

# Cyclic Strain Stimulates Proliferative Capacity, $\alpha_2$ Integrin by Human Articular Chondrocytes from Osteoarthritic Knee Joints

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**Abstract:** Articular cartilage, the load-bearing tissue in diarthrodial joints, is continually subjected to mechanical stimulation. Cartilage tissue consists of an extracellular matrix (ECM) and is sparsely populated by chondrocytes. Although chondrocytes comprise less than 10% of cartilage, these cells sense and respond to the mechanical stimuli. However, the effects of mechanical signals at the cellular level are still not fully defined. Moreover, the mechanisms by which chondrocytes respond to mechanical signals are not fully understood. The purpose of this study was to test the hypothesis that mechanical stimulation in the form of cyclic strain modulates proliferative capacity and integrin expression of chondrocytes from osteoarthritic knee joints. We also examined the effect of mechanical stimulation on integrin expression since it has been proposed to mediate the transduction of mechanical signals. Chondrocytes were isolated from human knees during total knee arthroplasty and were propagated in microcarrier spinner culture for 2 weeks. Cells were subsequently harvested and then plated onto flexible-bottom wells. They were then subjected to cyclic strain for 24 hr using a computer-controlled vacuum device, while replicate samples were maintained under static conditions. Proliferative capacity was determined by radiolabeled thymidine uptake. Phenotype and integrin expression were analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR). Cyclic strain increased proliferative capacity by about 30% of controls. RT-PCR analysis of mRNA expression showed that cyclic strain enhanced expression of collagen type II and aggrecan whereas Col I expression was unaltered. Parallel to enhancement of the chondrocytic phenotype, the expression of integrin  $\alpha_2$  subunit was also enhanced. In contrast, there was a slight but noticeable change in  $\alpha_5$  integrin expression and no change in the  $\beta_1$  integrin. These results demonstrate that mechanical stimulation by cyclic strain can directly alter proliferative capacity, phenotype expression, and integrin  $\alpha_2$  subunit expression by human chondrocytes. Our observations agree with previous findings for the behavior of chondrocytes subjected to a mechanical stress and also support the theory that integrins may participate in mediating the response of chondrocytes to their mechanical environment.

## Introduction

Articular cartilage, the load-bearing surface in diarthrodial joints, is subjected to repetitive mechanical stimuli. The

bulk of this tissue consists of extracellular matrix (ECM) made up primarily of a collagen type II framework filled with the hydrophilic proteoglycans. In articular cartilage, collagen type II and proteoglycans that form large molecular weight aggregates (aggrecans) enable the tissue to perform its biomechanical function. Comprising less than 5% of the cartilaginous tissue volume, chondrocytes sense and respond to their mechanical environment in the joint. The ability of chondrocytes to detect and react to mechanical stimuli is reflected by changes in their metabolic activity and in their synthesis–degradation of ECM macromolecules that constitute cartilage. Mechanical forces such as strain, compression, or shear have been shown to modulate the ability of chondrocytes to proliferate and produce extracellular matrix components (ECM) characteristic of hyaline cartilage. Proteoglycan synthesis can be stimulated via moderate exercise as shown by *in vivo* studies [28], while immobilization of a joint has the opposite effect [46,4]. Biosynthetic response of chondrocytes to mechanical stress has been examined with several *in vitro* studies (reviewed in [56]). Several experiments have shown that cartilage explants, when subjected to mechanical compression or hydrostatic pressure, or in which cultured chondrocytes were subjected to tensile loading, have shown that while static loading inhibits synthesis of matrix components, cyclic loading at certain frequencies can stimulate synthesis [13,22,26,28,32,36,45,47,49,55]. Several mechanical and physicochemical mechanisms have been hypothesized to be responsible for mediating this biosynthetic response [22,49,52,56]. Alterations in chondrocyte synthetic activity correlate with local tissue strains and cell deformation [7,55]. Earlier studies also demonstrated similar changes in cellular metabolism after exposure of chondrocytes seeded in agarose gels to static and dynamic compression [6,33]. Perturbation of the extracellular matrix milieu provided by the agarose gel appeared to have an impact on chondrocyte response to mechanical stimulation. These studies seem to indicate that cell–matrix interaction is critical in mediating the response of chondrocytes to mechanical stimuli.

Previous studies suggest that the integrins, a family of cell surface proteins, are involved in mediating communication between the chondrocytes and the extracellular ma-

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trix [31,37,51,58]. Integrins are heterodimeric, non-covalent complexes of  $\alpha$  and  $\beta$  subunits which act as transmembrane receptors for specific components of the extracellular matrix, thus linking the extracellular matrix with the cytoskeleton and, ultimately, the nucleus [12,39,53,57]. These molecules have been shown to mediate cell–matrix interactions in a variety of tissues, including cartilage, retina, vascular endothelium, and bone [25,53]. In adult human articular cartilage, the predominantly expressed integrins contain the  $\beta_1$  subunit [38,51,58]. In contrast, integrin  $\beta_3$  and  $\beta_5$  subunits are less detectable [37,58]. The  $\beta$  subunits dimerize with a variety of  $\alpha$  subunits, including  $\alpha_1$ ,  $\alpha_5$ ,  $\alpha_v$ , and  $\alpha_{10}$ , to form receptors that bind matrix proteins [9,37,44,51,58].

Previous investigators have explored the role of integrins in chondrocyte function. In fetal cartilage, cell–matrix interactions mediated by  $\beta_1$  integrins have been proposed to regulate chondrocyte differentiation [15,50]. In osteoarthritic cartilage,  $\beta_1$  expression may be associated with the severity of the lesion. The relative distribution of  $\alpha$  subunits is altered in osteoarthritic cartilage, while the level of  $\beta_1$  expression appears to be inversely correlated with the anatomic extent of the pathologic foci [30,31,38,44]. The observation that integrins bind to matrix proteins has also been thought mediate the production of degradative enzymes in both osteoarthritis and rheumatoid arthritis [2]. However, limited information has been available in defining the effect of mechanical strain on integrin expression by chondrocytes. Holmvall and colleagues reported variable effects of mechanical strain on  $\beta_1$  expression in adult bovine chondrocytes. They proposed that exposure to cyclic strain induces redistribution of integrins within the cell rather than de novo integrin synthesis.

In an earlier report, we described that human chondrocytes when subjected to cyclic strain did not show alteration in  $\beta_1$  expression [5] while expression of phenotype markers collagen type II and aggrecan were enhanced. These chondrocytes were retrieved from microcarrier suspension cultures, which facilitated retention of their chondrocytic phenotype [19]. Chondrocytes retrieved from monolayer cultures which favored dedifferentiation into fibroblastoid cells that produce collagen type I and low molecular weight proteoglycans also did not show increased  $\beta_1$  expression. However, these “fibroblastoid” chondrocytes showed decreased collagen type II and aggrecan expression with increased expression of collagen type I. Our observations appear to be in agreement with that of earlier reports by Holmvall [24] where cyclic strain did not elicit consistent alteration in  $\beta_1$  expression. In the present paper, we hypothesize that other integrin subunits such as the  $\alpha_2$  subunit may be more responsive to mechanical stimuli and may parallel the expression of cartilage phenotypic markers. The  $\alpha_2$  integrin subunit functions as receptor for collagen and is involved in collagen reorganization. The purpose of the present study was to investigate the effect of cyclic strain on the integrin, phenotypic expression and proliferative capacity of human articular chondrocytes harvested from microcarrier spinner cultures.

## Methods

### Preparation and propagation of articular chondrocytes

Non-fibrillated articular cartilage was obtained from the knees of three patients (ages 55–76) undergoing total knee arthroplasty for osteoarthritis. The protocol for the use of cartilage pieces in the study has been reviewed and approved by the Johns Hopkins Investigational Review Board. Chondrocytes were isolated by digestion of the tissue with collagenase A (Boehringer Mannheim, Mannheim, Germany) for 18–24 hr at 37°C. The cells were filtered through a wire mesh screen, washed twice with Hanks’ balanced salt solution (HBSS, Gibco, Grand Island, NY), and then directly on monolayer cultures in enriched Dulbecco’s minimal essential medium supplemented with 20% fetal calf serum until confluence. The cells were harvested by trypsinization, counted, and assayed for viability. Chondrocytes were subsequently seeded onto collagen microcarrier beads (Cellagen<sup>®</sup> 100–400  $\mu\text{m}$  derived from bovine corium; ICN, Cleveland, OH) at a density of  $4 \times 10^3$  chondrocytes/ $\text{cm}^2$  in a siliconized spinner flask. During the first 4 hr, the mixture was intermittently stirred for 2 min every 30 min at 25–30 rpm. The cell–microcarrier suspension was subsequently stirred at 45 rpm for another 4 hr. The speed was gradually increased to 60 rpm and then maintained at 60 rpm for 2 weeks. The final volume of the suspension culture was 3 mL per  $1.0 \times 10^6$  chondrocytes. To replenish the spinner cultures, the microcarriers were sedimented for 5 min and approximately 50% of the spent medium was replaced every 3 days. Spinner cultures were incubated at 37°C, 5%  $\text{CO}_2$  (refer to Fig. 1 for protocol).

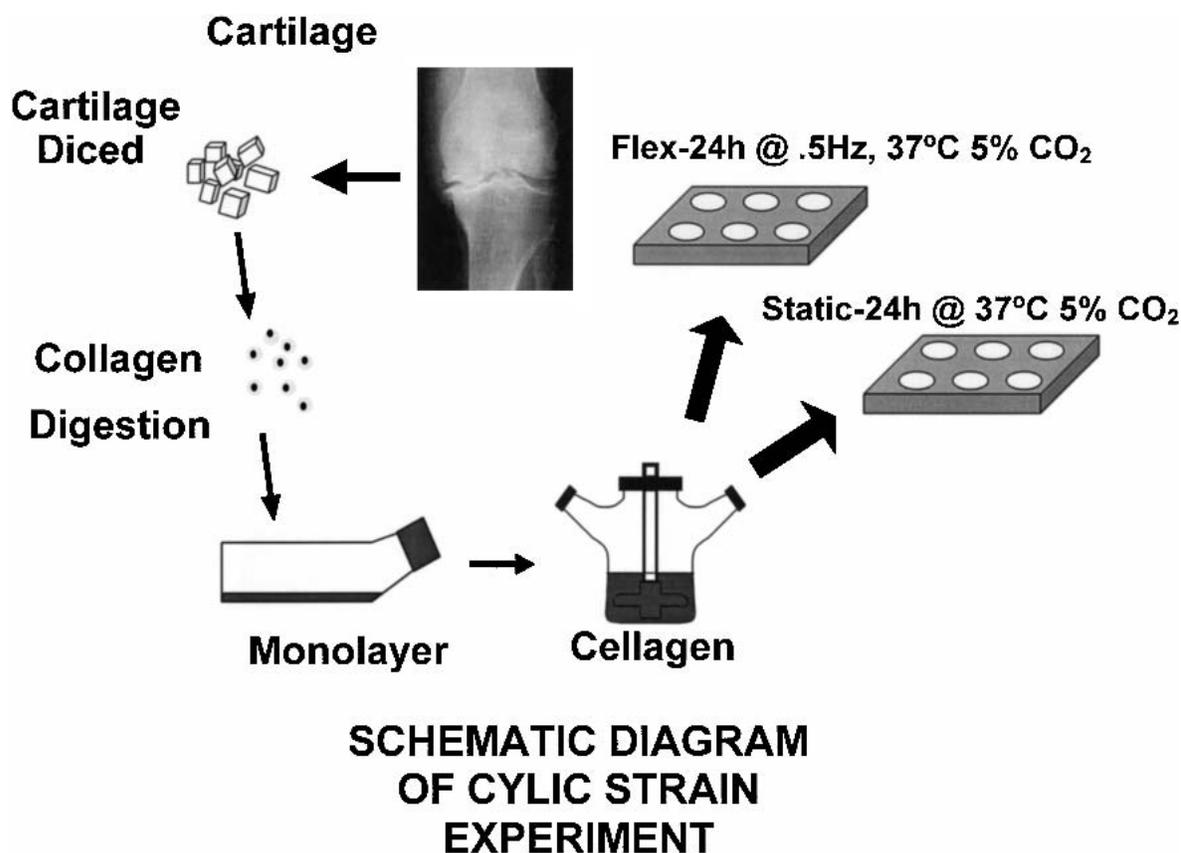
### Mechanical stimulation via cyclic strain

After 14 days in culture, chondrocytes were harvested from the microcarriers by collagenase digestion, or from monolayer culture by digestion with 0.25% trypsin, and plated onto type I collagen-coated flexible-bottom wells (Flex I plates, FlexCell International, McKeesport, PA) at a density of  $2 \times 10^5$  cells/1 mL medium per well. The wells were incubated at 37°C, 5%  $\text{CO}_2$ . After the cells had been allowed to adhere for 48 hr, the wells were subjected to cyclic strain at 0.5 Hz (1 sec of deformation alternating with 1 sec of relaxation) for 24 hr, using a computer-controlled vacuum strain apparatus (Flexercell Strain Unit, FlexCell International) with a vacuum pressure of  $-20$  kPa. Replicate samples were maintained under static conditions, with no applied cyclic strain (Fig. 1).

Approximately  $3.0 \cdot 10^6$  articular chondrocytes were frozen at  $-70^\circ\text{C}$  and used for semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis to verify the presence of integrins and specific chondrocytic phenotype markers. The remaining chondrocytes were used for phenotypic analysis using immunoperoxidase as well as thymidine uptake to assess proliferative capacity.

### Determination of cell proliferative capacity

After 18 hr, radiolabeled thymidine was added to flexed and static samples (1  $\mu\text{C}/\text{mL}$  of media). The samples were



**Fig. 1.** Schematic illustration of experimental protocol used in the present study. Chondrocytes were extracted from human knee cartilage and seeded into microcarrier spinner culture and monolayer culture. After 2 weeks in culture, cells were plated onto flexible-bottom silicone rubber wells and allowed to adhere for 48 hr. The wells were then mechanically flexed for 24 h; replicate samples were maintained under static conditions.

then flexed, and the control samples were left static for another 6 hr. The supernatant medium was collected, and the wells were washed twice with sodium chloride. Attached cells were retrieved by scraping of the wells. Assessment of the proliferative capacity was carried out by the determination of trichloroacetic (TCA) precipitable  $^3\text{H}$ -thymidine radioactivity in a Beckman multi-purpose scintillation counter (Model LS 6500). The results were presented as mean radioactivity expressed as counts per minute/ $10^5$  cells.

#### RNA extraction and analysis by RT-PCR

Total RNA was isolated by the TRIzol<sup>®</sup> (Life Technologies, Rockville, MD) reagent method. A total cDNA library was synthesized using the Advantage RT-PCR Kit (Clontech Laboratories, Palo Alto, CA) and Oligo (dT)<sub>18</sub> primer. The resulting RT product was expanded using the SuperTaq Plus (Ambion, Austin, TX) PCR kit and specific primers for collagen type II, type I, aggrecan,  $\beta_1$  integrin, and the housekeeping gene ribosomal RNA S14 subunit.

Two microliters of cDNA template was used in each PCR reaction. Primers for collagen type II were used for amplification (sense, 5' CAC CTT GGA CGC CAT GAA GGT 3'; antisense, 5' GTG AAC CTG CTA TTG CCC TCT 3') collagen type I (sense, 5' GAC GGG AGT TTC TCC TCG GGG TC 3'; antisense, 5' GAG TCT CCG GAT CAT CCA CGT C 3'), aggrecan (sense, 5' GGG TCA ACA GTG CCT

ATC AG 3'; antisense, 5' GGG TGT AGC GTG TAG AGA TG 3'),  $\beta_1$  integrin (sense, 5' GTT ACA CGG CTG CTG GTG TT 3'; antisense, 5' CTA CTG CTG ACT TAG GGA TAC 3'), and S14 (sense, 5' GGC AGA CCG AGA TGA ATC CTC A 3'; antisense, 5' CAG GTC CAG GGG GTC TTG GTC C 3'). PCR reactions for collagen type II, collagen type I, aggrecan, and S14 were conducted in a Perkin-Elmer thermal cycler. After initial treatment (75°C, 5 min; and 94°C, 1 min), 30 of the following cycles were performed: denaturation (94°C, 5 min), annealing (65°C, 15 sec), and extension (68°C, 3 min).  $\beta_1$  integrin was processed with an initial treatment (95°C, 3 min) followed by 35 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min), extension (72°C, 1 min), and an end step at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis, and densitometry was performed using the UN-SCAN-IT gel automated digitizing system (Silk Scientific Corporation).

#### Phenotypic analysis using immunoperoxidase technique

After the chondrocytes were incubated in Flex plates under static or dynamic condition, plates were washed free of media, fixed with 2% paraformaldehyde, and finally air-dried. To visualize the morphology of the chondrocytes seeded on the flexible membrane, pie pieces were cut and chondrocytes on the membrane were next immuno-

stained with monospecific antibodies for collagen types I and II (Fisher Scientific, Pittsburgh, PA) as well as chondroitin sulfate (ICN Biomedicals). Staining was visualized using the immunoperoxidase technique with diaminobenzidine as substrate, which produced a brownish color (Vector Laboratories Immunoassay Kit Brochure, Burlingame, CA). Cell preparations were counterstained with 0.5% toluidine blue. Human osteoblasts at passage 1 served as the positive immunostaining control for collagen type I. Human chondrocytes at passage 1 served as the positive immunostaining control for collagen type II. Human lymphocytes comprised the negative immunostaining controls for collagen types I and II. The specificity of the immunoperoxidase staining was verified by omitting the primary antibody. No staining occurred when the primary antibody was omitted.

#### Statistical analysis

Paired *t*-test was used to determine the significance ( $P < 0.05$ ) between samples using STATA software (SAS Institute, Cary, NC).

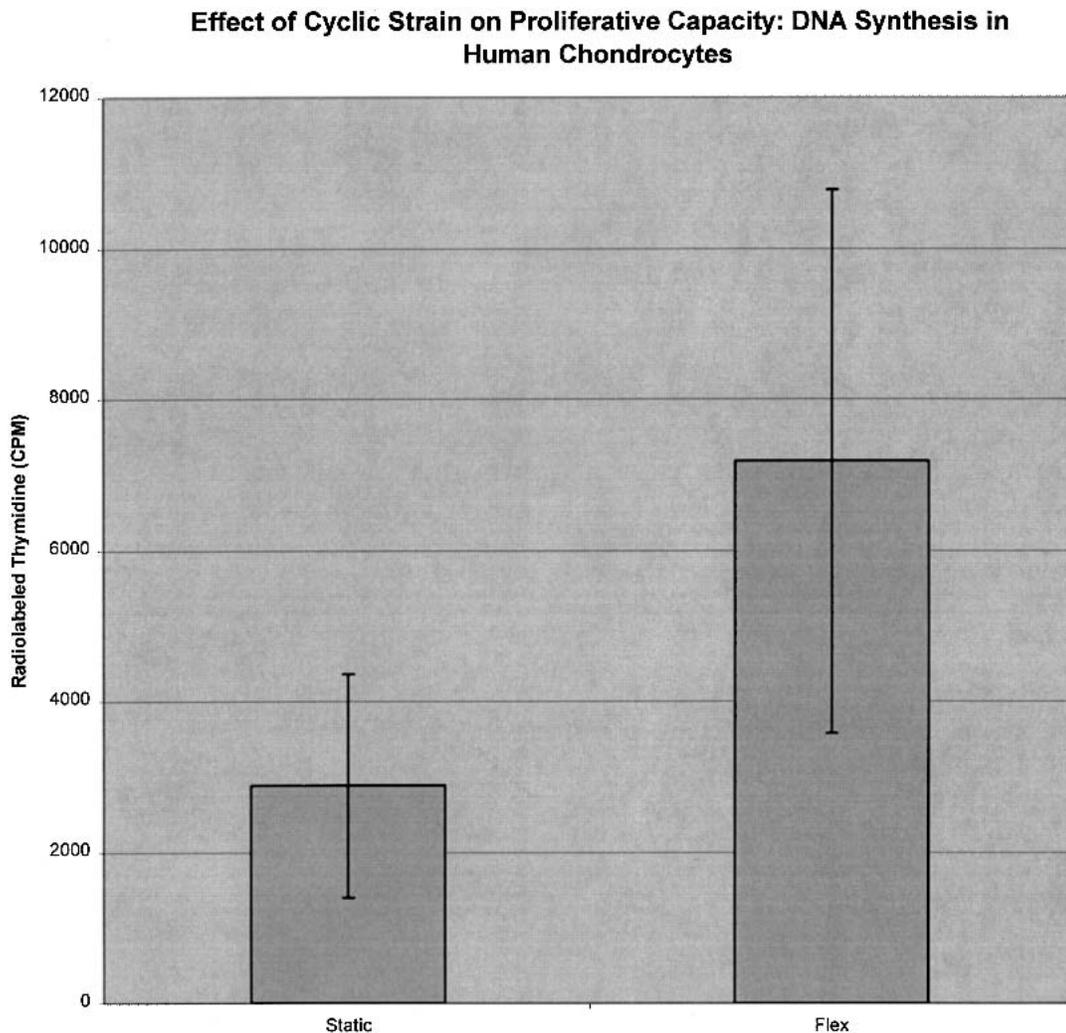
## Results

#### Effect of cyclic strain on proliferative capacity

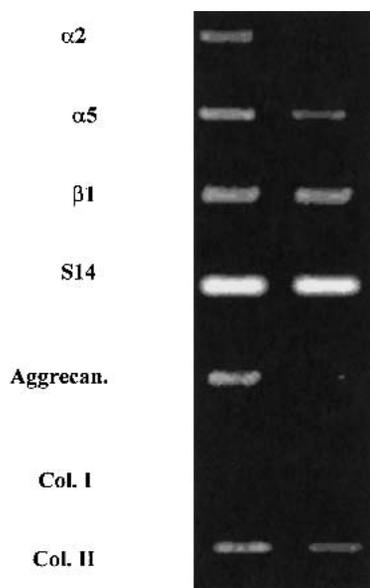
Figure 2 illustrates the average of proliferative capacities of the three chondrocyte lines studied. The mean CPM for the static cases was approximately 3,000 with a standard deviation of about 1,500. The flexed samples had a mean CPM of around 7,000 CPM with a standard deviation of 3,600. Our results indicate a trend toward an increase in proliferative capacity; however, the statistical analysis shows that there was no significant increase in proliferative capacity between the flexed and static samples ( $P > 0.05$ ).

#### Effect of cyclic strain on phenotype expression

Agarose gel electrophoresis following RT-PCR expansion revealed distinct bands corresponding to mRNA message for collagen types I and II, aggrecan,  $\alpha_2$ ,  $\alpha_5$ , and  $\beta_1$  subunits, and the "housekeeping" gene S14 (Fig. 3). The S14 bands appeared equal in intensity for both static and mechanically flexed cells, confirming that equal volumes of DNA had been loaded under each condition. Flexed samples



**Fig. 2.** Histograms of radiolabeled thymidine uptake was determined from three different patients. In each case, cells were seeded onto and spinner cultures. Following 24 hr of mechanical flexing, cells were labeled with  $^3\text{H}$ -thymidine and TCA precipitable counts were determined by enumeration with a Beckman scintillation counter.



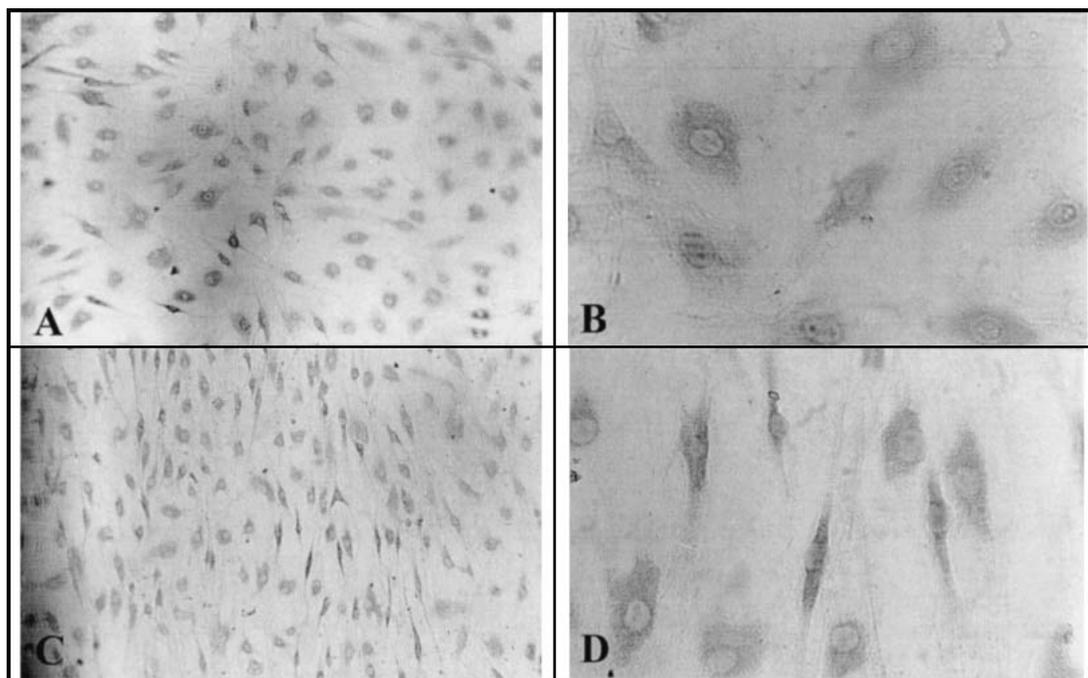
**Fig. 3.** Photograph (under ultraviolet light) of ethidium bromide-containing agarose gel following electrophoresis of RT-PCR products. Cells were propagated in microcarrier spinner cultures. Total RNA was extracted and RT-PCR performed using primers specific for type I collagen (Col I), type II collagen (Col 2), aggrecan (Agg), the “housekeeping” gene S14, and  $\beta_{\Phi 1}$  integrin subunit. Each pair of lanes represents the results from cells subjected to mechanical flexing (F) and maintained under static conditions (S).

showed an up-regulation of collagen type II as well as the  $\alpha_2$  subunit compared to the static samples as indicated by the more intense bands for the flexed samples. The  $\alpha_5$  subunit as well as aggrecan showed a slight yet noticeable

increase between the flexed and static samples as well. Both sample types showed low intensity for collagen type I, as expected for chondrocytes. An intense band corresponding to  $\beta_1$  message was seen, with no apparent difference between flexed and static conditions.

### Morphological and immunostaining patterns

Chondrocytes obtained from microcarrier suspension culture adhered readily onto the flexible surface of membranes. Upon incubation overnight under static conditions, chondrocytes appeared randomly distributed on the surface of the membrane (Fig. 4A). Some chondrocytes appeared stellate and rounded, whereas other most appeared to have spread out as seen at higher magnification (Fig. 4B). Chondrocytes in static culture showed positive staining for collagen type II, as shown by brown color in the cytoplasm, while the nuclei are counterstained with toluidine blue (Fig. 4A), which is more easily appreciated at higher magnification (Fig. 4B). Cyclic strain induced alignment of chondrocytes perpendicular to the strain vector (Fig. 4C). Cells appeared to be elongated and to have localized at the area of maximum strain, i.e., toward the edge of the plate. Flexed cells produced a darker shade of brown, indicating that there was an increase in protein expression for collagen type II (Fig. 4C,D). The immunostaining pattern for collagen type II was disseminated throughout the cytoplasm. There was no increase in the immunostaining pattern for collagen type I in chondrocytes kept under static conditions compared to flexed cells. There was also no noticeable difference in the expression of chondroitin sulfate between flexed and static samples.



**Fig. 4.** Representative micrographs of immunostained chondrocytes subjected to static or to cyclic strain using the Flexercell apparatus. Chondrocytes were immunostained with monospecific antibody to collagen type II and counterstained with toluidine blue as described above in Methods. (A) Chondrocytes kept in static condition (original magnification 100 $\times$ ); (B) chondrocytes kept in static condition (original magnification 400 $\times$ ); (C) chondrocytes subjected to flex-cyclic strain (original magnification 100 $\times$ ); (D) chondrocytes subjected to flex-cyclic strain (original magnification 400 $\times$ ).

## Discussion

We have discovered that cyclic strain up-regulated expression of chondrocyte marker collagen type II that is in parallel with enhancement of  $\alpha_2$  integrin expression. This upregulation of collagen type II and  $\alpha_2$  integrin expression seems to be more consistent and profound than promotion of proliferative capacity. This observation suggests that the  $\alpha_2$  integrin subunit may play a role in the regulation of chondrocyte function and proliferation by mechanical forces. We obtained chondrocytes from elderly patients with osteoarthritis, as these are the only consistent source of such tissue. Care was also taken to retrieve "healthy appearing" tissue with no gross evidence of fibrillation. Nevertheless, it is possible that cartilage samples may contain chondrocytes already undergoing degenerative changes due to osteoarthritis or aging. It has been noted that  $\beta_1$  expression correlates inversely with the degree of histological damage in osteoarthritic cartilage [31]. Studies also reported that integrin expression may be altered in osteoarthritic cartilage compared to normal tissue and may not reflect normal tissue from younger individuals.

It is well known that articular cartilage is subjected to a combination of mechanical compression and fluid shear during in vivo loading. Our in vitro model used chondrocytes that were plated onto flexible wells, which were subjected to cyclic deformation. In this model, there is a combination of cyclic strain due to chondrocytes adherence to the wells and fluid shear due to relative motion between the cells and the overlying medium. We used the vacuum strain unit used for the present study (Flexercell, FlexCell International) that has been extensively utilized to evaluate the effect of cyclic strain a variety of cell types [23,24,53]. The conditions used, such as amplitude and frequency of strain, were comparable to those reported and were selected within physiologic levels. Other systems reported to study the response of chondrocytes to cyclic strain noted that increased proteoglycan synthesis [15,55]. In spite of its limitations that do not allow evaluation of complex loading pattern experienced by intact cartilage in vivo, the cyclic strain model is a useful tool for analyze the ability of chondrocytes to sense and react to their mechanical environment. The strain profile applied by the FlexCell unit is known to be non-uniform over the surface of the flexible wells. The maximum strain occurs near the constrained outer edge while the lowest degree of strain occurs near the center [20]. Since our samples for analyses constituted a pool of cells subjected to a range of strain amplitudes, the observed results represent an average and may thus be less dramatic than if cells in areas of maximum strain were used. That a change in expression can be detected in response to a strain of only 1% demonstrates the high sensitivity of the chondrocyte response.

We have demonstrated that mechanical stimulation in the form of cyclic strain modulates  $\alpha_2$  integrin expression as well as collagen type II at the messenger level. However, the connection between this response and other intracellular processes, as well as other pathways by which chondrocytes

sense and react to mechanical stimuli still need to be defined. Insight into the mechanisms by which chondrocytes sense and react to mechanical stimuli may contribute to an understanding of the pathophysiology of disorders in the joint such as osteoarthritis.

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