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In vitro co-culture of epithelial cells and smooth muscle cells on aligned nanofibrous scaffolds

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ABSTRACT

Esophagus is a complex, hollow organ consisting of epithelial cells in the inner mucosal layer and smooth muscle cells in the outer muscle layer. In the present study, we have evaluated the *in vitro* co-culture of epithelial cells and smooth muscle cells on the aligned nanofibrous scaffold made of PHBV, PHBV-gelatin, PCL and PCL-gelatin developed through electrospinning using rotating drum collector. Epithelial cells were labeled with cell tracker green while the smooth muscle cells were labeled with cell tracker red. Labeled cells were seeded on the aligned nanofibers matrices and tracked using laser scanning confocal microscopy. The results demonstrate that both epithelial and smooth muscle cells attach, extend, and proliferate over these nanofibrous matrices. Confocal z-sectioning shows that epithelial and smooth muscle cells tend to separate into two distinct layers on a single nanofiber system mimicking the *in vivo* anatomy. Cell viability assay showed that both types of cells are viable and also interact with each other. The functional gene expression of respective cell types demonstrates that both epithelial and smooth muscle cells are phenotypically as well as functionally active when they were co-cultured. Thus the study highlighted that aligned nanofibrous scaffolds could be potential alternative graft for esophageal tissue regeneration.

1. Introduction

Congenital defects, trauma, and cancer are major complications which severely affect the function of esophagus [1–3]. Surgical, non-surgical and lengthening techniques are conventionally employed for the treatment of esophageal disorders [4,5]. Unfortunately, these approaches associated with post-operative complications like leakage, infection, stenosis negatively impacts the quality of patient's life [6]. Tissue engineered artificial constructs could be a prospective alternate strategy in the near future to address esophageal disorders. The lack of regenerative ability of the esophageal tissue and limited availability of the autologous grafts further necessitate development of tissue engineering strategies [7]. Literature survey suggests that decellularized tissues [6] and polymeric matrices [3,7] support the adhesion and proliferation of epithelial cells (ECs) [7,8], smooth muscle cells (SMCs) [9], and fibroblast cells [10] independently for the regeneration of esophagus tissues.

PHBV and PCL are aliphatic polyesters that have been extensively employed for various tissue engineering applications. PHBV has shown attractive properties that include good cellular biocompatibility, biodegradability, good oxygen permeability, and piezoelectricity, which aids cellular proliferation [11–14]. Further, the degradation products such as β-hydroxybutyric acid are normal metabolites of blood stream [12]. Similarly, PCL is a semi-crystalline biodegradable and biocompatible synthetic material that possesses higher mechanical strength required during the implantation of artificial esophagus [15,16]. Even though the nanofibrous topography of the PHBV and PCL mimick the native architecture of tissue, their hydrophobicity alters normal cell behavior. Gelatin is a natural protein widely used in medical and pharmaceutical applications [17]. It also possesses a cell recognition integrin binding RGD (arginine-glycine-aspartic acid) motif, which helps in better cellular adhesion and proliferation [18]. To achieve improved cell-scaffold interactions, we have blended PHBV/PCL with gelatin and the cellular behavior has been compared with the native PHBV/PCL system.

Esophagus has multiple layers and each layer comprises various cell types. Mucosal layer consists of non-keratinized stratified epithelium, which secretes mucus that makes the esophagus moist at the inner layer and guards the esophagus from mechanical damage [8]. The muscle layer is made of smooth muscle cells that are arranged in an *endo*-circular and *exo*-longitudinal orientation to aid peristalsis of esophagus during food bolus transport [4]. The adventitia is the outermost layer of

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the esophagus. It is necessary to investigate the potential of the nanofibrous scaffolds with both epithelial as well as smooth muscle cells since both cell types are essential for function of esophagus. Our previous studies demonstrate that both PCL as well as PHBV nanofibrous scaffolds support the adhesion and proliferation of epithelial and smooth muscle cells independently [19–23]. Though the scaffolds promoted the adhesion and proliferation of epithelial and smooth muscle cells independently, it is also important to investigate the interaction of epithelial cells and smooth muscle cells on a single scaffold for their influence on phenotypic morphology, proliferation capacity and gene expression levels.

Topography of the scaffolds plays a major role in the regulating the cell-fate processes such as adhesion, orientation and proliferation of cells [24]. Most of the studies revealed that cell orientation and proliferation was superior in the aligned nanofibrous scaffolds than the random nanofibrous scaffolds [25]. The present study focuses on the fabrication of oriented nanofibers for the co-culture of esophageal cells to partially mimic the native architecture of the esophagus tissue. Fiber orientation is considered to be an essential element to achieve oriented clusters of cells. Hence, this study focuses on the co-culture of human epithelial cells and smooth muscle cells on aligned nanofibrous scaffolds (PHBV, PHBV-gelatin, PCL and PCL-gelatin) and their cell-cell interactions compatibility were assessed.

2. Materials and methods

Poly(3–hydroxybutyrate–*co*–3–hydroxyvalerate) (PHBV; 12% hydroxyvalerate content; M_w 450 kDa, Goodfellow Cambridge Ltd, UK), poly(ε-caprolactone) (PCL, M_w 300 kDa, Lakeshore Biomaterials, USA), gelatin (M_w 50 kDa, Sigma Aldrich, USA), chloroform and dimethylformamide (DMF, Merck, India), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, Sigma Aldrich, USA) were used for fabrication of aligned nanofibrous scaffolds. Human non-keratinized, stratified, squamous epithelial cells and human smooth muscle cells were a kind gift from Sankara Nethralaya Eye Hospital, Chennai and Mohan Diabetic Research Foundation (MDRF), Chennai respectively. Dulbecco's Modified Eagle Medium (DMEM), DMEM/Ham's F12, epithelial cell growth supplement (S-009-5), fetal bovine serum (FBS), penicillin-streptomycin (P/S) and Dulbecco's phosphate-buffered saline (D-PBS) solution were purchased from Gibco, USA. Live-dead assay kit, cell tracker green (CMFDA), cell tracker red (CMTPX) were procured from Molecular probes, USA, and used in the co-culture experiment.

2.1. Preparation of aligned scaffolds

Aligned nanofibers were developed through electrospinning (Nano Fiber Electrospinning Unit, HO-NFES-040, Holmarc, India) under optimized conditions reported by our group earlier (Table S1) [21,23]. The surface morphology of aligned nanofibers was viewed under field-emission scanning electron microscope (FE-SEM, JSM 6701F, JEOL, Japan). The aligned nanofibrous scaffolds were vacuum dried for two days before being using for cell-culture study.

2.2. Labeling of epithelial cells

Epithelial cells (human non-keratinized, stratified, squamous cells) were cultured in an epithelial cell culture medium (ECs-CM) and the culture flask was maintained at 37 °C in a 5% CO_2 chamber. The ECs-CM consists of basal medium DMEM/F12 along with 1% epithelial growth supplement, 10% FBS, and 1% P/S (penicillin/streptomycin). Epithelial cells were labeled with cell tracker™ green CMFDA dye (5-chloromethyl derivative of fluorescein diacetate) by following the manufacturer's protocol. Briefly, after attaining cell confluence, the medium was removed, the cells washed with D-PBS and incubated with cell tracker green (5 μM of green dye in 2 mL of serum free media) for 45 min at 37 °C. After incubation, the dye was removed and further incubated with culture medium for 30 min at 37 °C. Culture medium was removed and washed with D-PBS. Labeled cells pass the dye to the daughter cells but not to the adjacent cells and the green dye labeled cells were used for the co-culture study.

2.3. Labeling of smooth muscle cells (SMCs)

SMCs were maintained in a smooth muscle cell culture medium (SMCs-CM) consisting of DMEM with 10% FBS, and 1% P/S. Culture flask was maintained under 5% CO_2 at 37 °C. Smooth muscle cells (SMCs) were labeled with cell tracker™ red CMTPX dye. The same labeling procedure was followed as for epithelial cell labeling and the red-labeled cells were used for the co-culture study.

2.4. Cell seeding and tracking

Aligned nanofibrous scaffolds (12 mm diameter) were sterilized with ultra violet radiation (UV) for 1 h at each side followed by incubated in 100% ethanol for 1 h. After sterilization, scaffolds were washed thrice with D-PBS and pre-incubated with culture medium overnight [26–28]. Labeled epithelial cells (2×10^5) and smooth muscle cells (2×10^5) were trypsinized, counted and used for co-culture tracking study. Co-cultured epithelial and smooth muscle cells were maintained in a mixture of both cell culture media at a 1:1 ratio. Cell seeded scaffolds were imaged using laser scanning confocal microscopy (FV 1000, Olympus, Japan).

2.5. Viability of co-cultured epithelial cells and smooth muscle cells

The viability of the cells cultured on the aligned nanofibers was assessed using live-dead assay kit [29]. Briefly, 50,000 unlabeled cells (25,000 epithelial cells and 25,000 smooth muscle cells) were seeded on the aligned nanofibrous scaffolds and cultured for 3 and 5 days. After 3 and 5 days, the scaffolds were removed, washed with D-PBS and incubated with calcein AM & ethidium bromide for 30 min at 37 °C. After incubation, scaffolds were washed with D-PBS and imaged using laser scanning confocal microscope (FV 1000, Olympus, Japan). Viable cells appeared green while dead cells appeared red.

2.6. Real-time RT-PCR for co-cultured epithelial cells and smooth muscle cells

Cell seeded scaffolds ($n = 3$) were digested with trizol (Gibco BRL, USA) followed by mixing with chloroform (Merck, India) and allowed to stand for 2 min. Samples were centrifuged at 12,000 rpm at 4 °C for 15 min. The organic phase (containing nucleic acids) was carefully collected and stabilized with 70% ethanol and the total RNA was separated from nucleic acids using QIA Shredder spin column (Qiagen, USA). Isolated RNA was subjected to Quantitect Reverse transcription kit (Qiagen, USA) to form cDNA using hexamers as a primer [30]. Cells specific primers used in the gene expression study were based on previous report [19,21]. Epithelial cell specific genes (cytokeratin 4 (CK 4), cytokeratin 15 (CK 15), collagen type IV, and laminin) and smooth muscle cell specific genes (alpha smooth muscle actin (α-actin), myosin heavy chain (MHC), collagen type I, and elastin) were quantified using SYBR (Bio-Rad, USA) through thermo cycler (Eppendorf AG22331, Germany). The gene expression was quantified using delta-delta CT method and values were calibrated with TCPS control and normalized with house-keeping gene (GAPDH, glyceraldehyde phosphate dehydrogenase). The product specificity was confirmed by melting curve analysis.

2.7. Statistics

Two-factor analysis of variance (two-way ANOVA) was performed to evaluate the significant difference between the scaffolds and time points for the gene expression study ($p < 0.05$).

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