Published in final edited form as:

Ultrasound Med Biol. 2011 November ; 37(11): 1853–1864. doi:10.1016/j.ultrasmedbio.2011.07.008.

Vascularization of Three-Dimensional Collagen Hydrogels Using Ultrasound Standing Wave Fields

Kelley A. Garvin, M.S.^{*,†}, Diane Dalecki, Ph.D.^{*,†,‡}, and Denise C. Hocking, Ph.D.^{*,†,§}

^{*}Department of Biomedical Engineering, University of Rochester, Rochester, New York

[†]The Rochester Center for Biomedical Ultrasound, University of Rochester, Rochester, New York

[‡]Department of Electrical and Computer Engineering, University of Rochester, Rochester, New York

[§]Department of Pharmacology and Physiology, University of Rochester, Rochester, New York

Abstract

The successful fabrication of large, three-dimensional (3D) tissues and organs in vitro requires the rapid development of a vascular network to maintain cell viability and tissue function. In this study, we utilized an application of ultrasound standing wave field (USWF) technology to vascularize 3D, collagen-based hydrogels in vitro. Acoustic radiation forces associated with USWF were used to non-invasively organize human endothelial cells into distinct, multicellular planar bands within 3D collagen gels. The formation and maturation of capillary-like endothelial cell sprouts was monitored over time and compared to sham-exposed collagen constructs which were characterized by a homogeneous cell distribution. USWF10 induced cell banding accelerated the formation and elongation of capillary-like sprouts, promoted collagen fiber alignment, and resulted in the maturation of endothelial cell sprouts into lumen-containing, anastomosing networks found throughout the entire volume of the collagen gel. USWF-induced endothelial cell networks contained large, arteriole-sized lumen areas that branched into smaller, capillary-sized structures indicating the development of vascular tree-like networks. In contrast, sprout formation was delayed in sham-exposed collagen gels, and endothelial cell networks were absent from sham gel centers and failed to develop into the vascular tree-like structures found in USWF-exposed constructs. Our results demonstrate that USWF technology leads to rapid and extensive vascularization of 3D collagen-based engineered tissue and therefore, provides a new strategy to vascularize engineered tissues in vitro.

Keywords

Ultrasound standing wave field; tissue engineering; collagen; endothelial cells; vascularization

© 2011 World Federation for Ultrasound in Medicine and Biology. Published by Elsevier Inc. All rights reserved.

Corresponding Author: Denise C. Hocking, Ph.D., Department of Pharmacology and Physiology, University of Rochester, 601 Elmwood Ave., Box 711, Rochester, NY 14642, denise_hocking@urmc.rochester.edu, Phone: 585-273-1770, Fax: 585-273-2652.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Developing technologies that enable the repair or replacement of diseased or injured tissues and organs is a major focus of tissue engineering (Langer and Vacanti 1993). Recent advances in this field include the engineering of skin (Priya et al. 2008), cartilage (Chung and Burdick 2008), and bladder tissue (Atala et al. 2006), which are relatively thin tissues that can rely on diffusion for the delivery of oxygen and nutrients (Folkman and Hochberg 1973; Griffith et al. 2005). The development of larger, more complex, three-dimensional (3D) tissues and organs has been limited by the need to create a vascular network within the tissue to supply essential oxygen and nutrients to maintain tissue viability and function (Folkman and Hochberg 1973; Griffith et al. 2005; Griffith and Naughton 2002; Khademhosseini et al. 2009; Mooney and Mikos 1999). As such, strategies that promote the development of vascular networks within 3D engineered tissues have the potential to significantly advance the fabrication of large organs and tissues in vitro.

Two general strategies for vascularizing engineered tissue are under development (Laschke et al. 2006; Lokmic and Mitchell 2008; Rouwkema et al. 2008). In vivo strategies depend upon the ingrowth of host blood vessels into an implanted, engineered tissue to form a vascular network within the tissue (Chen et al. 2007; Pandit et al. 1998; Richardson et al. 2001). However, host-dependent vascular ingrowth is a slow process that can compromise tissue viability (Mikos et al. 1993; Tremblay et al. 2005). Alternatively, in vitro strategies rely on the formation of a vascular network within the engineered tissue prior to implantation (Black et al. 1998; Chen et al. 2009; Levenburg et al. 2005; Tremblay et al. 2005). Tissue perfusion occurs once the engineered vessels anastomose with the host vasculature, a process that occurs more rapidly than host-dependent vascular ingrowth (Tremblay et al. 2005). Minimizing the time required for tissue perfusion after implantation is crucial to maintain tissue function. Thus, in vitro approaches are viewed as a promising strategy to vascularize engineered tissue.

At present, the most common approach used to promote in vitro vascularization of engineered tissue is to seed constructs with endothelial cells and induce the spontaneous organization of the cells into vascular structures using pro-angiogenic factors (Chen et al. 2009; Levenburg et al. 2005; Tremblay et al. 2005). However, endothelial cell responsiveness to the activities of pro-angiogenic factors is limited in the absence of cell-cell contacts (Korff and Augustin 1998). Correspondingly, aggregation of endothelial cells into multicellular spheroids renders the cells responsive to pro-angiogenic factors and prevents apoptosis (Korff and Augustin 1998). As such, endothelial cell spheroids have been increasingly used as a tool for tissue construct vascularization (Alajati et al. 2008; Korff and Augustin 1999; Korff et al. 2001; Wenger et al. 2004). Cell spheroid formation is also a relatively slow process that can take up to day to complete (Alajati et al. 2008; Korff and Augustin 1998; Korff and Augustin 1999; Korff et al. 2001; Wenger et al. 2004). In the current study, we utilized an ultrasound-based technology to rapidly (in minutes) generate spatially organized, multicellular planar aggregates of endothelial cells within collagen-based gels. We then characterized the use of this technology as a new approach for in vitro vascularization of collagen-based tissue constructs.

Acoustic radiation forces associated with an ultrasound standing wave field (USWF) can spatially organize particles or cells to defined locations in suspending media (Coakley et al. 1989; Dyson et al. 1974; Gol'dberg 1971; Gor'kov 1962; Gould and Coakley 1974; Whitworth and Coakley 1992). When an ultrasonic pressure wave is incident on an acoustic reflector, the reflected wave interferes with the incident wave resulting in the formation of an USWF. These fields are characterized by areas of maximum pressure, known as the pressure antinodes, as well as areas of minimum pressure, known as the pressure nodes.

When a particle or cell suspension is exposed to an USWF, acoustic radiation forces can actively direct particles or cells to the pressure nodes, resulting in the alignment of particles or cells into bands that are perpendicular to the direction of sound propagation and spaced at half-wavelength intervals (Coakley et al. 1989; Dyson et al. 1974; Gould and Coakley 1974; Whitworth and Coakley 1992).

Acoustic radiation forces that band particles in suspensions exist only during exposure to the USWF. Thus, suspending media that undergo phase conversions from liquid to solid states during USWF exposure have been used to maintain USWF-induced spatial organizations of particles after removal of the sound field (Gherardini et al. 2002; Gherardini et al. 2005; Saito et al. 1998; Saito et al. 1999). In our previous work, acoustic radiation forces associated with USWF were used to non-invasively control the spatial distribution of mouse embryonic fibroblasts within 3D collagen-based engineered tissues (Garvin et al. 2010). The conversion of soluble collagen into a polymerized gel during USWF exposure was used to maintain the spatial organization of cells after exposure. USWF-induced cell alignment increased cell contractility and resulted in enhanced cell-mediated extracellular matrix reorganization (Garvin et al. 2010).

In the current study, we investigated whether USWF-mediated organization of human endothelial cells could enhance endothelial cell function to promote in vitro vascularization of engineered tissue constructs. The formation and maturation of capillary-like sprouts within the polymerized collagen gels was monitored over time and compared to sham-exposed constructs. Our results indicate that USWF-induced endothelial cell aggregation promotes the rapid vascularization of collagen-based hydrogels.

Materials and Methods

Ultrasound exposure chamber

The experimental set-up for USWF exposures has been described (Garvin et al. 2010). The ultrasound source was a 1 MHz unfocused transducer (2.5 cm diameter), mounted at the bottom of a water tank (Fig. 1). A waveform generator (Model 33120A; Hewlett Packard, Palo Alto, CA), radio frequency power amplifier (Model 2100L; ENI, Rochester, NY), and attenuator (Model 837; Kay Elemetrics, Lincoln Park, NJ) were used to create a radio frequency signal to drive the transducer. Samples were contained in the wells of silicone elastomer-bottomed cell culture plates (FlexCell International, Hillsborough, NC). Well diameters were reduced to 1 cm using elastomer molds (Dow Corning, Midland, MI) (Garvin et al. 2010). Previous measurements demonstrated only 0.07 dB of attenuation of ultrasound at 1 MHz due to the Bioflex® plate (Garvin et al. 2010). The sample plates were affixed to a three-axis positioner (Series B4000 Unislide; Velmex, East Bloomfield, NY) to allow precise control over their location in the sound field. The air-media interface above the sample served as the acoustic reflector to generate the USWF within the sample volume.

Acoustic field calibrations, sample holder placement in the USWF, and measurements of the USWF within the sample holders have been described previously (Garvin et al. 2010). Prior to each experiment, the acoustic field was calibrated using a needle hydrophone (Model HNC-0400; Onda, Sunnyvale, CA) under traveling wave conditions. 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). At this axial location, the -6 dB transaxial beamwidth of the sound field was 1.2 cm (Garvin et al. 2010). Using coordinates obtained from the hydrophone measurements, the three-axis positioner was used to place the well bottoms of the cell culture plates near the air-water interface, 12.2 cm from the transducer (Fig. 1A).

Cell-embedded collagen gels

Human umbilical vein endothelial cells (HUVEC; Vec Technologies, Rensselaer, NY; pooled donor) were cultured in MCDB-131 growth medium (MCDB-131 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), L-glutamine (Invitrogen), and ENDO GRO[®] growth factor (Vec Technologies)). Neutralized type-I collagen solutions were prepared on ice by mixing collagen type-I (BD Biosciences, Bedford, MA; from rat tail) with 2X concentrated Dulbecco's modified Eagle's medium (DMEM; Invitrogen) and 1X DMEM such that the final mixture contained 1X DMEM and 0.8 mg/ml collagen (Garvin et al. 2010; Hocking et al. 2000). HUVEC were added to aliquots of the collagen solution at a final concentration of 1×10^6 cell/ml immediately prior to USWF exposure. Experiments were performed using HUVEC between passage 3 and 8.

USWF exposures

Aliquots of collagen/cell solutions were pipetted into each of two wells of the modified BioFlex[®] plate. One sample was exposed to a 1 MHz continuous wave USWF with 0.2 MPa peak pressure amplitude for 15 min at room temperature (Fig. 1B) (Garvin et al. 2010). The other sample was treated exactly as the USWF-exposed sample, but was not exposed to the USWF and served as the sham exposure condition (Fig. 1B). Ultrasound exposure conditions were chosen based on results of our previous studies, and the 15-min exposure duration was sufficient for collagen polymerization (Garvin et al. 2010). Following a 1 hr incubation at 37°C and 5% CO₂, an equal volume of MCDB-131 growth medium supplemented with 20 ng/ml phorbol myristate acetate (Ilan et al. 1998) (PMA; Sigma, St. Louis, MO) was added to each collagen gel. Collagen gels were maintained at 37°C and 5% CO₂ and media were changed daily.

Analysis of cell morphology and sprout formation

At various times, USWF- and sham-exposed collagen gel constructs were examined using an inverted microscope (Carl Zeiss MicroImaging, Thornwood, NY) and photographed using a digital camera (Model Infinity 2; Lumenera, Ottawa, ON, Canada). ImageJ software (NIH, Bethesda, MD) was used to quantify endothelial cell sprout length, which was defined as the length of straight-line vessel segments. Two separate images (fields of view) from each condition (USWF- and sham- exposed) from each of at least three different experiments were used to quantify sprout length on days 1, 4, 6, and 10 after exposure. Thiazolyl blue tetrazolium bromide (MTT) was used to visualize viable endothelial cells within the collagen gel constructs (Mosmann 1983).

Quantification of lumen area

USWF- and sham-exposed collagen gels were incubated at 37°C and 5% CO₂ for either 4 or 10 days and then fixed in 4% paraformaldehyde. Following routine histological processing, gels were embedded in paraffin for sectioning. Hematoxylin and eosin (H&E) staining was performed on 4 µm thick sections. Digital images of gel sections were collected and ImageJ software was used to quantify lumen area on each of 30 sections collected from 3 collagen gels (fabricated on different experimental days, 10 sections/gel) per condition per time point. Collagen gels used for lumen area quantification were step-sectioned to ensure at least 40 µm intervals between measurements.

Immunostaining and microscopy

Multiphoton immunofluorescence microscopy was used to visualize endothelial cell networks within collagen gels. USWF- exposed collagen gels were fixed and permeabilized (Hocking et al. 2000). Antibodies used were: anti-human CD31 monoclonal antibody (clone MEM-05; Invitrogen); anti-human Ki67 monoclonal antibody (BD Biosciences); Alexa

Fluor[®] 594-conjugated goat anti-mouse IgG (Invitrogen). Cell nuclei were labeled with 60 nM 4',6-diamidino-2-phenylindole (DAPI) dilactate (Invitrogen). Collagen gels were examined using an Olympus Fluoview 1000 AOM-MPM microscope equipped with a 25X, 1.05 NA water immersion lens (Olympus, Center Valley, PA). Samples were illuminated with 780 nm light generated by a Mai Tai HP Deep See Ti:Sa laser (Spectra-Physics, Mountain View, CA). The emitted light was detected with a photomultiplier tube using two bandpass filters - one with a 460 nm center wavelength (Filter FF01-460-80; Semrock, Rochester, NY) to visualize DAPI staining and the second with a 609 nm center wavelength (Filter FF01-609/54-25; Semrock) to visualize Alexa Fluor[®] 594 (CD31 or Ki67) staining simultaneously. Images were collected using a CMOS digital camera (Moticam 1000; Motic, Xiamen, China).

Collagen fibers were visualized using second-harmonic generation microscopy (Freund and Deutsch 1986; Roth and Freund 1979; Williams et al. 2005) as described (Garvin et al. 2010). Endothelial cells were simultaneously visualized by exploiting cellular auto-fluorescence (Garvin et al. 2010; Kirkpatrick et al. 2007). ImageJ software was used to measure the angle of collagen fibers relative to the direction of the endothelial cell sprouts in USWF-exposed samples and relative to the image bottom in sham samples. Five separate fields of view were captured in each of two USWF-exposed and two sham-exposed gels from each of three different experiments, and the angle of ten fibers per image near sprouts (in USWF images) or near rounded cells (in sham images) were used to quantify collagen organization (300 fibers total per condition).

Statistical analysis

Unless otherwise noted, all experiments were performed on three separate occasions with at least duplicate USWF- and sham-exposed samples in each experiment. Data are presented as mean $\pm$ SEM. Statistical comparisons between USWF- and sham-exposed conditions were performed using the Students *t*-test for paired samples in GraphPad Prism software (La Jolla, CA). Differences were considered significant for *p* values < 0.05 .

Results

USWF pattern endothelial cells into multicellular planar bands within 3D collagen gels

To demonstrate that acoustic radiation forces associated with an USWF could organize endothelial cells into planar bands within collagen gels, cells were suspended in an unpolymerized collagen type-I solution, and then exposed to a 1 MHz, continuous wave USWF with a peak pressure amplitude of 0.2 MPa. The collagen solution was allowed to polymerize during the 15 min exposure period in order to maintain the banded pattern of cells after removal of the sound field. Multiple bands of endothelial cells were observed throughout the height of 3D collagen gels exposed to the USWF (Fig. 2A; arrows). Adjacent cell bands were separated by the expected half-wavelength distance for a 1 MHz USWF ($\sim 750 \mu\text{m}$ (Garvin et al. 2010)). In contrast, sham-exposed samples were characterized by a random distribution of cells throughout the collagen gel (Fig. 2B). Viewed from the top, each endothelial cell band in USWF-exposed samples was a multicellular planar aggregate of cells (Fig. 2C; arrows), whereas endothelial cells were monodispersed in sham-exposed gels (Fig. 2D). These data show that USWF can organize endothelial cells into multicellular planar bands within 3D collagen gels.

Capillary-like sprouts emerge from USWF-induced cell bands and form anastomosing networks

To assess the effects of USWF exposure on endothelial cell function, USWF- and sham-exposed endothelial cell-embedded collagen gels were examined over time for changes in

cell morphology. Following USWF exposure, endothelial cells were organized into multicellular planar bands (Fig. 3A). In contrast, endothelial cells were observed as single, rounded cells in sham-exposed constructs (Fig. 3B). One day following USWF exposure, multiple endothelial cell sprouts originating from USWF-induced cell banded areas were clearly visible (Fig. 3C; arrow), whereas cells maintained a rounded morphology in sham-exposed gels (Fig. 3D). On day 4, sprouts in USWF-exposed samples increased in length and the formation of branches and interconnections between sprouts was observed (Fig. 3E). At this time point, endothelial cells in sham-exposed samples had just begun to adopt an elongated morphology (Fig. 3F). The elongated cells in sham-exposed samples persisted at day 6 and 10 and exhibited some intercellular connections (Fig. 3H and 3J). In contrast, on day 6, USWF-induced endothelial cell sprouts had formed anastomosing networks with both neighboring sprouts and adjacent cell bands (Fig. 3G). At day 10, the anastomosing networks in USWF-exposed samples had progressed into longer and thicker structures (Fig. 3I).

To directly compare endothelial cell sprout formation and elongation in USWF- and sham-exposed collagen gels, endothelial cell sprout length was measured on days 1, 4, 6, and 10. Approximately 22 hr after ultrasound exposure, endothelial cell sprouts having an average length of $95.0 \pm 10.4 \mu\text{m}$ had formed in USWF-exposed collagen constructs (Fig. 4; Day 1). In contrast, endothelial cell sprouts were absent in sham samples (Fig. 4; Day 1). The length of endothelial cell sprouts within USWF-exposed constructs continued to increase over the course of the 10-day incubation period (Fig. 4). Moreover, sprout length was significantly greater in USWF-exposed versus sham-exposed samples at each time point [Fig. 4; Day 4: $157.2 \pm 8.7 \mu\text{m}$ (USWF) vs. $66.5 \pm 5.0 \mu\text{m}$ (sham); Day 6: $240.9 \pm 6.1 \mu\text{m}$ (USWF) vs. $86.6 \pm 6.8 \mu\text{m}$ (sham); Day 10: $264.9 \pm 14.5 \mu\text{m}$ (USWF) vs. $115.0 \pm 7.4 \mu\text{m}$ (sham)]. These data demonstrate that endothelial cell sprout formation and elongation occur at earlier time points in USWF-exposed collagen gels as compared to sham constructs. Subsequent experiments focused on day 4 and day 10 time points to analyze early and late stages of capillary-like network progression, respectively.

Characterization of USWF-induced endothelial cell networks

To assess cell viability, MTT was added to USWF-exposed and sham samples on day 10. Reduction of MTT by metabolically-active cells results in the intracellular formation of purple formazan crystals (Mosmann 1983). As shown in Figure 5, both USWF- and sham-exposed constructs contained numerous MTT-positive cells, indicating high levels of cell viability. USWF-exposed constructs were characterized by extensive, anastomosing networks containing both long and thick sprouts that were found throughout the volume of the collagen gel (Fig. 5A). In contrast, MTT staining in day 10 sham-exposed constructs was concentrated at the gel periphery and at the bottom of the well, indicating an uneven distribution of cells in the non-patterned, sham-exposed collagen gels. Moreover, no detectable MTT staining was observed within the central portion of the sham-exposed constructs indicating a cell-free gel center (Fig. 5B). These data indicate that USWF exposure of endothelial cell-embedded collagen gels leads to the formation of viable, anastomosing, capillary-like networks throughout the 3D construct.

Histological cross-sections of USWF- and sham-exposed samples were next analyzed for the presence of cell-lined lumen. Lumen-containing, endothelial cell sprouts were observed in USWF-exposed constructs on days 4 and 10 (Fig. 6A and 6D; arrows). Observation of serial sections indicated that lumen-containing sprouts emerged from cell-banded regions (Fig. 6A; dotted line). At day 4 and day 10, the original USWF-induced cell bands were lined with elongated endothelial cells (Fig. 6A and 6D; arrowheads in insets), and the central portion of the cell-banded regions contained apoptotic bodies which gradually dispersed over time (Fig. 6A and 6D). At both early (day 4) and late (day 10) time points, endothelial

cell sprouts with lumens were observed in sham constructs (Fig. 6B and 6E; arrows and insets). However, consistent with data presented in Figure 5, lumen-containing sprouts of sham-exposed constructs were only observed at the gel periphery (Fig. 6B and 6E) and were absent from the gel center (Fig. 6C and 6F). These data indicate that USWF-induced cell patterning results in the formation of lumen-containing, capillary-like sprouts that emerge from cell bands which themselves become lined with elongated endothelial cells to form large vessel-like structures.

To quantify lumen size within USWF- and sham-exposed collagen constructs, lumen areas were measured on day 4 and day 10. USWF- and sham-exposed samples showed a similar distribution of capillary-sized ($0\text{--}500\text{ }\mu\text{m}^2$) and small arteriole-sized ($501\text{--}5,000\text{ }\mu\text{m}^2$) lumen areas on days 4 and 10 (Fig. 6G and 6H). In contrast, large arteriole-sized ($5,001\text{--}50,000\text{ }\mu\text{m}^2$) lumen areas were observed only in USWF-exposed samples at both time points (Fig. 6G and 6H). A larger percentage of vessel lumens were the size of small-arterioles on day 10 compared to day 4 vessels (Fig. 6G and 6H), confirming the observed increase in vessel width with time (Fig. 3). Taken together, these data indicate that USWF exposure results in the formation of vascular-like networks with large arteriole-sized vessels branching into smaller lumen-containing, capillary-like structures.

To confirm that USWF-induced endothelial cell sprouts are multicellular structures and to examine cell-cell adhesion formation, USWF-exposed constructs were stained with an anti-CD31 antibody and co-stained with the nuclear stain, DAPI. At both early (day 4) and late (day 10) time points, multiple, aligned cell nuclei were observed in endothelial cell sprouts within USWF-exposed gels (Fig. 7A and 7B), confirming that these endothelial sprouts were multicellular structures. Additionally, some CD31 staining localized to areas of cell-cell contact (Fig. 7; arrows) indicating that endothelial cells within the multicellular networks formed CD31-containing cell-cell adhesions with neighboring cells.

To visualize proliferating cells, USWF-exposed constructs were stained with an anti-Ki67 antibody; gels were co-stained with DAPI to visualize all cells within the constructs. Only DAPI staining was observed in USWF-exposed collagen gels on day 4 (Fig. 7C), indicating a lack of cell proliferation at this time point. In contrast, endothelial cells in USWF-exposed collagen gels stained positive for Ki67 on day 10, indicating the presence of proliferating cells (Fig. 7D; arrows to pink nuclei). In USWF-exposed constructs, proliferating endothelial cells were observed in sprouts emerging from USWF-induced cell-banded regions as well as in the layer of endothelial cells lining original USWF-induced cell bands (Fig. 7D; dotted line designates original cell band). These data indicate that the progression of endothelial cell sprouts into anastomosing networks is associated with endothelial cell proliferation within the collagen gels.

Collagen fibers surrounding endothelial cell sprouts align in the direction of sprout outgrowth

Sprouting angiogenesis is associated with endothelial cell-mediated extracellular matrix reorganization (Kirkpatrick et al. 2007; Korff and Augustin 1999; Sieminski et al. 2004; Vernon and Sage 1996). In particular, angiogenic sprouts and growing neovessels actively remodel surrounding collagen fibers resulting in collagen fiber alignment and condensation around the sprouting neovessels (Kirkpatrick et al. 2007; Korff and Augustin 1999). To examine the organization of collagen fibers within our endothelial cell-embedded collagen constructs, second-harmonic generation microscopy was used to visualize collagen fibers in USWF- and sham-exposed samples. At the onset of capillary sprout formation in USWF-exposed constructs (day 1), elongated collagen fibers were aligned in the direction of sprout outgrowth (Fig. 8A; arrow). Collagen fiber alignment extended into the collagen matrix well beyond the tip of the sprout (Fig. 8A). In contrast, collagen fibers in sham-exposed

constructs were organized randomly throughout the collagen gel (Fig. 8B). At day 4, fibrillar collagen was condensed along endothelial cell sprouts in USWF- (Fig. 8C; arrow) and sham-exposed (Fig. 8D; arrow) constructs, as seen by the bright second-harmonic generation signal at the sprout edge (Kirkpatrick et al. 2007). Additionally, alignment of collagen fibers at sprout tips was present at day 4 in both USWF- (Fig. 8C) and sham-exposed (Fig. 8D) samples. At day 10, the condensation of collagen to endothelial cell sprout edges became more apparent in both USWF- (Fig. 8E; arrows) and sham-exposed (Fig. 8F; arrow) collagen constructs. Observations in three-dimensions above and below the endothelial cell sprouts presented in Figures 8E and 8F revealed that the closely associated collagen fibers were aligned and condensed along the length of sprouts in both USWF- (Fig. 8G) and sham-exposed samples (Fig. 8H). These data demonstrate that USWF-exposed endothelial cells remodel the surrounding collagen matrix in a manner that is characteristic of collagen reorganization observed during natural capillary sprouting.

To quantify collagen fiber orientation within USWF- and sham-exposed constructs one day post-exposure, the angle of collagen fibers was measured relative to the sprout axis in USWF-exposed samples and relative to the horizontal image bottom in sham-exposed constructs. Collagen fibers near endothelial cell sprouts of USWF-exposed constructs were reorganized in the direction of sprout outgrowth (Fig. 8I). In contrast, there was no preferred direction of collagen fibril orientation near rounded cells in sham constructs (Fig. 8J). These data demonstrate that cell-mediated reorganization of extracellular matrix collagen into aligned fibrils in the direction of endothelial cell sprout propagation occurs at early time points in USWF-exposed constructs.

Discussion

In this study, we utilized USWF technology to rapidly fabricate vascularized 3D collagen-based, engineered tissue constructs in vitro. USWF-induced cell banding within collagen gels resulted in the formation of ~100 μ m-long endothelial cell sprouts and co-aligned collagen fibers within 1 day of ultrasound exposure. Within 6–10 days, the endothelial cell sprouts had grown into anastomosing, vascular networks that were found throughout the entire volume of the 3D collagen gel. USWF-exposed collagen constructs contained endothelial cell networks with large, arteriole-sized lumen areas that branched into smaller, capillary-sized, lumen-containing structures, indicating the formation of a complex tree-like network within these collagen constructs. In contrast, endothelial cell sprout formation in sham-exposed constructs was delayed until day 4; network structures found within the sham-exposed samples were absent from gel centers and lacked the tree-like complexity formed within USWF-exposed constructs.

Cell migration, proliferation, and extracellular matrix remodeling are three essential components of neovessel formation in vivo (Risau 1997). USWF-exposed constructs contained proliferating endothelial cells after 10 days in culture. In contrast, proliferating cells were not observed in day 4 collagen gel constructs. Thus, the initial increase in sprout length in USWF-exposed collagen gels was likely due to endothelial cell migration out of the banded areas without a significant contribution from cell proliferation. Extensive remodeling of collagen fibers into elongated, aligned fibrils was observed on day 1 in USWF-exposed constructs. Capillary formation in vitro has been shown to occur along tracks of aligned collagen fibers (Kirkpatrick et al. 2007; Korff and Augustin 1999). Therefore, the early cell-mediated collagen fibril reorganization observed in our USWF-exposed samples is consistent with the rapid migration of endothelial cell sprouts from banded areas along similarly aligned collagen fibers. Additionally, collagen condensation around capillaries is associated with sprout maturation (Kirkpatrick et al. 2007). Thus, the cell-mediated collagen condensation observed in USWF-exposed gels at days 4 and 10 is

consistent with the progression of the endothelial cell sprouts into more mature structures. The presence of proliferating cells at day 10 suggests that increases in sprout length and lumen diameter from day 4 to day 10 were due, at least in part, to cell proliferation. Taken together, these data provide evidence that USWF-mediated organization of endothelial cells into planar bands is sufficient to initiate a cascade of events similar to that which controls capillary formation in vivo (Folkman and Shing 1992; Risau 1997).

The formation of multicellular, planar aggregates of endothelial cells using USWF resulted in the formation of a vascular-like network throughout the entire collagen gel volume. By day 4, USWF-induced cell bands were lined with elongated endothelial cells, effectively producing vessels with large lumen areas from which smaller, lumen-containing, capillary-like structures emerged. Endothelial cells within the interior of the cell bands likely underwent apoptosis (Korff and Augustin 1998), and apoptotic bodies cleared from the lumen areas with time. These results, coupled with the finding that cell-cell adhesions are formed in endothelial cell-lined sprouting structures, suggest that USWF technology may be used to fabricate 3D constructs that contain vascular channels throughout the gel to accommodate unrestricted blood perfusion upon implantation. Similarly, immediate perfusion of USWF-fabricated tissue constructs in vivo could be established with surgical anastomosis of the large lumen areas to host vasculature upon implantation (Rouwkema et al. 2008).

In summary, our results demonstrate that non-invasive organization of endothelial cells using USWF accelerates the formation of capillary-like sprouts compared with sham-exposed collagen gels, and results in the maturation of sprouts into lumen-containing, branching networks throughout the complete volume of the collagen construct. Ultrasound technologies are non-destructive, non-ionizing, inexpensive, and can be adapted to a wide variety of fabrication processes. Design of USWF, by choice of ultrasound frequency and/or use of multiple transducer geometries, can produce more complex cell patterns within hydrogels. Thus, USWF technologies provide a novel approach to vascularize large 3D engineered tissues in vitro.

Acknowledgments

This work was supported in part by Grants EB008368 and EB008996 from the National Institutes of Health. We thank Dr. Karl Kasischke and Gheorghe Salahura (Multiphoton Core Facility, University of Rochester) for assistance with multiphoton microscopy.

References

- Alajati A, Laib AM, Weber H, Boos AM, Bartol A, Ikenberg K, Korff T, Zentgraf H, Obodozie C, Graeser R, Christian S, Finkenzeller G, Stark GB, Heroult M, Augustin HG. Spheroid-based engineering of a human vasculature in mice. *Nat Methods*. 2008; 5:439–445. [PubMed: 18391960]
- Atala A, Bauer SB, Soker S, Yoo JJ, Retik AB. Tissue-engineered autologous bladders for patients needing cystoplasty. *Lancet*. 2006; 367:1241–1246. [PubMed: 16631879]
- Black AF, Berthod F, L'heureux N, Germain L, Auger FA. In vitro reconstruction of a human capillary-like network in a tissue-engineered skin equivalent. *FASEB J*. 1998; 12:1331–1340. [PubMed: 9761776]
- Chen RR, Silva EA, Yuen WW, Mooney DJ. Spatio-temporal VEGF and PDGF delivery patterns blood vessel formation and maturation. *Pharm Res*. 2007; 24:258–264. [PubMed: 17191092]
- Chen X, Aledia AS, Ghajar CM, Griffith CK, Putnam AJ, Hughes CCW, George SC. Prevascularization of a fibrin-based tissue construct accelerates the formation of functional anastomosis with host vasculature. *Tissue Eng Part A*. 2009; 15:1363–1371. [PubMed: 18976155]
- Chung C, Burdick JA. Engineering cartilage tissue. *Adv Drug Deliv Rev*. 2008; 60:243–262. [PubMed: 17976858]

- Coakley WT, Bardsley DW, Grundy MA, Zamani F, Clarke DJ. Cell manipulation in ultrasonic standing wave fields. *J Chem Tech Biotechnol.* 1989; 44:43–62.
- Dyson M, Pond JB, Woodward B, Broadbent J. The production of blood cell stasis and endothelial cell damage in the blood vessels of chick embryos treated with ultrasound in a stationary wave field. *Ultrasound Med Biol.* 1974; 1:133–148. [PubMed: 4372758]
- Folkman J, Hochberg M. Self-regulation of growth in three dimensions. *J Exp Med.* 1973; 138:745–753. [PubMed: 4744009]
- Folkman J, Shing Y. Angiogenesis. *J Biol Chem.* 1992; 267:10931–10934. [PubMed: 1375931]
- Freund I, Deutsch M. Second-harmonic microscopy of biological tissue. *Opt Lett.* 1986; 11:94–96. [PubMed: 19730544]
- Garvin KA, Hocking DC, Dalecki D. Controlling the spatial organization of cells and extracellular matrix proteins in engineered tissues using ultrasound standing wave fields. *Ultrasound Med Biol.* 2010; 36:1919–1932. [PubMed: 20870341]
- Gherardini L, Radel S, Sielemann S, Doblhoff-Dier O, Groschl M, Benes E, McLoughlin AJ. A study of the spatial organisation of microbial cells in a gel matrix subjected to treatment with ultrasound standing waves. *Bioseparation.* 2002; 10:153–162. [PubMed: 12233739]
- Gherardini L, Cousins CM, Hawkes JJ, Spengler J, Radel S, Lawler H, Devic-Kuhar B, Groschl M, Coakley WT, McLoughlin AJ. A new immobilisation method to arrange particles in a gel matrix by ultrasound standing waves. *Ultrasound Med Biol.* 2005; 31:261–272. [PubMed: 15708466]
- Gol'dberg, ZA. Radiation forces acting on a particle in a sound field. In: Rozenberg, LD., editor. *High Intensity Ultrasonic Fields.* New York: Plenum Press; 1971. p. 109–117.
- Gor'kov LP. On the forces acting on a small particle in an acoustical field in an ideal fluid. *Sov Phys Dokl.* 1962; 6:773–775.
- Gould, RK.; Coakley, WT. The effects of acoustic forces on small particles in suspension. In: Bjorno, L., editor. *Finite amplitude wave effects in fluids: Proceedings of the 1973 Symposium.* Guildford: IPC Science and Technology Press Ltd; 1974. p. 252–257.
- Griffith CK, Miller C, Sainson RCA, Calvert JW, Jeon NL, Hughes CCW, George SC. Diffusion limits of an in vitro thick prevascularized tissue. *Tissue Eng.* 2005; 11:257–266. [PubMed: 15738680]
- Griffith LG, Naughton G. Tissue engineering-current challenges and expanding opportunities. *Science.* 2002; 295:1009–1014. [PubMed: 11834815]
- Hocking DC, Sottile J, Langenbach KJ. Stimulation of integrin-mediated cell contractility by fibronectin polymerization. *J Biol Chem.* 2000; 275:10673–10682. [PubMed: 10744764]
- Ilan N, Mahooti S, Madri JA. Distinct signal transduction pathways are utilized during the tube formation and survival phases of in vitro angiogenesis. *J Cell Sci.* 1998; 111:3621–3631. [PubMed: 9819353]
- Khademhosseini J, Vacanti J, Langer R. Progress in tissue engineering. *Sci Am.* 2009; 300:64–71. [PubMed: 19438051]
- Kirkpatrick ND, Andreou S, Hoying JB, Utzinger U. Live imaging of collagen remodeling during angiogenesis. *Am J Physiol Heart Circ Physiol.* 2007; 292:H3198, H3206. [PubMed: 17307995]
- Korff T, Augustin HG. Integration of endothelial cells into multicellular spheroids prevents apoptosis and induces differentiation. *J Cell Biol.* 1998; 143:1341–1352. [PubMed: 9832561]
- Korff T, Augustin HG. Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. *J Cell Sci.* 1999; 112:3249–3258. [PubMed: 10504330]
- Korff T, Kimmina S, Martiny-Baron G, Augustin HG. Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J.* 2001; 15:447–457. [PubMed: 11156960]
- Langer R, Vacanti JP. Tissue engineering. *Science.* 1993; 260:920–926. [PubMed: 8493529]
- Laschke MW, Harder Y, Amon M, Martin I, Farhadi J, Ring A, Torio-Padron N, Schramm R, Rucker M, Junker D, Haufel JM, Carvalho C, Heberer M, Germann G, Vollmar B, Menger MD. Angiogenesis in tissue engineering: breathing life into constructed tissue substitutes. *Tissue Eng.* 2006; 12:2093–2104. [PubMed: 16968151]

- Levenburg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC, Marini R, vanBlitterswijk CA, Mulligan RC, D'Amore PA, Langer R. Engineering vascularized skeletal muscle tissue. *Nat Biotechnol.* 2005; 23:879–884. [PubMed: 15965465]
- Lokmic Z, Mitchell GM. Engineering the microcirculation. *Tissue Eng Part B Rev.* 2008; 14:87–103. [PubMed: 18454636]
- Mikos AG, Leite SM, Vacanti JP, Langer R. Prevascularization of porous biodegradable polymers. *Biotechnol Bioeng.* 1993; 42:716–723. [PubMed: 18613104]
- Mooney DJ, Mikos AG. Growing new organs. *Sci Am.* 1999; 280:60–65. [PubMed: 10201117]
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; 65:55–63. [PubMed: 6606682]
- Pandit AS, Feldman DS, Caulfield J, Thompson A. Stimulation of angiogenesis by FGF-1 delivered through a modified fibrin scaffold. *Growth Factors.* 1998; 15:113–123. [PubMed: 9505167]
- Priya SG, Jungvid H, Kumar A. Skin tissue engineering for tissue repair and regeneration. *Tissue Eng Part B Rev.* 2008; 14:105–118. [PubMed: 18454637]
- Richardson TP, Peters MC, Ennett AB, Mooney DJ. Polymeric system for dual growth factor delivery. *Nat Biotechnol.* 2001; 19:1029–1034. [PubMed: 11689847]
- Risau W. Mechanisms of angiogenesis. *Nature.* 1997; 386:671–674. [PubMed: 9109485]
- Roth S, Freund I. Second harmonic generation in collagen. *J Chem Phys.* 1979; 70:1637–1643.
- Rouwkema J, Rivron NC, vanBlitterswijk CA. Vascularization in tissue engineering. *Trends Biotechnol.* 2008; 26:434–441. [PubMed: 18585808]
- Saito M, Daian T, Hayashi K, Izumida S. Fabrication of a polymer composite with periodic structure by the use of ultrasonic waves. *J Appl Phys.* 1998; 83:3490–3494.
- Saito M, Itagaki K, Hayashi K, Tsubata K. Composite materials with ultrasonically induced layer or lattice structure. *Jpn J Appl Phys.* 1999; 38:3028–3031.
- Sieminski AL, Hebbel RP, Gooch KJ. The relative magnitudes of endothelial force generation and matrix stiffness modulate capillary morphogenesis in vitro. *Exp Cell Res.* 2004; 297:574–584. [PubMed: 15212957]
- Tremblay P-L, Hudon V, Berthod F, Germain L, Auger F. Inosculation of tissue-engineered capillaries with the host's vasculature in a reconstructed skin transplanted on mice. *Am J Transplant.* 2005; 5:1002–1010. [PubMed: 15816880]
- Vernon RB, Sage EH. Contraction of fibrillar type I collagen by endothelial cells: a study in vitro. *J Cell Biochem.* 1996; 60:185–197. [PubMed: 8655629]
- Wenger A, Stahl A, Weber H, Finkenzeller G, Augustin HG, Stark GB, Kneser U. Modulation of in vitro angiogenesis in a three-dimensional spheroidal coculture model for bone tissue engineering. *Tissue Eng.* 2004; 10:1536–1547. [PubMed: 15588413]
- Whitworth G, Coakley WT. Particle column formation in a stationary ultrasonic field. *J Acoust Soc Am.* 1992; 91:79–85.
- Williams RM, Zipfel WR, Webb WW. Interpreting second-harmonic generation images of collagen I fibrils. *Biophys J.* 2005; 88:1377–1386. [PubMed: 15533922]

Fig. 1.

Schematics of experimental set-up and USWF-induced cell banding. A 1 MHz, 2.5 cm diameter unfocused transducer was positioned at the bottom of a plastic exposure tank filled with degassed, deionized water. The sound field propagated towards the cell suspension contained within wells of acoustically transparent silicone elastomer-bottomed cell culture plates. The air-media interface above the sample served as the acoustic reflector to generate an USWF within the sample volume. (A) Cells in suspension were homogeneously distributed prior to USWF exposure. (B) USWF exposure resulted in the formation of multicellular planar bands of cells at pressure nodal planes separated by cell-free internodal spaces. Sham samples, treated identically to USWF-exposed samples but not exposed to the sound field, maintained a random cell distribution.

Fig. 2.

Multicellular bands of endothelial cells form within 3D collagen gels following exposure to USWF. Endothelial cells were suspended in an unpolymerized collagen type-I solution and were exposed to a 1 MHz continuous wave USWF with 0.2 MPa peak pressure amplitude for 15 min. Sham samples were treated identically as USWF-exposed samples but were not subjected to the USWF. Representative phase-contrast images, taken 1 hr after USWF exposure, show the spatial distribution of cells within USWF-exposed (A, C) and sham-exposed (B, D) collagen gels that were examined from both the side (A, B) and top views (C, D). Arrows, endothelial cell band. Scale bars, 200 μ m.

Fig. 3.

Time course of neovessel formation following USWF exposure. Endothelial cells were suspended in an unpolymerized collagen type-I solution and were exposed to an USWF (1 MHz, continuous wave, 0.2 MPa peak pressure amplitude, 15 min duration) to promote the formation of multicellular bands of cells within collagen gels. Top view, phase-contrast images of USWF-exposed (A, C, E, G, I) and sham-exposed (B, D, F, H, J) collagen constructs were collected at day 0 (the day of USWF exposure; A, B), day 1 (C, D), day 4 (E, F), day 6 (G, H), or day 10 (I, J). The white arrow highlights the progression of one USWF-induced endothelial cell sprout through time. Scale bar, 100 μ m.

Fig. 4. USWF exposure accelerates neovessel formation and elongation within collagen gels. The length of endothelial cell sprouts in USWF- and sham-exposed collagen constructs was quantified as described in the Materials and Methods section using images collected on days 1, 4, 6, and 10. Elongated cells in sham-constructs were considered endothelial cell sprouts. Data are presented as average endothelial cell sprout length $\pm$ SEM ($n = 3$). *Significantly different from corresponding sham-exposed condition ($p < 0.05$).

Fig. 5.

Anastomosing networks form throughout USWF-exposed collagen constructs. Endothelial cells were suspended in unpolymerized collagen type-I solutions and exposed to an USWF as described in the legend to Fig 2. Following a 10-day incubation, MTT was added to USWF- (A) and sham-exposed (B) constructs to visualize viable cells (dark stain). Representative top view images are shown. Scale bar, 200 μ m.

Fig. 6. USWF-induced neovessels exhibit large lumen areas with smaller, branching lumen-containing capillaries. Endothelial cells were suspended in unpolymerized collagen type-I solutions and exposed to an USWF as described in the legend to Fig 2. Following a 4-day or 10-day incubation, USWF- and sham-exposed constructs were processed for histological sectioning. Representative images of H&E stained gel cross-sections are shown for USWF-exposed (A, D) and sham-exposed (B, C, E, F) constructs on day 4 (A, B, C) and day 10 (D, E, F). Panels B and E are representative images of the sham-exposed collagen gel periphery, while panels C and F are representative images of the cell-free central region of the sham-exposed gels. Insets in panels A, B, D, and E are higher magnification images of boxed regions to show elongated endothelial cells lining lumen areas. Arrows, endothelial cell sprouts; arrowheads in insets, elongated endothelial cells lining cell-banded regions; dotted line, lumen-containing sprout originated at cell-banded region. Scale bar, 100 μm . Inset

scale bar, 20 μm . (G, H) Lumen areas were quantified from images of day 4 (G) and day 10 (H) collagen gel cross-sections and then grouped into categories representing capillary-sized (0–500 μm^2), small arteriole-sized (501–5,000 μm^2), and large arteriole-sized (5,001–50,000 μm^2) lumen areas. Data are presented as average percentage of total lumen $\pm$ SEM ($n = 3$). *Significantly different from the sham condition within the same time point and size category ($p < 0.05$).

Fig. 7.

CD31-containing cell-cell adhesions form in multicellular, USWF-induced neovessels. Endothelial cells were suspended in unpolymerized collagen type-I solutions and exposed to an USWF as described in the legend to Fig 2. Following a 4-day or 10-day incubation, USWF-exposed constructs were processed for immunofluorescence microscopy. (A, B) CD31 was visualized by staining with a mouse anti-human CD31 monoclonal antibody (red). Cell nuclei were visualized by co-staining with DAPI (blue). Multiphoton microscopy was used to collect top view images along the z-axis in 1 μm slices. Images were then projected onto the z-plane using ImageJ software. Representative z-stack images are shown for day 4 (A) and day 10 (B) USWF-exposed collagen gels. Arrows, CD31 within areas of cell-cell contact. Scale bars, 50 μm . (C, D) Ki67 was visualized using a mouse anti-human Ki67 monoclonal antibody (red). Cell nuclei were visualized by co-staining with DAPI (blue). Multiphoton microscopy was used to collect top view images along the z-axis in 1

μm slices. Representative slices are shown for day 4 (C) and day 10 (D) USWF-exposed collagen gels. Arrows, proliferating endothelial cells; dotted line, USWF-induced cell band border. Scale bars, 50 μm .

Fig. 8.

Collagen tracts extend from USWF-induced endothelial cell bands. Endothelial cells were suspended in unpolymerized collagen type-I solutions and exposed to an USWF as described in the legend to Fig 2. Following a 1-day, 4-day, or 10-day incubation, second-harmonic generation microscopy was used to visualize collagen type-I fibers (red) within USWF- and sham-exposed constructs. Endothelial cells (green) were visualized simultaneously using intrinsic cellular autofluorescence. Top view images were collected along the z-axis in 1 μ m slices. Representative slices are shown for day 1 (A, B), day 4 (C, D), and day 10 (E, F) constructs that were either USWF- (A, C, E) or sham-exposed (B, D, F). For panels G and H, day 10 image slices were projected onto the z-plane using ImageJ software. Representative z-stack images for day 10 USWF- (G) and sham-exposed (H) samples show condensed, aligned collagen fibers surrounding the entire length of the

endothelial cell sprouts presented in panels E and F. Arrows, endothelial cell sprouts. Scale bar, 50 μm . (I, J) The angle of collagen fibers was quantified using day 1 images of USWF- and sham-exposed constructs. Fiber angle was measured relative to the sprouting direction in USWF-exposed constructs and relative to the image bottom in sham samples (yellow lines in insets). Data are presented as histograms to display the frequency of occurrence of fiber angles in USWF-(I) and sham-exposed (J) collagen gels.