Dynamic biophysical strain modulates proinflammatory gene induction in meniscal fibrochondrocytes

Mario Ferretti,* Shashi Madhavan,† James Deschner, Birgit Rath-Deschner, Ewa Wypasek, and Sudha Agarwal. Dynamic biophysical strain modulates proinflammatory gene induction in meniscal fibrochondrocytes. Am J Physiol Cell Physiol 290: C1610–C1615, 2006. First published February 1, 2006; doi:10.1152/ajpcell.00529.2005.—Fibrochondrocytes of meniscus adapt to changes in their biomechanical environment by mechanisms that are yet to be elucidated. In this study, the mechanoresponsiveness of fibrochondrocytes under normal and inflammatory conditions was investigated. Fibrochondrocytes from rat meniscus were exposed to dynamic tensile forces (DTF) at various magnitudes and frequencies. The mechanoresponsiveness was assessed by examining the expression of inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), and matrix metalloproteinase-13 mRNA expression. The mRNA and protein analyses revealed that DTF at magnitudes of 5% to 20% did not induce proinflammatory gene expression. IL-1β induced a rapid increase in the iNOS mRNA. DTF strongly repressed IL-1β-dependent iNOS induction in a magnitude-dependent manner. Exposure to 15% DTF resulted in >90% suppression of IL-1β-induced mRNA within 4 h and this suppression was sustained for the ensuing 20 h. The mechanosensitivity of fibrochondrocytes was also frequency dependent and maximal suppression of iNOS mRNA expression was observed at rapid frequencies of DTF compared with lower frequencies. Like iNOS, DTF also inhibited IL-1β-induced expression of proinflammatory mediators involved in joint inflammation. The examination of temporal effects of DTF revealed that 4- or 8-h exposure of DTF was sufficient for its sustained anti-inflammatory effects during the next 20 or 16 h, respectively. Our findings indicate that mechanical signals act as potent anti-inflammatory signals, where their magnitude and frequency are critical determinants of their actions. Furthermore, mechanical signals continue attenuating proinflammatory gene transcription for prolonged periods of time after their removal.

interleukin-1; biomechanical signals; cartilage; inflammation

THE MENISCUS OF THE KNEE, positioned between the articular surfaces of the femur and tibia, is crucial for the biomechanical stability of the joint. The geometry, type of extracellular matrix, and cells that synthesize the matrix of this fibrocartilage are well adapted to withstand repetitive biomechanical forces under physiological conditions (3, 26). The fibrochondrocytes of this cartilage synthesize matrix that consists of type I and type II collagens in addition to glycosaminoglycans to provide increased ability to endure tensile, compressive, and torsional forces during joint movement (2, 11, 21). Biomechanical forces regulate fibrochondrocyte functions in a complex manner (1, 3, 19, 21), and any changes in the magnitudes of these forces elicit profound effects (9, 10, 18, 19, 23, 28). Nonphysiological or traumatic loading of the joints can be injurious and induce upregulation of proinflammatory mediators like nitric oxide (NO), inducible NO synthase (iNOS), matrix metalloproteinase-1 (MMP-1), as well as downregulation of matrix proteins like proteoglycans, decorin, and collagen type II, in vitro (8, 10, 18, 24, 29). Similarly, in vivo, increased stress or damage to the meniscus can lead to progressive degenerative changes, leading to osteoarthritis and associated joint dysfunction, suggesting a role of mechanical signals in the pathogenesis of arthritic diseases (14, 16, 18, 22, 23).

The responses of fibrochondrocytes to biomechanical forces are little understood. However, dynamic mechanical stimulation of appropriate/physiological magnitudes is shown to significantly influence anabolic activities in articular chondrocytes (3). The support that these signals are reparative in nature comes from studies demonstrating that mechanical signals of low/physiological magnitudes are potent anti-inflammatory signals and inhibit IL-1β and tumor necrosis factor (TNF)-α-induced proinflammatory gene induction, induce matrix synthesis, and prevent dedifferentiation of chondrocytes (1, 6, 12, 17, 19, 28, 30). The fibrocartilage of the meniscus is notorious for its limited regenerative capacity, and therefore, salvage of damaged meniscus has been a major focus of clinical as well as experimental studies. Biomechanical signals in the form of active or passive motion to improve healing of the fibrocartilage have been tried with variable success (4, 5, 13, 15).

Recently, in a model of experimental antigen-induced arthritis, the integrity of meniscal cartilage was shown to be better preserved in joints exposed to controlled biomechanical loading (9, 13, 20). These observations suggest that appropriate mechanical forces can be used for the repair and rehabilitation of meniscal cartilage.

To develop appropriate therapeutic strategies for the repair or regeneration of damaged fibrocartilage in this study, we have made an attempt to understand the mechanisms of actions of biomechanical signals on the cells of fibrocartilage. It was our hypothesis that fibrochondrocytes of the meniscus respond to mechanical signals in a magnitude- and frequency-dependent manner. Furthermore, biomechanical signals suppress inflammation by inhibiting proinflammatory gene transcription, and these effects are sustained for extended periods of time. We show that signals generated by dynamic tensile forces play an important role in transcriptional regulation of proinflammatory genes in fibrochondrocytes.

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METHODS

Cell harvesting and culture. Menisci harvested from the knees of female Sprague-Dawley rats (200–250 g; Harlan) were minced and treated with 0.2% trypsin for 10 min, followed by 0.15% collagenase I (Worthington) digestion in a two-compartment digestion chamber for 2 h. All protocols were approved by the Institutional Animal Care and Use Committee at The Ohio State University. Fibrochondrocytes released were washed in TCM, composed of 50% DMEM/50% F-12 medium (Cellgro, Mediatech) supplemented with 10% defined fetal calf serum (Hyclone, Logan, UT) and penicillin/streptomycin (100 U·100 μg−1·ml−1), and cultured at 37°C and 5% CO2. Subsequently, the phenotype of meniscal cells was confirmed by synthesis of collagen type-I and -II, aggrecan, biglycan, and versican. The phenotype of meniscal cells was found to be stable during the first three passages.

Exposure of cells to dynamic tensile forces. Fibrochondrocytes (10^3/well) were transferred to collagen I-coated six-well Bioflex-II plates (Flexcell), and grown for 5 days to achieve 70–80% confluence. The medium was replaced with TCM containing 1% FCS, 20 h before the cells were subjected to DTF at various magnitudes (3, 6, 9, 12, 15, or 20%) and frequencies (4, 25, 50, 100 or 250 mHz) in a Flexcell strain unit (Flexcell) that exposes cells to equibiaxial tensile forces. In all experiments the load was given in a square waveform and remained the same at all frequencies and magnitudes (Fig. 1A). Cells were subjected to four different treatment regimens: 1) untreated cells, 2) cells exposed to recombinant human interleukin-1β (IL-1β; 1 ng/ml, Calbiochem), 3) cells exposed to DTF alone, and 4) cells exposed to both DTF and IL-1β (1 ng/ml) reproducibly induced iNOS mRNA expression and NO production (1, 12, 30).

RNA purification and real-time polymerase chain reaction. RNA was isolated from cells with RNeasy kit (Qiagen) according to manufacturer’s recommended protocols. Briefly, cells were scraped in a total of 350 μl of RLT buffer, and DNA shredded by being passed through a QiAshredder (Qiagen) column and were spun at 13,000 g for 2 min. Subsequently, the cell extracts were mixed with an equal volume of 70% ethyl alcohol, treated with DNase, and loaded on a silica gel column to bind RNA. The RNA bound to silica gel was thoroughly washed, and eluted with 50 μl of sterile water. The concentration of RNA was assessed by reading absorbance at 260/280 nm in a Biophotometer 6131 (Eppendorf).

Gene-specific primer sequences were selected using the Taqman Probe and Primer Design function of the Primer Express version 1.5 software (Applied Biosystems). The sense and antisense sequences of rat primers used are as follows: MMP-13 sense, 5’-GT-TCAAGGAAATCACGCTCTCTATGG-3’; antisense 5’-TG-GGTCACACTTTCTGTGTTTT-3’, probe 6-FAMd(CCAAG-GAGATGAAAGCCCCACCACTTAAGC)BHQ-1 (XM143345); TNF-α sense 5’-CCCCAGGACCTCACTCAGATC-3’; antisense 5’-TCGTAGCAAACCCAAACGACG-3’, probe 6-FAMd (TCCAGTGCAACCGCGTACC)BHQ-1(X66539); iNOS sense 5’-TTTCGCTTAATGCGGAAGGT-3’, antisense 5’-GCTTCC-GACTTTTCCTGTCTeA-3’, probe 6-FAMd(CGCGCTGACA-GCCACAGCT)BHQ-1(D44591). Reverse transcription reactions were carried out using 2 μg RNA and TaqMan reverse transcription reagents, followed by real-time PCR using TaqMan PCR Master Mix and ABI Prism 7700 Sequence Detection System (Applied Biosystems). Reactions were performed as follows: cycle I (1×): 95°C for 3 min; cycle II (50×): step 1 at 95°C for 0.3 min, followed by step 2 at 55°C for 0.3 min, and step 3 at 72°C for 30 min; cycle III at 40°C held. After amplification, melt curve analysis was obtained to ensure that primer dimers or nonspecific products had been eliminated or minimized. The data obtained by real-time PCR were analyzed by the comparative threshold cycle (Ct) method. In this method, the amount of the target, normalized to GAPDH, and relative to a calibrator (either untreated sample or IL-1β-stimulated cells), is given by 2^(-ΔΔCt), where ΔΔCt = ΔCt (sample) – ΔCt (calibrator), and ΔCt is the Ct of the target gene subtracted from the Ct of GAPDH.

Semiquantitative analysis of proteins by Western blot analysis. The semiquantitative estimation of proteins was carried out by Western blot analysis (1, 12). Briefly, cells exposed to various regimens of
mechanostimulation were lysed in ice-cold Tris-buffered saline containing protease inhibitor cocktail (Roche) and the extracted proteins were loaded on the SDS-10% acrylamide gels. The proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad) and identified by monoclonal mouse anti-MMP-13 IgG (1:1,000 dilution; Calbiochem), mouse anti-iNOS IgG (1:200 dilution; BD Biosciences), rabbit anti-TNF IgG (1:500 dilution; Biosource). Monoclonal mouse anti-β-actin IgG (1:20,000; Abcam) was used to reprobe the same blots as a standard. HRP-labeled donkey anti-mouse IgG (1:10,000 dilution; Chemicon) or goat anti-rabbit antibody (1:10,000 dilution; Santa Cruz) was used as secondary antibody. The presence of HRP was detected by Lumilip (Amersham). The semiquantitative expression of proteins was estimated in digital images of luminescence in bands of Western blots by Kodak Image Station 1000 and Kodak 1D image analysis software. Expression of β-actin was used to standardize the protein input in each lane of a blot (1, 30). The relative luminescence of bands in cells treated with IL-1β and DTF was estimated as the percentage of luminescence in the bands from cells treated with IL-1β alone.

Measurements of NO. NO measurements in the culture supernatants of fibrochondrocytes were quantified by a modified Griess reaction as described earlier (1, 12).

RESULTS
Mechanosensitivity of meniscal fibrochondrocytes to DTF at various magnitudes. Before the role of DTF on fibrochondrocytes was determined, we examined the effects of 0.1 to 25 ng/ml concentrations of IL-1β on the induction of NO in fibrochondrocytes. The optimal NO production was observed at 1 ng/ml, beyond which a plateau in NO production was observed (data not shown). To examine the effects of DTF on fibrochondrocytes, these cells were exposed to equibiaxial strain at various magnitudes and 0.05 Hz in the presence or absence of IL-1β. Fibrochondrocytes did not express iNOS mRNA constitutively. Exposure of fibrochondrocytes to IL-1β (1 ng/ml) exhibited a marked upregulation of iNOS mRNA and protein induction (Fig. 1, A and B). Meniscal cells, when exposed to DTF alone at 5%, 10%, 15%, or 20%, did not exhibit iNOS mRNA expression (data not shown). However, in the presence of IL-1β, DTF inhibited IL-1β-induced iNOS expression in a magnitude-dependent manner: as little as 5% equibiaxial strain inhibited 57%, whereas 15% DTF suppressed >98% of IL-1β-induced iNOS mRNA expression (Fig. 1A). In parallel experiments, Western blot analysis revealed that DTF also significantly inhibited iNOS synthesis in a dose-dependent manner, exhibiting an inhibition of 42% iNOS synthesis by 5% DTF and >95% iNOS synthesis by 15% DTF (Fig. 1B). The confirmatory studies analyzing NO production in culture supernatants of cells exposed to DTF for 36 h also showed a significant inhibition of IL-1β-induced NO production parallel to inhibition of iNOS synthesis (Fig. 1C).

Anti-inflammatory effects of DTF are frequency dependent. In the next series of experiments, we investigated whether the inhibitory effect of DTF on the IL-1β-induced iNOS mRNA expression is frequency dependent. Because DTF at a magnitude of 15% exhibited suboptimal suppression of IL-1β-dependent iNOS synthesis and NO production, the experiments were conducted at a magnitude of 15%. As shown in Fig. 1D, the frequency of DTF is as important in regulating proinflammatory gene expression as is magnitude. A frequency as rapid as 0.25 Hz significantly inhibited >48 ± 6% IL-1β-induced iNOS mRNA expression. Similar to the magnitudes of DTF, the effects of frequencies of biomechanical signals were also dose-dependent. A frequency of 0.025 Hz inhibited IL-1β-induced iNOS expression maximally. However, exposure of cells to decreasing frequencies of strain vs. rest demonstrated that 0.004 Hz minimally inhibited IL-1β-induced iNOS gene expression (Fig. 1D).

Dynamic tensile strain blocks IL-1β-induced synthesis of proinflammatory mediators. Because IL-1β upregulates multiple proinflammatory genes (14, 16), we next determined whether the effects of DTF at a magnitude of 20% DTF and a frequency of 0.05 Hz also blocked the synthesis of other proinflammatory proteins. TNF-α and MMP-13, representing two major categories of proteins, a cytokine and a matrix metalloproteinase involved in cartilage destruction (17, 19), were selected. Control cells and cells exposed to DTF alone for 4 or 24 h did not express mRNA for TNF-α or MMP-13. However, meniscal cells exposed to IL-1β exhibited a significant upregulation of TNF-α and MMP-13 induction. Quantitative analysis by real-time PCR revealed that co-exposure of cells to DTF for 4 or 24 h abolished >95% IL-1β-induced TNF-α and MMP-13 (Fig. 2A). As expected, in parallel experiments the synthesis of TNF-α and MMP-13 was also suppressed by the actions of DTF in IL-1β-treated cells after 24 h (Fig. 2, B and C).

Suppression of proinflammatory gene induction by DTF is sustained despite the presence of IL-1β. The observations that DTF supresses IL-1β-induced proinflammatory gene transcription in fibrochondrocytes prompted us to investigate the temporal regulation of IL-1β-induced genes by biomechanical signals. In these experiments, IL-1β was added to fibrochondrocytes at the start of the experiment and remained in the medium for 24 h. The cells were exposed to DTF (15%, 0.05 Hz) for the first 4, 8, 12, 16, or 20 h, followed by a period of rest for 20, 16, 12, 8, or 4 h, respectively. All cultures were analyzed at 24 h for the expression of iNOS mRNA. In addition, in the presence of IL-1β cells were exposed to DTF continuously for 4, 12, 16, or 20 h, and analyzed immediately after DTF exposure. As illustrated in Fig. 3A, in the presence of IL-1β continuous exposure of cells to DTF for 4, 12, 16, or 20 h exhibited significant suppression of IL-1β-induced iNOS gene induction. On the contrary, analysis of cells exposed to DTF for 4 h, followed by 20 h of rest, revealed that DTF suppressed <60% of IL-1β-induced iNOS expression. Analogous to these observations, DTF exposure for 8 h strongly suppressed (96% ± 5%) IL-1β induced iNOS gene induction during the ensuing 16 h (Fig. 3B). Surprisingly, exposure of cells to DTF for 12, 16, or 20 h, followed by rest, failed to block the IL-1β-induced iNOS gene expression, when analyzed after 12, 8, or 4 h of rest. As expected, continuous exposure of fibrochondrocytes to DTF for 24 h blocked the iNOS mRNA expression induced by IL-1β. Thus the anti-inflammatory effects of DTF are sustained after short-term exposures of fibrochondrocytes to DTF, whereas long-term exposures of DTF, followed by rest, did not block the actions of IL-1β in a sustained manner. On the other hand, continuous exposure of cells to DTF consistently blocks the actions of IL-1β (Fig. 3).

DISCUSSION
The findings of this study support the hypothesis that biomechanical signals regulate the proinflammatory gene expres-
Mechanoregulation of Meniscal Fibrochondrocytes

In fibrochondrocytes of the meniscus in a magnitude- and frequency-dependent manner. In this in vitro system, we have examined the responses of meniscal fibrochondrocytes to two factors, IL-1β that is involved in the pathogenesis of the meniscus and dynamic strain that is known to elicit rehabilitative effects on the cartilage (1, 3, 4, 7, 9, 13). The effects of IL-1 are mediated by NO; therefore, the expression of iNOS and resultant NO production was used as a marker of inflammation (10, 25). Signals generated by DTF of 5% to 20% are not perceived as traumatic signals in meniscal fibrochondrocytes. Contrarily, DTF at magnitudes of 5–20% are anti-inflammatory and suppress IL-1β-induced iNOS mRNA expression and consequently its synthesis and NO production in a magnitude-dependent manner. In these experiments, DTF blocked mRNA expression that was paralleled by inhibition of protein synthesis, suggesting that mechanical signals inhibit IL-1β-induced proinflammatory gene induction at transcriptional level. Present technical limitations imposed by the strain devices restrict the examination of the effects of magnitudes of DTF >20%. These findings are similar to those observed in articular chondrocytes, where tensile as well as compressive forces of low magnitudes are shown to exert anti-inflammatory effects (6, 10). However, fibrochondrocytes exhibit suppression of proinflammatory gene induction at 2- to 3-fold higher magnitudes of mechanical forces than articular chondrocytes (1, 12, 30).

Fibrochondrocytes serve discrete functions in a diarthroidal joint. In this cartilage, fibrochondrocytes constitute >20% of the tissue, and synthesize tough intercellular matrix comprising 20–25% collagens type I and II, and only 0.6% to 0.8% of the total collagen proteins. This tissue is responsible for the load-bearing and shock-absorbing function of the meniscus. In vitro, fibrochondrocytes respond to dynamic strain by releasing NO, which can modulate the expression of proinflammatory genes (10). The effects of DTF on the expression of IL-1β-induced matrix metalloproteinase-13 (MMP-13) and TNF-α in fibrochondrocytes are shown in Fig. 2. The expression of these genes is suppressed by DTF in the presence of IL-1β. Furthermore, the expression of iNOS mRNA is regulated by DTF, as shown in Fig. 3. The data are representative of 1 of 3 separate experiments performed in triplicate. The bars represent means ± SE. *P < 0.05, compared with IL-1β-treated cells.
glycosaminoglycans (2, 3, 11, 21, 22). While this collagen-rich stiffer matrix provides the ability to undergo tensile, compressive, torsion, and shear forces during loading, its >10 times lower glycosaminoglycan content may provide limited ability for hydrodynamic shock absorption. This may therefore lead to exposure of meniscal chondrocytes to higher magnitudes of biomechanical forces during normal joint function, and their ability to withstand 2- to 3-fold higher magnitudes of mechanical forces without perceiving them as a proinflammatory signal (27).

The frequency of mechanical signals is as important in regulating the responsiveness of cells as their magnitude. Mechanical forces of rapid frequencies (0.25 and 0.025 Hz) effectively inhibit IL-1β-induced proinflammatory responses, whereas a slower frequency (0.004 Hz) is less effective in revoking the IL-1β actions in meniscal fibrochondrocytes. While earlier studies (23, 29) have shown that static forces are proinflammatory to cartilage, the present findings suggest that signals at lower frequencies inhibit IL-1β-induced proinflammatory gene transcription but to a lesser extent than higher frequencies in fibrochondrocytes.

Injury or trauma to cartilage induces a 250-fold increase in the production of MMPs, and severalfold increase in the expression of cytokines and NO production (18, 23). Mechanical signals, despite the presence of IL-1β in the medium, block the expression of proinflammatory mediators involved in cartilage destruction. For example, TNF-α, the major mediator involved in the pathogenesis of rheumatoid arthritis and osteoarthritis, is upregulated in response to an injury (18, 23). DTF effectively inhibits synthesis of TNF-α by suppressing expression of its mRNA in the presence of exogenous IL-1β. Similarly, MMP-13, by initiating cleavage of collagen, is one of the major matrix-associated proteinases involved in the development of arthritic lesions. Downregulation of MMP-13 synthesis by DTF represents another mode of protective effects of biomechanical signals on inflamed joints. In this respect, the actions of DTF are similar to the inhibitors of inflammation known to be palliative to inflamed joints (3, 7), and suggest that DTF likely serves as a key signal in preserving the functional integrity of the inflamed fibrocartilage. Proinflammatory gene transcription by IL-1β is mediated by nuclear factor-κB (NF-κB) transcription factors, which activate a plethora of proinflammatory gene expression (1). In addition, the downregulation of proinflammatory responses by biomechanical signals has been shown to be mediated by inhibition of nuclear translocation of NF-κB in articular chondrocytes (1). Therefore, it is likely that inhibition of IL-1β-induced proinflammatory protein induction by biomechanical signals in fibrochondrocytes is also brought about by inhibition of nuclear translocation of NF-κB. In such a case, it is tempting to speculate that biomechanical signals may inhibit number of other proinflammatory genes controlled by NF-κB. The role of this pathway in the anti-inflammatory actions of biomechanical signals needs further elucidation.

The upregulation of proinflammatory molecules after a traumatic injury is sustained up to 72 h after its initiation (18). The experiments to examine the time-dependent effects of DTF have revealed that in the constant presence of IL-1β, 1) exposure of DTF for 4 or 8 h is sufficient to downregulate iNOS induction for the ensuing 20 or 16 h, 2) exposure of cells to DTF for 12, 16, or 20 h minimally suppresses IL-1β-induced iNOS induction when examined after a rest of 12, 8, or 4 h respectively, and 3) the effects of DTF are sustained from 4 to 24 h if DTF is applied to cells continuously. These observations suggest that long-lasting anti-inflammatory effects of DTF can be achieved in cells by exposure of cells from 4 to 8 h of DTF. Similarly, constant exposure of cells to DTF without rest results in a sustained suppression of proinflammatory responses. However, exposure of longer durations of DTF (12, 16, or 20 h), followed by a rest (8 or 4 h), does not have similar sustained effects on iNOS gene suppression. It is unknown whether this failure of longer durations of DTF followed by rest to repress iNOS induction is due to cellular fatigue after long exposures to mechanical signals. On this note, motion-based therapies in the form of continuous passive motion are known to be beneficial to inflamed joints and reduce pain and inflammation (4, 5, 13, 15). Our data also suggest that constant exposure to mechanical signals suppresses the IL-1-induced proinflammatory gene induction in a sustained manner. Furthermore, limited exposures to DTF can elicit long-lasting effects on the inhibition of proinflammatory gene induction. However, there is an optimal period of time that is sufficient for the effectiveness of biomechanical signals, beyond which the biomechanical signals become less effective. Whether therapies similar to continuous passive motion can also be utilized for shorter intervals without losing their effectiveness is yet to be determined.

Overall, this is the first evidence that dynamic tensile strain by blocking proinflammatory gene transcription acts as an anti-inflammatory signal on fibrochondrocytes of the meniscus. The mechanoresponsiveness of fibrochondrocytes varies according to the magnitude and frequency of the signals generated by the tensile forces. More importantly, the effectiveness of anti-inflammatory actions of biomechanical signals is sustained despite the surrounding proinflammatory environment but is dependent on the length of their exposure to cells. These in vitro findings may provide the molecular basis for the beneficial effects of motion-based therapies on inflamed joints. Further examination of these effects in vivo is essential for translating anti-inflammatory effects of biomechanical signals into successful therapeutic modalities.

GRANTS

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REFERENCES


