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ScienceDirect
Biochemical and Biophysical Research Communications 358 (2007) 692–697
BBRC
www.elsevier.com/locate/ybbrc

In vitro formation of capillary networks using optical lithographic techniques

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Received 16 April 2007
Available online 11 May 2007

Abstract

Tissue engineering approaches have been developed for vascular grafts, but success has been limited to arterial replacements of large-caliber vessels. We have developed an innovative technique to transplant engineered capillary networks by printing techniques. Endothelial cells were cultured on a patterned substrate, in which network patterns were generated by prior optical lithography. Subsequently, the patterned cells were transferred to extracellular matrix and tissue at which point they changed their morphologies and formed tubular structures. Microinjection of dye showed that the micrometer-scale tubular structure had *in vitro* flow potential. When capillary-like networks engineered on amnion membranes were transplanted into mice, we found blood cells inside of the lumen of the transplanted capillary-like structure. This is the first report of the *in vitro* formation of capillary networks using cell transfer technique, and this novel technique may open the way for development of rapid and effective blood perfusion systems in regenerative medicine.

Keywords: Capillary network; Endothelial cells; Off-set printing; Optical lithography; Tissue engineering

Tissue engineering emerged in the early 1990s to address the limitations of conventional tissue grafting. Engineering an artificial tissue generally requires that cells be seeded on a biodegradable scaffold [1]. In contrast, scaffold-free tissue engineering has been developed using a temperature-responsive polymer [2,3]. In both cases, one of the principal limitations to the 3-dimensional (3D) structure of an engineered tissue is oxygenation and nutrient transport, as vascularization into an engineered tissue requires at least 1 day to achieve [4,5]. In the absence of an internal vascular supply, viability of implanted cells embedded in an engineered scaffolding is maintained exclusively by oxygen diffusion from surrounding host tissues, and as a result, oxygen deprivation can result. Lack of vascularization after

Abbreviations: VEGF, vascular endothelial growth factor; BMCs, bone marrow-derived mononuclear cells; EPCs, endothelial progenitor cells; FAS, fluoro-alkyl-silane; TiO_2, titanium dioxide; BCAECs, bovine carotid arterial endothelial cells; HUVECs, human umbilical vein endothelial cells; FBS, fetal bovine serum; ECGF, endothelial cell growth factor; 3D, 3-dimensional.

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doi:10.1016/j.bbrc.2007.04.206

grafting has been suggested as one of the major contributors to poor engraftment and limited survival of transplanted tissues [6]. Potential solutions include engrafting blood vessels from other host tissues/organs after implantation of engineered tissues or co-culturing transplanted cells with endothelial cells in the scaffolds to accelerate the creation of a vascularized scaffold.

Angioplasty is an accepted therapy for the treatment of ischemic tissues. Patients suffering ischemic diseases require invasive mechanical procedures of angioplasty or graft bypass surgery to restore perfusion in diseased areas. However, use of these procedures is limited to larger blood vessels with diameters >1 mm [7,8]. Recently, to restore adequate perfusion in ischemic regions, promotion of neovascularization and angiogenesis has been developed therapeutically. The microvascular endothelium participates in neovascularization in a stepwise manner. During the initiation phase, endothelial cells respond to locally produced angiogenic factors such as vascular endothelial growth factor (VEGF) and upregulate the expression of extracellular proteinases. The next phase is characterized by the migration of endothelial cells through the basement membrane into the surrounding extracellular space where these cells proliferate, migrate, and form new capillary tubes. One possible method of vascularization is through gene or protein delivery of angiogenic agents such as VEGF. Another approach is cell therapy [9,10]. Progenitor cells have emerged as a new therapeutic option for various cardiovascular conditions. For this purpose, bone marrow-derived mononuclear cells (BMCs) or circulating endothelial progenitor cells (EPCs) have been obtained directly from the bone marrow or peripheral blood of the patient and expanded *ex vivo* before implantation. However, EPCs and BMCs do not give rise to blood vessels directly, and instead stimulate vascularization by secreting large amounts of cytokines to attract and stimulate endothelial cells [11].

In vitro formation of capillary networks for engraftment into ischemic regions may offer a significant advance for tissue implantation and angioplasty. Herein, we demonstrate the *in vitro* formation of capillary networks using photo-lithographic printing techniques [12–14]. Capillary beds generated in this way were examined for *in vivo* function.

Materials and methods

Isolation and culture of endothelial cells. Bovine carotid artery endothelial cells (BCAECs) were isolated from bovine carotid artery and maintained as previously described [15]. Cells were cultured with MEM (Life Technologies) containing 5% fetal bovine serum (FBS) (Life Technologies). Human umbilical vein endothelial cells (HUVECs) were isolated from cannulated human umbilical veins by treatment with collagenase and maintained in RPMI medium (Gibco) supplemented with 20% FBS, endothelial cell growth factor (ECGF) (Sigma) and Heparin-Na.

Photo-mask preparation. STK-03 titanium dioxide (TiO_2) photo-catalyst aqueous dispersion (Ishihara Sangyo) was diluted 33 wt% with isopropyl alcohol, stirred for 1 h and filtered using a 3.0 μm pore size polytetrafluoroethylene filter (Advantec). Cr-Quartz photo-masks with slit width 60 μm and slit interval 300 μm were cleaned using a VUM-3184 UV-ozone washing machine (Oak Manufacturing) to decompose low molecular weight adsorbents on the mask surface. The mask was coated with the TiO_2 dispersion by spin-coating at ~700 rpm for 15 s and then baking at 150 °C for 10 min.

Substrate preparation. NA35 polished glass substrates (NH Techno Glass) were cleaned using the VUM-3184 for 420 s. Next, 1.5 g of heptadecafluorodecyltrimethoxysilane solution (TSL-8233; GE Toshiba Silicone), 5.0 g of tetramethoxysilane solution (TSL-8114; GE Toshiba Silicone), and 2.4 g of 0.005 N HCl were mixed and stirred for 24 h at room temperature to make fluoro-alkyl-silane (FAS) mixture solution. The mixture was diluted with 1 wt% isopropyl alcohol, stirred for 15 min, filtered by Chromato-Disc (filter type 0.45 μm; Kurabo) and then coated onto the glass substrate by spin-coating at ~1000 rpm for 15 s. The FAS-coated substrate was then baked at 150 °C for 10 min.

Surface modification and patterning. Both TiO_2-coated photo-masks and FAS-coated substrates were immersed and sonicated in de-ionized water for ⩾5 min, then baked at 120 °C for 5 min. The TiO_2-coated side of the photo-mask was irradiated with UV for 15 min at irradiation energy of ~30 J/cm^2 to rejuvenate the photo-catalytic activity of TiO_2. The FAS-coated side of the glass substrate was placed facing and in contact with the TiO_2 layer of the photo-mask and UV-irradiated for several minutes through the mask to form hydrophilic regions on the FAS layer.

Microinjection. Pattern-forming blood vessels were examined for microinjection with fluorescent dye and calcein solution (100 μM) by micromanipulator (Leitz) using micropipets (Humagen Fertility Diagnostics). Live-images were recorded as 7.5 frames/s by using a VB-6000 inverted fluorescence microscope system (Keyence). Micropipets were inserted into the end of the tubular structure of pattern-forming blood vessels under bright conditions, and then calcein solution was injected. Video images were taken at different intervals. Animation or montage of live-images allowed us to follow the flow dynamics of the fluorescence solution. Observations were performed at 18–22 °C. For quenching fluorescence of calcein in medium, $CoCl_2$ solution (10 mM; WAKO) was added to the medium.

Cell labeling. After transplantation, living cells were labeled with calcein-AM (5 mM; molecular probes) for 30 min. Capillary-like tubular structures on Matrigel (Becton Dickinson) were scanned in 3D imaging (LSM510META; Carl-Zeiss) under a 40× objective lens.

Immuno-histochemistry. Engineered capillary-like tubular structures on Matrigel were fixed in 4% paraformaldehyde and stained with antibody against VE-cadherin (ALEXIS), then scanned in 3D imaging under a 40× objective lens.

Implantation into nude mice. Human tissues were handled according to the tenets of the Declaration of Helsinki, with the approval of University Review Boards. Human placenta was obtained from an elective Cesarean section performed on a woman who was seronegative for human immunodeficiency virus, human hepatitis types B and C and syphilis. Amnion membranes were prepared as described elsewhere [16]. BCAECs were trypsinized and labeled with PKH26 and seeded on pattern-forming substrates. After 16-h incubation, cell patterns on substrates were applied to the amnion membrane and medium containing 0.3% FBS was added. Capillaries were implanted into a bluntly dissected subcutaneous punch in the anterior abdominal wall of 6-week-old BALB/C nude mice. The wound was closed by suturing with 4–0 silk, then tissues were harvested 5 days later and analyzed under laser microscopy and conventional histology.

Results and discussion

Patterning of endothelial cells

Numerous processing techniques have been developed to design and fabricate 3D scaffolds for tissue-engineered implants [17]. Moreover, patterning of cultured cells on substrates has also been attempted using polylysine [18].