Cartilage Tissue Enhances Proteoglycan Retention by Nucleus Pulposus Cells In Vitro

Claudia J. Arana, Eleftherios P. Diamandis, and Rita A. Kandel

Objective. **To investigate the effect of cartilage on nucleus pulposus (NP) tissue in an in vitro model.**

Methods. **Cells were isolated from bovine NP or articular cartilage and allowed to form tissue in vitro. The NP tissue was grown either alone or in the presence of cartilage tissue (coculture) for up to 4 weeks and examined for histologic appearance, gene expression, and biochemical composition. For selected experiments, NP tissue was grown in coculture with fragments of cartilage end-plate.**

Results. **Coculture of in vitro–formed NP tissue with cartilage end-plate tissue resulted in a significant increase in proteoglycan content in the NP tissue by 2 weeks, compared with NP tissue grown alone. Substituting in vitro–formed cartilage tissue for cartilage end-plate also had a positive effect on the NP tissue, suggesting that it was an appropriate substitute for cartilage end-plate. Coculture of NP with in vitro–formed cartilage for 2 weeks increased aggrecan and collagen gene expression compared with that in NP tissue grown alone, and also reduced expression of matrix metalloproteinase 3 (MMP-3), MMP-13, and ADAMTS-5. NP cells from older and younger animals responded similarly to in vitro–formed cartilage. Expression of genes for tumor necrosis factor α (TNFα) and TACE in NP cells was higher when grown in the absence of cartilage. This corresponded with increased TNFα protein levels in the absence of cartilage.**

Conclusion. **The data suggest that chondrocytes may secrete a factor(s) that positively enhances tissue growth, perhaps by inhibiting TNFα production. This could be a potential mechanism explaining how loss of the cartilage end-plate may contribute to the development of NP degenerative changes.**

The intervertebral disc consists of 3 distinct tissues: a nucleus pulposus (NP) and anulus fibrosus sandwiched between 2 cartilage end-plates. The cartilage end-plate, which anchors the NP and inner anulus fibrosus to the vertebral body (1,2), is composed of a thin layer of hyaline cartilage, which is, on average, ~0.6 mm thick in humans (1,3). The end-plate plays an important role in maintaining NP cell viability by allowing diffusion of nutrients and oxygen either through blood vessels that extend from the vertebral body into the cartilage end-plate in younger individuals (3–6) or by direct diffusion through the cartilage in older individuals (6). The end-plate also prevents the NP from bulging into the adjacent vertebral body and absorbs the pressure resulting from mechanical loading of the spine (2,3).

Low back pain, often associated with intervertebral disc degeneration, has a lifetime prevalence of up to 80% in North America (7). The pathogenesis of this condition is not well understood, but it appears to be an irreversible process associated in part with aging. Both exogenous and endogenous factors have been shown to predispose to the development of the degenerative changes in the intervertebral disc.

There is increasing evidence that the cartilage end-plate plays an important role in the pathogenesis of degenerative disc disease (4,8). With aging, the cartilage end-plate can undergo calcification and may eventually be replaced by bone (8,9), which could impair diffusion across the end-plate. In vivo human studies have shown that transport of dyes into the disc across the cartilage end-plate–subchondral bone is reduced in degenerated discs (6,10,11). This impaired diffusion may compromise

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movement of nutrients across the cartilage end-plate and outward diffusion of degradation products and metabolic waste products, and these could be factors contributing to the development of disc abnormalities (12). Another consequence of this loss of permeability is a decline in oxygen levels in the nucleus, which would favor anaerobic metabolism, increased production of lactic acid, and reduced pH (5,13). The notion of a relationship between calcified cartilage end-plate and disc degeneration is also supported by findings of several animal studies. In a rabbit model of disc degeneration, Peng and colleagues found a positive correlation between the thickness of the calcified layer of the cartilage end-plate and the degree of disc degeneration (14), while Gruber et al showed a correlation between cartilage end-plate calcification and disc degeneration in the sand rat (15). In addition, there are 2 mouse models, one in which an allele of the α1 chain of type II collagen is inactivated and the other in which growth differentiation factor 8 is disrupted, in which cartilage end-plate calcification and disc degeneration develop (16,17).

Interestingly, reports in the literature suggest that the cartilage end-plate may contribute directly to changes that occur in the NP (11,18–20). Studies have shown that disruption of the cartilage end-plate by needle puncture can induce disc degeneration (18,19). In some individuals, cartilage end-plate injury leads to disc degeneration despite enhanced metabolite transport into the disc (11), suggesting that the degenerative process may be a result of tissue alterations in the cartilage end-plate rather than nutritional deficiency. In accordance with this, Lee et al demonstrated that NP cells can modulate their oxygen consumption (21), which suggests that these cells may not be as susceptible to the negative consequences of limited diffusion and hypoxia as had been suspected. Given the role of the cartilage end-plate in disc degeneration, it is possible that the cartilage end-plate directly influences the NP, which led us to hypothesize that the cartilage present in the end-plate has a positive effect on the metabolism of the NP. In this study we used an in vitro tissue culture system to investigate the effects of cartilage on the NP and the potential mechanisms regulating these changes.

MATERIALS AND METHODS

Substrate to support tissue formation. Porous substrates (diameter 4 mm, height 2 mm) used to support tissue formation were generated from calcium polyphosphate powder as described previously (22). The substrates were encased in Tygon tubing to generate a "well-like" structure and steril-ized by gamma irradiation (2.5 mrads), prior to placing in culture for cell seeding.

In vitro–formed tissue samples. To generate NP tissue in vitro, intervertebral disc tissue was aseptically excised from young (6–9 months) or older (18–24 months) bovine caudal spines as previously described (23). Discs from 3 animals were combined in order to have enough cells for each experiment. The NP was dissected out and subjected to sequential enzymatic digestion in 0.5% protease for 1 hour followed by 0.1% collagenase A overnight at 37°C. Cells were then resuspended in Ham's F-12 medium supplemented with 25 mM HEPES and 5% fetal bovine serum (FBS). Cells were seeded on the top surface of the porous substrate at a density of either 2×10^6 cells/substrate, 1×10^6 cells/substrate, or 0.5×10^6 cells/substrate. On day 2, cultures were transferred to Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. At the next medium change (day 5), the serum supplementation was increased to 20%. Ascorbic acid (100 μg/ml) was added to the medium from day 7 onward. The cells were maintained under these conditions for various amounts of time up to 4 weeks. The medium was changed every 2–3 days.

To generate hyaline cartilage tissue in vitro, chondrocytes were isolated from bovine metacarpophalangeal joint cartilage, subjected to sequential enzymatic digestion, resuspended in Ham's F-12 supplemented with 25 mM HEPES and 5% FBS, and seeded on the top surface of the substrate at a density of 80,000 cells/mm^2 as described previously (24). These were placed in coculture 2 days after seeding.

Coculture studies. The NP and chondrocyte constructs after 2 days of culture were placed side by side in a single well (12-well dish) containing 4 ml of medium. By this time point both cell types had formed a thin layer of tissue. The Tygon tubing surrounding the substrate was removed after 7 days in culture, and the constructs were grown for various amounts of time up to 4 weeks in DMEM as described above. The 2 constructs were not in contact and were transferred into new wells every week to ensure that chondrocytes could not grow out from the in vitro–formed cartilage and cross over to the NP cultures.

For the cartilage end-plate explant–NP coculture studies, cartilage end-plate tissue was harvested from bovine spines after removal of the intervertebral disc. To confirm that the tissue was cartilage end-plate, random fragments of tissue were evaluated histologically. The tissue fragments (total weight ~0.055 gm) were placed in coculture with in vitro–formed NP tissue (at 2 days), for 2 weeks. The cultures were grown in DMEM supplemented as described above.

Histologic evaluation. Tissue was removed from the substrate, fixed in 10% buffered formalin, and paraffin embedded. Five-micrometer sections were stained with either hematoxylin and eosin or toluidine blue and examined by light microscopy (23).

Determination of DNA content. The tissue was papain digested as described previously (23) and stored at –30°C until analyzed. The DNA content was determined using the Hoechst dye 33258 assay and fluorometry (excitation [λ] = 365 nm, emission [λ] = 458 nm) (23,24). All samples were corrected for autofluorescence. A standard curve was generated using calf thymus DNA (Sigma).

Proteoglycan and collagen content. Proteoglycan content was estimated in the papain digests using the dimethyl-

methylene blue dye binding assay and spectrophotometry (525 nm) as previously described (23). To determine collagen content, aliquots of the papain digests were hydrolyzed in $6N$ HCl for 18 hours at 110°C, after which the hydroxyproline content was determined using a chloramine T assay and spectrophotometry (560 nm) (23). The standard curve was generated using l-hydroxyproline (Sigma). Collagen content was ascertained with the assumption that hydroxyproline constitutes ~10% of the weight of collagen.

Evaluation of gene expression. The tissue was homogenized using a mortar and pestle in liquid nitrogen, and total RNA was extracted in TRIzol (Gibco BRL). Complementary DNA was synthesized using Superscript DNA polymerase (Invitrogen) in a DNA thermal cycler (iCycler), according to the instructions of the manufacturer (Bio-Rad). Relative gene expression was determined by semiquantitative polymerase chain reaction (PCR) using *Taq* polymerase (Qiagen) and sequence-specific primers in reactions designed to amplify the sequence of interest within the linear range. The primers for aggrecan, type II collagen, type I collagen, matrix metalloproteinase 1 (MMP-1), MMP-3, MMP-13, 18S ribosomal RNA (rRNA), and ADAMTS-5 have been reported previously (25). PCR products were separated using 2% agarose gels, stained with ethidium bromide, and visualized by ultraviolet transillumination. The level of gene expression was semiquantified by densitometry using LabWorks 4.0 Image analysis (UVP Bio-Imaging Systems) and corrected for 18S rRNA, which served as a housekeeping gene.

Real-time PCR analysis was performed for selected genes using an Eppendorf Mastercycler Realplex. The fold change in expression of the target genes (tumor necrosis factor α [TNFα], TACE, TNF receptor I [TNFRI], and TNFRII) relative to the housekeeping gene (18S rRNA) was determined, and expression calculated by the $2^{\Delta\Delta C_t}$ method.

Western blot analysis. The tissues were disrupted by dounce homogenization in radioimmunoprecipitation assay buffer (150 mM Tris HCl [pH 7.5], 150 mM NaCl, 1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 2 mM EDTA) supplemented with protease inhibitor cocktail (Mini-Complete; Roche Molecular Biochemicals). The protein content was determined (BCA protein assay; Pierce), and equivalent amounts of protein (25 mg) were resolved by electrophoresis on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioTrace NT; Pall Life Sciences) by electroblotting. Immunoblotting overnight at 4°C was performed using antibody reactive with MMP-13 (1:500; Oncogene Research Products) and reactivity detected using horseradish peroxidase–conjugated goat anti-mouse IgG secondary antibodies (1:1,000, Santa Cruz Biotechnology). An ECL Plus Western Blotting Detection System was used for visualization, according to the instructions of the manufacturer (Amersham Pharmacia Biotech) The blots were stripped and reprobed for actin (1:10,000) (AC-15; Sigma-Aldrich) to ensure equal loading of protein.

TNFα enzyme-linked immunosorbent assay (ELISA). After 2 weeks of culture, the medium was collected from NP tissue grown in the presence or absence of cartilage. TNFα levels were quantified by ELISA (Bovine TNFα ELISA Development Kit) according to the instructions of the manufacturer (Pierce). Briefly, coating antibody was reconstituted, and 100 μl was added to each well. The plate was incubated at room temperature overnight. Blocking buffer (4% bovine serum albumin [BSA], 5% sucrose in phosphate buffered saline [PBS; 300 μl]) was then added to each well and incubated for 1 hour at room temperature. One hundred microliters of either the standard or the samples was added to each well, incubated for 1 hour at room temperature, washed 3 times (0.05% Tween 20 in PBS [pH7.4]), and incubated with biotinylated detection antibody (1:100) for 1 hour at room temperature. After washing (6 washes), 5 ng/well of alkaline phosphatase–conjugated streptavidin solution (Jackson ImmunoResearch) in BSA (4% in PBS [pH 7.4]) was added to each well and incubated for 15 minutes at room temperature. Following washing (6 times with the wash buffer), 100 μl substrate buffer (0.1M Tris buffer [pH 9.1], 1 mM diflunisal phosphate, 0.1M NaCl, 1 mM MgCl$_2$) was added to each well and incubated for 10 minutes at room temperature. Developing solution (1M Tris base, 0.4M NaOH, 2 mM TbCl$_3$, 3 mM EDTA) was added to each well and incubated for 1 minute at room temperature, and fluorescence was measured using a time-resolved fluorometer (EnVision; Perkin-Elmer).

Statistical analysis. All experiments were performed 3 times in triplicate except the cell density experiment, which was performed in duplicate. Results from the experiments were pooled and expressed as the mean $\pm$ SEM. The data were analyzed by two-way analysis of variance when >2 variables were compared, and by t-test when only 2 groups were compared. All pairwise comparisons between groups were conducted using Tukey's highest significant difference post hoc test. P values less than 0.05 were considered significant.

RESULTS

Effects of cartilage end-plate on matrix accumulation by NP tissue in vitro. To determine if the cartilage end-plate affects NP tissue growth, NP tissue formed in vitro was cultured in the presence or absence of native cartilage end-plate in an explant culture. After 2 weeks of culture, NP tissue grown in the presence of cartilage end-plate contained 2.4-fold more proteoglycans than NP tissue grown in the absence of cartilage end-plate (coculture 19 $\pm$ 0.5 μg/μg DNA, control 8 $\pm$ 1.8 μg/μg DNA [mean $\pm$ SEM]; $P < 0.05$). There was a trend toward increased collagen in the NP tissue, but it did not reach significance (coculture 13 $\pm$ 0.6 μg/μg DNA, control 10 $\pm$ 1.9 μg/μg DNA). The presence of cartilage did not affect tissue cell number; there was no significant difference in DNA content in the NP tissue grown alone or in the presence of cartilage end-plate (coculture 14 $\pm$ 1.4 μg, control 11 $\pm$ 0.5 μg).

In vitro–formed cartilage mimics the effect of cartilage end-plate on NP tissue formation. Obtaining sufficient amounts of cartilage end-plate to perform numerous experiments is difficult due to the small amount of cartilage end-plate and its low cellularity. Since articular cartilage is easily attainable in large
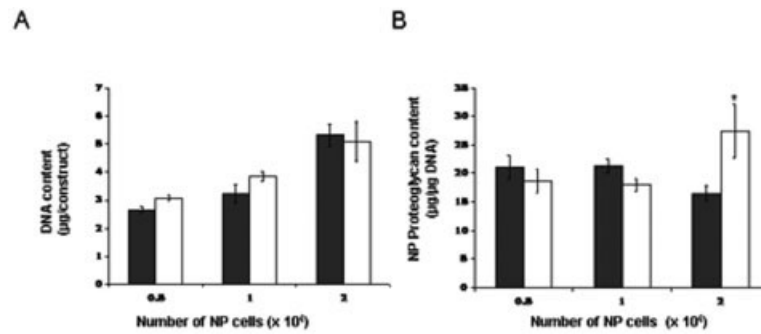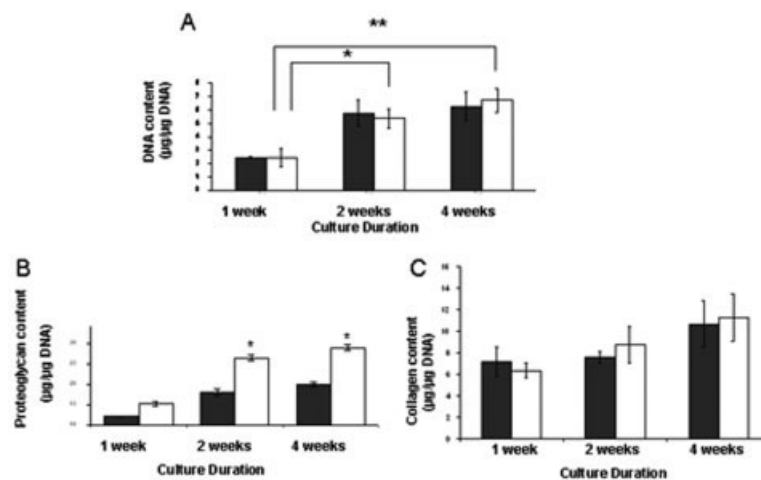
Figure 1. Effects of in vitro–formed cartilage on nucleus pulposus (NP) tissue formation. NP cells were seeded at various cell densities and grown for 2 weeks in the presence (open bars) or absence (solid bars) of in vitro–formed cartilage as described in Materials and Methods, and DNA content (**A**) and proteoglycan content (**B**) were determined. The experiments were performed 3 times with each condition tested in duplicate, and the results pooled. Values are the mean ± SEM (n = 6 samples per experimental condition). ∗ = $P < 0.05$ versus NP tissue grown in the absence of cartilage.

quantities and since cartilage end-plate is hyaline cartilage, we examined whether in vitro–formed cartilage had the same positive effect on NP tissue as cartilage end-plate, in order to determine if it would be a suitable substitute for cartilage end-plate. Coculture of NP with in vitro–formed cartilage for 2 weeks had no effect on tissue DNA content at the cell densities examined, similar to the findings with native cartilage end-plate. In contrast, proteoglycan content was significantly enhanced in NP tissue when it was grown in the presence

of in vitro–formed cartilage (Figure 1). This effect was seen only when the seeding density was twice that of the chondrocytes. At lower ratios (NP cells:chondrocytes 1:1 or 0.5:1), the presence of cartilage did not affect proteoglycan accumulation by NP cells.

Histologic findings. To determine if coculture affects the type of NP tissue that forms, the NP tissue was processed for histologic evaluation after 2 weeks of coculture. The tissue grown in the presence of in vitro–formed cartilage and that grown in the absence of in

Figure 2. Effects of cartilage on nucleus pulposus (NP) tissue over time. Bovine NP cells (2 million cells) were grown for up to 4 weeks in the presence (open bars) or absence (solid bars) of in vitro–formed cartilage. **A,** DNA content of NP tissue. ∗ = $P < 0.05$; ∗∗ = $P < 0.001$. **B,** Proteoglycan content of NP tissue. ∗ = $P < 0.05$ versus NP tissue grown in the absence of cartilage. **C,** Collagen content of NP tissue. The experiments were performed 3 times and the results pooled. Values are the mean ± SEM (n = 9 samples per experimental condition).

Table 1. Biochemical composition of tissue formed by nucleus pulposus cells of different ages in the presence or absence of cartilage tissue*

	Proteoglycan, μg/μg DNA	Collagen, μg/μg DNA
Older cells (age 18−24 months)		
Coculture	7.5 ± 0.8†	2.3 ± 0.6
Control	4.7 ± 0.4	2.6 ± 0.4
Younger cells (age <9 months)		
Coculture	25 ± 3.4†	9.5 ± 1.4
Control	17 ± 2.4	7.4 ± 1.2

* Values are the mean ± SEM (n = 9 per group).
† $P < 0.001$ versus in vitro−formed nucleus pulposus grown for 2 weeks in the absence of in vitro−formed cartilage tissue (control).

vitro–formed cartilage were similar morphologically. However, when cartilage was present the tissue exhibited increased toluidine blue staining, indicative of increased tissue proteoglycan content (results available at http://www.mshri.on.ca/bestt/).

Effects of cartilage on NP tissue over time. In order to characterize the effect of in vitro–formed cartilage tissue on NP tissue formed in vitro, these tissues were cocultured for 1, 2, or 4 weeks (Figure 2). The DNA content of the NP tissue increased significantly between 1 week and 2 weeks ($P < 0.05$), whether grown alone or in the presence of in vitro–formed cartilage. Figure 2A shows that the presence of in vitro–formed cartilage did not affect the DNA content of the NP tissue. Similarly, there was an increase in proteoglycan content in NP tissue between 1 week and 2 weeks, but significantly more proteoglycan was present

in the NP by the second week of coculture compared with NP tissue grown in the absence of cartilage ($P < 0.05$) (Figure 2B). There was also an increase in proteoglycan content between 1 week and 4 weeks when NP tissue was grown alone or in the presence of in vitro–formed cartilage ($P < 0.001$). There was no significant difference in collagen content between the NP tissues grown under either condition over the 4 weeks of culture (Figure 2C).

Age of the NP cells does not affect responsiveness of NP cells to cartilage. Using NP cells obtained from cows of 2 different ages, we examined whether the age of the animals would influence the responsiveness of the NP cells to the anabolic effect of in vitro–formed cartilage after 2 weeks of coculture. Although the NP tissue formed by the older cells (18–24 months of age) had reduced amounts of extracellular matrix when compared with tissue formed by the younger cells (<9 months of age) (Table 1), the proteoglycan content of the tissue formed by the older cells was significantly higher in NP tissue cocultured with in vitro–formed cartilage (mean ± SEM 7.5 ± 0.8 μg/μg DNA) compared with NP tissue grown in the absence of in vitro–formed cartilage (4.7 ± 0.4 μg/μg DNA). Furthermore, there was no significant difference in DNA content between the control and the cocultured NP tissues formed by the older cells (data not shown).

Effects of coculture of cartilage and NP on expression of genes for extracellular matrix molecules and matrix-degrading proteases. To investigate how the in vitro–formed cartilage was affecting NP cells, the
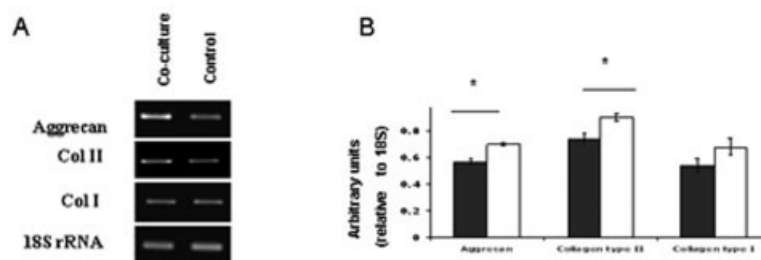
Figure 3. Effect of coculture of cartilage and nucleus pulposus (NP) on the expression of extracellular matrix genes (aggrecan, type II collagen [Col II], and type I collagen). Expression of extracellular matrix genes in NP tissue grown for 2 weeks in the presence (coculture) or absence (control) of in vitro–formed cartilage was analyzed by polymerase chain reaction. **A,** Representative agarose gel from an experiment that was performed 3 times. **B,** Densitometric semiquantification of gene expression, corrected for 18S ribosomal RNA (rRNA). Open bars and solid bars show results obtained in the presence and in the absence, respectively, of in vitro–formed cartilage. The experiments were performed 3 times. Values are the mean ± SEM (n = 9 samples per experimental condition) from 1 representative experiment. ∗ = $P < 0.05$.
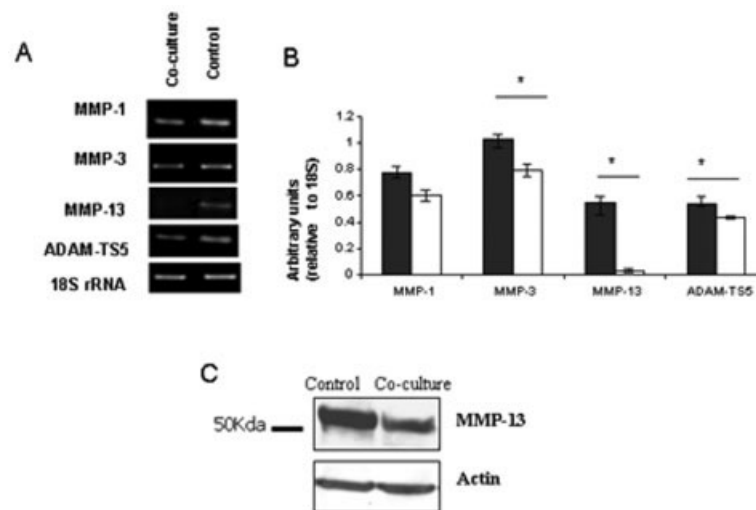
Figure 4. Effect of coculture of cartilage and nucleus pulposus (NP) on the expression of matrix-degrading enzymes. Expression of genes for matrix-degrading enzymes in NP tissue grown for 2 weeks in the presence (coculture) or absence (control) of in vitro–formed cartilage was analyzed by polymerase chain reaction. **A,** Representative agarose gel from an experiment that was performed 3 times. **B,** Densitometric semiquantification of gene expression, corrected for 18S ribosomal RNA (rRNA). Open bars and solid bars show results obtained in the presence and in the absence, respectively, of in vitro–formed cartilage. The experiments were performed 3 times and the results pooled. Values are the mean $\pm$ SEM (n = 6 samples per experimental condition). $*$ = $P < 0.05$. **C,** Western blot analysis of matrix metalloproteinase 13 (MMP-13) levels in NP tissue grown for 2 weeks in the presence or absence of in vitro–formed cartilage. The blots were stripped and stained for actin to confirm that equal amounts of protein had been loaded.

expression of genes for matrix molecules was examined. As shown in Figure 3, coculture of NP tissue with cartilage significantly enhanced expression of aggrecan (by a mean $\pm$ SEM of $17.8 \pm 1.8\%$) and type II collagen (by $19 \pm 1.3\%$) (both $P < 0.05$). There was no effect on type I collagen gene expression. Evaluation of the expression of genes for some of the proteases implicated in NP tissue degradation showed that the presence of in vitro–formed cartilage suppressed the expression of MMP-13, MMP-3, and ADAMTS-5 in NP cells (Figure 4). MMP-13 gene expression in the coculture was almost completely inhibited compared with control (grown in the absence of in vitro–formed cartilage) ($P < 0.05$). There was no significant change in MMP-1 gene expression compared with control. Western blot analysis confirmed that changes in MMP-13 gene expression resulted in changes in protein production: levels of MMP-13 protein were higher in NP tissue grown in the absence of in vitro–formed cartilage tissue (Figure 4C).

Effects of coculture of in vitro–formed cartilage and NP on expression of genes for TNFα and TACE. After observing that the expression of matrix molecules

and proteases in the NP tissue differed in the presence versus the absence of in vitro–formed cartilage, we examined the levels of expression of genes for TNF and its family members after 2 weeks of coculture. The NP cells expressed TNFα, TACE, TNFRI, and TNFRII. Coculture of in vitro–grown NP tissue with in vitro–formed cartilage tissue resulted in a significant decrease in TNFα as well as TACE gene expression ($P < 0.05$) compared with expression in NP tissue grown alone (Figure 5). There was a trend toward lower expression of TNFRI and TNFRII in the cocultured NP, but the decreases were not significant.

Effects of coculture of in vitro–formed cartilage and NP on TNFα protein levels. The amount of TNFα protein released into the culture media was measured by ELISA, to ascertain whether there were changes that paralleled those observed for TNFα gene expression. TNFα was detected in culture media of NP cells grown in the presence or absence of in vitro–formed cartilage. The amount of TNFα was significantly decreased when NP tissue was grown in the presence of in vitro–formed cartilage (mean $\pm$ SEM 84 ± 20.1 pg/ml) compared with
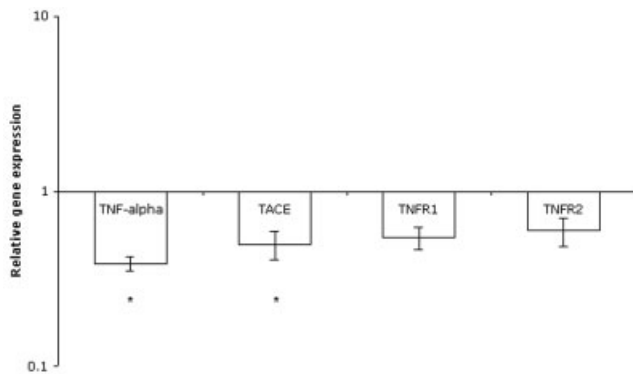
Figure 5. Expression of genes for tumor necrosis factor (TNF) signaling molecules in nucleus pulposus (NP) tissue. Expression of genes for TNF family members in NP tissue grown for 2 weeks in the presence of in vitro–formed cartilage was analyzed by polymerase chain reaction. Expression was normalized to the housekeeping gene (18S), and then the level of expression relative to that in NP tissue grown alone was determined using the $2^{\Delta\Delta C_t}$ method. The experiments were performed 3 times and the results pooled. Values are the mean ± SEM (n = 9 samples per experimental condition). ∗ = $P < 0.05$ versus control. TNFRI = TNF receptor I.

the level measured when NP was grown alone (180 ± 24.3 pg/ml) ($P < 0.05$).

DISCUSSION

This study demonstrated that the presence of cartilage enhanced proteoglycan accumulation by NP cells in an in vitro model system, as assessed biochemically. The appearance of the NP tissue, as determined by light microscopic examination, was not altered by coculture with in vitro–formed cartilage; the only difference was in the amount of extracellular matrix when compared with NP grown in the absence of in vitro–formed cartilage. This anabolic effect correlated with increased aggrecan and type II collagen gene expression as well as decreased expression of genes for several proteases, i.e., MMP-3, MMP-13, and ADAMTS-5. Western blot analysis demonstrated that the level of MMP-13 protein was decreased as well. The age of the NP cells did not affect their response to in vitro–formed cartilage since cells from older animals (skeletally mature cows 18–24 months of age) responded similarly, although to a lesser extent, when compared with cells obtained from younger animals (calves <9 months of age). The enhanced proteoglycan accumulation was not a result of a greater number of cells since there was no difference in DNA content in NP tissue grown in the presence or absence of cartilage.

Since expression of collagen, aggrecan, and proteases can be influenced by cytokines such as TNFα (25) and since this cytokine has been implicated in the pathogenesis of disc degeneration (26–28), we investigated for alterations in TNFα and its related signaling molecules. NP cells in the presence of cartilage expressed lower levels of TNFα and TACE messenger RNA, and there was less TNFα protein in the culture medium. Because the culture conditions utilized did not allow for tissue contact or cross-contamination of cells, this suggests that chondrocytes may mediate these alterations by producing a factor(s) that down-regulates expression of the proinflammatory cytokine TNFα. Although the role of TNFα in this process and the factors regulating its expression are unknown at present, there is evidence in the literature that this cytokine is elevated in degenerated human discs (26,28). Interestingly, Haschtmann et al were able to demonstrate that trauma to the cartilage end-plate, which likely causes cell death, resulted in an increase in TNFα gene expression in the NP (29).

It is not clear why this proteoglycan anabolic response was dependent on a ratio of NP cells to chondrocytes of at least 2:1. This may be a result of the in vitro conditions utilized or the need for a sufficient number of NP cells in order to detect a difference using a biochemical assay, or alternatively, it may be that a critical mass of cells/tissue is needed before the cells can respond to the factor(s). Further study is required to investigate this.

That in vitro–formed cartilage can influence NP cells is not entirely unexpected; there have been at least 3 studies showing that NP cells can be stimulated to up-regulate proteoglycan synthesis by other cell types (30–33). Aguiar et al (30) cocultured canine or bovine NP cells with notochordal cells obtained from adult canine discs and demonstrated an increase in proteoglycan production by NP cells. In another study, Erwin and colleagues cultured canine notochord cells on alginate beads under serum-free conditions to produce notochord cell–conditioned medium. This medium, which contains connective tissue growth factor, stimulated bovine disc cells to increase proteoglycan production and cell proliferation as compared with chondrocytes grown in media alone (31,32). Okuma et al (33) found that the viability of NP cells was enhanced when they were cocultured with anulus fibrosus cells. The ability of one cell type to influence another has also been observed in tissue other than the NP. For example, coculture of mesenchymal stromal cells with bone cells can induce differentiation of the mesenchymal stem cells (34).

It is possible that the anabolic effect observed was an artifact of the culture system. Although the composition of the extracellular matrix of the intervertebral disc cartilage end-plate is similar to that of articular cartilage since they are both hyaline cartilage and consist primarily of water, proteoglycans, and type II collagen (8), it is clear that in vitro–formed cartilage differs from the cartilage end-plate; these tissues have distinct physical properties as well as structural differences. However, the finding that the effect of in vitro–formed cartilage on NP tissue is similar to that of native cartilage end-plate suggests that the factor(s) released by these cells is dependent on cell phenotype and not on the source of the cartilage. Native NP tissue was not used in the present study since it is difficult to work with due to the tissue swelling that occurs when it is placed in vitro (35). Furthermore, our group showed previously that in vitro–formed NP resembled native NP tissue in terms of proteoglycan content and compressive mechanical properties (23). Thus, it would appear that the tissues (in vitro–formed NP and cartilage) are suitable to use as models for native NP and cartilage end-plate. However, we cannot rule out the possibility that the presence of a substrate may alter NP gene expression, since biomaterials have been shown to modulate cell function (36,37). Validation of our observations using human tissue in vivo is required.

The absence of a significant increase in collagen content in the cocultured NP tissue was unexpected since type II collagen gene expression was significantly increased. There are several possible explanations for this. First, it is known that changes in gene expression do not always result in protein changes. Second, although the expression of protease genes was decreased, it was not completely repressed except in the case of MMP-13, so it is possible that there was sufficient ongoing degradation to limit collagen accumulation. Third, given the small changes in gene expression, the limited sensitivity of the collagen quantification assay, and the duration of the culture period, changes in collagen content may have been too small to detect with our approach. We believe this latter explanation is the most likely since 2 weeks of culture and sufficient numbers of NP cells (2 million) were required before significant differences in proteoglycan content could be detected using a biochemical assay.

The findings of this study allow us to propose a mechanism by which cartilage end-plate may contribute to the development of aging or degenerative changes in the NP: As the cartilage end-plate calcifies and/or chondrocytes die, the cartilage end-plate no longer produces sufficient factor(s) that can modulate matrix retention by the NP cells. This is compounded, as we have described previously (38), by the fact that NP cells, as they age, produce more metalloproteinases constitutively. These two alterations could lead to NP tissue changes. There are several human studies whose findings would support this concept that changes in cartilage end-plate contribute to the development of disc degeneration. Nerlich et al found that changes in human cartilage end-plate commence around the age of 2 years, while the degenerative changes in the NP are seen after 10 years of age (39). Another study showed that degeneration of the cartilage end-plate precedes disc degenerative changes (40) and may even play a role in disc herniation (41).

In summary, in vitro–formed cartilage produces a factor(s) that enhances proteoglycan retention by NP cells. Identifying the mechanism that regulates the maintenance of NP tissue could lead to the development of a novel therapeutic approach to stimulate in vivo intervertebral disc repair. Furthermore, the present findings suggest that any attempt to regenerate the NP using tissue engineering methods may provide only temporary results unless the cartilage end-plate is also restored.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kandel had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study conception and design. Arana, Kandel.
Acquisition of data. Arana, Diamandis, Kandel.
Analysis and interpretation of data. Arana, Diamandis, Kandel.

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