Effects of vimentin disruption on the mechanoresponses of articular chondrocyte

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ABSTRACT

Human articular cartilage is subjected to repetitive mechanical loading during life time. As the only cellular component of articular cartilage, chondrocytes play a key role in the mechanotransduction within this tissue. The mechanoresponses of chondrocytes are largely determined by the cytoskeleton. Vimentin intermediate filaments, one of the major cytoskeletal components, have been shown to regulate chondrocyte phenotype. However, the contribution of vimentin in chondrocyte mechanoresponses remains less studied. In this study, we seeded goat articular chondrocytes on a soft polyacrylamide gel, and disrupted the vimentin cytoskeleton using acrylamide. Then we applied a transient stretch or compression to the cells, and measured the changes of cellular stiffness and traction forces using Optical Magnetic Twisting Cytometry and Traction Force Microscopy, respectively. In addition, to study the effects of vimentin disruption on the intracellular force generation, we treated the cells with a variety of reagents that are known to increase or decrease cytoskeletal tension. We found that, after a compression, the contractile moment and cellular stiffness were not affected in untreated chondrocytes, but were decreased in vimentin-disrupted chondrocytes; after a stretch, vimentin-disrupted chondrocytes showed a lower level of fluidization-resolidification response compared to untreated cells. Moreover, vimentin-disrupted chondrocytes didn't show much difference to control cells in responding to reagents that target actin and ROCK pathway, but showed a weaker response to histamine and isoproterenol. These findings confirmed chondrocyte vimentin as a major contributor in withstanding compressive loading, and its minor role in regulating cytoskeletal tension.

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1. Introduction

The cytoskeleton of chondrocyte consists of actin microfilaments, tubulin microtubules, and vimentin intermediate filaments (VIFs), all of which play an important role in maintaining chondrocyte biological as well as mechanical function. Actin predominately provides the cell with mechanical integrity (shape, stiffness, etc), and to some extent controls chondrocyte phenotype. It has been shown that the increase of actin stress fibers inhibits chondrogenic differentiation [1–3], and the disruption of stress fibers restores chondrocyte phenotype [4–6]. Tubulin is mainly responsible for intracytoplasmic transport [7]. Vimentin also contributes to chondrocyte phenotype, as disruption of vimentin in vitro results in reduction of type II collagen and aggrecan synthesis [8], and altered levels of vimentin are observed in situ in both human [9] and rat [10] osteoarthritic chondrocytes. Moreover, vimentin is thought to be involved in the mechanical function of chondrocyte, which is supported by the observation of increased vimentin expression in chondrocytes of weight-bearing cartilage [11].

However, the role of VIFs in the mechanical properties of chondrocytes is still unclear. Trickey et al. reported that disruption of VIFs doesn’t affect the stiffness of chondrocytes in 2D culture [12]. In contrast, Haudenschild et al. found that disruption of vimentin substantially reduce the stiffness of chondrocytes in 3D culture [13]. These results suggest a different organization of vimentin cytoskeleton between 2D and 3D culture of chondrocytes. Moreover, the contribution of vimentin cytoskeleton to the mechanoresponses of chondrocytes is less studied. Ofek et al. found that the removal of VIFs causes chondrocytes to become incompressible to compressive strains [14]. Note that in their study chondrocytes were isolated from extracellular matrix (ECM), which
might affect the force transmission from ECM to cytoskeleton. To systematically investigate the effects of vimentin disruption on chondrocyte mechanoresponses, in this study we seeded goat articular chondrocytes on polyacrylamide (PA) substrate, applied a transient stretch or compression, and compared the changes of cellular stiffness and traction force between acrylamide-treated cells and untreated control. In addition, effects of vimentin disruption on the intracellular force generation were examined by the real-time measurement of cellular stiffness upon administration of contractile or relaxation reagents. Our results would provide a comprehensive understanding in mechanoresponses of chondrocytes grown on a soft matrix to mechanical and chemical stimulation.

2. Materials and methods

2.1. Cell culture

Primary Spanish goat articular chondrocytes were kindly provided by Dr. Myron Spector (Harvard University). Cells were plated onto soft PA gel substrates at the density of 4000 per substrate in Advanced Dulbecco’s Modified Eagle Medium (DMEM; Gibco 12491) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone Technologies, Logan, UT), and 1% (v/v) penicillin/streptomycin (Life Technologies). Culture medium was changed every 2 days. Cells were serum starved overnight before measurement. To disrupt VIFs, chondrocytes were treated with 4 mM acrylamide for 24 h, as previous studies have shown that 4 mM acrylamide substantially disrupt vimentin network without interrupting other cytoskeletal components or inducing cytotoxic effect [12,13]. The following reagents were used to change cytoskeletal cohesion of chondrocytes: 20 μM histamine (Sigma–Aldrich), 10 ng/ml Interleukin-1β (IL-1β; R&D Systems), 10 ng/ml transforming growth factor-β1 (TGF-β1; R&D systems), 10 μM isoproterenol (Sigma–Aldrich), 10 μM Y-27632 (Sigma–Aldrich), and 0.1 μM latrunculin-A (Sigma–Aldrich).

2.2. Fabrication of PA substrates

PA gel substrates were prepared according to our previous studies [15,16]. The glass bottom of 35 mm dishes (P25–G–020–C, MatTek Corporation, MA) was treated with bind silane overnight, washed and air dried. Then, 300 μl gel solution consisting of 5% (v/v) acrylamide (Bio-Rad, Hercules, CA), 0.1% (v/v) bis-acrylamide (Bio-Rad), 0.6% (v/v) fluorescent bead suspension (0.5 μm, pink, Invitrogen, Eugene, OR) and ultrapure water was mixed with 5% (v/v) ammonia persulphate (Bio-Rad) and 0.05% (v/v) TMED (Bio-Rad) successively. The mixture was then added to the center of glass bottom of each dish and covered by a 25 mm circular coverslip (VWR) to yield gel with a final thickness of ~700 μm. After gel polymerization, the coverslips were gently removed with a forceps, and gels were surface-activated using 200 μl of 1 mM sulphonylsuccinimidyl-6-[4-azido-2-nitrophenyl]-amino]hexanoate ( Sulpho-SANPAH; Pierce, Rockford, IL) under UV light for 10 min. These gel substrates were then coated with type I collagen (0.1 mg/mL in PBS; Advanced BioMatrix, San Diego, CA) and stored overnight at 4 °C. On the next day, the collagen solution was removed, and the gels were kept in PBS at 4 °C till use. We set the ratio of acrylamide to bis-acrylamide at 5%: 0.1% to generate gel substrates with Young’s modulus of 4 kPa, as 4 kPa PA substrates showed good results in maintaining chondrocyte phenotype in vitro [17].

2.3. Optical Magnetic Twisting Cytometry

The stiffness of chondrocytes was probed using Optical Magnetic Twisting Cytometry (OMTC). Detailed descriptions of this method have been given elsewhere [18,19]. RGD-coated ferrimagnetic beads (4.5 μm in diameter) were incubated with cells for 20 min to allow the beads bind to cell surface receptors that link to the underlying cytoskeleton. Then, a gel substrate was mounted to microscope stage equipped with bead twisting setup. The beads were magnetized horizontally and then twisted in an oscillatory magnetic field with a frequency of 0.75 Hz. This exerted a sinusoidal torque that caused the beads to twist, with resulting back-and-forth horizontal translation. The motions of beads were recorded with Leica DMRB CCD camera. The specific torque (T) applied to a bead was computed as $T = mBν$, where $ν$ is the bead volume, $m$ is the bead magnetic moment, and $B$ is the applied magnetic field. The complex elastic modulus ($G’$) of the cell was computed from the Fourier transforms of the applied torque $T$ and of the resulting bead displacement ($d$), as given by $G’ = T/d’ = G’ + jG^*$, where $G'$ is the storage modulus, which we referred to as cellular stiffness (in Pa/nm), $G^*$ is the loss modulus, and $j^2 = -1$. Baseline cellular stiffness was denoted as $G_0$. Due to variability in $G_0$ from different experimental batches and groups, $G’$ was normalized to $G_0$ in each experiment so that the stiffness response to interventions could be compared.

2.4. Traction force microscopy

As described previously in Butler et al. [20], the traction field was calculated from a displacement vector map of changes in bead positions using Fourier transform traction cytometry (FTTC). This field was used to calculate the root-mean-square traction (RMST), which is a scalar measure of the cell’s net contractile strength. Compression/stretch experiments were conducted as follows: baseline images of the cell and beads were recorded and then homogeneous isotropic compression/stretch of 10% strain was applied. Bead images were collected immediately after and 10 min after compression, followed by a final reference image of the gel after the cell’s removal by trypsin. Compression and stretch were applied with annular indenters as described in Krishnan et al. [21].

2.5. Fluorescent staining

Cells were fixed in 4% paraformaldehyde in PBS for 10 min, and permeabilized in PBS containing 0.25% Triton X-100 (Sigma–Aldrich) for 10 min. Non-specific bindings were blocked with 1% bovine serum albumin (BSA; Sigma–Aldrich) in PBS for 30 min. For actin, fixed cells were stained with Alexa 594 Phalloidin (Molecular Probes) for 30 min at room temperature; for vimentin, fixed cells were incubated with mouse anti-vimentin primary antibody (1:100, Sigma–Aldrich) for 1 haat room temperature, and Alexa 488 donkey anti-mouse secondary antibody (1:400 dilution, Abcam) for another 1 h at room temperature for vimentin, fixed cells were incubated with mouse anti-vimentin primary antibody (1:100, Sigma–Aldrich) for 1 haat room temperature, and Alexa 488 donkey anti-mouse secondary antibody (1:400 dilution, Abcam) for another 1 h at room temperature in the dark. Nuclei were stained with 0.1 μg/mL 4’6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich) in PBS for 1 min. Immunostained preparations were observed by Leica DM 6000 B microscope, and images were acquired using Leica Application Suite Advanced Fluorescence (LAS AF). To quantify the intensity of stained actin and vimentin, at least three staining images for each experimental group were analyzed using ImageJ.

2.6. Statistical analysis

Statistical analysis was performed with SigmaStat 3.5 (Systat Software, Chicago, USA). Data of cell stiffness measurement were presented as mean ± SEM, and other data were presented as mean ± SD. A two-tailed Student t test was performed to determine the statistical significance between two groups. A value of $p < 0.05$
was considered statistically significant.

3. Results

3.1. Substrate stiffness affects vimentin distribution in chondrocytes

Fluorescent images of vimentin revealed that in chondrocytes grown on glass (stiffness at GPa range), the vimentin was evenly distributed throughout the cytoplasm (Fig. 1A, left), whereas in chondrocytes grown on 4 kPa PA gel, the vimentin network appeared more abundant around the nucleus and spread toward cell periphery (Fig. 1A, right). After 4 mM acrylamide treatment, the vimentin network in chondrocytes grown on 4 kPa PA gel retreated from cell periphery and was only localized around the nucleus (Fig. 1B). The relative fluorescent intensity of vimentin in acrylamide-treated chondrocytes was 62 ± 4% compared to that in untreated cells (Fig. 1C). Fluorescent staining of actin (Fig. 1D) was also carried out to confirm that vimentin disruption by acrylamide didn’t affect the level of actin (Fig. 1E).

3.2. Changes of chondrocyte traction forces in response to a single compression or stretch

After a transient compression, acrylamide-treated chondrocytes exhibited a decrease in both cellular traction force distribution and magnitude (Fig. 2A). Quantification of cell contractile strength using RMST showed that the contractile strength of untreated chondrocytes didn’t change, while the contractile strength of acrylamide-treated chondrocytes decreased (68% relative to control) immediately after compression, and didn’t fully recover (75% relative to control) after 10 min (Fig. 2B). Measurement of cell area using ImageJ showed that the cell area of both acrylamide-treated chondrocytes and untreated control didn’t change after compression (Fig. 2C). After a transient stretch, the traction force of chondrocytes in both groups decreased immediately and recovered within 10 min, indicating the stretch-induced fluidization and resolidification processes (Fig. 2D). The changes of RMST after stretch didn’t reveal any difference between acrylamide-treated chondrocytes and untreated control (Fig. 2E), and the cell area of chondrocytes in both groups didn’t change after stretch as well (Fig. 2F).

3.3. Changes of chondrocyte stiffness in response to a single compression or stretch

The stiffness of hundreds of chondrocytes was measured simultaneously using OMTC, a technique extensively used to measure material properties of cells (see diagram in Fig. 3A). The real time stiffness of chondrocytes was recorded from 30 s before to 300 s after compression or stretch (Fig. 3B), and the resulting stiffness of each cell was normalized to its own baseline stiffness. After a transient compression, the normalized stiffness of untreated chondrocytes fluctuated slightly around baseline within 300 s, indicating that cell stiffness didn’t change; while the normalized stiffness of acrylamide-treated chondrocytes was between 0.7 and 0.8, indicating a 20%–30% decrease in cell stiffness after compression (Fig. 3C). After a transient stretch, the normalized stiffness of chondrocytes in both groups immediately dropped to 0.6, and gradually recovered toward baseline, with untreated chondrocytes having a higher rate of recovery (Fig. 3D).

3.4. Changes of chondrocyte stiffness in response to drug stimulation

To regulate cytoskeletal tension, chondrocytes were exposed to a panel of drugs during OMTC measurement. The stiffness of normal chondrocytes decreased (Fig. 4A, blue) immediately in response to isoproterenol (relaxation agonist), Y27632 (inhibits

![Fig. 1. Fluorescent images of goat articular chondrocytes. (A) Vimentin staining of chondrocytes grown on glass (left) and 4 kPa polyacrylamide (PA) substrate. (B) Vimentin staining of untreated control (left) and acrylamide-treated chondrocytes grown on 4 kPa PA substrate. (C) Quantification of vimentin intensity, acrylamide-treated group (n = 3) was normalized by control group (n = 3). Data were presented as mean ± SD, *p < 0.05. (D) Actin staining of untreated control (left) and acrylamide-treated chondrocytes grown on 4 kPa PA substrate. (E) Quantification of actin intensity, acrylamide-treated group (n = 3) was normalized by control group (n = 3). Data were presented as mean ± SD.](image-url)
Rho/Rho-kinase pathway), or latrunculin-A (inhibits actin polymerization and disrupts microfilament organization), increased (Fig. 4B, blue) immediately in response to histamine (contractile agonist), TGF-β1, or IL-1β. The stiffness of vimentin-disrupted chondrocytes (Fig. 4AB, red) showed similar responses to Y27632, latrunculin-A, TGF-β1, or IL-1β, but weaker responses to isoproterenol and histamine.

4. Discussion

The load-bearing joints of human, such as hip, knee, and ankle, withstand a wide range of passive and active forces. It is the articular cartilage that absorbs and dissipates the forces to protect the connecting bones. This mechanical robustness relies on the cartilage matrix, which is synthesized and maintained by the chondrocytes under physiological loading. Therefore, chondrocytes play a key role in cartilage function, and abnormal mechanosensing and mechanoresponses of chondrocytes may be directly linked to cartilage pathology [22]. VIFs, as one of the three major filamentous polymers of the cytoskeleton, are known to be involved in mechanosensing of chondrocytes; however, their role in chondrocyte mechanics has not yet been completely understood. In this study, we compared the mechanoresponses between vimentin-disrupted chondrocytes and normal chondrocytes, so that the difference in our measurements reflected the specific mechanical contribution of vimentin. The principal findings of this study were (i) disruption of vimentin impaired the mechanoresponses (traction force and cellular stiffness) to compressive loading, but didn’t change much the responses to tensile loading, (ii) vimentin played a minor role in regulating cytoskeleton tension only when actin related activities were not inhibited.

In this study, we used PA gel as cell culture substrate. The gel together with its coated ECM protein provides the cell with a 3D microenvironment which is more suitable than 2D substrate in studying cell behavior [23]. In addition, the combination of PA gel and our custom-built indenting device makes it a powerful tool to study cell mechanics, especially cell mechanoresponses. The stiffness of PA gel can be easily tuned by adjusting the ratio of acrylamide to bis-acrylamide. By using this gel system, we [15] and others [17,24] have shown that substrate stiffness can greatly influence chondrocyte phenotype and behavior. However, the effect of substrate stiffness was not within the focus of this study, thus we used a single substrate stiffness (4 kPa) throughout. In spite of this, we still found that substrate stiffness influenced chondrocyte vimentin organization by comparing fluorescent images of chondrocytes grown on 4 kPa substrate to those on glass (Fig. 1A).

**Fig. 2.** Effects of vimentin disruption on the changes of cellular traction forces in response to compression or stretch. (A, D) Traction maps of single chondrocytes grown on 4 kPa substrate after a transient compression (A) or stretch (D). Inset: Phase-contrast images of the cell at corresponding time points. (B, E) Average root-mean-square traction (RMST) of chondrocytes before, immediately after, and 10 min after compression (B) or stretch (E). Data were presented as mean ± SD (n = 6, *p < 0.05). (C, F) Average cell area of chondrocytes before, immediately after, and 10 min after compression (C) or stretch (F). Data were presented as mean ± SD (n = 6).
PA gel is also an ideal cell substrate for traction force microscopy because it is transparent and elastic. The gel substrate we used here was thick enough (700 μm) for generating a 10% tensile or compressive strain. We monitored chondrocyte traction before, immediately after (0 min), and 10 min after compression or stretch. In response to compression, the traction force of vimentin-disrupted chondrocytes decreased, while the traction force of control cells remained unchanged, indicating an important role of vimentin in withstanding compressive loading. This is in accordance with the fact that vimentin is enriched in the loading-bearing region of cartilage [11], and the previous finding that vimentin disruption leads the chondrocyte to become...
incompressible [14]. Interestingly, in other cells such as smooth muscle cells [25], the major compression-bearing cytoskeleton components are microtubules rather than intermediate filaments. This might be due to the different mechanical functions of cells: smooth muscle cells are routinely subjected to stretch, while chondrocytes are mostly subjected to compression. However, a study comparing the mechanical contribution of vimentin and tubulin in chondrocytes is necessary for further investigation. In response to stretch, the stiffness of chondrocytes decreased at first, and then gradually recovered toward baseline. This “fluidization-resolidification” phenomenon has been observed in our previous study [16] and other studies in other cell types [21,26], and is established as universal cellular response to a transient stretch.

The changes of cellular stiffness were consistent with the changes of cellular traction in response to the compression or stretch, which further supported our finding that vimentin played a key role in withstanding a sudden compression not stretch. In addition, another mechanical function of vimentin we found here was to assist actin to regulate cytoskeleton tension. We stimulated chondrocytes with three drugs that were expected to increase cytoskeletal tension, the vimentin-disrupted cells showed similar changes of stiffness to control cells in responding to TGF-β1 and IL-1β, but weaker response to histamine. Similarly, then we stimulated chondrocytes with three drugs that were expected to decrease cytoskeletal tension, the vimentin-disrupted showed similar changes of stiffness to control cells in responding to Y27632 and latrunculin-A, but weaker response to isoproterenol. Since we have shown that both TGF-β1 and IL-1β regulate actin dynamics through Rho/ROCK pathway [15,27], and Y27632 is a ROCK inhibitor, latrunculin-A is an inhibitor of actin polymerization, therefore we concluded that cytoskeletal tension of chondrocytes was dependent mainly on actin and slightly on vimentin, and the minor role of vimentin can be neglected when actin dynamics was disturbed.

In conclusion, our study demonstrates that chondrocyte vimentin plays an important role in withstanding a transient compressive loading instead of tensile loading, and a minor role in regulation of cytoskeletal tension dominated by actin dynamics.

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Appendix A. Transparency document

Transparency document related to this article can be found at http://dx.doi.org/10.1016/j.jbrc.2015.11.083.

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