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Cyclic stretch enhances reorientation and differentiation of 3-D culture model of human airway smooth muscle

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ABSTRACT

Activation of airway smooth muscle (ASM) cells plays a central role in the pathophysiology of asthma. Because ASM is an important therapeutic target in asthma, it is beneficial to develop bioengineered ASM models available for assessing physiological and biophysical properties of ASM cells. In the physiological condition *in vivo*, ASM cells are surrounded by extracellular matrix (ECM) and exposed to mechanical stresses such as cyclic stretch. We utilized a 3-D culture model of human ASM cells embedded in type-I collagen gel. We further examined the effects of cyclic mechanical stretch, which mimics tidal breathing, on cell orientation and expression of contractile proteins of ASM cells within the 3-D gel. ASM cells in type-I collagen exhibited a tissue-like structure with actin stress fiber formation and intracellular Ca^{2+} mobilization in response to methacholine. Uniaxial cyclic stretching enhanced alignment of nuclei and actin stress fibers of ASM cells. Moreover, expression of mRNAs for contractile proteins such as α-smooth muscle actin, calponin, myosin heavy chain 11, and transgelin of stretched ASM cells was significantly higher than that under the static condition. Our findings suggest that mechanical force and interaction with ECM affects development of the ASM tissue-like construct and differentiation to the contractile phenotype in a 3-D culture model.

1. Introduction

Contraction of airway smooth muscle (ASM) plays a central role in airway narrowing in asthma. Increased ASM mass due to cell proliferation, hypertrophy, and migration is involved in the mechanism of pathophysiology of airway remodeling. Therefore, ASM is an important therapeutic target for airway diseases, specifically asthma and chronic obstructive pulmonary disease [1]. In order to uncover mechanisms underlying activation of ASM cells, two-dimensional (2-D) *in vitro* cultures of ASM cells have widely been used due to difficult availability of human ASM tissue samples [2–4]. However, ASM cells *in vivo* exist as a part of complex three-dimensional (3-D) structures with the extracellular matrix (ECM). Within the airway wall, ASM exists as an aligned population that wraps around the bronchiole in a helical fashion *in vivo* [5,6]. Due to this unique arrangement, the angle of orientation and cell alignment are major factors that determine the phenotypes and properties of ASM cells [7]. Therefore, development of bioengineered 3-D models of ASM tissues is warranted to assess functional properties for pharmacological and biophysical studies [8–10].

The lungs and airways are continually exposed to mechanical forces such as shear stress, compression, and stretch during tidal breathing and pulmonary circulation *in vivo*. These mechanical stresses are involved in the mechanisms underlying the normal physiology and development of the respiratory system and pathogenesis of asthma [11]. In 2-D culture models of ASM cells, cyclic stretch induces cell alignment perpendicular to the stretch axis with reorganization of the cytoskeleton [12,13]. However, the roles of mechanical stresses in the development of bioengineered 3-D models of ASM tissues are not known.

This study was designed to develop a 3-D model of ASM. For this purpose, human ASM cells were embedded in a collagen gel [9,10]. We further examined the effects of cyclic mechanical stretch, which mimics tidal breathing, on the regulation of cell orientation, formation of stress fibers, and phenotype. We postulated that when cultured three-dimensionally within collagen gel with cyclic stretch, ASM cells develop

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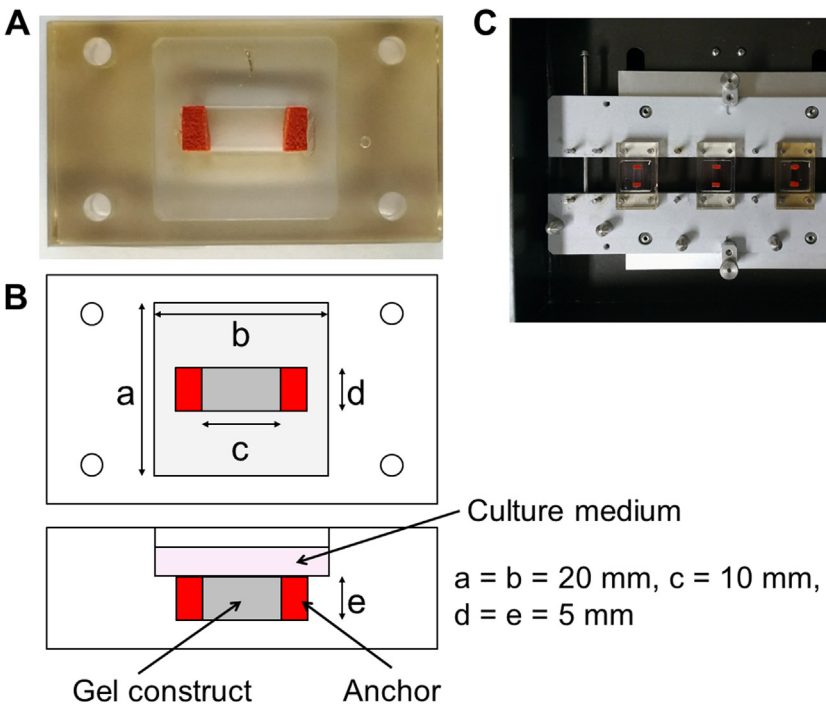

Fig. 1. Stretching chamber and stretch device. (A) An overview of the silicone chamber in which human airway smooth muscle (ASM) cells are cultured within a type-I collagen construct. **(B)** A scheme of the silicone chamber. **(C)** An overview of the stretch device (ST-140; Strex) on which three silicone chambers are fit. The arrow indicates stretch direction.

tissue-like behavior by upregulating expression of genes for contractile proteins.

2. Materials and methods

2.1. Cells

Primary cultures of normal human bronchial smooth muscle cells from three different donors were obtained from Lonza (Walkersville, MD) and maintained in SmGM-2 culture medium (Lonza) containing 5% fetal bovine serum (FBS) in an atmosphere of 5% CO_2 and 95% air at 37 °C [2,13,14]. Cells of passages 4–8 were used.

2.2. Fabrication of 3-D constructs

ASM (5×10^5/ml) cells were suspended in a solution of 2 mg/ml of type I collagen (Cellmatrix; Nitta Gelatin, Osaka, Japan) in SmGM-2 cell culture medium (Invitrogen, Carlsbad, CA) containing 5% FBS at room temperature. The solution was transferred into the well (10 mm in length x 5 mm in width x 5 mm in depth) of a silicone chamber with sponge anchors on both sides (STB-CH-3.5GS; Strex, Osaka, Japan) (Fig. 1**A and B**). The solution was able to infiltrate into the sponge. The gel was allowed to polymerize and attached to the sponge anchors by incubation at 37 °C for 15 min. After the gels were polymerized, 2 ml of SmGM-2 cell culture medium containing 5% FBS was added to the gel (Fig. 1**A**), then the medium was changed every other day.

2.3. Application of cyclic and static mechanical stretch

After the gel constructs were incubated for 24 h and polymerized, a uniaxial sinusoidal stretch of 12% strain at 30 cycle/min was applied for 48 h using a stretching apparatus driven by a computer-controlled stepping motor (ST-140; Strex) [2,13,15]. Briefly, one end of the chamber was attached to a fixed frame, while the other end was attached to a movable frame (Fig. 1**C**). The other two sides were free to move. The movable frame was connected to a motor driven shaft whose amplitude and frequency of stretch was controlled by a programmable microcomputer. Strain was calculated from the displacement of the silicone chamber before and after the stretch. Cells incubated under a static condition in the silicone chamber were used as a time-matched control.

2.4. Immunofluorescence staining

Cells grown within collagen gels were fixed with 4% formaldehyde for 30 min and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS for 30 min. This was followed by blocking with 1% bovine serum albumin (BSA) in PBS for 60 min. Then, the cells were incubated with a mouse polyclonal anti-α-SMA antibody (dilution 1:400, a2547; Sigma-Aldrich) in PBS containing 1% BSA overnight, washed, and further incubated with a goat anti-mouse secondary antibody (dilution 1:1000, A-11001; Thermo Fisher Scientific) for 60 min at room temperature. Filamentous actin (F-actin) and nuclei were stained with rhodamine-phalloidin (dilution 1:1000, R415; Thermo Fisher Scientific) and 4,6-diamino-2-phenylindole (DAPI) (dilution 1:1000, D523; Dojin, Kumamoto, Japan) for 60 min at room temperature. Immunofluorescence images were obtained using an upright laser scanning confocal microscope (A1RMP; Nikon, Tokyo, Japan), with a × 25/1.2 NA Plan Apo violet-corrected water immersion objective [16,17]. Images were obtained in 2 μm steps and up to 250 μm in depth.

2.5. Measurement of cell orientation

Images of the nuclei stained with DAPI were obtained using a confocal microscope with at least three arbitrarily selected visual fields. Optical volumes 517.6 μm x 517.6 μm x 200 μm were flattened into a single plane image. The orientation of each nucleus of the cell was measured as an angle (θ) of the long axis between 0° and 90° with respect to the stretch axis (Supplementary Fig. S1) using NIH ImageJ v1.33 software [13,15].

2.6. Quantitative real-time PCR

Gels were immersed in liquid nitrogen and then minced. Total cellular RNA was extracted using Trizol reagent (Thermo Fisher Scientific, Waltham, MA). RNA was reverse transcribed to cDNA using a Superscript III kit (Invitrogen, Carlsbad, CA). TaqMan Gene Expression Assays for α-smooth muscle actin (α-SMA) (*ACTA2*) (Hs00426835_g1),