

Laboratory Investigation

Notochordal Cells Influence Gene Expression of Inflammatory Mediators of Annulus Fibrosus Cells in Proinflammatory Cytokines Stimulation

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Objective : Notochordal cells in the intervertebral disc interact with nucleus pulposus (NP) cells and support the maintenance of disc homeostasis by regulation of matrix production. However, the influence of notochordal cells has not been evaluated in the annulus fibrosus (AF), which is the primary pain generator in the disc. We hypothesized that the notochordal cell has the capacity to modulate inflammatory mediators secreted by AF cells secondary to stimulation.

Methods : Notochordal and AF cells were isolated from adult New Zealand white rabbits. AF pellets were cultured with notochordal cell clusters or in notochordal cell-conditioned media (NCCM) for 24 or 48 hours with proinflammatory cytokines at varying concentrations. Gene expression in AF pellets were assayed for nitric oxide synthase (iNOS), cyclo-oxygenase (COX)-2, and interleukin (IL)-6 by real time reverse transcriptase polymerase chain reaction (RT-PCR).

Results : AF pellet in NCCM significantly decreased the iNOS and COX-2 messenger ribonucleic acid (mRNA) levels compared to AF pellets alone and AF pellets with notochordal cells ($p < 0.05$). AF pellet resulted in dose-dependent iNOS and COX-2 expression in response to IL-1 β stimulation, demonstrating that 1 ng/ml for 24 hours yielded a maximal response. AF pellet in NCCM significantly decreased the expression of iNOS and COX-2 in response to 1ng/ml IL-1 β stimulation at 24 hours ($p < 0.05$). There was no difference in IL-6 expression compared to AF pellets alone or AF pellets with notochordal cell clusters.

Conclusion : We conclude that soluble factors from notochordal cells mitigate the gene expression of inflammatory mediators in stimulated AF, as expected after annular injury, suggesting that notochordal cells could serve as a novel therapeutic approach in symptomatic disc development.

KEY WORDS : Notochordal cell · Annulus fibrosus · Inflammatory mediators · Real-time RT-PCR.

INTRODUCTION

The intervertebral disc develops from the embryonic mesenchymal and notochordal tissues²². It provides the primary load-bearing function of the vertebral column and is composed of three distinct structures : the nucleus pulposus (NP), a centrally located gelatinous tissue; the annulus fibrosus (AF), consisting of collagen fibril-rich concentric lamellae; and the end plate, which separates the NP and AF from the adjacent vertebral body⁷. The NP is critical to normal disc

health and function, and disc disease frequently begins with changes in the structure and composition of the nucleus. During disc degeneration, the nucleus undergoes organizational and biochemical changes that alter the mechanical function of the disc, and these changes eventually lead to tissue failure. The AF is populated by fibrochondrocyte-like cells of mesenchymal origin, and because peripheral nerve endings are in the outer layer of the AF, it is a major structure in symptomatic disc degeneration³. Furthermore, annular tearing with repair is an initial step in the development of discogenic pain². We previously identified nitric oxide, prostaglandin (PG) E2, and interleukin (IL)-6 as important inflammatory mediators in AF cell-macrophage interactions, and showed that a p38 mitogen-activated protein kinase inhibitor, SB202190, successfully suppressed PG secretion in AF cells exposed to proinflammatory cytokines^{12,13}.

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A rod-like notochord, surrounded by mesenchymal cells, plays an important role in the development of the vertebral column and AF during embryogenesis²³; the entrapped notochordal cells synthesize the NP primordium^{4,10}. The notochordal cells persist throughout adult life in some species, but disappear with maturation in other species²⁰. The notochordal cells in the NP typically disappear in humans, and the NP transforms from a notochordal structure to fibrocartilage by the second decade of life⁵. Notochordal cell loss and the associated loss of paracrine signals from the cells are important initial events in disc degeneration and aging²². Previous studies have suggested that cells from the NP may proliferate and synthesize extracellular matrix components in three-dimensional gel cultures, and that notochordal cells can stimulate matrix production from the NP by chondrocyte-like cells^{2,14}. A co-culture study of notochordal and AF cells demonstrated that notochordal cells activated AF cell metabolism and retarded disc degeneration when reinserted into discs¹⁸. However, the influence of notochordal cells on the production of pain-related inflammatory mediators in AF cells is not understood. In this study, we evaluated the ability of notochordal cells to modulate the gene expression of inflammatory mediators secreted by cytokine-stimulated AF cells.

MATERIALS AND METHODS

Cell isolation and culture

Discs were harvested from the lumbar spines of mature New Zealand white rabbits immediately postmortem, according to the our Institutional Animal Care and Use Committee. NP tissues were dissected from the specimens, washed with Hank's balanced salt solution (HBSS; GibcoBRL, Grand Island, NY, USA), and digested for 60 min in Ham's F-12 medium containing 1% penicillin/streptomycin (P/S), 5% fetal bovine serum (FBS), and 0.2% pronase (Calbiochem, La Jolla, CA). After digestion, the tissues were washed with HBSS and incubated overnight in 0.025% collagenase P (Sigma Chemical Co., St. Louis, MO, USA) at 37°C under gentle agitation. Cells from the digested tissues were passed through a sterile nylon mesh filter (70- μ m pore size), collected by centrifugation at 2,000 rpm for 5 min, re-suspended, and cultured in F-12 medium containing 10% FBS and 1% P/S in a 5% CO₂ atmosphere. As notochordal cell clusters did not adhere to the flasks until day 6, the clusters were separated from the chondrocyte-like cells on day 3.

The AFs were isolated from discs and placed in sterile F-12 medium containing 1% P/S and 5% FBS. AF tissues were washed three times with HBSS containing 1% P/S to remove blood and other contaminants prior to cell isolation. The tissues were minced and then digested in F-12 medium con-

taining 1% P/S, 5% FBS, and 0.2% pronase for 60 min at 37°C with gentle agitation. After digestion, the tissues were rinsed three times with HBSS containing 1% P/S and incubated overnight in F-12 medium containing 1% P/S, 5% FBS, and 0.025% collagenase P (Sigma Chemical Co.). A sterile nylon mesh filter (70- μ m pore size) was used to separate suspended cells from the remaining tissue debris. Isolated cells were collected by centrifugation at 2,000 rpm for 5 min, re-suspended in F-12 medium with 10% FBS and 1% P/S, placed in a 75-cm³ culture flask (VWR Scientific Products, Bridgeport, NJ), and incubated at 37°C in a humidified atmosphere of 5% CO₂. The culture medium was changed twice weekly, and cultures were grown to 95% confluence. Confluent cells were treated with trypsin, and the AF cells (2.5×10^5 cells) were re-suspended in culture medium. Aliquots of the cells were placed in individual conical polypropylene tubes (15 mL) with culture medium; the pellet aggregates were collected by centrifugation for 5 min at 2,000 rpm and incubated at 37°C for 7 days. The medium was changed every 2 to 3 days.

Culture protocol

Fig. 1 illustrates the culture protocol. For naïve AF pellet cultures, confluent AF cells were treated with trypsin, re-suspended in culture medium, placed in individual conical polypropylene tubes (15 mL), collected by centrifugation for 5 min at 2,000 rpm to form pellet aggregates (2.5×10^5 cells/pellet), and finally, incubated at 37°C for 7 days. For AF pellets co-cultured with notochordal cells clusters: Notochordal cell clusters were suspended in their culture medium, collected by centrifugation, washed with HBSS, re-suspended in serum starvation medium (F-12/Dulbecco's modified Eagle's medium with 1% FBS and 1% P/S). Cell clusters in serum starvation medium (10,000 clusters/mL) were placed in 24-well plates and incubated for 3 days. AF pellets were transferred to 24-well plates containing serum starvation medium (1 mL), incubated for 3 days, added to cell culture inserts (1- μ m pore size; Becton Dickson Labware, Franklin Lakes, NJ, USA) for stabilization, and placed in the wells containing notochordal cell clusters. For AF pellets cultured in notochordal cell-conditioned medium (NCCM): The medium from notochordal cells cultured in serum starvation medium for 3 days was filtered through a syringe-tip filter (0.25- μ m pore size), and the filtered NCCM was added to new 24-well plates (1 mL/well). AF pellets were added to the wells containing NCCM.

Stimulation with proinflammatory cytokines

Naïve AF pellets, AF pellets in NCCM, and AF pellets co-cultured with notochordal cells were incubated for 24 or 48 h

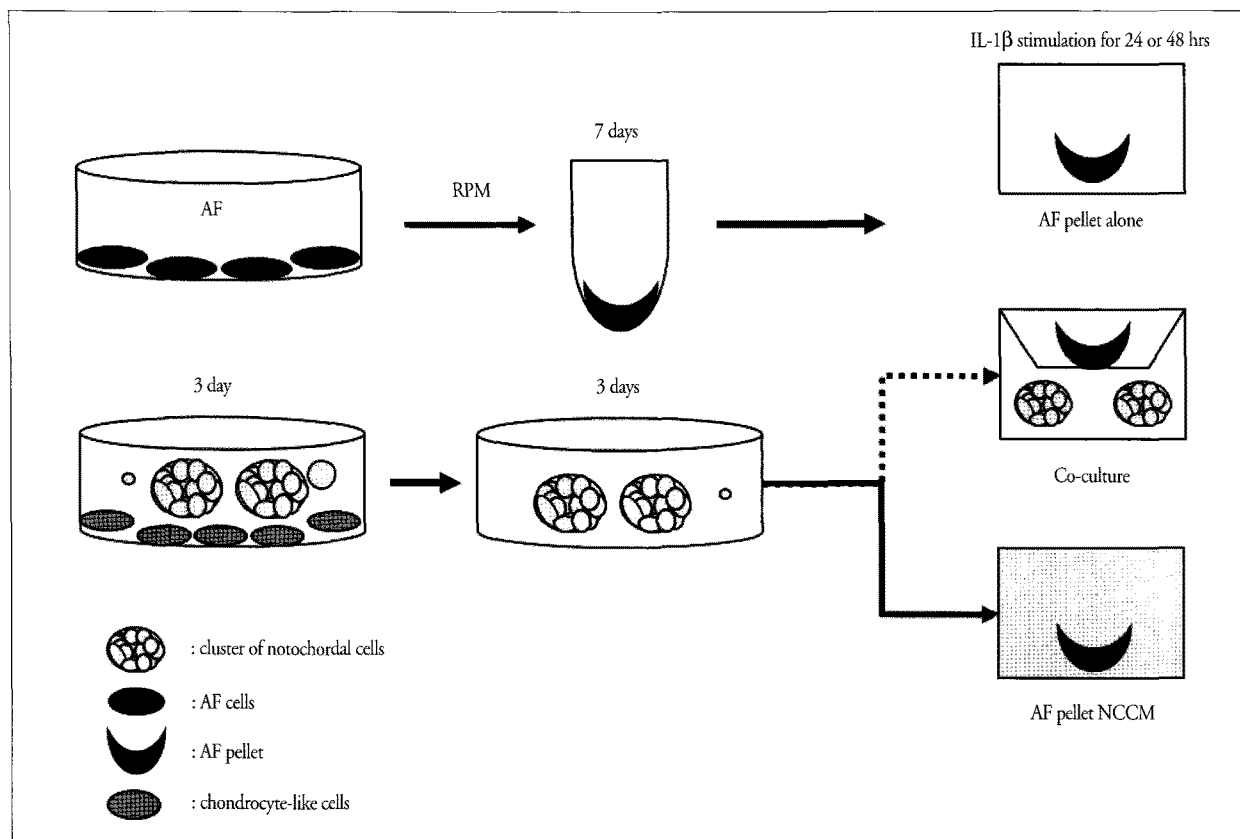


Fig. 1. Schematic diagram of experiments. Confluent AF cells were trypsinized, re-suspended in culture media, and placed in individual polypropylene conical tubes (15 mL) with culture medium. AF cells were centrifuged for 5 min at 2,000 rpm to form pellet aggregate (2.5×10^5 cells per pellet) and were incubated at 37°C for 7 days. Notochordal cell clusters in culture flask supernatants were centrifuged and counted in serum-starved media, F-12/DMEM with 1% FBS, and 1% P/S. Clusters (10,000 clusters per mL) were placed in 24-well plates. AF pellet was moved to 24-well plates containing serum-starved media (1 mL) and incubated. AF pellet was added to cell culture inserts (1 μm pore size, Becton Dickinson Labware, Franklin Lakes, NJ, USA) after 3 days for stabilization in wells containing notochordal cells clusters. Supernatants of notochordal cells cultured in serum-starved media for 3 days were filtered by syringe tip filter (0.25 μm pore size) and added to new 24-plate wells to evaluate AF pellet in notochordal cell-conditioned media (NCCM). AF pellet was added containing NCCM (1 mL). AF : annulus fibrosus, DMEM : Dulbecco Modified Eagle Medium, FBS : fasting blood sugar.

with recombinant human tumor necrosis factor- α (rhTNF- α ; Sigma Chemical Co.) at various concentrations (1, 10, and 100 ng/mL) or with recombinant human interleukin-1 β (rhIL-1 β ; Sigma Chemical Co.) at various concentrations (0.1, 1, and 10 ng/mL) to determine the appropriate concentration of each proinflammatory cytokine for maximal cell response. After stimulation with cytokines, the AF pellets and conditioned media were stored at 80°C for ribonucleic acid (RNA) assays and enzyme-linked immunosorbent assays (ELISAs), respectively.

RNA extraction

Total RNA was extracted from the AF pellets using an easy-spin kit (iNtRON, Seongnam, Korea), according to the manufacturer's instructions. In brief, easy-BLUE (1 mL) was added to prepared sample cells (2×10^6 cells) and mixed by vortexing for 30 s at room temperature. Residual protein was extracted by adding chloroform (200 μL), followed by mixing and centrifugation at 13,000 rpm for 10 min at 4°C . The

aqueous supernatant phase was collected, and the RNA was precipitated and resuspended in the same volume of binding buffer, with gentle mixing and incubation at room temperature for 1 min. The solution was loaded onto a column and eluted by centrifugation for 30 s at 13,000 rpm. The flow-through was discarded after centrifugation, and the column was washed with washing buffer A (700 μL), followed by washing buffer B (700 μL). The collection tubes were discarded, and the column membrane was dried by centrifugation for 1 min at 13,000 rpm. The column was placed in clean 1.5-mL tube, and elution buffer (50 μL) was added directly to the membrane. The column was incubated at room temperature for 1 min and then centrifuged for 1 min at 13,000 rpm. The total RNA pellet was dissolved in ultra-pure water, and the RNA quantity and quality were measured at 260 and 280 nm using a spectrophotometer.

Real-time polymerase chain reaction

The cDNA was synthesized from total RNA (1 μg) with a

High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA), according to the manufacturer's instructions. A SYBR Premix Ex Tag quantitative PCR kit (Takara, Japan) and an ABI Prism 7000 detection system were used to quantify the gene expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and IL-6, using GAPDH as a control. The PCR, in a total volume of 25 μ L, consisted of 40 cycles of 5 s at 95°C and 31 s at 60°C. The primer sequences were as follows : GAPDH, 5'-GATGC TGGTGCCGAGTAC-3' and 5'-GCTGAGATGATGAC CCTTTTGG-3'; iNOS, 5'-CCCCTTCAACGGCTG GTA-3' and 5'-TCTGTGACGGCCTGATCTTTC-3'; COX-2, 5'-CAAAGTCTCCTGAAACCCACTC-3' and 5'-GCTATTGACGATGTTCCAGACTCC-3'; IL-6, 5'-CTCAAGACGACCACGATCCA-3' and 5'-AAGGACA CCCGCACTCCAT-3'.

Statistical analysis

Individual cell preparations included cells from 10 or more rabbits. Each experiment was performed three times, in duplicate or triplicate wells, with cells from different cell preparations. The data are presented as mean \pm SEM. Student's t-

test was used to analyze the data. He statistical significance was established at $p < 0.05$.

RESULTS

Gross and microscopic analyses

Isolated notochordal cells were densely aggregated and existed as clusters (5-20 cells/cluster) (Fig. 2A). The AF cells assumed a morphology reminiscent of fibroblasts when plated as a monolayer. After 5 days in growth medium, the cells formed spherical aggregates with a diameter of 1-2 mm that freely floated as a unit (Fig. 2B). When pelleted at 2,000 rpm in a conical tube and examined histologically, the AF cells in the outer layer were observed encasing a dense cell aggregate, while the inner cell mass was sparse, compared with the outer region (Fig. 2C).

Gene expression in AF pellets co-cultured with notochordal cell clusters or cultured in NCCM

The mRNA expression levels of iNOS and COX-2 were increased in the AF pellets co-cultured with notochordal cell clusters (iNOS, 1.71 \pm 0.74 fold; COX-2, 1.25 \pm 0.28 fold)

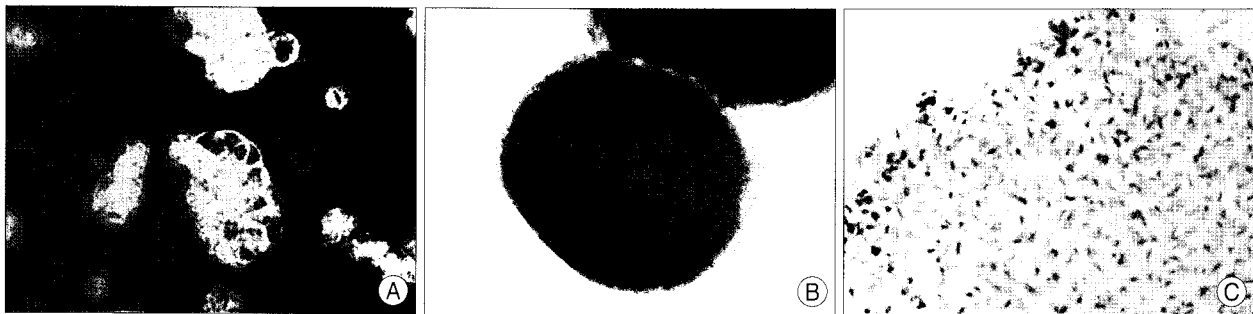


Fig. 2. Microscopic findings of notochordal cell clusters and AF pellet. A : Isolated notochordal cells were densely aggregated and floated in the media as clusters (inverted microscopy, $\times 20$). B : The cells formed a 1-2 mm spherical aggregate after pelleting at 2,000 rpm in a conical tube ($\times 20$). C : AF pellet in the outer layer were observed encasing the dense aggregation of fibroblast-like cells while the inner cell mass was sparse compared to the outer region (H & E, $\times 100$). AF : annulus fibrosus.

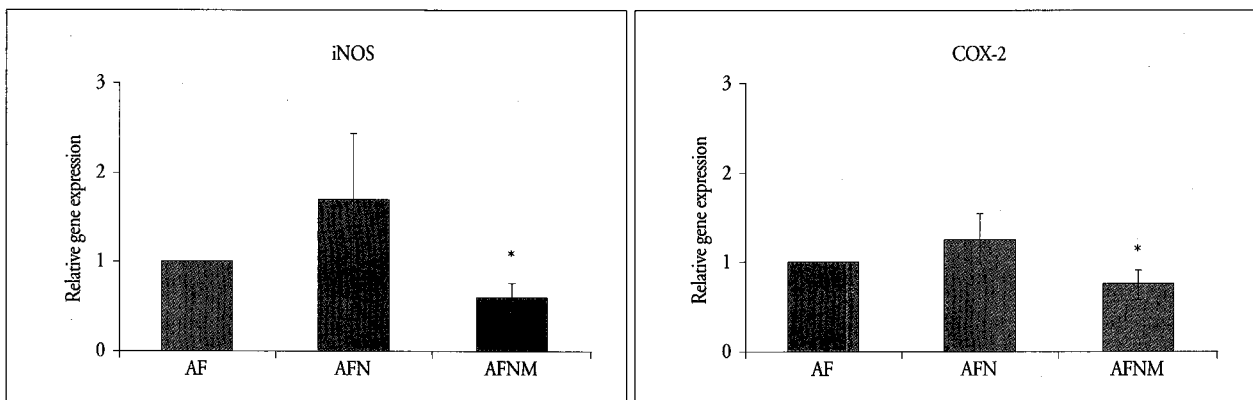


Fig. 3. Gene expression in AF pellet with notochordal cell clusters or in NCCM. AF pellet in NCCM significantly decreased the iNOS and COX-2 mRNA levels compared to AF pellet alone and AF pellet with notochordal cells (* $p < 0.05$). AF : naive AF pellet, AFN : AF pellet co-cultured with notochordal cells clusters, AFNM : AF pellet cultured in NCCM. AF : annulus fibrosus, COX-2 : cyclo-oxygenase, iNOS : nitric oxide synthase, mRNA : messenger ribonucleic acid, NCCM : notochordal cell-conditioned media.

compared with the levels in the AF pellets alone ($p > 0.05$). The AF pellets cultured in NCCM showed significantly stabilized mRNA levels of iNOS (0.61 ± 0.18 fold) and COX-2 (0.76 ± 0.14 fold) ($p < 0.05$), as shown in Fig. 3. No IL-6 mRNA was detected in the AF pellets, regardless of notochordal influence (data not shown).

Gene expression in AF pellets in response to TNF- α or IL- β stimulation

Naïve AF pellets cultured alone were stimulated with TNF- α and IL-1 β for 24 or 48 h. There was no significant difference in total mRNA between the control and the TNF- α -stimulated or IL-1 β -stimulated AF pellets (data not shown). With IL-1 β (1 ng/mL) stimulation for 24 h, the mRNA levels of both iNOS and COX-2 were increased, by 1454.84 ± 817.55 fold and 38.19 ± 6.78 fold, respectively, in the AF pellets. The iNOS expression level was higher than that of COX-2, and a 24-h stimulation by IL-1 β yielded a greater response than a 48-h stimulation (Fig. 4A, B).

IL-1 β stimulation for 24 h significantly increased both iNOS and COX-2 mRNA levels, with increases of $52.3 \pm$

25.7 fold and 1.57 ± 0.07 fold, respectively, at 0.1 ng/mL IL-1 β and increases of 65.1 ± 27.33 fold and 2.99 ± 0.73 fold, respectively, at 1 ng/mL IL-1 β . No further increase of the mRNA levels was observed at 10 ng/mL IL-1 β (Fig. 4C, D).

The levels of iNOS and COX-2 mRNA were not increased in the AF pellets exposed to 1, 10, or 100 ng/mL TNF- α for 24 h.

IL-1 β -stimulated gene expression in AF pellets co-cultured with notochordal cell clusters or cultured in NCCM

AF pellets in NCCM showed a significantly decreased expression of iNOS (0.79 ± 0.11 fold) and COX-2 (0.57 ± 0.06 fold) mRNA in response to IL-1 β stimulation, compared with the AF pellets alone or co-cultured with notochordal cell clusters (iNOS : 1.23 ± 0.13 fold, COX-2 : 1.05 ± 0.20 fold) (Fig. 5A, B). The expression level of IL-6 in the AF pellets did not differ among the three culture conditions (Fig. 5C). Furthermore, using a commercially available ELISA kit (Usnlife Sciences & Technology Co., Wuhan, China), we could not detect IL-6 in the media from any of

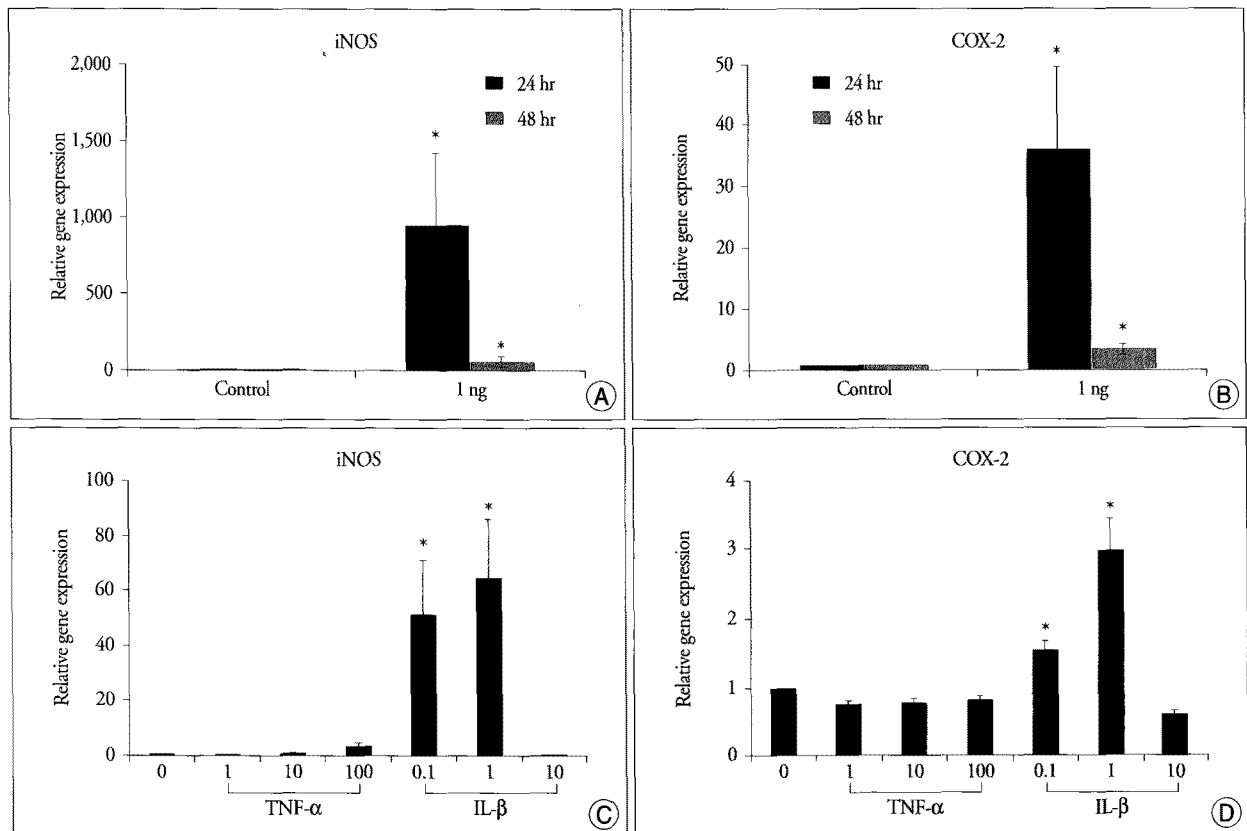


Fig. 4. Gene expression in naïve AF cells by proinflammatory cytokines. A and B : Naïve AF pellet significantly increased iNOS and COX-2 gene expression at 24 hours compared to 48 hours in stimulation of 1 ng/mL IL-1 β ($*p < 0.05$). (C and D). The iNOS and COX-2 mRNA levels significantly increased at 0.1 ng/mL IL-1 β stimulation at 24 hrs and 1 ng/mL IL-1 β stimulation ($*p < 0.05$). The iNOS and COX-2 mRNA levels did not increase in AF pellet in response to TNF- α . AF : annulus fibrosus, COX-2 : cyclo-oxygenase, IL : interleukin, iNOS : nitric oxide synthase, mRNA : messenger ribonucleic acid, TNF- α : tumor necrosis factor-alpha.

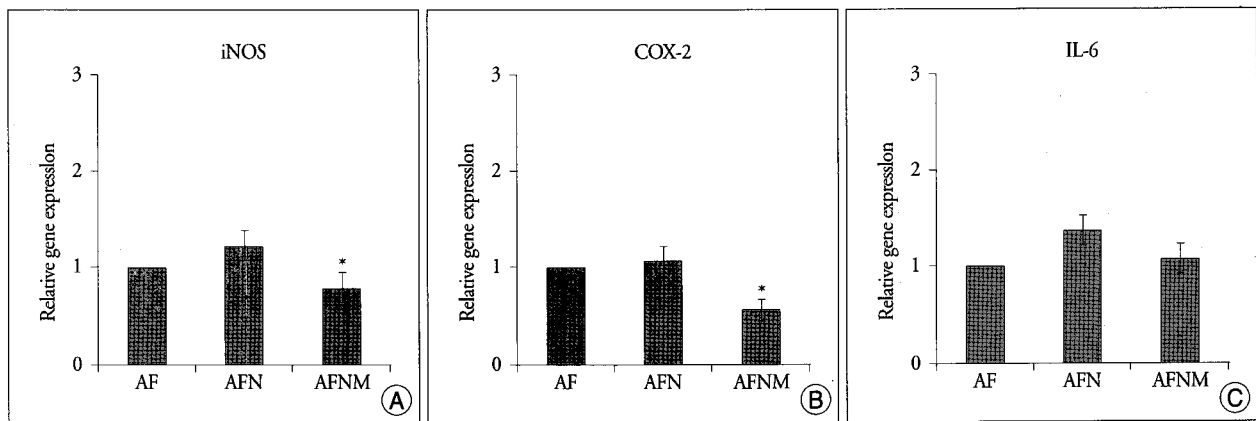


Fig. 5. Gene expression in each group by IL-1 β stimulation. A and B : AF pellet in NCCM significantly decreased the expression of iNOS and COX-2 in response to 1 ng/mL IL-1 β stimulation (* $p < 0.05$). C : There was no difference in IL-6 expression compared to AF pellet alone or AF pellet with notochordal cell clusters. AF : naïve AF pellet, AFN : AF pellet co-cultured with notochordal cells clusters, AFNM : AF pellet cultured in NCCM. AF : annulus fibrosus, COX-2 : cyclo-oxygenase, IL : interleukin, iNOS : nitric oxide synthase, NCCM : notochordal cell-conditioned media.

the three AF pellet cultures.

DISCUSSION

Intervertebral disc degeneration is common in patients with lower back pain. Discogenic pain results from a prior injury and biological repair of the outer AF²⁴. In a previous study, abundant macrophages in symptomatic discs were strongly associated with inflammatory reactions, granulation tissue formation, and discogenic lower back pain²⁴. Macrophages enhance the secretion of inflammatory mediators in AF cells. The complex biological interactions between macrophages and naïve AF cells, and their roles in the pathogenesis of painful disc degeneration, have not been carefully evaluated. However, several studies have demonstrated that various inflammatory mediators, including TNF- α ^{17,21}, IL-1 β ^{20,24}, IL-6^{19,21}, IL-8⁸, and nitric oxide (NO)¹¹, are present in degenerative disc tissue.

Our results demonstrate that at 1 ng/mL, IL-1 β elicits iNOS and COX-2 gene expression. A recent study identified NO (generated by iNOS after stimulation) in the granulation tissue surrounding the disc and demonstrated that NO can produce thermal hyperalgesia in the AF⁴. Our previous studies showed that co-culture of AF cells with macrophages produced large amounts of NO, suggesting that the presence of macrophages may also play a critical role in discogenic pain via NO regulation¹². COX-2 is an inducible enzyme and is abundant in activated macrophages and other cells at inflammatory sites; COX-2 expression increases prostaglandin E2 (PGE2) levels¹. Allodynia, a prominent feature of neuropathic pain and inflammation, may be induced by PGE2 through capsaicin-sensitive or -insensitive fibers¹⁵. Therefore, PGE2 may also serve as a potential mediator for the development of discogenic pain. Naïve AF cells secreted large amounts of

PGE2 and PGF2 α after stimulation by proinflammatory cytokines, and this response was enhanced by macrophage exposure, suggesting that hypersensitization through PG-mediated inflammatory reactions may occur in the disc¹³.

Non-chondrodystrophic dogs are relatively resistant to degenerative disc disease, compared with chondrodystrophic strains⁹. The primary difference between the strains is the maintenance of the notochord cell population within the intervertebral disc nucleus in non-chondrodystrophic dogs¹⁶. Erwin et al.⁶ reported that notochordal cell supernatants from non-chondrodystrophic canine disc NPs upregulated disc-derived chondrocyte proteoglycan production in a dose-dependent manner and that gene expression for the essential extracellular matrix proteoglycans, aggrecan and versican, were upregulated after 24 h in serum-free NCCM¹⁰. Our study is the first report describing the anti-inflammatory influence of notochordal cells on AF cells. Gene expression of inflammatory mediators was significantly decreased in AF pellets cultured in NCCM, with or without stimulation, compared with the expression in naïve AF pellets. Compared with AF pellets cultured in NCCM, AF pellets co-cultured with notochordal cell clusters showed greater expression of inflammatory mediator mRNA, suggesting that cell-to-cell interactions between AF and notochordal cells may result in inflammatory reactions that override the anti-inflammatory effects of soluble NCCM factors.

CONCLUSION

AF and notochordal cell interactions did not decrease the mRNA expression of inflammatory mediators, whereas soluble factors from notochordal cells did mitigate the expression of inflammatory mediator genes in stimulated AFs, suggesting the efficacy of using notochordal cells as a novel therapeutic

tic agent in symptomatic disc degeneration.

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