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The effect of bioreactor induced vibrational stimulation on extracellular matrix production from human derived fibroblasts

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ABSTRACT

To study the affect of mechanical stimuli on human laryngeal fibroblasts, we developed bioreactors capable of vibrating cell seeded substrates at frequencies and displacements comparable to measured phonation values in human subjects. In addition, we developed a means of harvesting the secreted matrix as a bulk biomaterial by removing the polymer foam using an organic solvent. Using the system human derived laryngeal fibroblasts were subjected to vibrational stimuli (100 Hz) for 1–21 days. Following mechanical conditioning, extracellular matrix and matrix related gene expression, cytokine production, matrix protein accumulation, and construct material properties were assessed with DNA microarray, enzyme linked immunosorbent, indirect immunofluorescent, and uni-axial tensile assays respectively. The results show that vocal fold-like vibrational stimuli is sufficient to influence the expression of several key matrix and matrix related genes, enhance the secretion of the profibrotic cytokine TGFβ1, increase the accumulation of the extracellular matrix proteins, fibronectin and collagen type 1, as well as enhance construct stiffness compared to non-stimulated controls. Our results demonstrate that high frequency substrate vibration, like cyclic strain, can accelerate matrix deposition from human derived laryngeal fibroblasts. The study supports the notion that preconditioning regimens using human cells may be useful for producing cell derived biomaterials for therapeutic application.

1. Introduction

Injuries to the vocal chords can have devastating consequences both professionally and socially. In challenging cases where voice therapy or traditional surgical practices are not successful in restoring speech, the repair or replacement of the damaged tissue with a graft is needed. Current surgical repair techniques utilizing various synthetic materials have their limitations [1,2], which is why the development of an engineered replacement tissue deserves consideration. We believe that the development of such a graft will be facilitated by an understanding of the factors that influence tissue assembly *in vitro*. One factor, mechanical stimulation, has been shown to influence *in vitro* matrix production and has been used to accelerate matrix deposition in various tissue engineering strategies.

Available evidence indicates that fibroblasts contain the molecular machinery needed to convert mechanical stimuli into responses that influence tissue remodeling [3]. Of the diverse responses identified, those factors that influence tissue assembly are of particular interest to the tissue engineering community. Specifically, the fact that mechanical regulation of extracellular matrix homeostasis and matrix related cytokine production have been observed in a variety of cell types by mechanical stimuli points to the possible benefits of developing mechanically stimulated *invitro* tissue assembly methods [4–7]. Indeed, engineered vascular structures and connective tissues, employing pulsatile flow and cyclic stretch bioreactors, respectively, have been reported [8,9]. We, therefore, suspect that the repair of damaged vocal chords also may benefit from approaches that cultivate tissue within a bioreactor that mimics all or part of the mechanical environment that the vocal chords experience during normal use.

The site of vocal transduction is the vocal fold, a highly dynamic tissue exposed to low frequency stretching and high frequency vibration during vocalization. To sustain phonation stresses the mechanical properties of the vocal folds must be maintained at appropriate levels. To properly regulate the mechanical properties, we hypothesize that vocal fold fibroblasts sense the mechanical stimulation generated during speech and translate this information into adaptive responses such as an increase or decrease in matrix production as a means of maintaining homeostasis.

Previously, we have reported that laryngeal fibroblast cell seeded scaffolds conditioned with low frequency cyclic strain increase matrix synthesis and the expression of several key matrix related gene products [10]. After one week of repeated stimulation,

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strain conditioned laryngeal fibroblasts show increased collagen accumulation and were significantly stiffer than non-mechanically stimulated controls. To build upon these observations, the present studies were designed to determine whether the mechanical stimulation encountered during substrate vibration also acts as a stimulus for matrix production. Here we report on the design, characterization and use of bioreactors to study the influence of high frequency substrate vibration on human laryngeal fibroblasts extracellular matrix production *in vitro*.

2. Materials and methods

2.1. Vibrational bioreactor

To study the affect of high frequency, oscillating mechanical stimulation on cell behavior a bioreactor was designed, fabricated, and characterized which contains different sample holders (Fig. 1). In each case, a low mass substrate was displaced back and forth along a horizontal axis from an equilibrium position by an electromagnetic voice coil actuator (BEI Kimco, Vista, CA) powered by a sinusoidal voltage supplied and controlled by an analog output board and control software (Labview, National Instruments, Austin, TX) running on a PC. The software allows the user to control the frequency of voltage change, the displacement or distance of the vibration cycle and the duration of the duty cycle. The voice coil actuator was attached to a holder that contains a standard tissue culture multi-well plate (Fig. 1A), or a custom fabricated single sample culture well (dia. = 25 mm) (Fig. 1B). The base of the single well (Fig. 1B), was either a planar solid sheet of polydimethylsiloxane (PDMS) or a planar open celled polyurethane foam sheet. These were press fit into a holder as shown in (Fig. 2). In all three different substrates were used.

To measure the degree of substrate displacement as a function of frequency of applied voltage, we used fluorescent microspheres (Molecular Probes, OR) that were attached to the surface of the various substrates. When viewed microscopically, during a typical sinusoidal voltage cycle the spheres were observed as bright linear streaks, which traced the back and forth motion of each substrate (Fig. 3A). Using image analysis software (ImagePro, Media Cybernetics, MD), the displacement was measured as a function of frequency of applied voltage (Hz) as the distance between the peak heights of the maximum relative fluorescence intensity (Fig. 3B,C; $N = 5$, each condition). In addition, the maximum acceleration was calculated as the second derivative assuming a sinusoidal displacement pattern with time (Fig. 3C).

2.2. Three-dimensional substrates

Three-dimensional substrates were prepared as previously described [10]. Briefly, polyurethane pellets (Tecoflex SG-80A, Thermedics; 6.75 G) were dissolved in 39.1 ml of dimethylacetamide (DMAC) overnight at 60 °C. Pluronic 10R5 (BASF, 18.95 ml) was added dropwise to the solution and thoroughly mixed. The polymer solution was lowered to 46 °C, pipetted into delrin molds 0.5 mm deep, 40 mm wide and 40 mm in length. The polymer containing molds were cooled for 2.5 min in a dry-ice/ethanol bath, and precipitated in 25 °C distilled water overnight. The substrates were gently pulled out of the molds which yielded foam sheets. These were rinsed in DI water for 48 h, frozen to −80 °C, and lyophilized. Lyophilized sheets (40 × 40 × 0.5 mm) were cold ethylene oxide sterilized at the University of Utah Hospital sterilization facility and stored at room temperature until used.

2.3. Experimental approach overview

The experiments were designed to assess cellular responses that could influence tissue assembly. Specific experiments included (1) assessment of matrix and matrix related gene expression using a DNA microarray; (2) measurement of soluble cytokine levels in conditioned media using enzyme linked immunosorbent assays (ELISA), (3) evaluation of matrix protein deposition on conditioned samples using indirect immunofluorescent labeling, and (4) measurement of conditioned sample material properties of harvested cell-derived extracellular matrix using uni-axial tensile testing.

2.4. Cell culture conditions

For all experiments, human laryngeal fibroblasts that were isolated from a single patient from passage 4–6 were used. Frozen cells were thawed, plated in T-175 flasks, and grown to confluence in DMEM/F12 with 10% FBS and 25 μg/ml gentamicin. Confluent flasks were disassociated with 0.25% trypsin and 1 mM EDTA in HBSS, centrifuged, and resuspended in serum free DMEM/F12. Cells were plated onto either 6 well plates (DNA microarray), planar PDMS membranes (cytokine levels), or porous polyurethane scaffolds (matrix accumulation and mechanical testing). The fabrication of the polyurethane scaffolds has been described previously [11]. To facilitate cell attachment to the PDMS membranes and polyurethane scaffolds, the substrates were immersed overnight in a solution of 20 μg/ml bovine fibronectin in phosphate buffer saline (PBS), and rinsed with sterile distilled water (DI). Once plated, cells were maintained in serum free Sato media [12] for 48 h prior to conditioning. Under these conditions, both the six well plate and PDMS planar substrates were confluent. Similarly, cells were densely distributed throughout the porous scaffolds. Prior to conditioning, serum free media was changed to DMEM/F12 supplemented with 1 mM ascorbic acid (Sigma) and 10% FBS.

2.5. Gene expression analysis

With the bioreactor configured for a commercially available six well plate (Fig. 1A), a confluent six well plate of human laryngeal fibroblasts was vibrated at 100 Hz, a frequency that falls within the male speaking range [13], for 14 min distributed over a 6 h period. The stimulation pattern consisted of 1.5 s of vibration followed by a 30 s rest period. This was repeated for 6 h and then followed by 18 h of rest. The 6 h conditioning period was repeated once for 3 days. A static confluent six well plate that was not vibrated was maintained as a control condition. At the completion of the experiment, total RNA was isolated from the vibrated and static control samples using Trizol reagent. Sample mRNA was amplified into aRNA using the RiboAmp (Molecular Devices, Sunnyvale, CA) amplification process. Cy3 and Cy5 labeled cDNA was reverse transcribed from vibrated and control sample aRNA, respectively by the University of Utah DNA microarray facility. Labeled cDNA was hybridized to a microarray slide containing 9600 human gene clones in duplicate. Fluorescence ratios (green:red) were used to quantify differences in gene expression between the vibrated and non-vibrated controls. The mean expression ratio and standard deviation was calculated for the population of genes tested. Matrix and matrix related genes of interest with expression ratios two standard deviations above the mean were identified and were reported.

2.6. Measurement of media cytokine levels

With the bioreactors converted to accommodate a single culture well (Fig. 1B), four separate samples with PDMS membrane bases were vibrated at 100 Hz. The conditioning pattern consisted of 1 s of vibration followed by a 2 s static period, repeated for 6 h, which was followed by an 18 h rest period. After 24 h, conditioned media from the vibrated and static control cultures ($n = 4$ samples/condition) was collected. Following collection, cells were fixed, permeabilized, and cell nuclei were stained with DAPI. Fifteen macroscopic fields (200×) were used to estimate the cell density and total cell number for each culture well.

Media levels for the profibrotic cytokine transforming growth factor beta1 (TGFβ-1) were determined using a sandwich ELISA. Ninety-six well high protein

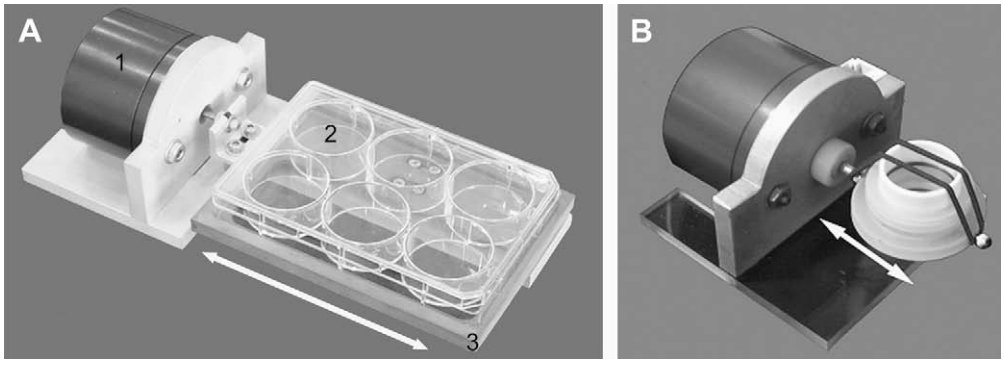

Fig. 1. Bioreactor for high frequency substrate vibration for use with commercial multi-well plate (A) showing (1) the voice coil actuator (2) 6 well culture plate, and (3) base. With a configuration change, the bioreactor can also accommodate a custom designed low mass culture well (B).