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Controlling the spatial organization of cells and extracellular matrix proteins in engineered tissues using ultrasound standing wave fields

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Abstract

Tissue engineering holds great potential for saving the lives of thousands of organ transplant patients who die each year while waiting for donor organs. However, to successfully fabricate tissues and organs *in vitro*, methodologies that recreate appropriate extracellular microenvironments to promote tissue regeneration are needed. In this study, we have developed an application of ultrasound standing wave field (USWF) technology to the field of tissue engineering. Acoustic radiation forces associated with USWF were used to non-invasively control the spatial distribution of mammalian cells and cell-bound extracellular matrix proteins within three-dimensional collagen-based engineered tissues. Cells were suspended in unpolymerized collagen solutions and were exposed to a continuous wave USWF, generated using a 1 MHz source, for 15 min at room temperature. Collagen polymerization occurred during USWF exposure resulting in the formation of three-dimensional collagen gels with distinct bands of aggregated cells. The density of cell bands was dependent on both the initial cell concentration and the pressure amplitude of the USWF. Importantly, USWF exposure did not decrease cell viability, but rather enhanced cell function. Alignment of cells into loosely clustered, planar cell bands significantly increased levels of cell-mediated collagen gel contraction and collagen fiber reorganization as compared to sham-exposed samples with a homogeneous cell distribution. Additionally, the extracellular matrix protein, fibronectin, was localized to cell banded areas by binding the protein to the cell surface prior to USWF exposure. By controlling cell and extracellular organization, this application of USWF technology is a promising approach for engineering tissues *in vitro*.

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Keywords

Ultrasound; standing wave field; acoustic radiation force; tissue engineering; extracellular matrix; collagen; fibronectin

INTRODUCTION

Tissue engineering is a potentially revolutionary approach for replacing diseased or damaged tissues and organs (Langer and Vacanti 1993). During the past decade, several promising tissue engineering strategies have emerged, including injecting autologous or allogenic cells directly into damaged tissue, implanting tissue analogs generated *in vitro* from cultured cells, and stimulating tissue regeneration *in situ* (Griffith and Naughton 2002). To date, these strategies have met with limited success. With native tissue remodeling, the three-dimensional (3D) extracellular matrix provides cells with critical biomechanical and biochemical signals that mediate cell adhesion, control cell function and, in turn, guide tissue development. As such, it has become increasingly clear that recreating the appropriate microenvironment for engineered tissues is a key step to converting basic tissue engineering strategies into successful clinical treatments. When tissue engineering is viewed from the perspective of engineering the cell's natural microenvironment, technologies that can specifically control cell and extracellular matrix organization hold great potential for engineering tissues *in vitro*.

Technologies currently in development to organize cells and proteins into complex patterns can be divided into two general categories. In the first approach, micropatterning of cell-adhesive contacts using extracellular matrix proteins coated onto microfabricated stamps by photolithography or microcontact printing is used to direct cell adhesion into pre-designed patterns. In the second approach, a force is applied to cells to direct cell movement to a desired location. The applied force can be optical, magnetic, electrokinetic, or fluidic (Lin et al. 2006). In the current study, we examine the ability of acoustic radiation forces associated with ultrasound standing wave fields to control the spatial distribution of cells and the extracellular matrix protein, fibronectin, in a collagen-based model tissue.

When an ultrasonic pressure wave is incident on an acoustic reflector, the reflected wave interferes with the incident wave resulting in the development of an ultrasound standing wave field (USWF). An USWF is characterized by areas of maximum pressure, known as pressure antinodes, and areas of zero pressure, known as pressure nodes. Exposure of particle or cell suspensions to an USWF can result in the alignment of particles or cells into bands that are perpendicular to the direction of sound propagation and that are spaced at half-wavelength intervals (Coakley et al. 1989; Dyson et al. 1974; Gould and Coakley 1974; Whitworth and Coakley 1992). A primary acoustic radiation force, (F_{rad}), generated along the direction of sound propagation in the USWF, is largely responsible for this movement. F_{rad} is defined as

$$F_{rad} = \left(\frac{-\pi P_o^2 V \beta_o}{2\lambda} \right) * \phi * \sin \left(\frac{4\pi z}{\lambda} \right) \quad (1)$$

where P_o is the USWF peak pressure amplitude, V is the spherical particle volume, λ is the wavelength of the sound field, z is the perpendicular distance on axis from pressure nodal planes, and ϕ is an acoustic contrast factor given by

$$\phi = \frac{5\rho_p - 2\rho_o}{2\rho_p + \rho_o} - \frac{\beta_p}{\beta_o} \quad (2)$$

where ρ_p and β_p are the density and compressibility of the particles or cells, and ρ_o and β_o are the density and compressibility of the suspending medium, respectively (Gol'dberg 1971; Gor'kov 1962; Gould and Coakley 1974).

Applications of USWF in biotechnology use the radiation forces associated with USWF to aggregate cells at defined locations within suspending media (Coakley 1997; Coakley et al. 2000). Exposure of cell suspensions to USWF can result in cellular aggregation at areas of minimum acoustic pressure (the pressure nodes) (Coakley et al. 1989; Dyson et al. 1974; Gould and Coakley 1974; Whitworth and Coakley 1992). Ultrasonic filtration systems use USWF to sediment large aggregates of cells from their suspending media (Hawkes et al. 1997; Limaye and Coakley 1998). USWF may be used to manipulate cells within microfluidic devices for various applications (Wiklund et al. 2006). Additionally, half-wavelength USWF devices have been used to create cellular aggregates in suspension in order to study cell behavior following aggregation (Bazou et al. 2005; Bazou et al. 2006; Edwards et al. 2010; Kuznetsova et al. 2009). Subsequent sedimentation and removal of cell aggregates from similar USWF devices have been used to develop cell culture systems (Bazou et al. 2008; Hultström et al. 2007; Liu et al. 2007).

The acoustic radiation forces that band particles exist only during application of the USWF. Suspending media that undergo phase conversions from liquid to solid states during USWF exposure have been used to maintain the USWF-induced banded distribution. For example, USWF have been used to align yeast and red blood cells in polyacrylamide, alginate, and agar (Gherardini et al. 2002; Gherardini et al. 2005). Others have localized acrylic particles in polysiloxane resin (Saito et al. 1998; Saito et al. 1999). In this way, the banded pattern of particles may be retained after removal of the sound field.

In this study, we demonstrate that USWF technology can be used to organize mammalian cells and extracellular matrix proteins at defined spatial locations within collagen-based, 3D tissue constructs. The conversion of soluble type-I collagen to polymerized gel during USWF exposure was used to maintain the 3D spatial organization of cells after exposure. We present data indicating that fabricating tissue constructs in this manner can enhance cell function and extracellular matrix organization, and discuss applications of this technology to the field of tissue engineering.

MATERIALS AND METHODS

Experimental Set-up

The experimental set-up used for all USWF exposures is depicted in Figure 1A. A plastic exposure tank (36 × 20 × 18 cm) was filled with degassed, deionized water at room temperature. The acoustic source consisted of a 1 MHz unfocused transducer, fabricated from a 2.5 cm diameter piezoceramic disk. The transducer was mounted on the bottom of the water tank. The signal driving the transducer was generated by a waveform generator (Model 33120A, Hewlett Packard, Palo Alto, CA, USA), RF power amplifier (Model 2100L, ENI, Rochester, NY, USA), and an attenuator (Model 837, Kay Elemetrics Corp., Lincoln Park, NJ, USA). Samples were contained within the wells of a modified silicone elastomer-bottomed cell culture plate (BioFlex® culture plates, FlexCell International Corporation, Hillsborough, NC, USA). These sample holders were mounted to a three-axis positioner (Series B4000 Unislide, Velmex Inc., East Bloomfield, NY, USA) to allow

precise control over their location within the sound field. The air interface above the samples was used as the acoustic reflector to generate an USWF within the sample volume.

Sample Holder Preparation

The BioFlex® culture plates used as sample holders for our investigations are depicted in Figure 1B. They were modified from the manufacturer's form by reducing the diameter of 3 wells per plate from 4 cm to 1 cm using Sylgard® 184 silicone elastomer (Dow Corning Corporation, Midland, MI, USA). Through this modification, the diameter of the sample was comparable in size to the -6 dB beam width at the exposure location. The two-part silicone elastomer was mixed in a 10:1 ratio as recommended by the manufacturer's instructions. The solution was degassed at room temperature using a vacuum chamber (Model 5830, National Appliance Company, Portland, OR, USA) and was subsequently poured around 1 cm diameter Teflon® mandrels (Dupont, Wilmington, DE, USA) that were placed at the center of the 3 wells of interest. Following curing of the silicone elastomer at 20°C for 48 hr, the mandrels were carefully removed to leave a 1 cm diameter sample space within 3 wells of each BioFlex® culture plate (Fig. 1B).

Attenuation Measurements

The acoustic attenuations of the silicone elastomer well bottom of the BioFlex® plates, the Sylgard® 184 silicone elastomer, and standard tissue culture polystyrene (Corning/Costar, Cambridge, MA, USA) were measured using an insertion loss technique. Using the water tank set-up, each material was inserted into the acoustic path between the unfocused 2.5 cm diameter, 1 MHz transducer and a hydrophone (either a bilaminar PVDF membrane hydrophone (Marconi Research Center, Chelmsford, England) or a needle hydrophone (Model HNC-0400, Onda Corporation, Sunnyvale, CA, USA)). Peak positive and peak negative pressure amplitudes were measured using the hydrophone and a digital oscilloscope (Model 9310AM, LeCroy, Chestnut Ridge, NY, USA) in the presence and absence of each material for various source amplitudes. The thickness of each material was measured using calipers. The acoustic attenuation coefficient (in dB/MHz/cm) was calculated for each material.

Absorption/Heating Measurements

The acoustic absorption coefficient of Sylgard® 184 silicone elastomer was measured using a thermocouple technique. Briefly, a 50 µm copper-constantan thermocouple was embedded in a sample of Sylgard® 184 silicone elastomer. Using the water tank set-up, the active element of the embedded thermocouple was positioned at the focus of a 1 MHz transducer fabricated from a 3.8 cm diameter plane, piezoceramic disk cemented to the back of a plano-concave lens. A laboratory thermometer (Model BAT-4, Bailey Instruments Co. Inc., Saddle Brook, NJ, USA) and digital oscilloscope were used to monitor the thermocouple output for various pulsing parameters and exposure amplitudes. For each exposure condition, the initial rate of temperature rise in the sample and the spatial peak temporal average intensity (I_{spta}) were measured and used to calculate the absorption coefficient. The calculated absorption coefficients from each exposure condition were averaged to determine the acoustic absorption coefficient (in dB/cm) of Sylgard® 184 silicone elastomer at 1 MHz.

Temperature changes in the collagen/cell samples were also monitored during USWF exposure using a 50 µm copper-constantan thermocouple. Thermocouple output was monitored using a digital laboratory thermometer (Model BAT-12, Physitemp Instruments Inc., Clifton, NJ, USA), sensitive to changes of 0.1°C, over the duration of USWF exposures.

Acoustic Field Measurements

Using the water tank set-up, axial and transaxial spatial distributions of pressure from the 1 MHz, 2.5 cm diameter unfocused transducer were measured under USWF exposure conditions in both the presence and absence of the sample holder. The Onda needle hydrophone, connected to a three-axis positioner, and a digital oscilloscope were used to measure the acoustic pressure. The sample holder was placed in the far-field with the well bottoms situated at an axial distance of 12.2 cm from the transducer. Axial spatial distributions of pressure were measured through a 0.5 cm distance below the air interface in 0.1 mm intervals. The 0.5 cm distance approximates the height of the collagen samples used in our investigations. Transaxial spatial distributions of pressure were measured at an axial distance of 12.2 cm from the transducer in 0.1 mm intervals. A sinusoidal pulse of 50 μ s duration was employed and peak positive pressures were measured for each position.

Acoustic Field Calibrations

Prior to each experiment, the acoustic field was calibrated using either the Marconi membrane hydrophone or the Onda needle hydrophone under traveling wave conditions. Hydrophones were calibrated regularly using the steel sphere radiometer technique (Dunn et al. 1977). Acoustic pressure was measured in the far-field at an axial distance of 12.2 cm from the transducer (where samples were located during USWF exposure). Coordinates from the exposure site to a fixed pointer were determined using the three-axis positioner and were used to position the center of the lower, left-hand well of the sample holder at the exposure site (bottom of the well was 12.2 cm from the transducer). Some water was removed from the tank such that the sample holder was located at the exposure site without full submersion.

Cell Culture

Fibronectin-null mouse embryonic myofibroblasts (obtained from Dr. Jane Sottile, University of Rochester) were used for all experiments. These cells do not produce fibronectin and have been adapted to grow under serum-free conditions (Sottile et al. 1998). Cells were routinely cultured in a 1:1 mixture of AimV (Invitrogen, Carlsbad, CA, USA) and Cellgro (Mediatech, Herndon, VA, USA) on tissue culture dishes pre-coated with collagen type-I. These media do not require serum supplementation. Thus, no source of fibronectin is present during routine culture. On the day of USWF exposure, fibronectin-null cells were harvested from monolayer culture by treatment with 0.08% trypsin (Invitrogen) and 0.5 mM EDTA in PBS. Trypsin activity was neutralized with 2 mg/ml soybean trypsin inhibitor (STI; Sigma, St. Louis, MO, USA). Cells were washed one time with 1 mg/ml STI in PBS and were then resuspended in a 1:1 mixture of AimV/Cellgro.

Collagen Solution Preparation

A neutralized type-I collagen solution was prepared on ice by mixing collagen type-I (isolated from rat tail tendons (Windsor et al. 2002)) with 2X concentrated Dulbecco's modified Eagle's medium (DMEM; Invitrogen) and 1X DMEM containing HEPES so that the final mixture consisted of 0.8 mg/ml collagen and 1X DMEM (Hocking et al. 2000). Both the 1X and 2X DMEM media were degassed in a vacuum chamber for 30 min under sterile conditions prior to incorporation into the collagen mixture.

USWF Exposures

Fibronectin-null cells were added to aliquots of neutralized type-I collagen solutions on ice at various final concentrations immediately prior to USWF exposure. Aliquots (400 μ l) of the collagen/cell solution were then loaded into two of the 1 cm diameter Sylgard® 184 silicone elastomer molded wells of the BioFlex® plate. For "no-cell" samples, an equal

volume of AimV/Cellgro was added in place of fibronectin-null cells and aliquots were loaded into a third well. The collagen/cell solution in the left-hand well of each plate was exposed to a 1 MHz, continuous wave USWF for 15 min at room temperature. The two other samples in the plate (right-hand side) served as sham control wells that were treated exactly as the exposed sample but were not exposed to the USWF. The 15 min exposure duration was sufficient to promote collagen polymerization at room temperature. Following USWF exposure, collagen gels were incubated for 1 hr at 37°C and 8% CO₂ to allow for complete collagen polymerization. An equal volume (400 µl) of DMEM was then added to wells containing collagen gels. In some experiments, collagen/cell and collagen/no-cell solutions were incubated for 1 hr at 37°C and 8% CO₂ in the sample holders to allow collagen polymerization before USWF exposure.

Cell Viability Assay

Thiazolyl blue tetrazolium bromide (MTT) was used to assess cell viability (Mosmann 1983). At various time points after USWF exposure, collagen gels were incubated with 5.3 mM MTT (USB Corporation, Cleveland, OH, USA) for 4 hr at 37°C and 8% CO₂. Gels were then digested with 0.77 mg/ml collagenase (from *Clostridium histolyticum*, type-I, Sigma) and formazan crystals were dissolved using acidified isopropanol (0.04 N HCl). Absorbance measurements at 570 nm and 700 nm (background) were determined using a spectrophotometer. MTT absorbance was calculated by subtracting background absorbance values and non-specific reduction of MTT in no-cell gels from the 570 nm readings. There was a linear relationship between cell number and MTT absorbance. This assay is sensitive to differences of 5000 cells and greater (data not shown).

Collagen Gel Contraction Assays

The extent of collagen gel contraction was determined using two established methods. For *volumetric* gel contraction assays, collagen gels were scored around their edges to form free-floating gels. After an additional 20 hr of incubation at 37°C and 8% CO₂, the gels were removed from the wells and weighed (Model B303, Mettler Toledo, Columbus, OH, USA). Volumetric collagen gel contraction was calculated as a decrease in gel weight as compared to the control, no-cell gel weight (Hocking et al. 2000). For *radial* gel contraction assays, collagen gel diameters were measured using a 10X inspection microscope equipped with a calibrated eyepiece micrometer. Two measurements were recorded for each gel and averaged to calculate gel diameter. Investigators measuring diameters were blinded to exposure conditions. Radial collagen gel contraction was calculated as a decrease in gel diameter as compared to the original gel diameter of 1 cm (Tingstrom et al. 1992).

Soluble Fibronectin Binding

Fibronectin-null cells in suspension (2×10^7 cell/ml) were incubated with 100 µg/ml of Alexa Fluor® 488-labeled human, plasma-derived fibronectin (FN-488; labeled according to manufacturer's instructions) in the presence of 1 mM MnCl₂ for 30 min at room temperature (Akiyama and Yamada 1985; Mastrangelo et al. 1999). Cells were washed twice with AimV/Cellgro to remove unbound fibronectin and were then added to neutralized type-I collagen solutions and exposed to an USWF as described above. In other experiments, 10 µg/ml of FN-488 was added to neutralized type-I collagen solutions in the absence of cells and exposed to an USWF as described above.

Microscopy

One hour after USWF exposure, cell-embedded collagen gels were examined using an Olympus IX70 inverted microscope (Center Valley, PA, USA) with a 4X phase-contrast objective and were photographed using a digital camera (Spot RT Slider, Model 2.3.1,

Diagnostic Instruments Inc., Sterling Heights, MI, USA). FN-488 was visualized using epifluorescence microscopy. Gels were flipped on their side to visualize cell bands through the height of the cylindrical sample. For volumetric collagen gel contraction experiments, gels were imaged after obtaining weight data. Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA) was used to measure the linear distance between fibronectin-null cell bands within collagen gels. Pixel distance was converted to micron values using a micrometer calibration. A total of 10 distances were measured on each of 20 different images collected from 3 different experiments.

To visualize type-I collagen fibers, cell-embedded collagen gels were examined using second-harmonic generation microscopy (Freund and Deutsch 1986; Roth and Freund 1979; Williams et al. 2005). One hour after USWF exposure, gels were fixed in 4% paraformaldehyde for 1 hr at room temperature. Second-harmonic generation microscopy was performed using an Olympus Fluoview 1000 AOM-MPM microscope equipped with a 25X, 1.05 NA water immersion lens (Olympus). Samples were illuminated with 780 nm light generated by a Mai Tai HP Deep See Ti:Sa laser (Spectra-Physics, Mountain View, CA, USA) and the emitted light was detected with a photomultiplier tube using a bandpass filter with a 390 nm center wavelength (Filter FF01-390/40-25, Semrock, Inc., Rochester, NY, USA). Fibronectin-null cells were simultaneously visualized using a second bandpass filter with a 519 nm center wavelength (Filter BA 495-540 HQ from MPFC1, Olympus) by exploiting the intrinsic auto-fluorescence of cells (Monici 2005). Cell-embedded collagen gels were photographed using a CMOS digital camera (Moticam 1000, Motic, China).

Statistical Analyses

Data are presented as the mean $\pm$ SEM. Statistical comparisons between USWF-exposed and sham experimental conditions were performed using either the Student's *t* test for paired samples or one-way analysis of variance in GraphPad Prism software (La Jolla, CA, USA). Differences were considered significant for *p* values < 0.05 .

RESULTS

Characterization of Sample Holders

The measured ultrasound attenuation of standard polystyrene multi-well tissue culture plates was 4.5 ± 0.7 dB/MHz/cm ($n = 3$). Due to the significant attenuation of the sound field by polystyrene plates, silicone elastomer-bottomed plates were investigated as possible sample holders for our studies. The measured acoustic attenuation of the silicone elastomer well bottom (thickness = 1 mm) of the BioFlex® plates was only 0.7 ± 0.2 dB/MHz/cm ($n = 5$) indicating that there is negligible attenuation (0.07 dB at 1 MHz) of the sound field due to the presence of the BioFlex® sample holders.

The measured ultrasound attenuation of the Sylgard® 184 silicone elastomer molding material was 2.4 ± 0.04 dB/MHz/cm ($n = 3$). Sound absorption at 1 MHz (1.4 ± 0.03 dB/cm; $n = 3$) was found to contribute ~60% of this attenuation. Thermocouple measurements monitoring sample temperature during USWF exposure indicated that temperatures of collagen/cell samples never exceeded that of room temperature. Therefore, BioFlex® plates modified with Sylgard® 184 silicone elastomer molds (Fig. 1B) were chosen as sample holders for our investigations because they did not significantly interfere with the sound field.

Characterization of Acoustic Fields Generated in Experimental Set-up

Axial spatial distributions of pressure were measured in both the presence and absence of the BioFlex® sample holders and are shown in Figure 2. Well-developed USWF,

characterized by pressure nodes and antinodes, were found in both the free field (Fig. 2A) and within the sample space (Fig. 2B). Peak pressure amplitudes of these USWF were ~0.2 MPa, and therefore, as expected (Blackstock 2000), were double the transducer output pressure amplitude of 0.1 MPa. Additionally, the measured distance between pressure nodes in both beam patterns was ~0.7 mm. This finding is consistent with the expected half-wavelength spacing between pressure nodes for a 1 MHz USWF generated in water (0.75 mm). Furthermore, the transaxial -6 dB beam width was 1.2 cm in the free field, and this beam width was not affected by the presence of the sample holder (data not shown). Taken together, these data provide further evidence indicating that the BioFlex® plates modified with the Sylgard® 184 silicone elastomer molds did not significantly interfere with the sound field, and thus are appropriate sample holders for our investigations.

USWF Exposure Induces the Formation of Cell Bands in 3D Collagen Gels

To determine if USWF radiation forces could manipulate cell organization in 3D collagen gels, fibronectin-null cells, suspended in unpolymerized type-I collagen solutions, were either exposed to, or not exposed to (sham samples), a 1 MHz, continuous wave USWF with 0.2 MPa peak pressure amplitude using the experimental set-up depicted in Figure 1A. Fibronectin-null cells do not produce fibronectin and are grown in serum-free conditions (Sottile et al. 1998). These cells were chosen for our initial studies to differentiate the effects of USWF on the localization of cells and the extracellular matrix protein, fibronectin (discussed in Figure 9, below). Collagen solutions were allowed to polymerize during the 15 min exposure to maintain the USWF-induced cell distribution after removal of the ultrasound field. Cell distribution was then analyzed throughout the collagen gels using phase-contrast microscopy (Fig. 3). Images shown are representative of our results. Collagen gels polymerized in the presence of the USWF showed a distinct banded pattern characterized by the localization of cells to the pressure nodes of the USWF (Fig. 3 panels B and C), while sham samples exhibited a homogeneous cell distribution (Fig. 3 panel A). The mean of the measured distance between cell bands was $657 \pm 15 \mu\text{m}$. Increasing the initial concentration of cells in the collagen solutions led to the formation of denser cell bands at the nodal planes (compare Figures 3B and 3C). These data indicate that an USWF can spatially organize cells within 3D collagen gels, and that the extent of the banded pattern of cells is dependent on cell concentration.

To evaluate potential adverse effects of USWF exposure on cell viability, cell number was quantified using MTT. No differences in cell number were observed between USWF-exposed and sham-exposed cell-embedded collagen gels 20 hr after exposure (Fig. 4). These findings indicate that an USWF can alter the spatial organization of cells within 3D collagen gels without decreasing cell viability.

To estimate the magnitude of radiation force exerted on the cells in the applied USWF, Equation 1 was used to calculate the maximum F_{rad} . The acoustic exposure parameters and the physical properties of the cells and the suspending collagen medium used for the calculation are listed in Table 1. Results of this calculation indicated that the cells were subjected to a maximum radiation force of approximately 2.2 pN.

USWF-Induced Formation of Cell Bands Enhances Cell-Mediated Collagen Gel Contraction

The biomechanical properties of normal human tissue and engineered tissue constructs are in part dictated by the organization of the extracellular matrix comprising that tissue or tissue construct (Vogel and Sheetz 2006). In turn, extracellular matrix organization is partly influenced by cell-derived forces. These forces are exerted on matrix components through intracellular tension generation due to cytoskeletal contractility (Hinz and Gabbiani 2003; Hocking et al. 2000; Lee et al. 1998). Cell-mediated collagen gel contraction is a common

measure of extracellular matrix remodeling by cells (Korff and Augustin 1999; Sieminski et al. 2004; Vernon and Sage 1996). Thus, to determine if a change in the spatial distribution of cells affects cell-mediated extracellular matrix remodeling, a collagen gel contraction assay was used to compare the extent of collagen gel contraction between USWF cell-organized gels and sham gels.

Fibronectin-null cells, suspended in unpolymerized collagen type-I solutions, were either exposed to, or not exposed to (sham), an USWF (0.2 MPa peak pressure, 1 MHz source) using the experimental set-up depicted in Figure 1A. Following an overnight incubation, sham-exposed gels contracted 11.5 ± 1.7 % (Fig. 5A). In contrast, samples exposed to the USWF contracted 22.9 ± 2.1 % (Fig. 5A). Thus, a two-fold increase in the contraction of collagen gels with USWF-induced cell organization was found as compared to sham gels with a homogeneous cell distribution (Fig. 5A, B). Additional experiments were conducted to determine whether the increase in collagen gel contraction was due to cell alignment or to a direct effect of USWF exposure on cell tension generation. According to theory, increasing the viscosity of the suspending medium inhibits USWF-induced movement of cells to the pressure nodes (Coakley et al. 1989). Based on this prediction, collagen gels with a homogeneous distribution of fibronectin-null cells were allowed to polymerize prior to USWF exposure. Microscopic analyses confirmed the lack of localization of cells to the nodal planes (Fig. 5D). Importantly, no differences in collagen gel contraction between USWF-exposed and sham samples were observed (Fig. 5C). These data indicate that aligning cells with an USWF can enhance cell-mediated collagen matrix remodeling.

The Formation of Cell Bands Depends on USWF Pressure Amplitude

The magnitude of the acoustic radiation force (F_{rad}) in an USWF has a second order dependence on pressure amplitude (P_o) and, as such, changing P_o will affect the movement of cells to the pressure nodes (Eq. 1). Theoretical analysis of the forces acting on particles in an USWF predicts a threshold pressure for banding below which particles will not accumulate on the nodal planes (Coakley et al. 1989). To determine the threshold pressure amplitude necessary to achieve cell banding within collagen gels, fibronectin-null cells suspended in type-I collagen solutions were exposed during the polymerization process to an USWF of various peak pressure amplitudes. As shown in Figure 6, homogeneous cell distributions were observed within sham-exposed gels, as well as gels fabricated using an USWF with peak pressure amplitudes of 0.02 and 0.05 MPa. Exposing samples to an USWF with peak pressure amplitude of 0.1 MPa resulted in the formation of cell bands, indicating that the threshold pressure for USWF-induced cell banding in our system is ~ 0.1 MPa ($F_{rad\ max} = 0.55$ pN) (Fig. 6). When an USWF with pressure amplitude of 0.2 MPa was used to fabricate the collagen gels, cell bands appeared more dense (Fig. 6). With a pressure amplitude of 0.3 MPa, the resulting cell bands were thicker and more localized to the center of the gel (Fig. 6), likely due to the influence of secondary lateral acoustic radiation forces acting within the pressure nodal planes (Spengler et al. 2003). These findings indicate that different USWF pressure amplitudes lead to variations in the patterns of banded cells within collagen gels.

USWF Pressure Amplitude Has a Biphasic Effect on Cell-Mediated Collagen Gel Contraction

Our data indicate that USWF-induced cell organization enhances cell-mediated collagen gel contraction, and that the extent of cell banding is affected by the USWF pressure amplitude. To determine if different cell banded patterns affect the extent of collagen gel contraction, radial collagen gel contraction assays were used to compare levels of collagen gel contraction among gels fabricated at the six different USWF pressure amplitudes shown in Figure 6. No differences in collagen gel contraction were observed among sham-exposed

samples and samples exposed to either 0.02 or 0.05 MPa (Fig. 7A) where cells remained in a homogeneous distribution (Fig. 6). In contrast, a significant 1.5-fold increase in gel contraction occurred at 0.1 MPa (Fig. 7A), the pressure threshold for cell banding, where cells first become aligned into planar bands (Fig. 6). These results, obtained using gel diameter measurements, are similar to those reported in Figure 5 using volumetric contraction assays, and therefore, provide additional evidence that USWF-induced cell banding enhances cell-mediated collagen gel contraction and matrix reorganization.

As the USWF pressure amplitude was increased above 0.1 MPa, collagen gel contraction levels decreased (Fig. 7A). At 0.3 MPa, a 30% decrease in contraction as compared to sham levels was observed (Fig. 7A). There were no significant differences in the number of viable cells between sham-exposed and 0.3 MPa USWF-exposed samples (Fig. 7B). Therefore, the decrease in collagen gel contraction was not due to cell death, but was more likely due to effects of the cell banded pattern that occurred at 0.3 MPa. These findings indicate that the effect of USWF pressure amplitude on cell-mediated collagen gel contraction is biphasic. We attribute this biphasic effect on collagen gel contraction to the decrease in cell-extracellular matrix contacts formed as cell bands become more dense above the threshold pressure.

To directly assess collagen matrix organization relative to the cell banded areas formed using USWF of various pressure amplitudes, second-harmonic generation microscopy imaging of collagen fibers was performed. As shown in Figure 8, short collagen fibrils were randomly organized in sham-exposed cell-embedded collagen gels and in cell-embedded collagen gels exposed to either 0.02 or 0.05 MPa where cells remained in a homogeneous distribution. Exposure to 0.1 MPa resulted in areas of the gels where cells were loosely clustered and collagen fibrils were more elongated (Fig. 8). These data clearly show that cells aligned into planar bands at the pressure threshold for cell banding have reorganized their surrounding collagen matrix, and thus, provide further evidence that USWF-induced cell banding enhances cell-mediated collagen matrix remodeling.

Extensive areas of cell bands were clearly visible in collagen gels exposed to 0.2 or 0.3 MPa, and short collagen fibers surrounding these areas were randomly oriented (Fig. 8). These results indicate that as the USWF pressure amplitude increases beyond the pressure threshold for cell banding and cell bands become more dense, cell-mediated collagen matrix reorganization decreases. Therefore, the effect of USWF pressure amplitude on cell-mediated collagen matrix reorganization is biphasic and as such, these data both parallel and support our data showing that USWF pressure amplitude has a biphasic effect on collagen gel contraction. Taken together, these data indicate that radiation forces associated with an USWF can indirectly influence the relative location of extracellular proteins and thus, can be used to control extracellular matrix-dependent functions essential to tissue formation.

USWF Localize Cell-Bound Protein to Cell Bands in 3D Collagen Gels

Successful tissue engineering depends upon the stimulation of key cell functions, including cell proliferation, migration, and differentiation. These processes are influenced by a variety of soluble and insoluble factors, including growth factors, cytokines, and extracellular matrix proteins (Langer and Vacanti 1993). The extracellular matrix protein, fibronectin, stimulates cell growth, migration, and contractility (Hocking et al. 2000; Hocking and Chang 2003; Sottile et al. 1998). Concentrating stimulatory proteins to the cell banded areas of collagen gels may be a useful approach to stimulate cell function.

The radiation force exerted on a spherical particle in a standing wave field is proportional to the particle volume (Eq. 1). Soluble fibronectin molecules that are 20 nm in diameter (Vuillard et al. 1990) experience a maximum primary radiation force of $\sim 4.5 \times 10^{-8}$ pN when

exposed to an USWF with parameters shown to promote cell banding (Fig. 3 and Table 1). As such, soluble fibronectin molecules will not localize to the pressure nodes of the USWF used in this study. This idea was confirmed in our system by including Alexa Fluor® 488-labeled fibronectin (FN-488) in unpolymerized type-I collagen solutions and allowing polymerization to occur during USWF exposure. Epifluorescence microscopy images show a homogeneous distribution of labeled fibronectin remaining after USWF exposure (Fig. 9A).

To facilitate localization of fibronectin to the cell bands where it may influence cell functions, soluble FN-488 molecules were bound to fibronectin-null cells prior to USWF exposure. Binding of FN-488 to cells was confirmed by western blot analysis (data not shown). FN-488-bound cells were then added to unpolymerized type-I collagen solutions and exposed to an USWF with a pressure amplitude of 0.2 MPa. Epifluorescent microscopic analysis showed co-localization of fibronectin molecules to USWF-induced cell bands within the polymerized collagen gels (Fig. 9B) indicating that USWF radiation forces can influence the spatial organization of cell-bound proteins within 3D collagen gels.

DISCUSSION

We have developed the use of ultrasound standing wave fields as a non-invasive technology for organizing cells and cell-bound proteins within tissue engineered biomaterials. In this study, we show that acoustic radiation forces associated with an USWF can be used to organize both mammalian cells and cell-associated proteins into discrete bands within collagen hydrogels. The density of the USWF-aligned cell bands was dependent on both cell number and pressure amplitude. Exposure of cells to USWF parameters utilized in the current study did not decrease cell viability. Furthermore, the USWF-aligned cell bands were stable for at least 20 hr.

Under appropriate conditions, the organization of cells into bands led to an increase in cell-mediated collagen gel contraction, as measured by both volumetric and radial changes, demonstrating an increase in cell function in response to cell alignment. The increase in collagen gel contraction in response to USWF exposure did not occur if the collagen/cell samples were allowed to polymerize prior to USWF exposure, strongly suggesting that the increases in cell contractility and collagen fibril reorganization were mediated by the organization of cells into bands and were not an indirect effect of ultrasound exposure on individual cells. The extent of collagen contraction was dependent upon the spatial distribution of cells in the gel. No increase in collagen gel contraction was observed in response to USWF exposure at pressure amplitudes that did not produce cell banding. At the other extreme, no increase in collagen gel contraction was observed in response to USWF at pressure amplitudes that produced densely packed cell bands. However, exposure of samples to an USWF that led to the clustering of cells into planar bands within the gel resulted in a significant increase in collagen contraction above sham gels with a homogenous cell distribution.

Clustering of cells into planar bands in response to an USWF also led to changes in collagen fibril organization and length. Second-harmonic generation microscopic images showed that short collagen fibers were randomly organized in gels exposed to USWF at pressure amplitudes that did not produce cell banding, as well as pressure amplitudes that produced densely packed cell bands. In contrast, elongated collagen fibers were observed within loosely clustered cell banded areas indicating enhanced cell-mediated collagen matrix remodeling in these samples. These results are consistent with the biphasic results of the collagen gel contraction investigations. Hence, an important downstream effect of USWF-mediated cell alignment is enhanced extracellular matrix remodeling.

Acoustic radiation forces exerted on extracellular matrix proteins were too small to directly organize proteins in the system used in this study. However, the use of an USWF can indirectly affect the organization of proteins in the extracellular matrix by two avenues. First, as explained in the paragraph above, increased collagen fibril length and organization was observed in gels exposed to an USWF at pressure amplitudes that increased collagen gel contraction. Thus, a downstream effect of USWF-induced cell banding is the resultant cellular remodeling of the surrounding extracellular matrix. Second, we demonstrated that the extracellular matrix protein, fibronectin, could be aligned into bands within the collagen gel using an USWF if the fibronectin molecules were first bound to the cell surface. Thus, USWF technology can be used to spatially organize cells within engineered tissues and to co-locate active or inactive cell-bound molecules.

The use of an USWF has numerous advantages as a non-invasive technology to spatially organize cells and cell-bound molecules in engineered tissues, and thereby influence cell function. The acoustic radiation force acts directly on the cells, and thus, the approach does not require any prior modification of the cell surface. Various hydrogels that undergo phase transitions could be adapted to this technique. Similarly, various cell types or combinations could be used to engineer different tissue types. Changing frequency of the ultrasound field will affect spacing of cell bands, and multiple transducers could be used to produce more complex patterns of cells within engineered biomaterials. For example, USWF-induced banding of human endothelial cells can give rise to a vascular network within a 3D construct (Garvin et al. 2010). Another potential use of this technology is the production of dermal grafts using dermal fibroblasts or mesenchymal stem cells. Controlling cell patterning, cell function, and extracellular matrix organization are primary challenges to successfully engineering functional tissues and organs *in vitro* (Khademhosseini et al. 2009). Thus, the use of USWF to specifically control cell organization, function, and extracellular matrix remodeling within 3D artificial constructs has the potential to address these current challenges to tissue engineering.

SUMMARY

This study reports on the development of an application of USWF technology to the field of tissue engineering. Acoustic radiation forces associated with USWF were used to spatially organize cells and cell-bound proteins into distinct bands within 3D collagen gels. USWF-induced cell alignment increased cell contractility and resulted in enhanced cell-mediated extracellular matrix reorganization. By specifically controlling cell and extracellular matrix organization, this technology holds great potential for advancing the fabrication of complex engineered tissues *in vitro*.

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Figure 1. Schematic of the Experimental Set-up and Sample Holders used for USWF Exposures (A). An unfocused, 1 MHz, 2.5 cm diameter transducer was mounted on the bottom of a plastic exposure tank filled with degassed, deionized water. The acoustic fields were generated using a signal generator, RF power amplifier, and an attenuator to create an RF signal to drive the transducer. Using a three-axis positioner, the sample holders were placed with well bottoms situated at the water-air interface 12.2 cm from the transducer such that the center of the lower, left-hand well was on the transducer axis. (B). Silicone elastomer-bottomed BioFlex® culture plates were modified from their original form by reducing the diameter of 3 wells per plate to 1 cm using Sylgard® 184 silicone elastomer molds. The left-hand well contained the sample exposed to USWF. The right-hand wells served as sham control wells for cell-containing and non-cell-containing collagen samples.

Figure 2. Measurement and Characterization of Acoustic Field

Hydrophone measurements were used to determine the axial spatial distributions of pressure in the free field (A) and in the presence of the BioFlex® sample holders (B) in the water tank set-up. The USWF was generated using a 1 MHz source with 0.1 MPa output pressure amplitude. Measurements were recorded through a 0.5 cm distance below the air interface in 0.1 mm intervals. Surface tension effects prevented measurements directly at the air-water interface and therefore, measurements were started at the first pressure antinode.

Figure 3. USWF Exposure Induces the Formation of Cells Bands in 3D Collagen Gels

Fibronectin-null cells were suspended in neutralized collagen type-I solutions on ice at either 2×10^5 cell/ml (A, B) or 4×10^6 cell/ml (C). Samples were exposed at room temperature for 15 min to a continuous wave USWF with 0.2 MPa peak pressure amplitude generated using a 1 MHz source. Sham gels were treated identically to USWF samples but were not exposed to the sound field. Cell-embedded collagen gels were imaged as described in Materials and Methods. Representative phase-contrast images, taken 1 hr after USWF exposure, are shown. Scale bar, 200 μ m.

Figure 4. USWF Exposure Does Not Decrease Cell Viability

Fibronectin-null cells were suspended in collagen type-I solutions (2×10^5 cell/ml) and exposed at room temperature for 15 min to a continuous wave USWF with 0.2 MPa peak pressure amplitude generated using a 1 MHz source. Following a 20 hr incubation at 37°C and 8% CO₂, cell viability was determined using MTT as described in Materials and Methods. Data are presented as average fold difference in absorbance $\pm$ SEM normalized to sham average absorbance values ($n = 4$; $p > 0.05$).

Figure 5. Cell-Mediated Collagen Gel Contraction is Increased After USWF-Induced Formation of Cell Bands

Fibronectin-null cells (2×10^5 cell/ml) were suspended in collagen type-I solutions and exposed to a continuous wave USWF (0.2 MPa peak pressure, 1 MHz source) for 15 min at room temperature during the collagen polymerization process (A, B). In C and D, cell-containing collagen type-I solutions were allowed to polymerize at 37°C for 1 hr and then exposed to the USWF. Following exposure, collagen gels were incubated for an additional 20 hr at 37°C and 8% CO₂ and then removed from the wells and weighed. Data are presented as average percent volumetric gel contraction $\pm$ SEM (A, $n = 25$; C, $n = 28$). Representative phase-contrast images of USWF-exposed (right) and sham gels (left) are shown in B (exposed to USWF during collagen polymerization) and D (exposed to USWF after collagen polymerization). The * indicates a difference from sham group ($p < 0.05$). Scale bar, 200 μ m.

Figure 6. Cell Banding is a Function of USWF Pressure Amplitude

Fibronectin-null cells (4×10^6 cell/ml) were suspended in collagen type-I solutions and exposed at room temperature for 15 min to a continuous wave USWF (1 MHz source) with various peak pressure amplitudes. Representative phase-contrast images, from one of four experiments, indicate cell banding in samples exposed to 0.1 MPa and above. Scale bar, 200 μm .

Figure 7. USWF Pressure Amplitude Leads to a Biphasic Effect on Cell-Mediated Collagen Gel Contraction

(A). Fibronectin-null cells (4×10^6 cell/ml) suspended in collagen type-I solutions were exposed at room temperature for 15 min to a continuous wave USWF (1 MHz source) with various peak pressure amplitudes. Following 1 hr incubation at 37°C and 8% CO_2 , gel diameters were measured. Data are presented as average percent radial gel contraction $\pm$ SEM and are normalized to the sham condition ($n = 10$). The * indicates a difference from sham group ($p < 0.05$). (B). Fibronectin-null cells (2×10^5 cell/ml) suspended in collagen type-I solutions were exposed at room temperature for 15 min to a continuous wave USWF (1 MHz source) with peak pressure amplitude of 0.3 MPa. Following 1 hr incubation at

37°C and 8% CO₂, cell viability was assessed using MTT. Data are presented as average fold difference in absorbance $\pm$ SEM normalized to sham average absorbance values ($n = 3$; $p > 0.05$).

Figure 8. USWF Pressure Amplitude Has a Biphasic Effect on Cell-Mediated Collagen Reorganization

Fibronectin-null cells (4×10^6 cell/ml) suspended in collagen type-I solutions were exposed at room temperature for 15 min to a continuous wave USWF (1 MHz source) with various peak pressure amplitudes. Following 1 hr incubation at 37°C and 8% CO₂, gels were fixed with 4% paraformaldehyde and imaged using second-harmonic generation microscopy as described in Materials and Methods. Representative merged images, from one of three experiments, are shown (red - collagen I fibers; green - cells). Scale bar, 100 μ m.

Figure 9. USWF Exposure Localizes Cell-Bound Fibronectin to Cell Bands

(A). FN-488 (10 $\mu\text{g/ml}$) was added to collagen type-I solutions in the absence of cells and exposed at room temperature for 15 min to a continuous wave USWF with a peak pressure amplitude of 0.2 MPa (1 MHz source). Fibronectin distribution was analyzed using epifluorescence microscopy. In B, FN-488 was incubated with fibronectin-null cells as described in Materials and Methods. FN-488-bound cells were then suspended in collagen type-I solutions at 4×10^6 cell/ml and exposed to the USWF described above. Cell and fibronectin distribution were analyzed using phase-contrast and epifluorescence microscopy, respectively. Representative images, taken 1 hr after USWF exposure, are shown. Scale bar, 200 μm .

Table 1

Parameters used for calculation of primary acoustic radiation force (F_{rad}) using Equation 1.

Parameters (units)	Numerical Value	Source/Reference
P_o (MPa)	0.2	Hydrophone measurement
V (μm^3)	900 (cells) 4.2×10^{-6} (FN*)	Assuming spherical particles
r (μm)	6 (cells) 0.01 (FN)	Cell radius measured for rounded fibronectin-null cells in suspension; FN radius (Vuillard et al. 1990)
β_o (1/Pa)	4.4×10^{-10}	Calculation from $\beta = 1/c^2 \rho$
β_p (1/Pa)	4.1×10^{-10} (cells) 3.1×10^{-10} (FN)	
c_o (m/s)	1500	
ρ_o (kg/m ³)	1000	
λ (μm)	1500	Assuming collagen media has properties of water at room temperature
f (MHz)	1	Chosen frequency for our studies
ϕ	0.13 (cells) 0.58 (FN)	Using Equation 2
c_p (m/s)	1529 (cells) 1540 (FN)	Cell value (Taggart et al. 2007); FN value assumed to be sound speed in human soft tissue (Bamber 1998)
ρ_p (kg/m ³)	1050 (cells) 1350 (FN)	Cell value assumed to be dominated by cytoplasm taken as low concentration saline (Baddour et al. 2005); FN value (Fischer et al. 2004)
z (μm)	0-750	Axial distance between two pressure nodal planes ($\lambda/2$)
F_{rad} max (pN)	± 2.2 (cells) $\pm 4.5 \times 10^{-8}$ (FN)	Calculated using Equation 1 at $z = \lambda/8$ and $3\lambda/8$

*, soluble fibronectin molecules (FN)