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MECHANOTRANSDUCTION VIA INTEGRINS AND INTERLEUKIN-4 RESULTS IN ALTERED AGGRECAN AND MATRIX METALLOPROTEINASE 3 GENE EXPRESSION IN NORMAL, BUT NOT OSTEOARTHRITIC, HUMAN ARTICULAR CHONDROCYTES

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Objective. **To determine molecular events in the regulation of messenger RNA (mRNA) of cartilage matrix molecules and proteases by mechanical stimulation of chondrocytes from normal human articular cartilage and to ascertain whether similar regulatory systems are present in chondrocytes from osteoarthritic (OA) cartilage.**

Methods. **Chondrocytes extracted from macroscopically and microscopically normal and OA cartilage were mechanically stimulated in the presence or absence of GRGDSP or GRADSP oligopeptides, neutralizing interleukin-4 (IL-4) antibodies, gadolinium, or apamin. The relative levels of mRNA for aggrecan, tenascin, matrix metalloproteinase 1 (MMP-1), MMP-3, and tissue inhibitor of metalloproteinases 1 (TIMP-1) were determined by semiquantitative reverse transcription–polymerase chain reaction at several time points up to 24 hours poststimulation, using GAPDH as a control.**

Results. **Normal chondrocytes showed an increase in aggrecan mRNA and a decrease in MMP-3 mRNA within 1 hour following stimulation, with a return to baseline levels within 24 hours. These changes were blocked by GRGDSP, IL-4 antibodies, and gadolinium, but were unaffected by apamin. In contrast, chondrocytes isolated from OA cartilage showed no change in aggrecan or MMP-3 mRNA levels following mechanical stimulation. The mRNA levels of tenascin, MMP-1, and TIMP-1 were unaltered in mechanically stimulated normal and OA chondrocytes.**

Conclusion. **Mechanical stimulation of human articular chondrocytes in vitro results in increased levels of aggrecan mRNA and decreased levels of MMP-3 mRNA. The transduction process involves integrins, stretch-activated ion channels, and IL-4. This chondroprotective response is absent in chondrocytes from OA cartilage. Abnormalities of mechanotransduction leading to aberrant chondrocyte activity in diseased articular cartilage may be important in the progression of OA.**

Mechanical forces are important in both the maintenance of articular cartilage (1) and the development of osteoarthritis (OA) (2). In vivo and in vitro studies have supported the idea that optimal mechanical stimulation maintains articular cartilage structure and function, whereas abnormal mechanical forces lead to loss of cartilage and the onset of OA. Static immobilization of joints in vivo leads to cartilage breakdown (3), while increased or abnormal loading of joints leads to cartilage matrix degradation and tissue remodeling and, eventually, to the onset and progression of OA (4).

In vitro studies, using cartilage explants, monolayer, and 3-dimensional culture systems, have been used by different investigators to study the biochemical, molecular, and cellular responses of chondrocytes to both static and intermittent mechanical stimuli. Responses of chondrocytes to mechanical stimuli that have been identified include changes in cell membrane potential, increased levels of intracellular calcium and cAMP, and altered synthesis of total proteoglycans and glycosaminoglycans (5,6). Recently, load-controlled compression of bovine chondrocytes has been shown to

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result in increased aggrecan gene expression (7) and to influence fibronectin production (8).

The signal transduction mechanisms by which these mechanical signals are recognized by chondrocytes and translated into molecular and biochemical responses are poorly understood. Recent evidence suggests that integrins act as mechanoreceptors in a variety of different cell types, including chondrocytes and bone cells (9–11). Integrins are heterodimeric transmembrane proteins that are composed of an α and a β subunit (12). The extracellular domains form the ligand-binding domain that interacts with the cell matrix, while the cytoplasmic tails are associated with the actin cytoskeleton and various associated proteins, including focal adhesion kinase and paxillin, and thus provide a means for transducing extracellular mechanical signals into intracellular biochemical effects (13). Integrins have been shown to be involved in the rapid activation of extracellular signal–regulated kinase 2 in mechanically stimulated rat cardiac fibroblasts (14), and increased tyrosine kinase activity in osteoblasts and endothelial cells following mechanical stress (15). In coronary arterioles, shear stress–induced vasodilation and alterations in intracellular calcium concentrations are both mediated by integrins (16,17).

We have developed an in vitro system for mechanically stimulating cells in monolayer culture, which has allowed detailed examination of human articular chondrocyte mechanotransduction (11,18,19). Chondrocytes from human articular cartilage show changes in membrane potential following cyclic mechanical stimulation. The electrophysiologic response is dependent on the frequency of stimulation and on whether the chondrocytes have been isolated from macroscopically and microscopically normal or OA cartilage. Stimulation at 0.33 Hz (2 seconds on, 1 second off) for 20 minutes at 37°C results in a membrane hyperpolarization response of chondrocytes from normal cartilage as the result of activation of small-conductance apamin-sensitive calcium-activated potassium (SK) channels. In contrast, chondrocytes from OA cartilage show a membrane depolarization response with the same frequency and degree of mechanical stimulation, suggesting aberrant mechanotransduction in disease.

We have used this electrophysiologic response to elucidate signaling pathways activated in normal human articular chondrocytes by mechanical stimulation. The hyperpolarization response of normal chondrocytes to cyclic stimulation at 0.33 Hz is mediated via $\alpha5\beta1$ integrins (11) and involves the actin cytoskeleton, stretch-activated ion channels (SACs), and activation of tyrosine kinases, with subsequent release of interleukin-4 (IL-4). IL-4 then acts through an autocrine/paracrine loop to activate phospholipase C/protein kinase C pathways, which in turn lead to activation of SK channels (11,19,20).

The present study was undertaken to identify whether the mechanotransduction pathway we have identified in normal human chondrocytes, which results in membrane hyperpolarization, is involved in the regulation of cartilage structural and remodeling molecules, such as aggrecan and matrix metalloproteinases (MMPs). In addition, we wished to establish whether similar mechanotransduction pathways were present in chondrocytes from OA cartilage.

MATERIALS AND METHODS

Isolation and culture of chondrocytes. Knee joints were obtained at hospital autopsy (with relatives' consent), opened, and graded macroscopically for the presence or absence of OA using the Collins/McElligott system (21). Donors had died of a variety of diseases unrelated to the locomotor system and were undergoing routine hospital autopsy.

For normal cartilage (Collins/McElligott grade 0), a total of 15 cases were studied: 7 males and 8 females, with a mean donor age of 71 years (range 49–89 years). A minimum of 3 donors was used for each experiment. For OA cartilage, 6 cases were studied, with a Collins/McElligott grade of 1 (1 donor), 2 (2 donors), or 3 (3 donors). Articular cartilage was removed from either macroscopically normal or abnormal parts of the joint for isolation of chondrocytes by enzymatic release (10). Isolated cells were seeded at a concentration of 5×10^4 cells/ml into 55-mm diameter tissue culture dishes containing Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. Primary, nonconfluent cultures of chondrocytes were used in all experiments.

Morphologically, the cells from both normal and OA cartilage were typically flattened, with a polygonal cell shape, and did not show the fibroblastic appearance of dedifferentiated chondrocytes. In addition, reverse transcription–polymerase chain reaction (RT-PCR) and immunohistochemistry showed expression of cartilage-specific molecules, including type II collagen and aggrecan (11,19).

Mechanical stimulation. The technique used has previously been described in detail (11,19). Briefly, flexible plastic tissue culture dishes were placed in a sealed pressure chamber with inlet and outlet ports. The chamber was pressurized with nitrogen gas from a cylinder, the frequency of pressurization being dictated by an electronic timer controlling the inlet and outlet valves. The standard regimen of cyclic pressurization used in this study consisted of pressure pulses of 1 atmosphere above atmospheric pressure, at a frequency of 0.33 Hz (2 seconds on, 1 second off) for 20 minutes. Cyclic pressurization in this system has been shown to induce deformation and strain on the base of the plastic tissue culture dish and its adherent

Table 1. Primer sequences used for semiquantitative RT-PCR*

Primer	5′ sequence (5′ to 3′)	3′ sequence (5′ to 3′)
GAPDH	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC
Aggrecan	TGAGGAGGGCTGGAACAAGTACC	GGAGGTGGTAATTGCAGGGAACA
Tenascin	CTGAGACGCAAAAACGGACGC	GAGTGTTCGTGGCCCTTCCAGTGG
MMP-1	CTGTTCAGGGACAGAATGTGC	CAGCTATTAGCTTTCTGGAG
MMP-3	AGATGATATAAATGGCATTCAG	CTCCAACTGTGAAGATCCAG
TIMP-1	GACACCAGAAGTCAACCAGACC	GACTGCCAGGTGCACAGCCC

* RT-PCR = reverse transcription–polymerase chain reaction; MMP = matrix metalloproteinase; TIMP-1 = tissue inhibitor of metalloproteinases 1.

cells (19). A pressure of 1 atmosphere results in 3,200 microstrain on the base of the dish.

Experimental protocol. Dishes of chondrocytes were placed in serum-free media overnight, then stimulated for 20 minutes at 0.33 Hz in the presence or absence of reagents previously shown to inhibit the membrane hyperpolarization response of normal human chondrocytes to 0.33 Hz stimulation (11,19). The reagents used were as follows: 10 μM gadolinium (Sigma, Poole, UK), a blocker of SACs (22); 4.9 μM apamin (Sigma), an inhibitor of SK channels (23); 1 μg/ml of neutralizing anti–IL-4 antibody (R&D Systems, Abingdon, UK); and 50 μg/ml of GRGDSP (Calbiochem, Nottingham, UK), a peptide that competes for integrin-ligand RGD-binding sites, or 50 μg/ml of GRADSP (Calbiochem), a control peptide for GRGDSP. Following stimulation, dishes were incubated at 37°C for 0 (control), 1, 3, 6, and 24 hours in serum-free media, then total RNA was extracted.

RNA extraction. Total RNA was extracted from cultured chondrocytes as described in the Micro RNA Isolation kit (Stratagene, Amsterdam, The Netherlands), using a denaturing buffer of 4M guanidine thiocyanate, 0.75M sodium citrate, 10% (weight/volume) lauroylsarcosine, and 7.2 μl/ml of β-mercaptoethanol. The quantity of RNA isolated was determined by the absorbance reading at 260 nm on a spectrophotometer (GeneQuant; Pharmacia, St. Albans, UK).

RT-PCR and gel analysis. Prior to complementary DNA (cDNA) synthesis, all RNA samples were incubated with DNase I (Life Technologies, Paisley, UK) for 15 minutes in the presence of an RNase inhibitor (Pharmacia). Template cDNA was synthesized using 0.5 μg of RNA, Superscript II, and oligo$(dT)_{12\text{-}18}$ (Life Technologies) according to the manufacturer's instructions. The primers used for the PCR reactions are given in Table 1.

A typical 20-μl PCR reaction contained 20 mM ammonium sulfate, 75 mM Tris HCl, pH 8.8, 0.01% (v/v) Tween 20, 1 μM of each primer, 2 μl of cDNA, 100 μM dNTPs, 0.1% (w/v) bovine serum albumin, 0.25 units of *Taq* polymerase (Biogene, Kimbolton, UK). The magnesium chloride concentrations for each primer pair were as follows: for GAPDH 2.5 mM, for MMP-1 2 mM, and for aggrecan, tenascin, MMP-3, and tissue inhibitor of metalloproteinases 1 (TIMP-1) 1.25 mM.

The following protocol was used for all aggrecan, tenascin, MMP-1, and MMP-3 reactions: 94°C for 3 minutes; 24 cycles at 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute 30 seconds. For reactions involving TIMP-1, 28 cycles were used.

PCR products were analyzed by electrophoresis using a 1% (w/v) agarose gel stained with ethidium bromide, and the intensity of each band was measured under ultraviolet fluorescence using EASY Image analysis software (Scotlab, Coatbridge, UK). The ratio of intensities of the bands for the product of interest compared with the housekeeping gene GAPDH were calculated and compared. Each donor was tested in duplicate, and a minimum of 3 donors was used for each experiment.

Cell viability staining. To check for altered levels of apoptosis, chondrocytes were stained with acridine orange (Sigma) or annexin V (Oncogene, Nottingham, UK). Prior to staining, chondrocytes were incubated in the presence of RGD- or RAD-containing peptides for 3 hours, and then stimulated at 0.33 Hz for 20 minutes. Unstimulated cells and cells not incubated with RGD- or RAD-containing peptides were used as controls. In the case of acridine orange, following stimulation the media was poured off and the cells rinsed in phosphate buffered saline (PBS). Acridine orange solution (100 μg/ml in PBS) was added to each dish, and the cells viewed immediately under a fluorescence microscope. Annexin V staining was carried out according to the manufacturer's protocol (Oncogene).

Statistical analysis. The mean, standard deviation, and standard error of the mean were calculated for each time point. Student's t-test was used to evaluate whether there was statistical significance between each of the points calculated.

RESULTS

Altered levels of aggrecan and MMP-3 in normal, but not OA, chondrocytes following mechanical stimulation at 0.33 Hz. To investigate whether mechanical stimulation in our in vitro system influences messenger

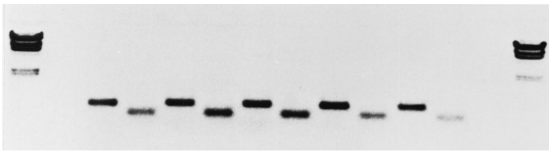

Figure 1. An agarose gel of GAPDH and aggrecan cDNA levels in normal chondrocytes following semiquantitative reverse transcription–polymerase chain reaction. Lane M, markers; lanes a, c, e, g, and i, GAPDH; lanes b, d, f, h, and j, aggrecan; lanes a and b, control (0 hours); lanes c and d, 1 hour after pressure-induced strain (PIS); lanes e and f, 3 hours post-PIS; lanes g and h, 6 hours post-PIS; lanes i and j, 24 hours post-PIS.

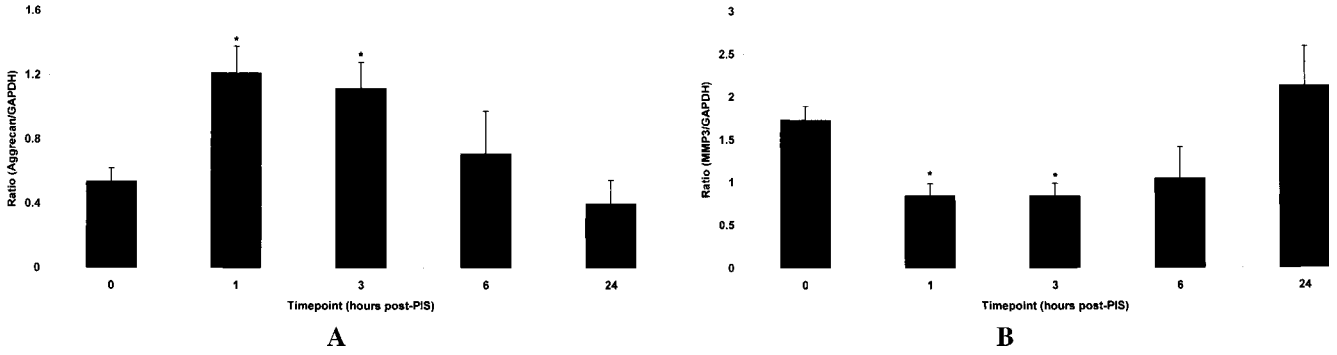

Figure 2. Effect of 0.33-Hz cyclic pressure-induced strain (PIS) for 20 minutes on the production of **A,** aggrecan mRNA (n = 15) and **B,** matrix metalloproteinase 3 (MMP3) mRNA (n = 14) over a 24-hour period in normal human articular chondrocytes, as determined by semiquantitative reverse transcription–polymerase chain reaction, with GAPDH as the control (∗ = $P < 0.001$). Values are the mean and SEM.

RNA (mRNA) levels, chondrocytes from normal and OA cartilage were stimulated at 0.33 Hz for 20 minutes, and total RNA was extracted immediately (time point 0) or at 1, 3, 6, or 24 hours following stimulation, and used for semiquantitative RT-PCR (Figure 1).

Significant changes in aggrecan and MMP-3 mRNA levels were identified in normal articular chondrocytes following mechanical stimulation at 0.33 Hz (Figure 2). Within 1 hour following stimulation at 0.33 Hz, relative levels of aggrecan mRNA had increased by 124%, with a return to baseline levels by 24 hours (Figure 2A). The relative levels of MMP-3 mRNA decreased over a similar time course, having decreased to 51% of the levels in resting cells 1 hour after stimulation (Figure 2B). In contrast, chondrocytes from OA cartilage showed no significant change in the relative levels of aggrecan or MMP-3 mRNA under the same conditions (Figure 3).

There was no significant change in levels of mRNA for tenascin, MMP-1, or TIMP-1 in either normal or OA chondrocytes following the mechanical stimulation (Table 2).

Effect of RGD-containing peptides on the molecular response of normal chondrocytes to stimulation at 0.33 Hz. To investigate the role of integrins in the regulation of aggrecan and MMP-3 following mechanical stimulation at 0.33 Hz, chondrocytes from normal cartilage of 3 donors were incubated with the synthetic hexapeptide GRGDSP or the control peptide GRADSP for 30 minutes prior to stimulation. The presence of GRGDSP or GRADSP did not significantly affect the basal ratios of aggrecan or MMP-3 to GAPDH mRNA.

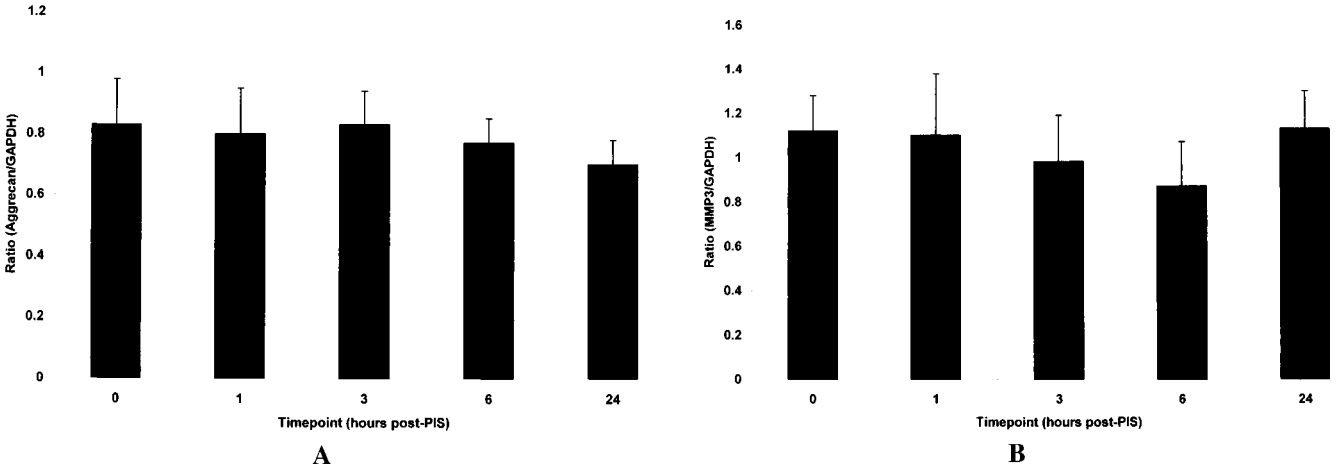

Figure 3. Effect of 0.33-Hz cyclic pressure-induced strain (PIS) for 20 minutes on the production of **A,** aggrecan mRNA (n = 6) and **B,** matrix metalloproteinase 3 (MMP3) mRNA (n = 6) over a 24-hour period in osteoarthritic human articular chondrocytes, as determined by semiquantitative reverse transcription–polymerase chain reaction, with GAPDH as the control. Values are the mean and SEM.

Table 2. Effect of 0.33 Hz of cyclic PIS for 20 minutes on the production of tenascin, MMP-1, and TIMP-1 mRNA over a 24-hour period in normal and OA human articular chondrocytes*

	mRNA ratio to GAPDH (hours post-PIS)				
	0	1	3	6	24
Normal					
Tenascin	1.28 ± 0.23	1.21 ± 0.20	0.95 ± 0.10	1.10 ± 0.20	1.12 ± 0.27
MMP-1	1.16 ± 0.24	0.78 ± 0.12	1.22 ± 0.41	1.04 ± 0.28	1.47 ± 0.22
TIMP-1	1.08 ± 0.24	1.12 ± 0.31	1.27 ± 0.27	1.07 ± 0.27	0.96 ± 0.25
OA					
Tenascin	1.70 ± 0.39	1.67 ± 0.30	1.69 ± 0.16	1.16 ± 0.19	1.48 ± 0.28
MMP-1	1.10 ± 0.23	0.85 ± 0.20	0.84 ± 0.13	0.66 ± 0.17	1.07 ± 0.40
TIMP-1	1.68 ± 0.23	1.48 ± 0.16	1.48 ± 0.09	1.41 ± 0.13	1.58 ± 0.15

* Values were determined by semiquantitative reverse transcription–polymerase chain reaction, with GAPDH as the control (n = 3 normal samples; n = 6 osteoarthritis [OA] samples). PIS = pressure-induced strain; MMP-1 = matrix metalloproteinase 1; TIMP-1 = tissue inhibitor of metalloproteinases 1.

Following incubation with GRGDSP and 20 minutes of stimulation at 0.33 Hz, no significant change in the levels of either aggrecan or MMP-3 mRNA was seen (Table 3). In the presence of the control peptide GRADSP, which has no effect on integrin signaling, an increase of aggrecan mRNA by 108% and a decrease of MMP-3 mRNA by 44% were seen (Table 3).

The presence of RGD-containing peptides had no effect on the apoptosis count or cell viability, as determined by acridine orange or annexin V staining (data not shown).

Involvement of IL-4 in the regulation of aggrecan and MMP-3 mRNA in mechanically stimulated chondrocytes. IL-4 activity is necessary for the electrophysiologic response of normal human chondrocytes to 0.33 Hz of stimulation. To investigate the possible role of IL-4 in the regulation of aggrecan and MMP-3 mRNA, chondrocytes isolated from the normal cartilage of 3 donors were incubated with neutralizing anti–IL-4 antibodies for 10 minutes before being stimulated at 0.33 Hz for 20 minutes. The basal levels of aggrecan and MMP-3 were unaffected by the presence of the antibodies. Following mechanical stimulation in the presence of IL-4 antibodies, chondrocytes showed no significant change in the mRNA ratios for aggrecan or MMP-3 at 1 or 3 hours poststimulation. In control, untreated chondrocytes, mechanical stimulation increased levels of aggrecan mRNA by 70% and decreased MMP-3 mRNA by 63% (Table 4).

Prevention of 0.33-Hz mechanical stimulation–induced changes in aggrecan and MMP-3 mRNA by blockade of SACs, but not SK channels. Gadolinium, a blocker of SACs, and apamin, a blocker of SK channels, were incubated with chondrocytes for 10 minutes prior

Table 3. Effect of 0.33 Hz of cyclic PIS for 20 minutes in the presence of GRADSP or GRGDSP oligopeptides on the production of aggrecan and MMP-3 mRNA over a 3-hour period in normal human articular chondrocytes*

	mRNA ratio to GAPDH (hours post-PIS)			% change at 1 hour
	0	1	3	
Aggrecan				
GRADSP	0.49 ± 0.18	1.02 ± 0.25†	0.96 ± 0.28†	108
GRGDSP	0.51 ± 0.11	0.54 ± 0.15	0.53 ± 0.12	5.9
MMP-3				
GRADSP	1.79 ± 0.23	1.00 ± 0.29†	1.00 ± 0.29†	−44
GRGDSP	1.78 ± 0.24	1.81 ± 0.21	1.66 ± 0.14	1.7

* Values were determined by semiquantitative reverse transcription–polymerase chain reaction, with GAPDH as the control (n = 3 samples). PIS = pressure-induced strain; MMP-3 = matrix metalloproteinase 3.
† $P < 0.02$ versus time 0.

Table 4. Effect of 0.33 Hz of cyclic PIS for 20 minutes in the presence of neutralizing IL-4 antibody on the production of aggrecan and MMP-3 mRNA over a 3-hour period in normal human articular chondrocytes*

	mRNA ratio to GAPDH (hours post-PIS)			% change at 1 hour
	0	1	3	
Aggrecan				
No antibody	0.84 ± 0.14	1.43 ± 0.24†	1.56 ± 0.23†	70
IL-4 antibody	0.80 ± 0.16	0.78 ± 0.17	0.77 ± 0.19	−2.5
MMP-3				
No antibody	1.67 ± 0.08	0.62 ± 0.11‡	0.57 ± 0.06‡	−63
IL-4 antibody	1.61 ± 0.10	1.59 ± 0.09	1.52 ± 0.12	−1.2

* Values were determined by semiquantitative reverse transcription–polymerase chain reaction, with GAPDH as the control (n = 3 samples). PIS = pressure-induced strain; IL-4 = interleukin-4; MMP-3 = matrix metalloproteinase 3.
† $P < 0.02$ versus time 0.
‡ $P < 0.0001$ versus time 0.

Table 5. Effect of 0.33 Hz of cyclic PIS for 20 minutes in the presence of gadolinium on the production of aggrecan and MMP-3 mRNA over a 3-hour period in normal human articular chondrocytes*

	mRNA ratio to GAPDH (hours post-PIS)			% change
	0	1	3	at 1 hour
Aggrecan				
Control	0.37 ± 0.06	0.66 ± 0.09†	0.58 ± 0.06†	78
Gadolinium	0.40 ± 0.07	0.40 ± 0.07	0.38 ± 0.06	0
MMP-3				
Control	1.73 ± 0.03	0.70 ± 0.18†	0.70 ± 0.17†	−60
Gadolinium	1.72 ± 0.02	1.66 ± 0.04	1.73 ± 0.06	−3.5

* Values were determined by semiquantitative reverse transcription–polymerase chain reaction, with GAPDH as the control (n = 3 samples). PIS = pressure-induced strain; MMP-3 = matrix metalloproteinase 3.
† $P < 0.02$ versus time 0.

to mechanical stimulation. Following stimulation at 0.33 Hz in the presence of gadolinium, there was no significant change in levels of aggrecan or MMP-3 mRNA at 1 or 3 hours poststimulation (Table 5). In control, untreated chondrocytes, mRNA ratios for aggrecan increased by 78% at 1 hour poststimulation, while the MMP-3 ratios decreased by 60% (Table 5).

In the presence of apamin, mechanical stimulation at 0.33 Hz resulted in an increase of aggrecan mRNA levels (48%) at 1 hour poststimulation, while MMP-3 levels were decreased by 64% at 1 hour (Table 6). Control chondrocytes stimulated in the absence of apamin showed a 43% increase in aggrecan mRNA at 1 hour, and a 67% decrease in MMP-3 mRNA ratios. The presence of gadolinium and apamin did not affect the basal levels of either aggrecan or MMP-3 mRNA relative to GAPDH.

Table 6. Effect of 0.33 Hz of cyclic PIS for 20 minutes in the presence of apamin on the production of aggrecan and MMP-3 mRNA over a 3-hour period in normal human articular chondrocytes*

	mRNA ratio to GAPDH (hours post-PIS)			% change
	0	1	3	at 1 hour
Aggrecan				
Control	0.44 ± 0.04	0.63 ± 0.05†	0.69 ± 0.09†	43
Apamin	0.44 ± 0.05	0.65 ± 0.12†	0.70 ± 0.09†	48
MMP-3				
Control	1.52 ± 0.30	0.50 ± 0.02†	0.69 ± 0.18†	−67
Apamin	1.49 ± 0.27	0.53 ± 0.16†	0.68 ± 0.15†	−64

* Values were determined by semiquantitative reverse transcription–polymerase chain reaction, with GAPDH as the control (n = 3 samples). PIS = pressure-induced strain; MMP-3 = matrix metalloproteinase 3.
† $P < 0.04$ versus time 0.

DISCUSSION

We have examined, by semiquantitative RT-PCR, the effect of 0.33 Hz of mechanical stimulation of human articular chondrocytes in vitro on levels of mRNA for aggrecan, tenascin, MMPs 1 and 3, and TIMP-1 over a 24-hour time course. The results have shown that in normal articular chondrocytes, aggrecan mRNA levels are increased and MMP-3 mRNA levels are decreased within 1 hour of stimulation, with a return to baseline levels within 24 hours. Under the same conditions, there are no significant changes in the levels of mRNA for tenascin, MMP-1, and TIMP-1. The transduction pathway involved in the production of these responses involves integrins, SACs, and IL-4. SK channels, which are required for the membrane hyperpolarization response of chondrocytes to mechanical stimulation at 0.33 Hz, are not required for the observed changes in aggrecan and MMP-3 mRNA.

The present study was undertaken with subconfluent monolayer cultures of human articular chondrocytes, where the cells are attached to a rigid substratum. The interactions of these cells with extracellular matrix molecules and organization of the actin cytoskeleton will not necessarily be identical to that of chondrocytes in vitro, which are suspended in a matrix gel. Indeed, the organization of the actin cytoskeleton of chondrocytes in long-term primary culture or following passage is known to change with increased development of stress fibers (24). This is associated with alteration of cell morphology, with chondrocytes becoming more fibroblastic and spindle shaped. In an attempt to minimize dedifferentiation, we used chondrocytes which had been in monolayer culture for up to 10 days. Phalloidin staining shows that these cells do not express well-developed stress fibers (11), and they have an actin cytoskeletal structure similar to that of chondrocytes in the cartilage matrix (25). Nevertheless, more subtle changes in the actin cytoskeleton and its association with other cytoplasmic and membrane proteins cannot be excluded.

Previous studies by other groups of investigators have shown that dynamic mechanical stimulation or hydrostatic pressurization of normal bovine cartilage explants leads to an increase in aggrecan mRNA levels and total proteoglycan synthesis (7,26), whereas static loading decreases synthesis of proteoglycans (27). Our results are consistent with those studies and indicate that dynamic mechanical stimulation of primary monolayer cultures of human articular chondrocytes results in an increase in the levels of mRNA for aggrecan, the major proteoglycan of cartilage. Such molecular changes, if

reflected in vivo, in association with the accompanying decrease in levels of MMP-3 mRNA, would support the idea that an appropriate dynamic mechanical stimulus leads to an anabolic response of chondrocytes in normal articular cartilage. This potentially chondroprotective response was absent in chondrocytes from OA cartilage. This may represent a general defect in the response of OA chondrocytes to mechanical stimulation. It remains possible, however, that OA chondrocytes have adapted to the altered mechanical environment in vivo and that this altered phenotype is maintained, at least short term, in vitro. In such a situation, OA chondrocytes may only show anabolic responses at other, as yet undefined, loads and frequencies of mechanical stimulation.

The mechanisms by which chondrocytes transduce a mechanical stimulus into biochemical signals and cellular responses are being defined. Evidence is increasing of a role for integrins in mechanotransduction in many tissues. In our model system, $\alpha5\beta1$ integrin has been shown to be involved in the electrophysiologic response of human articular chondrocytes to 0.33 Hz mechanical stimulation. The integrin $\alpha5\beta1$ is a receptor for fibronectin, to which it binds via an RGD motif. RGD-containing peptides compete with the RGD motifs of the extracellular matrix proteins for the ligand-binding sites of integrins, and so reduce the effective binding and signaling through integrins. Inhibition of the molecular responses with RGD-containing peptides supports roles for integrins in the metabolic effects of mechanical stimulation on chondrocyte function and would be consistent with our other studies, which have shown that mechanotransduction occurs via $\alpha5\beta1$ (11).

Integrin signaling is complex, involving a variety of intracellular signaling molecules, the actin cytoskeleton, and membrane ion channels. The electrophysiologic response of human articular chondrocytes to 0.33 Hz mechanical stimulation involves the activation of a signaling cascade involving $\alpha5\beta1$, SACs, tyrosine kinases, secretion of IL-4, and opening of SK channels. The involvement of integrins and SACs in the transduction of the mechanical stimulus that leads to changes in levels of aggrecan and MMP-3 mRNA is consistent with the proposal of Watson (28) that integrins and ion channels form a complex at the cell membrane to act as mechanotransducers. Blockade of the function of either component leads to inhibition of signal transduction. Sadoshima and Izumo (29) have also proposed the formation of SAC and integrin complexes as a mechanism for the response of cardiac myocytes to stretch, and the resultant release of angiotensin from the cells.

IL-4 is expressed in normal articular cartilage and has chondroprotective effects. IL-4 has been shown to inhibit IL-1–induced production of MMPs 1 and 3 by chondrocytes (30–32). Nemoto et al (32) suggested that this response was either due to an antagonistic effect of IL-4 on the IL-1 signaling pathway or the suppression of IL-1 synthesis. The present study indicates that as part of the mechanotransduction pathway activated by mechanical stimulation at 0.33 Hz, IL-4 can selectively regulate levels of mRNA for molecules involved in the maintenance of cartilage homeostasis.

The changes in levels of aggrecan and MMP-3 mRNA following mechanical stimulation at 0.33 Hz occurred in the presence of apamin, which blocks SK channel activity and membrane hyperpolarization. SK channels play important roles in excitable cells, and generation of long-lasting hyperpolarization, slow afterpolarization is essential for normal neurotransmission (33). The role of these ion channels in the regulation of chondrocyte function is, however, unclear.

It is also unclear why chondrocytes from OA articular cartilage do not show the same response as cells from normal cartilage. Chondrocytes from OA cartilage are recognized as showing a range of phenotypic differences in comparison with normal articular chondrocytes. These changes in phenotype, including altered expression of integrins, cytokines, and growth factors, as well as production of extracellular matrix, are believed to represent a reparative or remodeling response of chondrocytes following damage to cartilage (34). The range of integrin and other adhesion molecules expressed by chondrocytes is modified in OA, increasing the potential for altered cell–matrix interactions (34–36). There are also changes in the expression of a number of extracellular matrix molecules, such as fibronectin and tenascin (37,38), which function as potential integrin ligands, and these may also influence integrin-mediated signaling (39).

Furthermore, changes in the production of inflammatory mediators, including IL-1, tumor necrosis factor, and nitric oxide (NO), by OA chondrocytes may modify integrin-mediated signaling, either by modulating chondrocyte integrin expression (40) or by regulating intracellular signaling. Both NO and NO-producing cytokines, including IL-1α, exert profound effects on fibronectin–$\alpha5\beta1$ signaling in bovine chondrocytes (41). NO inhibits fibronectin-mediated proteoglycan production by inhibiting actin polymerization and translocation of focal adhesion kinase and RhoA to the site of $\alpha5\beta1$ ligation. Binding of fibronectin to chondrocytes is unaffected, consistent with the effects of NO being the result of modulation of integrin intracellular signaling rather

than by influencing cell–matrix interactions. Changes in autocrine/paracrine cytokine activity may influence the intracellular signaling processes that occur via $\alpha 5\beta 1$ integrin following mechanical stimulation, directly or indirectly through the production of NO.

Regulated production of matrix molecules and matrix degradative enzymes and their activity are important in the control of normal articular cartilage structure and function. Altered production of these molecules, with a bias toward increased tissue breakdown over formation, is seen in OA. Integrin-associated signaling pathways, activation of SACs, and autocrine/paracrine activity of IL-4 are involved in the anabolic response of human articular chondrocytes to dynamic mechanical stimulation in an in vitro model. This response is absent in chondrocytes from OA cartilage. Disordered mechanotransduction and inappropriate cellular responses may contribute to cartilage breakdown in OA.

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