Biomaterials 35 (2014) 5129–5137

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The effect of scaffold macroporosity on angiogenesis and cell survival in tissue-engineered smooth muscle

Christopher M. Walthers [a], Alireza K. Nazemi [a], Shilpy L. Patel [a], Benjamin M. Wu [a,b], James C.Y. Dunn [a,c,*]

[a] Department of Bioengineering, University of California, Los Angeles, CA 90095, USA
[b] Department of Advanced Prosthodontics, Biomaterials, and Hospital Dentistry, University of California, Los Angeles, CA, USA
[c] Department of Surgery, University of California, Los Angeles, CA, USA

ARTICLE INFO

Article history:
Received 16 January 2014
Accepted 12 March 2014
Available online 30 March 2014

Keywords:
Angiogenesis
Polycaprolactone
Porosity
Smooth muscle constructs
Tissue engineering

ABSTRACT

Angiogenesis and survival of cells within thick scaffolds is a major concern in tissue engineering. The purpose of this study is to increase the survival of intestinal smooth muscle cells (SMCs) in implanted tissue-engineered constructs. We incorporated 250-μm pores in multi-layered, electrospun scaffolds with a macroporosity ranging from 15% to 25% to facilitate angiogenesis. The survival of green fluorescent protein (GFP)-expressing SMCs was evaluated after 2 weeks of implantation. Whereas host cellular infiltration was similar in scaffolds with different macroporosities, blood vessel development increased with increasing macroporosity. Scaffolds with 25% macropores had the most GFP-expressing SMCs, which correlated with the highest degree of angiogenesis over 1 mm away from the outermost layer. The 25% macroporous group exceeded a critical threshold of macropore connectivity, accelerating angiogenesis and improving implanted cell survival in a tissue-engineered smooth muscle construct.

1. Introduction

Many tissue engineering applications employ scaffolds seeded with cells that grow during *in vitro* culture prior to implantation. In recent years, there have been many advances in tissue engineering of three-dimensional scaffolds. In such large scaffolds, the diffusion of nutrients and oxygen becomes an important consideration, and rapid vascular infiltration is necessary *in vivo* to sustain seeded cells following implantation [1]. By diffusion alone, thick scaffolds can only sustain cells within a few hundred μm of the liquid–scaffold interface *in vitro*, a limitation which is closely approximated *in vivo* [2,3]. Various techniques to improving vascular conduction have been studied [4], including use of growth factors [5–7], bioactive materials [8,9], microfabrication [10–12], angiogenic cells [13–15], decellularized organs with intact microvasculature [16,17], non-biological factors [18], and even oxygen-producing biomaterials [19]. Microfabrication is especially important since improved conduction pathways can accelerate or improve other efforts to increase angiogenesis in implants [20].

Porosity of scaffolds used in tissue engineering have long been known to affect angiogenesis and the development of functional tissue including structural tissues such as bone [21,22] and cartilage [23,24], as well as soft tissues such as the urethra [25], liver [26], skin [27,28], muscle [29], vascular structures [30], and soft tissue fillers [31]. In a previous report, we explored the role of pore size in electrospun polycaprolactone scaffolds using laser cut holes of various diameter ranging from 80 to 300 μm while maintaining a 15% macroporosity by area [32]. Larger diameter pores led to an increase in cellular infiltration and vascular development compared to controls without a significant change in material properties. In that study, the optimal density of the 300-μm pores, however, was not explored. Ideally, a porous electrospun scaffolds should have a large surface area for cell attachment, yet enough macropores to allow rapid angiogenesis. The density of macropores necessary to allow sufficient angiogenesis to sustain implanted cell survival within electrospun scaffolds remains unclear [33].

Many diseases affecting the gastrointestinal tract leave insufficient functioning intestine for normal absorption, resulting in a condition known as intestinal failure [34]. Without methods for curing these conditions, medical options are limited to reducing

* Corresponding author. Department of Bioengineering, University of California, Los Angeles, 420 Westwood Plaza, Room 5121 Engineering V, Los Angeles, CA 90095, USA.
E-mail address: jdunn@mednet.ucla.edu (J.C.Y. Dunn).

http://dx.doi.org/10.1016/j.biomaterials.2014.03.025
0142-9612/

symptoms, contributing to unacceptably high morbidity and mortality rates [35,36]. Tissue engineering is an appealing alternative for creating new intestine. While much progress has been made on growing the intestinal epithelium, less has been done with the intestinal smooth muscle [37–39]. The intestinal smooth muscle is a multi-layered tissue composed of an inner circular layer beneath the submucosa and an outer longitudinal layer adjacent to the serosa. Smooth muscle is critical for the transport of waste and nutrients through the gastrointestinal tract. Functional tissue-engineered smooth muscle that can generate the contraction strength necessary for peristalsis has been difficult to produce. Even the strongest tissue-engineered muscles are much weaker than normal muscle, suggesting the need for thicker scaffolds, which will further increase the need for rapid vascular infiltration and better mimic of the multi-layered intestine [1,33,40,41]. Furthermore, survival and maturity of intestinal smooth muscle cells (SMCs) *in vivo* is accompanied by angiogenesis [42,43], suggesting that a pro-angiogenic biomaterial may enable the development of a mature smooth muscle.

We sought to determine the optimal macroporosity to generate a critical amount of angiogenesis necessary to sustain a population of implanted SMCs within multi-layered scaffolds. Macroporosity was generated by laser-cutting of electrospun sheets, and cell-seeded sheets were rolled into a multi-layered tube. Following 2 weeks of implantation, the scaffolds were evaluated for cellular infiltration, vascular in-growth, and survival of implanted GFP-expressing SMCs.

2. Materials and methods

2.1. Fabrication of poly ε-caprolactone (PCL) scaffolds

Polymer solution was made from 11% w/w ester capped PCL (Lactel Absorbable Polymers, Birmingham, AL) in hexafluoro-2-propanol (Sigma, St. Louis, MO). Our electrospinning technique was described in detail previously [32]. Following the electrospinning, the designs of PCL scaffolds were drawn with Adobe Illustrator, and scaffolds were cut from the whole electrospun PCL sheet using a VersaLASER 2.3 laser cutting system (Universal Laser Systems, Scottsdale, AZ). Scaffolds for *in vivo* experiments were fabricated with a regular pattern to create pores in the PCL sheet. The pattern was drawn with 150-μm circles to accommodate melting, which created 250-μm pores with a 75-μm thick ring of solid polymer melt around the perimeter. After the pores were made, a second pattern was run to cut the perimeter of the scaffold to create a rectangular 45 mm × 9.5 mm sheet. Rows of pores were offset to create a staggered series of columns, and the distance between each pore was varied to create different porosities. Additionally, each row was 0.5 mm below the previous row. The laser was set to a power of 25× and a speed of 50×, with three consecutive scans over each substrate.

2.2. Macroporosity measurements

Scaffold macroporosity was determined using a VHX-2000 digital microscope (Keyence, Itasca, IL). Three images of each scaffold were taken at 300× magnification. Macroporosity was determined using ImageJ software to threshold and calculate the area lacking polymer in each picture.

2.3. Collagen coating

PCL scaffolds were loaded into a plasma chamber (Harrick Plasma, Ithaca, NY), at 500 mTorr and were etched for 1.5 min at high power. Scaffolds were then sterilized in 70% ethanol for 30 min, rehydrated with decreasing percentages of ethanol and finally in phosphate buffered saline (PBS). Scaffolds were then immediately coated overnight at 37 °C with 500 μL of a 0.25 mg/mL collagen (Purecol, Advanced Biomatrix, San Diego, CA) solution neutralized with .1 N sodium hydroxide to pH 7.4 and diluted in PBS. The coated scaffolds were rinsed 3 times in PBS for 5 min and then soaked in cell-culture medium until cell seeding.

2.4. GFP-expressing SMC seeding of the scaffold

2.4.1. Culture of GFP-expressing SMCs

Transgenic GFP-expressing Lewis rats were obtained from a breeding colony maintained by UCLA's Department of Laboratory Animal Medicine and were sacrificed with approval of Institutional Review Board. SMC isolation was described in a previous publication [42]. Following digestion, collagenase was quenched in cell-culture medium consisting of Dulbecco's Modified Eagle's Media (DMEM, Life Technologies, Carlsbad, CA) with 10% Fetal Bovine Serum (FBS, Life Technologies), and 1× ABAM (Life Technologies).

2.4.2. GFP-expressing SMC seeding

Primary SMCs were counted with a hemacytometer and were seeded at 250,000 cells/cm^2 on PCL scaffolds within a custom polydimethylsiloxane (PDMS, Dow Corning, Elizabethtown, KY) cell seeding tray shaped to fit the scaffolds. Cell-culture medium was changed every two days after washing with PBS and scaffolds were maintained in a 37 °C humidified incubator with 10% carbon dioxide for two weeks prior to implantation.

2.5. Scaffold implantation

2.5.1. Scaffold preparation

On the day of implantation, scaffolds containing cells were washed once with PBS, and a 9.5 mm section of sterile #6 silicone catheter (Bard, Covington, GA) was used to provide structural support for wrapping the scaffold in to a multi-layered tube. The catheter formed the lumen of the tube and prevented the scaffold from collapsing. Each scaffold was wrapped six times around the catheter with the cell-seeded side facing outwards. After wrapping, each scaffold was secured to the catheter with a 6–0 prolene suture (Ethicon, San Angelo, TX) and the ends were plugged with silicone rubber (Dow Corning, Midland, MI) to prevent cell infiltration from the scaffold edges. The rolled scaffolds were rinsed and stored in DMEM on ice until implantation. Scaffold wrapping is demonstrated in Fig. 2.

2.5.2. Implantation

All surgical procedures were done in accordance with UCLA's Animal Research Committee protocol # 2004-197-21. The surgical procedure was described in detail in a previous publication [32]. At specimen retrieval, each scaffold was cut in to four equal-sized cylinders, and two segments were used for qPCR while the other two were fixed in formalin for histology.

2.6. Immunohistochemistry

2.6.1. Immunostaining of GFP-expressing SMCs

The scaffolds fixed in formalin were processed for paraffin embedding and sectioning by UCLA's Translational Pathology Core Laboratory (TPCL). Slides were stained with hematoxylin and eosin (H&E). Normal intestine and GFP-expressing adult intestine were fixed, sectioned, and stained as controls. Each sample was cut into 5-μm sections with two sections per slide; one section on each slide was used for staining, while the second was used as a control with no primary antibody added. Slides were washed with xylene to strip away the paraffin and rehydrated with progressively decreasing concentrations of ethanol in water.

For immunofluorescence, antigen retrieval was performed in Citra solution (Biogenix, Fremont, CA) for 20 min at 100 °C followed by 20 min of cooling at room temperature. Samples were permeabilized with 0.5% Triton X and were washed with PBS-tween twice. A hydrophobic barrier was traced around all samples with a PAP pen (Abcam, Cambridge, MA). Non-specific staining was blocked with a solution of 2% bovine serum albumin and 4% goat serum in PBS-tween for 1 h. Following blocking, slides were incubated overnight at 4 °C in primary antibody diluted 1:50 in blocking solution. Antibodies for GFP were purchased from Clontech (Mountain View, CA). Excess primary antibody was washed away with three more PBS-tween rinses before a 30 min incubation with Alexafluor 488 goat anti-mouse secondary (Life Technologies) diluted 1:200 in blocking solution. Finally, slides were rinsed three more times in PBS-tween before adding a mounting media with added DAPI (Vector, Burlingame, CA) to facilitate nuclei visualization. Fluorescent images were taken with an Olympus IX71 microscope with cellSens software (Olympus, Center Valley, PA).

2.6.2. Immunohistochemical staining of von-Willebrand factor

Immunohistological staining for von-Willebrand Factor (vWF) was performed to quantify blood vessel infiltration in the scaffolds. Following paraffin removal in xylene and rehydration in decreasing concentrations of ethanol, antigen retrieval was performed in 0.05% trypsin-EDTA solution (Life Technologies) in PBS at 37 °C for 15 min. Slides were rinsed in PBS and the endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 30 min before drawing a hydrophobic barrier around each section. Samples were again rinsed in PBS and incubated for 1 h in blocking solution consisting of 4% horse serum and 2% BSA in PBS-tween. Immediately after blocking, samples were incubated overnight in a 1:500 dilution of polyclonal rabbit anti-human vWF antibody (Dako, Carpinteria, CA) in blocking solution. Biotinylated horse anti-rabbit secondary antibodies (Vector Labs, Burlingame, CA) were diluted 1:200 in blocking solution and incubated for 30 min at room temperature prior to a 30-min incubation in horseradish peroxidase streptavidin (Vector). Finally, 3,3′-Diaminobenzidine (DAB) chromogen (Vector) was developed for 10 min before counterstaining in hematoxylin. Lastly, samples coverslipped with VectaMount (Vector).

2.6.3. Quantification of staining

Fluorescent images of DAPI- or GFP-stained sections were taken from four sides of each scaffold section for each implant. Scaffold images were then manually separated in to three parts representing the outermost ("serosal") two layers, the middle two layers ("middle"), and the innermost ("luminal") layers. Each part was quantified by area using ImageJ. The four images from each section were added, and the area for each section was averaged with all the sections for the same