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Changes in surface topologies of chondrocytes subjected to mechanical forces: an AFM analysis

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Abstract

The cartilage is composed of chondrocytes embedded in a matrix of collagen fibrils interspersed within a network of proteoglycans and is constantly exposed to biomechanical forces during normal joint movement. Characterization of the surface morphology, cytoskeletal structure, adherence and elastic properties of these mechanotransductive cells are crucial in understanding the effects of mechanical forces around a cell and how a cell responds to changes in its physical environment. In this work, we employed the atomic force microscope (AFM) to image cultured chondrocytes before and after subjecting them to mechanical forces in the presence or absence of interleukin-1 β to mimic inflammatory conditions. Nanoscale imaging and quantitative measurements from AFM data revealed that there are distinct changes in cell surface topology and cytoskeleton arrangement in the cells following treatment with mechanical forces, IL-1 β or both. Our findings for the first time demonstrate that cultured chondrocytes are amenable to high-resolution AFM imaging and dynamic tensile forces may help overcome the effect of inflammatory factors on chondrocyte response.

Keywords

chondrocytes; mechanical strain; AFM; cytoskeleton

INTRODUCTION

Arthritic diseases are chronic inflammatory diseases of the joints associated with significant cartilage erosion resulting in compromised joint function. Increased production of cytokines including interleukin-1 β (IL-1 β) by the synoviocytes and chondrocytes provide evidence for their involvement in the pathogenesis of arthritic diseases, Weissmann (2006). These cytokines upregulate transcription of proinflammatory genes to initiate cartilage destruction and amplify immune responses, Moreland (2004), Ji H (2002), Kay (2004), LeGrand (2001). Although, antiinflammatory drugs are the choice treatment, the therapeutic potential of joint mobilization in restoring joint function is increasingly being recognized Renner (2006), Sharma (2007), Strombeck (2007), Wolf (2007). It is understood that the signals associated with joint

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mobilization are essential for cartilage homeostasis, as well as its repair and smooth functioning of the joints, Ferretti et al. (2006).

To understand how mechanical stresses affect articular cartilage functions, a number of studies have been performed both in vivo as well as on cartilage explants and chondrocytes monolayer culture, Mobasheri et. al.(2002), Guilak et. al. (2006). Load induced deformation of cartilage matrix can cause alterations in hydrostatic pressure, ionic and osmotic composition, interstitial fluid and streaming potentials. Chondrocytes embedded in the cartilage matrix constantly experience these stimuli, Ateshian (2007). These mechanosensitive cells then respond by bringing about changes in the gene expression, protein synthesis, matrix composition and ultimately biomechanical competence of the tissue, Sharma et. al.(2007), Garcia et al. (1999), Goldmann et al. (2002). At high or traumatic magnitudes biomechanical signals trigger expression of proinflammatory genes in chondrocytes, and at low physiological magnitudes these signals are potent inhibitors of IL-1 β dependent proinflammatory gene transcription, Hsieh et al. (2005), Ingber (1997), Agarwal et al. (2004). Furthermore, at low magnitudes these signals induce proteoglycan and collagen type II synthesis essential for cartilage homeostasis and repair, Agarwal et al. (2001), Gassner et al. (2000), Xu et al. (2000). During both processes, chondrocytes react with surrounding matrix, which results in alterations in their morphology, surface topography, gene expression and cytoskeletal organization. Cell surface proteins, receptors, and ion channels serve as the key interface between the cytoskeleton and the interaction of chondrocytes with the peri or extra-cellular matrix. Their density, distribution, and clustering crucially affect the cells response to external or internal stimuli. While biochemical assays are useful in revealing the expression level of these proteins Guilak et. al. (2006), Madhavan et. al. (2007), Salter D. (2004), ultrastructural studies can provide unique information regarding their localization with respect to cell-membrane or cytoskeletal features. However, as yet neither the changes in superficial topography of chondrocytes, nor the arrangement of cytoskeleton in response to biomechanical forces and/or inflammatory factors has yet been investigated at high magnifications.

Atomic force microscopy (AFM) can serve as a valuable tool to elucidate the ultrastructural changes in cell-surface topography at the nanoscale level to appreciate changes in the distribution of cell-surface molecules that accompany biochemical or biomechanical signal-induced surface-changes. These changes may potentially provide novel insights into the control of cell shape and their interactions with pericellular matrix during activation of chemical and mechanical signaling pathways. Secondly, AFM can serve as a valuable tool to provide insights into remodeling of the actin cytoskeleton and resulting changes in the cell such as in its mechanical properties, membrane tension and mechanosensitivity.

In this work, to investigate the effects of inflammation and joint mobilization at the ultrastructural level, we have examined the changes in surface topography of chondrocytes in response to mechanical strain and proinflammatory environment. Using fluorescence light microscopy and AFM we investigated the changes in topological details of cultured chondrocytes following application of dynamic tensile forces and/or interleukin-1 (IL-1 β). Our aims were to (1) to elucidate qualitative and qualitative changes in the surface topology of chondrocytes under above conditions, (2) to elucidate accompanying cytoskeletal remodeling, and (3) to investigate whether DTF can induce topological and/or cytoskeletal changes even in the presence of IL-1 β . These investigations would provide novel insights into the physical and morphological properties of chondrocytes and how they may interact with the pericellular matrix in healthy and diseased tissue.

EXPERIMENTAL METHODS

Cell culture

Chondrocytes isolated from the superficial layers of the articular cartilage from the knees of 10–12 weeks old Sprague-Dawley rats were grown in HAM's/F12 (Cellgro®, VA) containing 10% FBS (Hyclone®, UT) and used between 2nd and 3rd passage. Subsequently, cells were grown on collagen I coated flexible bottom plate (Flexercell International, NC) to 80% confluence and subjected to dynamic tensile forces (DTF) of 3% magnitude at 0.25 Hz for 24 hours, which is in the physiological range, Xu et. al. (2000). In addition, to examine the changes that may occur in chondrocytes due to mechanical and/or proinflammatory signals, these cells were also treated with interleukin-1 (IL-1 β) (1 ng/ml) for 24 hrs or concurrently with IL-1 β + DTF for 24 hrs. Unstretched cells were used as controls.

Fluorescence Microscopy

To analyze chondrocyte cytoskeleton arrangement, pie shaped slices of the flexible membranes containing cultured chondrocytes were cut, fixed with formaldehyde, and the chondrocytes were permeabilized in PBS containing 0.5% Triton X-100 for 30 min. The cells were stained with FITC-conjugated phalloidin (1:200) (Invitrogen Corporation, CA) for 20 min to visualize filaments of β -actin. Cell nuclei were stained by incubating the samples with 1 μ g/ml DAPI in PBS for 1 minute, then washed 2 times with PBS to remove the triton-X 100. The images were acquired using an Axioplan 2 fluorescence microscope (Carl Zeiss, NY).

Atomic Force Microscopy (AFM)

AFM imaging was performed on glutaraldehyde fixed sections of flexible membranes using a Multimode AFM instrument (Veeco, Santa Barbara, CA) equipped with a Nanoscope IIIa controller. Sections of membrane were glued with epoxy to metal specimen discs and imaged under PBS in Tapping Mode. NP-S silicon nitride probes (Veeco, Santa Barbara, CA) with a nominal tip radius of 10 nm and spring constant of 0.32 N/m were used at the resonant frequency of ~ 9–10 KHz in fluid. Images were acquired at 512 $\times$ 512 lines per scan direction using a scan rate of 0.8 to 1.5 Hz. Images were recorded for the height and amplitude channels. The height images were flattened using the Nanoscope software. Vertical height of the cell surface features was estimated using the “Section analysis” tool of the Nanoscope software on the AFM height images. At least 3 independent specimens in each experimental condition were observed under AFM and data was collected from at least n=25 different cells for each sample. At least n=5 different regions from AFM images, encompassing a surface area of 100 to 400 μ m² on the cell-surface were analyzed for each sample to estimate the density and the size of granular structures. The statistical analyses done for particle size and particle density was weighted using one-way ANOVA, with samples considered correlated and significance at the p<0.01 level.

Analysis of β -actin expression

To examine the expression of β -actin, total RNA was extracted from the cells using the RNeasy Kit (Qiagen Inc, Valencia, CA) immediately following termination of DTF. A total of 1 μ g of RNA was reverse transcribed using the Superscript III Reverse Transcriptase Kit (Invitrogen). Expression of β -actin and Glyceraldehyde phosphate dehydrogenase (GAPDH, used as an internal control) was detected by end-point RT-PCR as described earlier (Deschner et al., 2006). Custom-designed (Primer Express, Applied Biosystems, CA), gene-specific primers were used to amplify the cDNA in Platinum PCR SuperMix (Invitrogen). The primers used were for β -actin (429 bp): forward: TGCTATGTTGCCCTAGACTTCG and reverse: CTTGCTGATCCACATCTGCTG; and for GAPDH (323 bp) forward 3'AGACAGCCGCATCTTCTTGT5' and reverse 3'TACTCAGCACCAGCATCACC5'. The

synthesis of β -actin by chondrocytes exposed to various treatments was assessed by Western Blot analysis using rabbit anti β -actin antibodies (1:5000 dilution; Abcam, CA) and horse radish peroxidase labeled goat anti rabbit IgG (1: 2000 dilution; Santa Cruz Biotechnology, CA) (Deschner et al 2006).

RESULTS

Dynamic tensile forces (DTF) changes the surface topography of chondrocytes

AFM imaging of cell surfaces from three independent experiments showed that native chondrocytes had a relatively smooth surface with very fine granular structures present on the cell-surface (figure 1a). In cells treated with IL1 β , the cell-surface topography was similar with a few larger globular structures also present on the cell surfaces (figure 1 b). When both these samples were subjected to DTF for 24 hrs, striking changes in cell-surface topography were observed (figure 1 c, d). The stretched cells showed a marked increase in the number of granular structures on their surface, as compared to their unstretched counterparts. As shown in figure 2, while the unstretched native and IL1 β treated cells had a cell-surface particle density of 0.4 particles/ μm^2 , the stretched cells had an average of 0.75 particles/ μm^2 on their surfaces showing a two-fold increase in particle density.

A quantitative analysis of the size distribution of these granular structures was performed by measuring the vertical height of clearly identifiable granules above the cell surface for n=50 granules (figure 3). As seen in figure 3 and figure 4, there were significant changes in the size distribution of cell-surface granules for stretched or IL1 β treated cells as compared to native cells. The average granular size for native cells was 74 ± 21 nm while that for DTF or IL1 β treated cells was 147 ± 50 and 104 ± 27 nm respectively. IL1 β treated cells when subjected to DTF, did not differ significantly in their size distribution but had a slightly higher average size of the granular structures (125 ± 30 nm).

Dynamic tensile forces (DTF) modulates cytoskeletal remodeling in chondrocytes

Both fluorescence microscopy and AFM images of cells showed that the cytoskeletal arrangement in chondrocytes was modified in response DTF treatment both in the absence or presence of IL1 β . Staining for the cytoskeletal β -actin showed that the density and distribution of actin was similar for IL1 β treated or native cells (figure 5a and b). However, treatment with DTF for both these samples, led to enhanced actin polymerization with the actin fibers localized in the peripheral region of the cells (Fig 5 c–d). AFM images confirmed these observations and further revealed that native or IL1 β treated cells when subjected to DTF have a well-formed cytoskeleton which extends to the peripheral regions (figure 6a–d). No changes in mRNA or protein expression levels of β -actin were observed in these experiments (figure 6e).

DISCUSSION

Our investigations reveal that mechanical stress in the form of dynamic tensile forces (DTF) influences the cell-surface topography of native or IL1 β -treated chondrocytes isolated from the superficial layer of cartilage. In particular, our AFM investigations have provided two novel insights: (a) treatment with DTF induces increased density and sizes of granular structures on the cell surface for both native and IL1 β treated cells and (b) distribution of β -actin filaments is remodeled in response to DTF for both native and IL1 β treated chondrocytes.

Several biochemical changes have been shown by us and others to result after subjecting the chondrocytes to mechanical strain or pro-inflammatory factors, Long (2007), Agarwal et al. (2006), Xu et al. (2000). Prolonged exposure (> 24 hrs) of IL-1 β to chondrocytes as performed in our studies has been reported to enhance the expression of a number of cell-surface receptors

like the urokinase-type plasminogen activator receptor (uPAR) and membrane bound matrix metalloproteases (MMPs), Schwab et. al. (2004), Lyons-Giordano et. al. (1991), Saas et. al. (2006), complement regulatory proteins (CRPs) CD46, CD55 and CD59 mRNA, Hyc et. al. (2003), chemokine receptor CCR-5, Yuan et. al. (2001), CD44 and the proteinase-activated receptors (PAR-2), Xiang et. al. (2006). Thus the increase in the size of the granules observed on our IL1 β treated chondrocyte surface as compared to native cells could be explained by the increased expression of the above cell surface receptors and/or their complexes.

Mechanotransduction pathways on the other hand utilize cell-surface receptors like $\alpha_5\beta_1$ -integrin, discoidin domain receptor 2 (DDR2), Shyu et. al. (2005), Lam et al (2007) or stretch-activated ion channels (SAC), Lee et. al. (2000). Furthermore, mechanostimulation of chondrocytes also leads to reorganization of focal adhesion contacts, Takahasi et. al. (2003) or can stimulate matrix metabolism leading to increased aggrecan, glycosaminoglycans (GAGs) and collagen synthesis rate, Sharma et. al. (2007), Nugent et. al. (2006), Stoltz et. al. (2000), Guilak et. al. (2006). Thus, in our studies the increase in the number and size of granular structures on the cell surface by DTF may reflect the increased secretion of the pericellular matrix components, upregulation and/or clustering of cell-surface receptors or focal adhesion complexes. In our studies both DTF and IL1- β induced an increase in the average size of granular structures on the cell surface but only DTF could increase the density of granules present on the cell surface, suggesting that upregulation of cell-surface molecules by IL1 β and DTF may be regulated differently and DTF can result in biochemical changes even despite the presence of IL-1 β .

Further work involving specific labeling of cell-surface molecules coupled with AFM analysis would help ascertain the relative quantities and time course of expression of these surface complexes. AFM topographic imaging and surface roughness measurements have served as a valuable tool to quantify the distribution of specific membrane proteins on cell surfaces when labeled with antibodies, Kienberger et. al. (2006), such as, the cystic fibrosis transmembrane conductance regulator protein on erythrocyte membranes, Schillers et. al. (2007) and CD34, CD44 or CD29 antigens on the surfaces of adipose tissue derived mesenchymal stem cells (MSCs), Shu et. al. (2007). High-resolution AFM imaging coupled with AFM force spectroscopy can identify the exact location and distribution of cell-surface proteins such as Angiotensin II, Li G. et al (2005), Osteopontin, Ron et. al. (2007) and vascular endothelial growth factor (VEGF) receptor, Almqvist et. al. (2004). Our findings for the first time demonstrate that cultured chondrocytes are amenable to high-resolution AFM imaging and a quantitative analysis of the distribution of cell-surface molecules can provide new insights into the chondrocyte response to mechanical or inflammatory factors.

Chondrocytes sense and adapt to external mechanical stimuli through the actin cytoskeleton which also provides the cell its mechanical support and is linked to the focal adhesion complexes. For example, in response to changes in mechanical environment, actin together with talin, α -actinin, filamin, vinculin, and integrins undergoes reorganization and realignment at the cell surface, to allow communication with the extracellular matrix and thus to regulate cellular responses. Changes in the actin organization in chondrocytes have been reported after exposure to hypoosmotic stimulation or hydrostatic forces Guilak et. al. (2002), Chao et. al. (2006), Fioravanti et. al. (2003 & 2005) and Knight et al (2006). However, limited studies exist on the effect of mechanical strains at physiological levels on actin remodeling in chondrocytes. Campbell et al have demonstrated that, cyclic uniaxial compressive loading leads to net peripheral actin breakdown in articular chondrocytes with no significant change in expression of actin-binding proteins. In contrast, oscillatory tension causes an enhanced peripheral actin organization in cellular projections in chondrocytes, Vanderploeg et. al. (2004). Our results that DTF enhances peripheral actin remodeling support the hypothesis that tensile forces play an important role on the peripheral actin remodeling Vanderploeg et al (2004) and phenotypic

differentiation of chondrocytes, Takahashi et. al. (2003). In both earlier, Knight et al. (2006) and our present studies, changes in actin organization were not associated with changes in actin gene expression suggesting that the response is due to a remodeling of existing actin.

It has been reported earlier that the expression and/or organization of cytoskeletal proteins, is affected by IL-1, Guilak et. al. (2002), Pritchard et. al. (2006), Vinal et al (2002). Further, non-OA chondrocytes are known to exhibit a higher elastic modulus and viscosity as compared to OA cells, Trickey et. al. (2004). In our observations chondrocytes cultured on collagen-coated flexible membranes, failed to show significant remodeling of actin cytoskeleton upon 24 hrs exposure to IL-1 β as compared to native cells. One possible explanation could be the transient nature of IL-1 β effects as suggested by Pritchard et. al. (2006), wherein IL-1 β induced changes in actin can sensitize the cell to further IL-1 exposure through clustering and subsequent internalization of membrane receptors, Singh et. al. (1999). Nevertheless, our results demonstrate that DTF can induce enhanced peripheral actin remodeling even in the presence of proinflammatory stimulus, like IL-1 β , suggesting that mechanical forces may play a crucial role in restoring chondrocyte stiffness and mechanotransduction properties in OA.

As discussed above, mechanical forces and proinflammatory mediators are known to differ significantly in inducing biochemical, cytoskeletal, and topographical changes on chondrocytes. While our earlier studies have elucidated certain biochemical changes induced by DTF on IL-1 β treated cells, our current study revealed that prolonged exposure of chondrocytes to DTF in the physiological range may help overcome the catabolic effects of pro-inflammatory factors by restoring the chondrocyte mechanical properties and its capacity for cell-matrix interaction.

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Figure 1.

AFM amplitude images of native chondrocytes and those subjected to IL1 β treatment and/or stretch (DTF) for 24 hrs as indicated. The surface morphology of stretched cells is more granular as compared to unstretched cells. Remodeling of the cytoskeleton can also be observed for DTF treated cells as compared to their unstretched counterparts.

Figure 2.

The average number of granules per 100 μm^2 surface area of the cells (as in figure 1) is quantified. Samples with DTF have an increased granular density as compared to unstretched cells with $p < 0.01$.

Figure 3.

High magnification AFM height images showing relative sizes of granular structures on the surfaces of DTF treated cells. Inset in each image shows a quantitative vertical profile along a line across the AFM image, which enables measurement of the height of granular structures. Statistical distribution of the sizes of granular structures for unstretched cells (black) vs. DTF treated (grey) is shown in for (e) native and (f) IL1- β treated cells.

Figure 4.

Average particle size for cell-surface granules ascertained from AFM images for various cell samples as indicated. Samples with DTF have a higher average particle size than their unstretched counterparts with $p < 0.01$.

Figure 5. Fluorescence microscopy images showing arrangement of FITC-stained (green) β -actin filaments in (a) native, (b) IL-1 β treated, (c) DTF treated and (d) DTF and IL-1 β treated chondrocytes. Nuclei were stained with DAPI, shown in blue.

Figure 6.

Segments from AFM amplitude images showing cell cytoskeleton along the cell edges for (a) native, (b) DTF, (c) IL1 β and (d) IL1 β and DTF treated cells. Dominant and well-defined cytoskeletal fibers are observed at cell edges for all DTF treated cells, which are absent in their unstretched counterparts. (e) Expression of β -actin protein (top lane) and mRNA (middle lane) in control untreated chondrocytes, or chondrocytes exposed to IL-1 β (1 ng/ml), DTF (6% at 0.05 Hz), or IL-1 β and DTF. Cells were treated for 12 h for protein or 4 h for mRNA analysis. GAPDH (Bottom lane) was used as an internal control.