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Physiological pulsatile flow culture conditions to generate functional endothelium on a sulfated silk fibroin nanofibrous scaffold

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ABSTRACT

Many studies have demonstrated that *in vitro* shear stress conditioning of endothelial cell-seeded small-diameter vascular grafts can improve cell retention and function. However, the laminar flow and pulsatile flow conditions which are commonly used in vascular tissue engineering and hemodynamic studies are quite different from the actual physiological pulsatile flow which is pulsatile in nature with typical pressure and flow waveforms. The actual physiological pulsatile flow leading to temporal and spatial variations of the wall shear stress may result in different phenotypes and functions of ECs. Thus, the aim of this study is to find out the best *in vitro* dynamic culture conditions to generate functional endothelium on sulfated silk fibroin nanofibrous scaffolds for small-diameter vascular tissue engineering. Rat aortic endothelial cells (RAECs) were seeded on sulfated silk fibroin nanofibrous scaffolds and cultured under three different patterns of flow conditioning, e.g., steady laminar flow (SLF), sinusoidal flow (SF), or physiological pulsatile flow (PPF) representative of a typical femoral distal pulse wave *in vivo* for up to 24 h. Cell morphology, cytoskeleton alignment, fibronectin assembly, apoptosis, and retention on the scaffolds were investigated and were compared between three different patterns of flow conditioning. The results showed that ECs responded differentially to different exposure time and different flow patterns. The actual PPF conditioning demonstrated excellent EC retention on sulfated silk fibroin scaffolds in comparison with SLF and SF, in addition to the alignment of cells in the direction of fluid flow, the formation of denser and regular F-actin microfilament bundles in the same direction, the assembly of thicker and highly crosslinked fibronectin, and the significant inhibition of cell apoptosis. Therefore, the actual PPF conditioning might contribute importantly to the generation of functional endothelium on a sulfated silk fibroin nanofibrous scaffold and thereby yield a thromboresistant luminal surface.

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1. Introduction

Since clinically available vascular prostheses do not offer satisfactory outcomes for the small-diameter (<6 mm) vessel replacements, the need for engineered small-diameter vascular grafts is real and urgent. A wide variety of natural proteins and synthetic scaffold materials have been combined with vascular cells to create functional small-diameter grafts. The main reason for the long-term failure of small-diameter vascular grafts is due to the occlusion (thrombus formation) in the early phase followed by continuous and excessive tissue ingrowths (intimal hyperplasia) in the chronic phase [1]. The seeding of vascular endothelial cells (ECs) onto the lumen of vascular grafts in order to yield a thromboresistant surface and so reduce the high incidence of graft occlusion is one approach that has been proposed to address this problem [2–4]. However, attempts to seed ECs on current vascular prosthesis materials is problematic, a major concern being the low number of ECs and proportion of the surface that remains endothelialised surface on exposure to fluid shear stresses representative of blood flow in the peripheral circulation. It was shown that most EC losses occurred within the first hour of flow and varies

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from 5% to 85% [5,6]. As a result, the plasma proteins will immediately deposit on the denuded areas and subsequently make the foreign materials attractive for platelet adhesion and aggregation [7,8]. Thus any denuded areas on the luminal surface of vascular wall are probable sites for thrombus formation. Occlusive thrombus can quickly lead to graft failure and potentially catastrophic downstream consequences including myocardial infarction and limb ischemia [9].

Due to the low cell number remained, the cell retention and function on vascular grafts has to be improved for clinical applications. It is necessary to grow these grafts *in vitro* for a period before graft implantation to achieve higher EC coverage and better retention. To address EC attachment and function, cell-adhesive proteins such as fibronectin, vitronectin, laminin, and collagen VIII have been used to modify the surface of scaffold materials [10,11]. Nevertheless, while scaffold materials have been coated with cell-adhesive proteins with some success, some modifications also provide good substrates for platelet adhesion and aggregation [12,13]. Moreover, when exposed to the shear stress *in vivo*, the ECs bound to some of these biomolecules are not yet stable enough to assure that 100 percent of the scaffold surface is covered by a confluent endothelium.

On the other hand, many biomechanics studies have revealed that shear stress is an important factor in regulating the EC functions [14,15]. ECs subjected to the shear stress are able to convert mechanical stimuli into intracellular signals that affect cellular functions. Ott et al. showed that *in vitro* long-term shear stress promoted EC adhesion with a reorganization of the actin cytoskeleton, attachment plaque formation, and extracellular matrix (ECM) production when the cells were seeded on polypropylene hollow fibers [16]. Zhang et al. seeded human umbilical vein ECs onto the lumen of living tissue conduits and increased the shear stress applied on the cells step by step. The results showed that completely confluent monolayer ECs were elongated, and were oriented parallel to the flow direction [17]. Helmlinger et al. found that ECs can respond to different sinusoidal flow conditions by changing their morphologies [18]. In another study, Dardik et al. seeded rat aortic endothelial cells (RAECs) on the inner lumen of polyurethane grafts and exposed to shear stress for 6 days *in vitro* before implanted into syngeneic rats [19]. The *in vivo* results showed that immediate graft thrombosis was inhibited and confluent endothelium was presented 3 months after implantation. These findings suggested that *in vitro* shear stress conditioning of EC-seeded vascular grafts could improve cell retention and function once placed into a hemodynamically active environment. While many studies using different modes of shear forces such as laminar flow and sinusoidal pulsatile flow have provided considerable insights, none of them has applied an actual physiological pulsatile wave of small-diameter artery to ECs. Actually, most of these flow patterns are very different from the actual physiological small-diameter pulsatile flow which has the pulsatile nature of blood flow and asymmetric shape of the velocity profile [15,20]. The actual physiological pulsatile flow leading to temporal and spatial variations of the wall shear stress may result in different phenotypes and functions of ECs. Therefore, we believe it is essential to engineer small-diameter vascular grafts under the actual small-diameter arterial pulsatile flow conditions which can function in actual physiologic spatial and temporal dynamics of shear stress.

In our previous studies, ECs demonstrated strong attachment to sulfated silk fibroin nanofibrous scaffolds and proliferated well with high expression of phenotype-related marker genes and proteins under static culture condition [21]. In the present study, a physiological pulsatile flow bioreactor was designed to generate a typical femoral distal pulse wave *in vivo*. RAECs were seeded on sulfated silk fibroin nanofibrous scaffolds and cultured under three different patterns of flow conditioning, e.g., steady laminar flow, sinusoidal flow, or physiological pulsatile flow. The aim of this study is to find out the best *in vitro* dynamic culture conditions to generate functional endothelium on sulfated silk fibroin nanofibrous scaffolds for small-diameter vascular tissue engineering. Cell morphology, cytoskeleton alignment, fibronectin assembly, apoptosis, and retention on the scaffolds were investigated and were compared between three different patterns of flow conditioning.

2. Materials and methods

2.1. Preparation of sulfated silk fibroin

Raw Bombyx mori silk fibers were supplied from Suzhou Maoda Textile Co. Ltd. The raw silk fibers were degummed in an aqueous solution of 0.1% (w/v) Na_2CO_3 with temperature of between 98 and 100 °C. After 0.5 h, the aqueous solution was refreshed; this process was repeated thrice until the majority of the sericin has been removed. And then, silk fibroin were dissolved in $CaCl_2$–CH_3CH_2OH–H_2O (mole ratio = 1:2:8) at 78 ± 2 °C with continuous stirring and subsequently dialyzed against distilled water using a SnakeSkin Pleated Dialysis Tubing (PIERCE, MWCO 3500) at room temperature. Finally, the silk fibroin solution was freeze-dried for 24 h to form silk fibroin sponges and kept in a vacuum drying desiccator for future use.

The silk fibroin sponges were treated in a glass beaker with a solution prepared by gradually adding 10 ml of chlorosulfonic acid (Sigma, USA) to 60 ml of pyridine (Sigma, USA) in an ice bath. The reaction system was gradually heated to 80 °C in a thermostatically controlled bath and kept at constant temperature for 1 h with stirring. After reaction, 200 ml of distilled water was added to the system. Subsequently, the solution was neutralized by equivalent molar NaOH solution. The insoluble portion was removed by vacuum filtration; the soluble portion was precipitated with 500 ml of ethanol. The precipitate was harvested by centrifugation and dissolved with a small amount of water. And then, it was dialyzed against distilled water using a SnakeSkin Pleated Dialysis Tubing (PIERCE, MWCO 3500) for desalting. After freeze drying, sulfated silk fibroin was stored in a vacuum drying dessicator until use.

2.2. Electrospinning of sulfated silk fibroins

Sulfated silk fibroin was dissolved in hexafluoro-2-propanol (HFIP; Fluka Chemie GmBH, Germany) to generate a 10% (wt/v) solution. Electrospinning was performed with a steel capillary tube with a 1.5 mm inside diameter tip mounted on an adjustable, electrically insulated stand. The capillary tube was maintained at a high electric potential for electrospinning and was mounted in the parallel plate geometry. The capillary tube was connected to a syringe filled with the sulfated silk fibroin/HFIP solution. A constant volume flow rate of 0.8 ml/h was maintained using a syringe pump. High voltage of 10 kV was applied when the solution was drawn into fibers and was collected on rectangular slides kept at a distance of 13 cm from the needle tip. And then, the electrospun nanofibrous scaffolds were treated with 100% methanol for 10 min to induce a β-sheet conformational transition, which results in insolubility in water [21].

2.3. Cell culture

Experiments involving Sprague Dawley rats were carried out in strict accordance with guidelines for the Care and Use of Laboratory Animals of the Beijing Municipal Science & Technology Commission. The protocol was approved by the Ethics Review Committee for Animal Experimentation of the Peking University (SYXK (Beijing) 2006-0025). Surgeries were performed under phenobarbital anesthesia, and all efforts were made to minimize suffering.

Sprague Dawley rats (100–150 g) were purchased from Department of Laboratory Animal Science of Peking University (Beijing, China), where the use of animal was approved by the local Ethics Committee. RAECs were isolated and cultured by primary explants culture technique as previously described [22,23]. In brief, the thoracic aorta was isolated and adhered to plastic dishes (Corning, USA) while vascular intima downward. The vascular explants were cultured with M199 medium (Gibco, USA), supplemented with 100U/ml penicillin, 100 mg/ml streptomycin, 20% fetal bovine serum (FBS, Gibco, USA) and 50μ/ml heparin (Ameresco, USA) in cell incubator at 37 °C with 5% CO_2. Fibroblasts contamination was mechanically removed by a sterile scraper under inverted microscope. RAECs were cultured up to 80% confluence, and then were subcultured into plastic dishes (1:2). The cells were identified by their cobblestone appearance and confirmed by staining with a specific antibody to CD31 as described previously [24].

2.4. Flow exposure experiments

A self-designed physiological pulsatile flow tissue engineering bioreactor was used to apply different flow conditions to cultured cells in this research, which consists of a tissue culture system and a pulsatile flow system (Fig. 1A and B). The

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