The Effect of Ultrasound Stimulation on the Cytoskeletal Organization of Chondrocytes Seeded in Three-Dimensional Matrices

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Key Words

Actin cytoskeleton · Biomaterial · Cartilage, articular · Cartilage research · Cell-cell and cell-matrix interactions · Cytoskeleton · Cytoskeleton interaction · Tissue engineering, cartilage and bone

Abstract

The impact of low-intensity diffuse ultrasound (LIDUS) stimulation on the cytoskeletal organization of chondrocytes seeded in three-dimensional (3D) scaffolds was evaluated. Chondrocytes seeded on 3D chitosan matrices were exposed to LIDUS at 5.0 MHz (approx. 15 kPa, 51 s, 4 applications/day) in order to study the organization of actin, tubulin and vimentin. The results showed that actin presented a punctate cytosolic distribution and tubulin presented a quasiparallel organization of microtubules, whereas vimentin distribution was unaffected. Chondrocytes seeded on 3D scaffolds responded to US stimulation by the disruption of actin stress fibers and were sensitive to the presence of Rho-activated kinase (ROCK) inhibitor (Y27632). The gene expression of ROCK-I, a key element in the formation of stress fibers and mDia1, was significantly upregulated under the application of US. We conclude that the results of both the cytoskeletal analyses and gene expression support the argument that the presence of punctate actin upon US stimulation was accompanied by the upregulation of the RhoA/ROCK pathway.

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Introduction

The mechanisms by which ultrasound (US) stimulation induces mechanical stress may involve (1) receptor-mediated transmembrane signaling mechanisms, (2) activation of the stretch receptor ion channels [Mobasheri et al., 2002] and (3) a redistribution of the cytoskeletal elements coupled with a change in cellular morphology. We confine our research to the impact of US stimulation on the morphology and cytoskeletal organization of chondrocytes maintained in three-dimensional (3D) scaffolds. The cytoskeleton of a cell is an organized network of three main protein fibers, namely microfilaments (MFs), microtubules (MTs) and intermediate fil-

Abbreviations used in this paper

3D	three dimensional
DAPI	4',6-diamidino-2-phenylindole
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IFs	intermediate filaments
LIDUS	low-intensity diffuse ultrasound
MFs	microfilaments
MTs	microtubules
ROCK-I	Rho-activated kinase-1
US	ultrasound

aments (IFs), each having specific cellular functions [Herrmann et al., 2007]. MFs, which are mainly composed of β -actin subunits [Blain, 2009], provide mechanical strength to the cell and act as a linker between transmembrane and cytoplasmic proteins. MTs, which are assemblies of α - and β -tubulin dimers, are involved in the distribution of organelles and also drive ciliary movements, intracellular transport and cell division [Herrmann et al., 2007; Blain, 2009]. IFs, which are cell type specific, connect the nuclear envelope to the cell membrane [Goldman et al., 2008]. The latter have been reported to be involved in both signal transduction via integrins and in the direct transduction of cellular deformations to the nucleus [Langelier et al., 2000]. Mesenchymal cells and chondrocytes contain vimentin as the IFs [Benjamin et al., 1994].

Chondrocytes are mechanoresponsive cells, a feature that is often exploited to grow tissue-engineered cartilage in vitro cultures under the influence of dynamic compression, rotation, hydrostatic pressure and US [Fronzo et al., 1996; Carver and Heath, 1999a, b; Martin et al., 2000; Yamaoka et al., 2006]. There has been keen interest in evaluating the effects of external stimuli on the biosynthetic activity of chondrocytes [Li et al., 2003; Ng et al., 2007]. The organization of the cytoskeleton under mechanical stress has been evaluated as well, since the chondrocyte cytoskeleton is known to play a fundamental role in the control of the chondrocyte phenotype and in the interactions with the extracellular matrix. The cytoskeletal organization and composition of normal chondrocytes has been reported in detail [Langelier et al., 2000]; i.e. the distribution of actin is punctate and dense at the peripheral cortex [Durrant et al., 1999; Langelier et al., 2000; Trickey et al., 2004; Knight et al., 2006]; MTs have been observed as a mesh spanning through the cytoplasm [Blain, 2009], while IFs have been observed to form a mesh spanning through the cell nucleus to the cell membrane.

Chondrocytes subjected to mechanical compression (0.5–4 MPa) undergo changes in the cytoskeleton. Under the applied load, the distribution of actin was not observed to change whereas the appearance of the vimentin network changed from being diffuse in appearance to an organized network [Durrant et al., 1999]. Changes in actin and tubulin signal intensity upon the application of continuous high pressure (24 MPa) have been reported [Fioravanti et al., 2005]. Actin remodeling has been noted in cells subjected to compression or hydrostatic pressure under either static or cyclical conditions. With the exception of cells exposed to cyclical hydrostatic pressure, in

all cases cytoskeletal organization has been observed to return to control levels 1 h after loading [Knight et al., 2006]. Further, stimulation of primary human skin fibroblasts via US [Langelier et al., 2000] has been shown to impact cytoskeletal organization, while low-intensity diffuse ultrasound (LIDUS) induced the formation of actin stress fibers, which was mediated by the activation of RhoA [Zhou et al., 2004].

The effect of LIDUS stimulation on the proliferation and protein and gene expression of chondrocytes seeded on 3D chitosan scaffolds has been reported elsewhere [Noriega et al., 2007; Hasanova et al., 2010]. Specifically, the gross morphology of chondrocytes was observed to be impacted by the frequency of the US signal employed [Noriega et al., 2007; Hasanova et al., 2007]. We hypothesize that US stimulation of chondrocytes triggers or facilitates an intracellular response which leads to a rearrangement of the cytoskeletal elements. We focus on tasks that will permit an evaluation of the impact of US stimulation on the cytoskeletal organization of chondrocytes maintained in 3D scaffolds. The rationale underlying these experiments is that cytoskeletal elements play a significant role in mechanotransduction and that mechanical stimuli can affect the actin cytoskeleton [Campbell et al., 2007]. Thus, a study of the in situ distribution and physiological 3D organization of chondrocyte cytoskeletal networks (MFs, MTs and vimentin IFs) upon US stimulation is essential for a better understanding of the role of the cytoskeleton under mechanical stimulation.

Materials and Methods

Scaffold Preparation and Cell Seeding

Chitosan scaffolds were prepared by the freeze drying and lyophilization method (FDL) as detailed elsewhere [Hasanova et al., 2010], sterilized and prewetted with DMEM/F-12 with 10% FBS prior to cell seeding experiments. Discarded tissue from the shoulder joints of young calves was obtained from a local abattoir, and articular chondrocytes were extracted using the procedure detailed elsewhere [Hasanova et al., 2010]. Articular chondrocytes were grown in DMEM/F-12 in the presence of 10% fetal calf serum (GIBCO), 1% nonessential amino acids (GIBCO), 2 mM L-glutamine and 1.0% penicillin-streptomycin (Sigma Chemical Company, St. Louis, Mo., USA). All cells were grown at 37°C under a 5% CO₂-humidified atmosphere.

Prewetted scaffold discs (5 × 2.5 mm) were seeded with bovine chondrocytes from passage 3 at a seeding density of 3 × 10⁴ cells/scaffold, with 6 scaffolds per well in a 6-well tissue culture plate. One plate with 36 scaffolds represented one test condition. Typically, 15 μ l of a 2.0 × 10⁶ cells/ml stock solution was pipetted onto each scaffold, and the plates were kept in the CO₂ incubator at 37°C and 95% relative humidity for 3 h. Next, 8 ml of fresh me-

Table 1. Sequence of the primers used in RT-PCR

Gene	Accession number	Forward primer	Reverse primer	Size, bp
mDia1	XM_595101	5'-TTGCAAGAAAATGTGCAAGC-3'	5'-AACGATGCTCAGCAGGAAC-3'	331
RhoA	NM_176645	5'-AACAGGATTGGTGCTTTTGG-3'	5'-AGTGCAGAGGAGGGCTGTTA-3'	368
ROCK-I	AY_052529	5'-GCTCGAGAGAAGGCTGAAAATAG-3'	5'-CATCTGCCCTTCATTCCCTCTG-3'	342
GAPDH	XR_027343	5'-ACCCAGAAGACTGTGGATGG-3'	5'-CCCAGCATCGAAGGTAGAAG-3'	323

dium was added on top of the scaffolds and maintained for 20 h in the incubator. The scaffolds were transferred to a new tissue culture polystyrene plate with 5–8 ml of fresh complete medium per well and placed in the incubator. Control treatments did not include US stimulation and were handled similarly to US-treated specimens. The medium was changed on alternate days. Unseeded disks were included as a control.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Viability Assay

Fifty microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (1 mg/ml in phenol red-free medium) was added to each well of a 96-well plate that contained a scaffold, followed by 4 h of incubation in the CO₂ incubator. The solution was then removed, and 100 µl of isopropanol per well was added. Upon incubation for 30 min, the absorbance at 570 nm was determined. Six to 8 samples for each condition were analyzed.

US Stimulation

LIDUS was used to excite the chondrocytes seeded in scaffolds. However, unlike in previous studies, the total application time was quite long relative to the maximum travel time across the well (approx. 35 µs), meaning that the cells were excited in an incoherent (diffuse) manner due to the numerous multiple reflections from the boundaries of the well that the US waves experienced. The maximum intensity was quantified using a needle hydrophone (Onda Corp., Sunnyvale, Calif., USA). Because the waves multiply-reflect from the boundaries, the maximum intensity was found by moving the hydrophone to many positions within the well and observing the complete time domain signal. The total stimulation time was chosen such that the number of cycles was approximately constant and of the same order as in previous work [Parvizi et al., 1999]. Nonstimulated cell-seeded scaffolds served as controls (denoted as control) and were handled identically to the LIDUS-stimulated scaffolds. The LIDUS excitation was sinusoidal at 5.0 MHz for 51 s with a maximum pressure amplitude of approximately 15 kPa at any spatial position.

Chondrocytes seeded in 3D scaffolds were maintained in culture for 3 days and then stimulated with the US signal as indicated. The US was introduced into the wells using a nonfocused, immersion transducer (V300, Panametrics, Waltham, Mass., USA) with a diameter of 0.5 inches and a center frequency of 5 MHz, as described previously [Noriega et al., 2007]. US was applied 1, 2, 4 or 8 times per day. The time interval between applications was 7, 3 and 1 h for the ×2, ×4 and ×8 application frequencies, respectively. The US transducer was directly applied over the medium above the scaffolds; the probe was positioned 10.5 mm above the well's bottom, and the height of the medium in the well

was kept at 12.0 mm. The probe was sterilized with ethanol before each application.

Typically, a total of $1.0\text{--}5.0 \times 10^6$ chondrocyte cells was collected from 36 scaffolds (one test plate, one condition) after treatment. The cells were immediately frozen in liquid nitrogen and stored at -80°C until further use.

Rho-Activated Kinase-1 Inhibitor

Rho-activated kinase-1 (ROCK-I) inhibitor (Y27632; 1254, TOCRIS, Mo., USA) was added to each well of a 6-well tissue culture polystyrene plate at a final concentration of 20 µM. The inhibitor was added every time the medium was changed on the plates subjected to the inhibition experiment.

mRNA Gene Expression Analysis

RNA was extracted from liquid nitrogen-frozen cells using 1 ml of Trizol reagent (Invitrogen) and 250 µg/ml glycogen (Invitrogen) added as carrier. The integrity and purity of the isolated RNA, typically about 10–15 µg, were assessed by gel electrophoresis and A260/A280 absorbance ratios. Two micrograms of mRNA was used for making the cDNA templates. About 80–150 ng of RNA/cDNA (cDNA corresponding to 80–150 ng of total RNA) from each sample was used for PCR amplification of all genes. RNA samples (approx. 2 µg) were pretreated with 3 units of DNase I using the DNA-free kit (Invitrogen) for 20 min at room temperature, after which the enzyme was inactivated at 65°C in the presence of 2 mM EDTA. Then, the DNase-free RNA preparations were used for synthesis of the first-strand cDNA with 50 ng of random hexamer primers and a SuperScript II Reverse Transcriptase-based kit (Invitrogen, Calif., USA) at 42°C for 50 min. cDNA templates [1 µl for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or 3 µl for the remainder of the genes] were expanded using the Tag DNA polymerase (Invitrogen) in each PCR reaction with 31 cycles (for GAPDH) or 35–37 cycles (for the genes of interest) of denaturation at 94°C for 45 s, a 55–60°C annealing period of 30 s, a 72°C extension period of 1 min and a final extension at 72°C for 10 min using the forward and reverse gene-specific primers shown in table 1. GAPDH, a housekeeping gene, was used as a loading control. A series of PCR reactions was performed with the number of cycles as the variable to find the optimal cycle number for each individual gene. The optimal number of cycles for GAPDH was found to be 31, and for the other genes assayed, it was found to be between 35 and 37 cycles. The PCR products were analyzed on 1% agarose gels and stained with ethidium bromide. The band intensities on ethidium bromide-stained gels were quantified with the Alpha Innotech 8800 gel imaging system using Alpha EaseFC Software (Alpha Innotech, Santa Clara, Calif., USA).

Fig. 1. Analysis of the molecular responses of chondrocytes cultured in chitosan scaffolds to US stimulation. A 5.0-MHz US signal (approx. 15 kPa) was applied 1, 2, 4 and 8 times per day, for 51 s per application for one day. The mRNA levels of the indicated genes (RhoA, ROCK-I and mDia1) were measured by RT-PCR using the specific primers listed in table 1. The GAPDH gene was used as a loading control. Cells from seeded scaffolds that were not subjected to US stimulation served as controls (C). Data were normalized to the controls and are reported as the mean of three independent estimations with error bars, where an error bar represents 1 standard deviation.

Chondrocyte Cell Morphology on the Samples

For scanning electron microscopy, the cells seeded in scaffolds were cross-linked with 2.5% glutaraldehyde (Sigma) in phosphate-buffered saline (PBS) for 30 min, rinsed with deionized water and gradually dehydrated with a series of ethanol solutions. Hexamethyldisilazane (Fisher, Pa., USA) was used to remove 100% ethanol. Samples were sputter coated with Au-Pd prior to examination under scanning electron microscopy (Hitachi, S-3000N variable pressure, Japan). A voltage of 15 kV was used to visualize the samples.

Cytoskeleton Immunostaining for Confocal Microscopy Visualization

For fluorescent staining, the cells seeded in scaffolds were fixed in 4% paraformaldehyde in PBS penetrated with 0.1% Triton X-100 (Sigma) in PBS. Scaffolds were sectioned and incubated with antitubulin antibody (T4026, Sigma-Aldrich; dilution 1:200) for 12 h, rinsed and labeled first with Alexa-488 (A11001, Invitrogen; dilution 1:200) for 12 h, then copiously rinsed and labeled with actin-specific phalloidin-633 (A22284, Invitrogen; dilution 1:200) and Cy3-labeled antivimentin (C9080, Sigma-AI-

Fig. 2. Effect of ROCK-I inhibitor (Y27632) on cell viability. US signal was applied to cell-seeded chitosan scaffolds as follows: 4 times per day, 51 s per application. Upon completion of the experiment, the cells seeded on scaffolds were subjected to US stimulation and the cellular viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Fig. 3. Effect of ROCK-I inhibitor (Y26732) on cell gross morphology. US signal was applied to cell-seeded chitosan scaffolds 4 times per day for 51 s per application, and the scaffolds were maintained in culture in the presence or absence of the ROCK-I inhibitor Y26732. Upon completion of the experiment, the cell-seeded scaffolds were fixed and visualized by scanning electron microscopy. **a** Control cells in the absence of Y26732. **b** Control cells in the presence of Y26732. **c** US-stimulated cells in the absence of Y26732. **d** US-stimulated cells in the presence of Y26732. Scale bars = 2.5 μm .

drich; dilution 1:100) antibodies for 12 h more. Prior to confocal microscopy, the nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; D1306, Invitrogen; 0.05 mg/ml; excitation/emission maxima: 358/461 nm) before mounting on coverslips. A confocal laser scanning microscope (Olympus Inverted, Olympus IX 81) was used to obtain the images. Laser lines 405 (DAPI), 488 (Alexa-488), 543 (Cy3) and 633 (Alexa-633) were used sequentially to obtain the corresponding scans. Acquisition functions including 3D construction, 'z' series sectioning, time series observations, sequential laser scan and image analysis functions were used as appropriate. ImageJ™ software was used to build the montage of images out of the z slices obtained for each condition.

Quantification Analysis

Band intensities were quantified by densitometry using ImageQuant software (v5.2, Molecular Dynamics, Sunnyvale, Calif., USA). The values reported were normalized to unstimulated controls. For the analysis of the US stimulation effects on mRNA levels, the data represented the mean and standard deviation values of 3 independent estimations.

Statistical Analyses

Unless otherwise indicated, all experiments were run at least 3 times. One-way analysis of variance followed by Tukey's post hoc test was performed for statistical analysis of data. $p < 0.05$ (compared to control) was considered significant.

Results

While US has been shown to impact cartilage function at the cellular level [Parvizi et al., 1999; Nishikori et al., 2002; Zhang et al., 2002; Zhang et al., 2003], more research is necessary to understand the effect of US stimulation on chondrocytes seeded and maintained in 3D scaffolds, which are better representatives of in vitro culture of chondrocytes. To stimulate chondrocytes seeded in 3D scaffolds, this study used a continuous US wave for a predetermined time interval (5.0 HZ, 51 s, approx. 15 kPa) rather than pulsed US (1.5 MHz, 6–40 min) as used in previous studies.

Gene Expression of RhoA, ROCK-I and mDia1

ROCK-I is a key element in the formation of stress fibers; hence, the gene expression of both ROCK-I and mDia1 were analyzed to ascertain the role, if any, of ROCK-I and mDia1 in cellular activity (fig. 1). The impact of US stimulation on mRNA expression was examined by RT-PCR as a function of the frequency of US application and normalized with respect to GAPDH. The results are presented in figure 1a. Compared to the control, a higher level of ROCK-I, RhoA and mDia1 mRNA

Fig. 4. Visualization of chondrocyte cytoskeleton. US signal was applied to cell-seeded chitosan scaffolds 4 times per day for 51 s per application, and the scaffolds were maintained in culture in the presence or absence of the ROCK-I inhibitor Y26732. Upon completion of the experiment, the cell-seeded scaffolds were stained for actin, tubulin and vimentin and were visualized by confocal microscopy. **a** Actin distribution in control, nonstimulated cells. **b** Actin cytoskeleton in control cells in the presence of Y26732 inhibitor. **c** Actin distribution in US-stimulated cells in the absence of Y26732 inhibitor. **d** Distribution of actin in cells

subjected to US stimulation in the presence of Y26732 inhibitor. **e** Tubulin in control cells. **f** Tubulin distribution in control cells in the presence of Y26732 inhibitor. **g** Tubulin distribution in US-stimulated cells in the absence of Y26732 inhibitor. **h** Tubulin in US-stimulated cells with Y26732 inhibitor. **i** Vimentin distribution in control cells in the absence of Y26732 inhibitor. **j** Vimentin distribution in control cells in the presence of Y26732 inhibitor. **k** Vimentin distribution in US-stimulated cells in the absence of Y26732 inhibitor. **l** Vimentin distribution in US-stimulated cells in the presence of Y26732 inhibitor. Scale bars = 8 μ m.

expression was observed when the cells were stimulated with US 4 and 8 times per day, respectively. As reported elsewhere, the cells stimulated 4 times per day showed a significant increase in viability compared to control ($p < 0.01$) [Hasanova et al., 2010], while the cells stimulated 8 times per day were not statistically different from controls. Therefore, the cells were stimulated 4 times per day with the specified US regimen in all further experiments.

The observation that the gene expression of RhoA and ROCK-I was impacted by US led to two hypotheses, namely that an effect on actin would be visible upon US

stimulation and that ROCK-I would be one of the main effectors of US on the cytoskeleton. To test these hypotheses, the cells were seeded in scaffolds and US stimulation was applied both in the presence and absence of Y26732, a ROCK-I inhibitor which has been shown to disrupt actin filaments [Riveline et al., 2001].

Determination of Cellular Viability

Figure 2 shows the cell viability both in the presence and absence of the ROCK-I inhibitor, indicating that the addition of Y26732 did not negatively impact the viability.

Fig. 5. a Visualization of actin cytoskeleton Z stacks montage of non-US-stimulated cells. Montage made out of z slices of images taken from control cells grown on FDL chitosan scaffolds for 3 days in the absence of the actin inhibitor Y27632.

The high cellular viability ($p < 0.01$) obtained in US-stimulated cells was in agreement with previous results [Noriega et al., 2007].

Scanning Electron Microscopy

Figure 3 shows the gross morphology of the cells. Figure 3a shows that the control cells without Y27632 presented a rounded morphology with some development of lamellipodia. Figure 3b shows that the control cells with Y27632 presented a morphology of long thin extensions, which is characteristic of cells with inhibited ROCK-I expression [Darenfed et al., 2007]. Figure 3c shows that the US-stimulated cells without Y27632 presented a morphology of spindle-like shapes and the presence of long processes. Figure 3d shows that the US-stimulated cells with Y27632 presented a morphology of much longer thin extensions than the nonstimulated control cells with Y27632.

Cytoskeletal Analysis

Confocal microscopy was used to assess the distribution and organization of the chondrocyte cytoskeleton. Determinations were made before and after US stimulation and in the presence and absence of the Y27632 inhibitor. Figure 4 shows the confocal images obtained. Figure 4a (control cells) shows the presence of the long actin fibers which ran the length of the cells and an actin mesh surrounding the cells. Figure 4b shows that the control cells with Y27632 had punctate actin, and in some cases only membrane accumulated actin and no fibers were detected, which confirms the disruption of actin fibers by the inhibitor. Figure 4c shows that in US-stimulated cells without Y27632, the actin structure appeared to be non-organized and exhibited punctate F-actin located in the membrane and cytosol. There were only a few long actin filaments along with mesh-like actin structures; however, no stress fibers were observed. Figure 4d shows that the

Fig. 5. b Visualization of actin cytoskeleton Z stacks montage of non-US-stimulated cells with Y27632 inhibitor. Montage made out of z slices of images taken from control cells grown on FDL chitosan scaffolds for 3 days in the presence of the actin inhibitor Y26732.

distribution of actin in US-stimulated cells in the presence of Y27632 demonstrated some stress fibers as well as the presence of some long processes and microspikes.

Figure 4e shows the distribution of tubulin in the control cells without Y27632, which formed a mesh-like structure with fibers spanning the whole length of the cells. Figure 4f shows that the tubulin organization in the control cells with Y27632 was not impacted, although some fiber bundling was evident. Figure 4g shows that the tubulin network in the US-stimulated cells was intact and that there was no disruption in the organization of the tubulin bundles. However, with US stimulation, the tubulin seemed to acquire some organization in the form of parallel fibers. Figure 4h shows that the distribution of tubulin in US-stimulated cells with Y27632 exhibited features similar to those seen in figure 4e.

Figure 4i shows the distribution of vimentin in control cells without Y27632. Vimentin was observed to form a mesh-like structure around and in very close contact with the nucleus spanning the whole cytoplasm, which was similar to the distribution reported elsewhere in native chondrocytes [Durrant et al., 1999]. Figure 4j shows that the overall distribution of vimentin in control cells with Y27632 was not impacted, although some fiber bundling was evident. Figure 4k shows that the network distribution of vimentin in US-stimulated cells without Y27632 was intact and that no fiber disruption was evident. Figure 4l shows that the network distribution of vimentin in US-stimulated cells with Y27632 showed no significant change. Finally, no significant difference was noted in the images of DAPI-stained nuclei between the various study groups (images not shown).

Fig. 5. c Visualization of actin cytoskeleton Z stacks montage of US-stimulated cells. Montage made out of z slices of confocal images taken from US-stimulated cells without the Y26732 inhibitor. The arrows indicate cytoplasmic punctate actin.

Figure 5a–d shows the montage made out of the z slices from the confocal images. Figure 5a shows the z slices of control cells without Y27632. Actin distribution was noted to be cortical, with some faint organization in a mesh-like structure. Figure 5b shows the montage made out of the control cells with Y27632; the cell shape was completely changed, i.e. flattened, and there were more cell processes. Moreover, the actin filaments were completely disrupted and the actin distribution was even throughout the cytoskeleton. Figure 5c shows the montage made out of the z slices from the confocal images taken from US-stimulated cells without Y27632; the cell shape was also elongated. The arrows on the images show some cytoplasmic punctate actin [Haudenschild et al., 2008]. Figure 5d shows that the montage made out of the z slices from the confocal images taken from US-stimulated cells with Y27632 was similar to that for the control cells with Y27632, except that the cells were stellar in shape and not spindle-like. Further,

actin stress fibers and cytoplasmic actin were not detected.

In order to evaluate the time-dependent effect of US, the cytoskeleton organization and chondrocytes seeded on scaffolds were stimulated with US (5 MHz, 4 applications/day) for 1, 3 and 7 days. Figure 6a–c shows the images of tubulin cytoskeleton for the non-US-stimulated control cells after 1, 3 and 7 days, respectively. MTs were noted to span the length of the cell, from the MT organizing center to the ends of the cells, as can be observed in the insets. No evident organization of MTs was observed. Figure 6d–f shows the images of tubulin cytoskeleton for the US-stimulated cells after 1, 3 and 7 days, respectively. MTs showed a weak organization and seemed to start acquiring a parallel distribution. No obvious change in the organization and distribution of vimentin was observed between the control and US-stimulated cells (images not shown).

Fig. 5. d Visualization of actin cytoskeleton Z stacks montage of US-stimulated cells with Y27632 inhibitor. Montage made out of z slices of confocal images taken from US-stimulated cells with the Y27632 inhibitor added.

The actin cytoskeleton was also analyzed, and observations similar to those in figure 5 were noted, i.e. the distribution of actin in the control cells was cortical, with some mesh-like structures, whereas US-stimulated cells had stress fibers that were mostly disrupted.

Discussion

It has been postulated that the Rho family of small GTPases and downstream targets such as ROCK-I and mDia1 regulate cytoskeletal organization [Watanabe et al., 1999], in particular the formation of stress fibers and focal adhesion complexes [Riveline et al., 2001]. The organization and redistribution of cytoskeletal elements, notably actin, were noted to be impacted by US stimulation. The distribution of actin in chondrocytes seeded on 3D chitosan-based scaffolds without any US stimulation,

which served as controls, resembled the distribution of actin in 3D agarose gel-based matrices, where the actin was observed to be cortical and formed a mesh-like structure around the cytoplasm [Idowu et al., 2000; Knight et al., 2006]. As anticipated, the addition of the actin inhibitor Y27632 disrupted the actin organization and stress fibers in control cells and resulted in an elongated cellular morphology. Surprisingly, cells subjected to US stimulation also exhibited an altered actin distribution, namely no fiber-like structures and an elongated shape when compared to the control cells. The actin structure in the cells that were subjected to US stimulation in the presence of Y27632 inhibitor was noted to be similar to the non-stimulated cells that were cultured in the presence of the actin inhibitor (fig. 5d). The actin structure was similar in both the US-stimulated and nonstimulated cells with Y27632 (fig. 5d).

Fig. 6. Visualization of the tubulin cytoskeleton organization. US signal was applied to cell-seeded chitosan scaffolds 4 times per day for 51 s per application, and the scaffolds were maintained in culture for 1, 3 and 7 days. **a–c** Images of control cells after 1, 3 and 7 days. **d–f** Images of US-stimulated cells after 1, 3 and 7 days.

Previous work has reported on the reorganization of actin when chondrocytes embedded in agarose were subjected to dynamic compression (0.5 Hz at 5–15% strain, simulating a sinusoidal wave) [Haudenschild et al., 2008]. Specifically, cytoskeletal rearrangements of actin, which mainly included a significant increase in F-punctate actin, were noted, and interestingly, Rho kinase appeared to mediate the observed changes in actin organization [Haudenschild et al., 2008]. For example, upon dynamic compression for 10 min, a 3-fold increase in the levels of Rho-GTP and punctate, cytosolic F-actin were noted when compared to the control cells. Interestingly, no punctate actin was detected when the cells were subjected to dynamic compression in the presence of the ROCK-I inhibitor Y27632 [Haudenschild et al., 2008]. This observation lends credence to the idea that ROCK is involved in the reorganization of actin. As shown in figure 5c, our study found cytosolic punctate actin in US-stimulated cells compared to the control cells, where cortical actin was noted. We suggest that this supports the hypotheses

that chondrocytes seeded on 3D scaffolds respond to US stimulation by the disruption of actin stress fibers and perhaps in a ROCK-I-dependent manner. Interestingly, as noted in figure 1, both the ROCK-I and the RhoA genes were significantly upregulated under the application of US. Both the cytoskeletal analyses and the gene expression results support the theory that the presence of punctate actin upon US stimulation is accompanied by the up-regulation of the RhoA/ROCK-I pathway.

In contrast to our observations, the development of actin stress fibers [Zhou et al., 2004] along with an up-regulation of the RhoA/ROCK-I pathway has been reported when monolayer cultures of human fibroblasts are subjected to pulsed US (1.5 MHz, 1.0-kHz repeat, 10 min/application). We believe that the differences can be attributed to the different geometry of the support matrix, namely two-dimensional versus 3D support. In an independent experiment, we observed distinct actin stress fibers and focal adhesions when chondrocytes were cultured on glass slides for 3 days (data not shown). In con-

trast, we observed a mixed population of cells with varying actin distributions, with the most representative distribution being cortical actin, when chondrocytes were cultured in 3D chitosan-based scaffolds.

A significant result of this study is the upregulation in the gene expression of mDia1. One unexpected observation is the quasiparallel organization of MTs in the presence of a US field. The self-organization of MTs in vitro has been shown to be gravity dependent [Papaseit et al., 2000; Tabony et al., 2002], while numerical simulations of MT orientations suggest that the self-organization derives from the formation of the anisotropic chemical trails produced by the growth and shrinking of MTs. The process of tubulin polymerization and depolymerization creates concentration gradients that can activate and inhibit the formation of neighboring MTs and induce MT organization [Tabony et al., 2002]. Self-organization has been proven to be dependent on confinement limits [Pinot et al., 2009], forces [Janson et al., 2003] and also on the presence of cations [Needleman et al., 2004]. The prediction that self-organization could be dependent on weak external fields [Tabony et al., 2002] is in line with

our observations, i.e. MT organization was affected by US, and MT alignment was noted in cells that underwent US stimulation.

In conclusion, LIDUS (approx. 15 kPa) was shown to modulate the cytoskeleton organization of bovine chondrocytes maintained in 3D matrices. RT-PCR analysis revealed that US stimulation treatment affected the expression of RhoA, ROCK-I and mDia1. Our results confirm the important role of the cytoskeleton in mediating the cellular responses to external stimuli.

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