Tissue Engineering of Smooth Muscle under a Mechanically Dynamic Condition

KIM, BYUNG-SOO1*, SUNG IN JEONG1, SEUNG-WOO CHO1,3, JANETA NIKOLOVSKI4, DAVID J. MOONEY4, SOO HONG LEE2, OJU JEON, TAE WAN KIM1, SANG HYUN LIM1, YOO SUN HONG2, CHA YONG CHOI1, YOUNG MOO LEE1, SOO HYUN KIM2, AND YOUNG HA KIM2

1Department of Chemical Engineering, Hanyang University, Seoul 133-791, Korea
2Biomaterials Research Center, Korea Institute of Science and Technology, Seoul 130-650, Korea
3School of Chemical Engineering, Seoul National University, Seoul 151-742, Korea
4Departments of Biomedical Engineering, Chemical Engineering, Biologic and Materials Sciences, University of Michigan, Ann Arbor, MI 48109, U.S.A.
5Department of Cardiovascular Surgery, Korea University College of Medicine, Seoul 120-752, Korea

Received: March 8, 2003
Accepted: October 8, 2003

Abstract In order for engineered tissues to find clinical utility, the engineered tissues must function appropriately. However, smooth muscle (SM) tissues engineered in vitro with a conventional tissue engineering technique may not exhibit contractile functions, because smooth muscle cells (SMCs) cultured in vitro typically revert from a contractile, differentiated phenotype to a synthetic, nondifferentiated phenotype and lose their ability to contract. SMCs in vivo typically reside in mechanically dynamic environments. We hypothesized that cyclic mechanical stretch induces the features of SMCs in vitro engineered tissues to be similar to those of SMCs in native tissues. To test the hypothesis, aortic SMCs were seeded onto elastic, three-dimensional scaffolds and cultured in vitro under a cyclic mechanical stretching condition for 4 weeks. A significant cell alignment in a direction parallel to the cyclic stretching direction was found in the SM tissues exposed to cyclic stretching. The cellular alignment and alignment direction were consistent with those of native vascular SM tissues, in which SMCs in vivo align in the radial direction (parallel to stretching direction). In control tissues (SM tissues engineered without stretching), cells randomly aligned. The expression of SMα-actin and SM myosin heavy chain, phenotypic markers of SMCs in a contractile state, was upregulated in the stretched tissues by 2.5- and 2.0-fold, respectively, compared to SMCs in the control tissues. The cellular features of alignment and contractile phenotype of SMCs in the SM tissues engineered under a mechanically dynamic environment could allow the engineered SM tissues to exhibit contractile functions.

Key words: Tissue engineering, smooth muscle cell, cyclic mechanical stretch

Tissue engineering offers the possibility of creating functional new tissues that may replace lost or malfunctioning organs or tissues [10]. This approach may provide an alternative to organ and tissue transplantation therapies, both of which suffer from a severe limitation of supply [8, 12]. In the tissue engineering approach, cultured cells are seeded onto a suitable scaffold, and either allowed to develop into a new tissue in vitro, or transplanted into a patient to create a new tissue that replaces the functions of the lost or deficient tissue [5]. Smooth muscle (SM) is a functionally critical component of a variety of cardiovascular, gastrointestinal, and urinary tissues. Any attempt to engineer these tissues must include the development of functional SM. Importantly, to utilize engineered tissues clinically, the engineered tissues must function appropriately.

To engineer functional tissues, the transplanted cells must be provided with microenvironments that are similar to those of the native tissues in vivo during the process of new tissue development in vitro [5]. For tissue engineering of functional SM, mechanical stretch may be a crucial microenvironmental factor. Smooth muscle cells (SMCs) in SM-containing tissues (e.g., blood vessels, intestines, and bladder) in vivo are constantly exposed to cyclic mechanical stretch. Many studies have shown that mechanical stretch significantly regulates the phenotype of SMCs in two-dimensional culture systems [1, 4, 14, 16]. Recently, we have demonstrated that cyclic mechanical stretch enhanced...
the development and function of engineered smooth muscle (SM) tissues [6].

In the present study, we tested the hypothesis that cyclic mechanical stretch induces the phenotype of SMCs in *in vitro* engineered tissues to be similar to that of SMCs in native tissues *in vivo*. SMCs *in vivo* normally align in a specific direction and exist in a contractile or differentiated state, which is critical for contractile functions of SM [9]. *In vitro* cultured SMCs, however, do not align in a specific direction and often lose the contractile phenotype [2, 17]. To test the hypothesis, aortic SMCs were seeded onto elastic, three-dimensional scaffolds and subjected to cyclic mechanical stretch. In control experiments, SMCs on scaffolds were cultured without mechanical stretch. Subsequently, we examined the cellular alignment and the expression of SM α-actin and SM myosin heavy chain, phenotypic markers of SMCs in a contractile state [17], in the engineered SM tissues.

**MATERIALS AND METHODS**

**Scaffold Fabrication**
Scaffolds were fabricated from poly(glycolide-co-ε-caprolactone) (PGCL) using a previously described solvent casting and particulate leaching method [13]. The copolymer was generously provided by Biomaterials Research Center in Korea Institute of Science and Technology, Seoul, Korea. The copolymer contained an equal molar ratio of glycolide and ε-caprolactone, and had a molecular weight of 121,000. NaCl particles (100–200 µm), which served as porogens, were added to a PGCL solution in chloroform (5% w/v), and mixed homogeneously. The polymer-salt mixture was cast onto glass plates. The solvent was then allowed to evaporate for 48 h, and residual solvent was removed by drying under vacuum for 24 h. The resulting PGCL/salt composite membranes were immersed in distilled deionized water with shaking for 3 days to leach out the salt, resulting in the formation of porous scaffolds. Prior to use, the scaffolds were sterilized with ethylene oxide gas.

**Scaffold Characterization**
Scaffold morphology was studied with a scanning electron microscope (SEM, Hitachi, Tokyo, Japan). Samples were coated with gold using a sputter-coater (Eiko IB3, Tokyo, Japan), and the microscope was operated at 15 kV to image samples.

Dynamic mechanical tests (n=3) were performed using a custom-made mechanical stretch apparatus (Fig. 1), as previously described [7]. The scaffolds were subjected to cyclic stretch at an amplitude of 5% of initial scaffold length and at a frequency of 1 Hz in PBS solution at 37°C.

We chose these cyclic strain amplitudes and frequencies because it has been reported that these conditions are similar to those experienced by SM tissues *in vivo* [9]. The elastic properties of the scaffolds were determined by measuring the permanent deformation of the scaffolds following repetitive stretching.

**Smooth Muscle Cell Isolation and Culture**
SMCs were isolated from rat aortas according to the previously published technique [15]. In brief, the descending aortas of 3.5-kg female New Zealand white rabbits, after the removal of endothelium, adventitia, fat, and connective tissue, were cut into multiple small pieces and incubated under agitation with an orbital shaker (60 rpm) for 90 min at 37°C in a sterile conical flask containing an enzymatic dissociation buffer. This buffer contained 0.125 mg/ml elastase (Sigma, St. Louis, MO, U.S.A.), 1.0 mg/ml collagenase (CLS type I, 204 units/mg, Worthington Biochemical Corp., Freehold, NJ, U.S.A.), 0.250 mg/ml soybean trypsin inhibitor (type 1-S, Sigma), and 2.0 mg/ml crystallized bovine serum albumin (BSA, Gibco, Gaithersburg, MD, U.S.A.). Following the complete dissolution of the matrix, the resultant cell suspension was filtered through a 100 µm Nitex filter (Tetko, Inc., Briarcliff Manor, NY, U.S.A.) and centrifuged at 200 ×g for 5 min. The pellet was resuspended in growth medium consisting of Medium 199 (Gibco) supplemented with 10% (v/v) FBS (Gibco), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco), and 0.1 mg/ml streptomycin (Gibco), and cultured in tissue culture flasks (25 cm²). SMCs passaged twice were used in this study.

![Fig. 1. Apparatus utilized to subject scaffolds to cyclic mechanical stretch.](image-url)
Seeding of Cells onto Scaffolds and Subsequent Culture under Cyclic Stretching Condition

To seed SMCs onto PGCL scaffolds (40×10×2 mm), 0.8 ml of a SMC suspension containing 2.0×10^7 cells was injected into the scaffolds in tissue culture dishes. After 1, 2, and 6 h, 0.5, 1, and 30 ml of the culture medium was added to each scaffold, respectively. The seeded polymer scaffolds were maintained in a humidified 5% CO₂ atmosphere for 2 days with the medium changed every day. SMCs in the scaffolds were subjected to cyclic stretch in vitro using a custom-made cyclic stretching unit in a humidified incubator with 5% CO₂ at 37°C (Fig. 1). The seeded scaffolds were subjected to cyclic strain at a frequency of 1 Hz (1 cycle per second) and an amplitude of 5% of initial scaffold length for 4 weeks. As a control, seeded scaffolds were fixed at only one end of the clamps and moved back and forth at the same frequency and amplitude as the mechanical stretching conditions.

Cell Alignment Analysis

Cell alignment in engineered SM tissues was determined by SEM examinations. Specimens were fixed in 1% (v/v) glutaraldehyde and 0.1% (v/v) formaldehyde for 30 min and 24 h, respectively, dehydrated with a graded ethanol series, and dried. The dried samples were mounted on aluminum supports and sputter coated with gold. A scanning electron microscope was operated to image samples. More than 300 cells in 10 SEM photographs were examined for cell alignment.

Western Blot Analysis

To obtain total proteins, engineered tissues were crushed manually and dissolved in a lysis buffer (pH 7.4) containing 25 mM Tris, 0.4 M NaCl, 0.5% (w/v) SDS, and protease inhibitors [10 mg/ml aprotinin (Sigma), 10 mg/ml leupeptin (Sigma), 5 mg/ml pepstatin (Sigma), and 0.5 M phenylmethylsulfonyl fluoride (Sigma)]. Tissues were vortexed, boiled for 5 min, and centrifuged. To determine sample volumes representing the same amounts of total proteins from each tissue sample, supernatants were removed and analyzed by spectrophotometrically measuring absorbance at 280 nm. Protein samples were mixed with Laemmli sample buffer, loaded and separated by SDS-PAGE on 10% resolving gels for SM α-actin. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL, U.S.A.). Transfer was carried out at a constant voltage of 100 V in a cold room for 1 h in a buffer system (5.03 g Tris base, 14.5 g glycine, 0.5 g SDS, and 200 ml methanol in 1 l). Membranes were blocked with 5% skim milk in TBS-T (Tris buffered saline with 0.1% Tween-20, pH 7.6) for 1 h and probed with mouse anti-SM α-actin antibody (DAKO, Carpinteria, CA, U.S.A., 1:100 dilution with TBS-T) and mouse anti-SM myosin heavy chain antibody (DAKO, 1:100) for 1 h at room temperature. Membranes were washed, probed with 1:1,000 diluted horseradish peroxidase conjugated anti-mouse IgG secondary antibody (Sigma) for 1 h and visualized by an enhanced chemiluminescence system (Intron Biotechnology, Inc., Sungnam, Korea). Luminescence was recorded onto X-ray film (Fuji Super RX, Fujifilm Medical Systems, Tokyo, Japan). Band intensity was analyzed by imaging densitometer (Model GS-690, BioRad, Hercules, CA, U.S.A.). Bands were quantified densitometrically by scanning films into Scion Image (NIH Image, Scion Corporation, Maryland, U.S.A.), and analyzed (n=3).

RESULTS AND DISCUSSION

Appropriate scaffolds were first prepared as a three-dimensional cell culture substrate for tissue engineering of SM under a cyclic mechanical stretch condition. Scaffolds fabricated from PGCL (Fig. 2) were chosen as a three-dimensional cell culture substrate in this study. These scaffolds are biodegradable and biocompatible [3], which is important for new tissue formation following cell seeding and culture. In addition, these scaffolds were found to have elastic mechanical properties, which is a prerequisite for subjecting the cell-seeded scaffolds to cyclic mechanical stretch. The elastic properties were evaluated by measuring recovery after cyclic stretching of PGCL scaffolds. PGCL scaffolds were subjected to cyclic loading at 5% amplitude and 1 Hz frequency in PBS (pH 7.4) at 37°C for 4 weeks using a custom-made apparatus (Fig. 1). The scaffolds displayed no permanent deformation.

Fig. 2. SEM photomicrograph of PGCL scaffold. The porous, three-dimensional scaffold allows for cell adhesion and subsequent three-dimensional tissue formation.

The size bar indicates 50 µm.
Next, we investigated the effects of cyclic mechanical stretch on SMC alignment in engineered tissues. Seeded SMCs adhered well to PGCL scaffolds (Fig. 3). Subsequently, SMCs cultured in PGCL scaffolds were exposed to cyclic mechanical stretch for 4 weeks using a custom-made bioreactor (Fig. 1), and SEM examination indicated that the application of cyclic mechanical stretch induced a significant cell alignment, as compared with control tissues (SM tissues engineered without stretching) (Fig. 4). The cell alignment direction was in parallel to the cyclic mechanical stretch direction. The vascular SMC alignment and alignment direction in this study were consistent with those of native vascular SM tissues, in which SMCs align in the radial direction (parallel to stretching direction) [2, 9]. The induction of SMC alignment in this study was also consistent with previous results in which SMCs in two-dimensional culture systems were exposed to cyclic mechanical stretch, and the alignment direction in two-dimensional culture systems was nearly perpendicular to the stretching direction [2]. In our previous study, histological examination showed that cyclic strain induced the alignment of SMCs present in the inner parts of engineered tissues in a direction parallel to the stretching direction [6].

The effects of cyclic mechanical stretch on the phenotype of vascular SMCs in engineered tissues were next assessed. SMCs in tissues engineered under a cyclic stretch condition exhibited an increased expression of SM α-actin and SM myosin heavy chain (Fig. 5), which are phenotypic markers of SMCs in a contractile phenotype [17]. Densitometric examination indicated that the expression of SM α-actin and SM myosin heavy chain was 2.5 and 2.0 times greater, respectively, in tissues exposed to cyclic stretching than in control tissues. This result suggests that cyclic mechanical stretching induces a differentiated, contractile phenotype in SMCs in engineered tissues. This is consistent with our previous results that SMCs in tissues engineered under cyclic strain contained cellular organelles of contractile phenotype, as analyzed by transmission electron microscopy, while cells in control tissues exhibited the properties of the synthetic, nondifferentiated phenotype [7].

Engineered tissues must be functional in order to be utilized clinically, and mechanical stimulation may be critical for tissue engineering of functional SM. An important function of the SM element in many tissues (e.g., blood vessels and intestines) is to contract in order to regulate blood pressures or transfer fluids. In the present study, mechanical stimulation induced cellular alignment and contractile phenotype in SMCs in engineered tissues, which may allow the engineered SM tissues to exhibit contractile functions. Cell alignment in tissues would make tissue contraction feasible, because the contraction of randomly aligned cells in tissues would not result in tissue contraction (Fig. 6). In order for SMCs to contract, the cell phenotype must be a differentiated, contractile phenotype. SMCs in vivo normally exist in a contractile or differentiated state, whereas in vitro cultured SMCs lose their ability to contract [17]. Mechanical stimulation, to which SMCs in vivo are exposed, has been found to be a strong regulator of SMC phenotype and crucial for the
vitro tissue engineering process. Microenvironments of native SMCs in native SM tissues, it might be necessary to provide the stimulation for engineered SM tissues to exhibit the functions of the engineered SM tissues. SM tissues stretching exhibited cellular alignment and upregulated contractile phenotype and induced cellular alignment. This would allow the engineered SM tissues to exhibit contractile functions. In contrast, in vitro cultured SMCs in engineered tissues without mechanical stimulation did not align, resulting in no contraction of the SM tissues.

Maintenance of SMCs in a differentiated state [11, 14]. The SM tissues engineered without mechanical stimulation (control tissues) in this study had no cell alignment and decreased feature of contractile phenotype, thus resulting in no contraction of these tissues (Fig. 6). We are currently conducting experiments to investigate the effects of cellular alignment and contractile phenotype on contractile functions of engineered SM tissues.

In conclusion, tissue engineering of SM under a mechanically dynamic condition resulted in cellular features that resembled those of SMCs in SM tissues in vivo. Specifically, SMCs in tissues engineered under cyclic stretching exhibited cellular alignment and upregulated expression of SM α-actin and SM myosin heavy chain. These cellular features could result in contractile functions of the engineered SM tissues. SM tissues in vivo reside in mechanically dynamic environments. Therefore, in order for engineered SM tissues to exhibit the functions of the native SM tissues, it might be necessary to provide the microenvironments of native SMCs in vivo during the in vitro tissue engineering process.

Acknowledgment

This work was supported by Korea Research Foundation Grant (2001-003-E00307).

REFERENCES