows a similar binding of p65 to κB sites with or without IL-1β, suggesting that there may be a change in the

FIGURE 7. Expression analysis of β1, α5, and αV integrin subunits in human degenerative disc samples. A–C, real time RT-PCR analysis of human degenerate disc samples (n = 34, grades ≤3 = 11, grades 4 and 5 = 23) shows a trend of increasing expression of integrin subunits β1 (A), α5 (B), and αV (C). The data are represented as box and whisker plots. Each box represents the 75th–25th percentile of values, the line inside each box represents the median value separating the upper and lower quartiles, whiskers show the maximum and minimum values excluding outliers in each set, and dots denote outliers that fall outside of 1.5 times the upper or lower quartile range, respectively. D–F, a positive correlation in mRNA levels between β1 and α5 (ρ = 0.0038 (D), β1 and αV (ρ = 0.0038) (E), and αV and α5 (ρ = 2E-07) (F) was found in human degenerate disc samples.
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nature/composition of the assembled p65 complex in presence of the cytokine. Our results are in agreement with studies showing CCN2 suppression by inflammatory cytokines in other cell types, including chondrocytes, where prostaglandin E₂, IL-1β, and TNF-α are inhibitory (27–30). Unlike in NP cells, some studies have reported the involvement of NF-κB signaling in the induction of CCN2 in response to LPS, mechanical stretch, and hepatocyte growth factor (25, 31, 32). Thus, our results suggest that although p65-mediated gene repression is less common, it is context- and cell type-dependent (33).

Based on the established anabolic function of CCN2 in the NP cells and its known function in regulating growth factor and cytokine signaling in other systems, we determined whether CCN2 could modulate the catabolic effects of IL-1β on NP cells. Previous studies by our group and others have implicated several IL-1β-induced genes in the progression of intervertebral disc degeneration, including matrix degrading enzymes, MMP-3, and ADAMTS-5, as well as PHD3 and syndecan 4 (3, 11). Nonetheless, a strong positive correlation was observed between the expressions of these integrin subunits (33). Additionally, studies demonstrate the altered function of α5β1 during degeneration (47, 48). Le Maitre et al. (47) showed that mechanotransduction through α5β1 integrin was impaired in degenerate human NP cells, whereas Xia and Zhu (48) found that the fibronectin fragments that accumulate in the degenerative disc cause an increase in MMP-9 and -13 through α5β1 integrin signaling. Taken together, these data implicate the importance of CCN2-integrin signaling in the maintenance of matrix homeostasis in NP cells, with both the anabolic and anti-catabolic effects of CCN2 mediated through binding to both αβ3 and α5β1 integrins.

Because of their diverse and important biological functions, several investigators have begun to examine the differential expression of integrins between nondegenerate and degenerate or herniated discs and the functional consequences of altered expression. These studies show that the β1 integrin, especially the α5β1 heterodimer, is essential for NP cell attachment to fibronectin, collagen II, and lamin isoforms (LM-111 and LM-511), with additional contribution from α1, α2, and α3 integrin subunits (43–45). In the context of the disc degeneration, one study reported an increase in α5β1 integrin expression in herniated disc tissue; it was speculated that the increased expression in fibronectin and collagen I was a result of altered cell-matrix interactions (46). Further evidence of altered expression came from our own studies that showed a trend of increasing expression of β1, α5, and αV integrin subunits in severely degenerated human disc samples (grade 4 and 5) compared with moderately degenerated discs (grade 2 and 3). These trends failed to reach statistical significance because of large patient to patient variations as observed in previous studies of human disc tissues (3, 11). Nonetheless, a strong positive correlation was observed between the expressions of these integrin subunits. Additionally, studies demonstrate the altered function of α5β1 during degeneration (47, 48). Le Maitre et al. (47) showed that mechanotransduction through α5β1 integrin was impaired in degenerate human NP cells, whereas Xia and Zhu (48) found that the fibronectin fragments that accumulate in the degenerative disc cause an increase in MMP-9 and -13 through α5β1. Moreover, it is important to note that along with altered integrin expression and function, CCN2 levels are also elevated during disc degeneration (11, 22). These findings beg the questions: Is CCN2 able to exert anti-catabolic effects in degenerate state? Alternatively in the degenerate disc, does the altered integrin expression and matrix composition change the cellular response to CCN2?

It is possible that in healthy NP cells, interactions between CCN2 and α5β1, as well as αβ3, keep the basal expression of catabolic molecules in check, and during degeneration the increase in matrix degrading enzymes, and thus matrix fragments, compromise this interaction. These fragments outcompete CCN2 for integrin binding, possibly because of the higher affinity of these integrins for fibronectin, and shift the balance of signaling toward catabolism. Intriguingly, the results of our inhibitor experiments suggest that when α5β1 or αβ3 integ-
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Healthy NP

Degenerate NP

FIGURE 8. The role of CCN2 in healthy and degenerate NP. In the healthy NP, CCN2 interacts with α5β1 and αvβ3 integrins to maintain basal transcription of extracellular matrix genes, aggrecan (ACAN) and collagen II (COLII), while keeping the transcription of catabolic genes, MMPs, and ADAMTS, in check. TGF-β in the healthy state also promotes CCN2 and the matrix gene expression, resulting in an anabolic contribution toward matrix homeostasis. In the degenerate NP, an increase in inflammatory cytokines, IL-1β and TNF-α, drives the increase in catabolic, matrix degrading enzymes, MMPs and ADAMTS, and suppresses CCN2 through NF-κB signaling. Increased TGF-β in degeneration can override CCN2 suppression by cytokines and lead to excess CCN2, which will interact with a differential set of receptors induced in degeneration, including the heparan sulfate proteoglycan (HSPG), syndecan 4 (SYND4), other integrins, and increased α5β1. At the same time, the production of fibronectin fragments is induced in degeneration and induces catabolic gene expression by the interaction with α5β1, reducing the availability of this integrin for CCN2 interaction as well. It is possible that downstream signaling from these differential interactions, involving CCN2, could contribute toward the overall shift to catabolism in degeneration, unlike in the healthy state, where CCN2 interaction with α5β1 and αvβ3 integrins promotes anti-catabolic/anabolic effects. Thus, the differential effects of CCN2 could be due to the difference in the receptors it engages.
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combination of both and the consequences of altered interaction between CCN2 and cell surface receptors during disc degeneration will be important in testing this hypothesis.

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