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# Pro-inflammatory stimulation of meniscus cells increases production of matrix metalloproteinases and additional catabolic factors involved in osteoarthritis pathogenesis

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SUMMARY

*Objective:* Meniscus injury increases the risk of osteoarthritis; however, the biologic mechanism remains unknown. We hypothesized that pro-inflammatory stimulation of meniscus would increase production of matrix-degrading enzymes, cytokines and chemokines which cause joint tissue destruction and could contribute to osteoarthritis development.
*Design:* Meniscus and cartilage tissue from healthy tissue donors and total knee arthroplasties (TKAs) was cultured. Primary cell cultures were stimulated with pro-inflammatory factors [IL-1β, IL-6, or fibronectin fragments (FnF)] and cellular responses were analyzed by real-time PCR, protein arrays and immunoblots. To determine if NF-κB was required for MMP production, meniscus cultures were treated with inflammatory factors with and without the NF-κB inhibitor, hypoestoxide.
*Results:* Normal and osteoarthritic meniscus cells increased their MMP secretion in response to stimulation, but specific patterns emerged that were unique to each stimulus with the greatest number of MMPs expressed in response to FnF. Meniscus collagen and connective tissue growth factor (CTGF) gene expression was reduced. Expression of cytokines (IL-1α, IL-1β, IL-6), chemokines (IL-8, CXCL1, CXCL2, CSF1) and components of the NF-κB and tumor necrosis factor (TNF) family were significantly increased. Cytokine and chemokine protein production was also increased by stimulation. When primary cell cultures were treated with hypoestoxide in conjunction with pro-inflammatory stimulation, p65 activation was reduced as were MMP-1 and MMP-3 production.
*Conclusions:* Pro-inflammatory stimulation of meniscus cells increased matrix metalloproteinase production and catabolic gene expression. The meniscus could have an active biologic role in osteoarthritis development following joint injury through increased production of cytokines, chemokines, and matrix-degrading enzymes.


## Introduction

Meniscus injury is known to increase the risk of osteoarthritis. Untreated meniscus tears have an odds ratio of 5.7 for the development of radiographic osteoarthritis[1]. Even after partial meniscectomy, the relative risk (RR) for osteoarthritis increases following both degenerative tears (RR 7.0) and traumatic tears (RR 2.7)[2,3]. Successful repairs may lead to resumption of sports activity and decreased incidence of osteoarthritis[4]; however, many tears are not amenable to repair secondary to the tissue's minimal vasculature. This increased risk is historically attributed to changes in knee biomechanics due to meniscus deficiency[3,5,6].

The impact of cytokine stimulation on articular cartilage and subsequent extracellular matrix degradation is well documented[7–9]; however, the role of the meniscus in this process is unclear. The knee joint functions as an organ with a shared environment comprised of cartilage, synovium, ligaments and the meniscus. The meniscus is consequently exposed to inflammatory factors produced by knee tissues in response to acute or chronic injury and this exposure likely impacts meniscus biology. Certain aspects of meniscus biology are pathologically altered in meniscus injury and in the development of osteoarthritis[10–18]. Thus, the meniscus likely also has a biologic role in osteoarthritis development through the production of matrix-degrading enzymes and inflammatory

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factors. We hypothesized that inflammatory factors associated with joint injury would stimulate menisci to increase production of matrix-degrading enzymes, cytokines and chemokines which could contribute to joint tissue destruction and subsequent development of osteoarthritis.

## Materials and methods

### *Knee tissue acquisition*

Our institutional review board approved this protocol. Normal human meniscus specimens ($n = 18$ menisci from $n = 18$ donors 25–65 years old) were procured through the National Disease and Research Interchange (NDRI, Philadelphia, PA) or the Gift of Hope Organ and Tissue Donor Network (Elmhurst, IL) while osteoarthritic menisci were obtained from patients undergoing total knee arthroplasty (TKA) for osteoarthritis ($n = 36$ menisci from $n = 36$ donors 44–83 years old). Synovial tissue was removed. Meniscus tissue was macroscopically graded according to a modified International Cartilage Research Society Cartilage Morphology Score (Table SI). All normal meniscus specimens were a grade zero or one, while all but one osteoarthritic meniscus was a grade three or four (one osteoarthritic meniscus received a morphology grade two). Articular cartilage from TKA bone cuts was processed as previously described[7]. All comparisons between chondrocytes and meniscus cells used tissue from the same donor.

### *Cell culture*

Normal and osteoarthritic human meniscus and articular chondrocytes were isolated using our laboratory's tissue digestion and processing methods and primary cells cultured to confluence as described[7]. Prior to stimulation, primary cultures were incubated overnight in serum-free media (DMEM/F12) and then treated for either 6 or 24 h with one of the following: 10 ng/ml IL-1β; 10 ng/ml IL-6 with 25 ng/ml soluble IL-6 receptor; or TGF-α 20 ng/ml (all from R and D Systems) or fibronectin fragments (FnF), a recombinant fragment of fibronectin protein containing domains 7–10 of full length fibronectin (at 1 μM; gift from Harold Erickson, Duke University). For the NF-κB time course study, cells were stimulated with FnF (1 μM) for 15, 30, 45, 60, or 90 min with and without 30 min pretreatment with the NF-κB inhibitor, hypoestoxide (25 μM, Sigma). Cell lysates of nuclear and cytoplasmic fractions collected. Nuclear preparations were processed using the NE-PER fraction kit (Pierce Scientific) according to the manufacturer's instructions. For additional NF-κB studies, cell cultures were stimulated with cytokines at the aforementioned concentration with and without hypoestoxide (25 μM, Sigma) and cell lysates were collected and analyzed using immunoblot. Media was collected for MMP analysis and cells were harvested by scraping in either Trizol (Invitrogen) for RNA isolation or lysis buffer [lysis buffer (Cell Signal Technologies) plus Phosphatase Inhibitor Cocktail 2 (Sigma) and phenylmethanesulfonyl fluoride (Sigma)] for protein analysis.

### *Gene and protein analysis*

RNA was quantified (Nanodrop, ThermoScientific) and verified (BioAnalyzer Chip, Agilent) to ensure high quality RNA (RIN > 6). The reverse-transcription PCR generated cDNA (RetroScript Kit, Ambion). Real-time PCR was performed using the Applied Biosystems 7900HT thermocycler with TaqMan Universal PCR MasterMix and TaqMan Gene Assay (Applied Biosystems: mmp1 Hs00899658_m1; mmp3 Hs00968305_m1; GAPDH Hs02758991_g1). Data was analyzed using the ΔΔCT method in Microsoft Excel (Microsoft).

For quantitative real-time PCR arrays, RNA was harvested as above and purified using the RNEasy Mini kit (Qiagen, #74104). The purified RNA was then used for the extracellular matrix and adhesion PCR array (SABiosciences, #PAHS-013ZA-12) or NF-κB target gene PCR array (SABiosciences, #PAHS-225ZA-12) and the manufacturer's optimized master-mix (SABiosciences, #330522) for the Applied Biosystems 7900HT thermocycler according to the manufacturer's protocol.

For protein analysis, cell media was loaded in equal volumes (1:1 in Lamelli Sample Buffer, 5% β-mercaptoethanol; BioRad), separated by SDS-PAGE (BioRad), transferred to nitrocellulose (Odyssey, Invitrogen) and probed with the primary antibody [anti-MMP1 (PAB12708, Abnova); anti-MMP3 (AB2963, Millipore); anti-MMP8 (MAB3316, Millipore); anti-MMP13 (AB84594, Abcam)] and secondary antibody (CellSignal). Blots were visualized with chemiluminescence (Amersham ECL, GE Life Sciences). Since no known control exists for meniscus secreted proteins, loading was controlled by loading an equal volume of media from wells that had equivalent cell numbers verified by total protein content. Media was analyzed with an MMP Protein Array (#AAH-MMP-1, RayBiotech) or the Cytokine Array (#AAH-CYT-5, RayBiotech). For the NF-κB experiments, immunoblots were probed for phosphorylated-p65, then stripped and probed for total-p65, and then finally β-actin as the loading control. For nuclear preparations, blots were also probed for Lamin B (a nuclear protein) and lactate dehydrogenase (a cytoplasmic protein) to demonstrate the integrity of the fractions. Processed films were imported into Photoshop v7.0 (Adobe) and labeled. Densitometry was completed with ImageJ 1.44p (NIH).

### *Statistical analysis*

Statistical analysis was performed with SigmaPlot v10.0 (Systat Software) and Prism v5.02 (GraphPad Software, Inc.). Real-time PCR arrays were analyzed in Microsoft Excel (Microsoft) using the standard ΔΔCt method normalized to endogenous housekeeping genes in array-specific analysis templates (SABiosciences, http://www.sabiosciences.com/pcrarraydataanalysis.php). The template employed the Student's $t$ test for replicates of four individual donors with significance of $P \leq 0.05$. We accepted this analysis method with the understanding that we did not account for multiple comparisons. A small number of genes may have been found to be significantly different because of the total number of genes analyzed; however, this limitation was accepted because we chose to analyze related genes of either extracellular matrix proteins or the NF-κB family and the arrays were used for hypothesis generation within targeted gene families rather than hypothesis testing for any individual gene.

The effects of cytokine stimulation on MMP-1 and MMP-3 gene expression in normal and osteoarthritic menisci were compared using a multivariate analysis of variance (MANOVA). *Post-hoc* tests were performed when group effects were found to be significant. A *post-hoc* two-tailed Dunnett's test was performed when appropriate to compare cytokine treatments to the unstimulated control, since we did not attempt to rank cell response to the different cytokine treatments.

Immunoblot densitometry was reported with the 95% confidence intervals and analyzed using ANOVAs. We reported Bonferroni corrections for multiple comparisons. Significance was set at $P \leq 0.05$.

## Results

### *Response of normal meniscus to pro-inflammatory factors*

Normal meniscus cell cultures were stimulated with pro-inflammatory factors to evaluate alterations in extracellular matrix