Isolation and differentiation of porcine progenitor cells into endothelial-like cells for vascular tissue engineering

Josephine B. Allen, Sadiya Khan, Delara Motlagh, María Concepción Serrano, Asha Shekaran, Guillermo A. Ameer
Northwestern University, Evanston, IL USA

Introduction

Tissue engineered vascular grafts may one day provide a solution to many of the limitations associated with the use of synthetic vascular grafts and vein autografts. In order for vascular tissue engineering to become a clinical reality, scaffolds and cell sources that meet rigorous scientific as well as social criteria will be required. Several laboratories have been investigating various scaffold materials and cell sources in an attempt to meet the requirements of an ideal vascular graft [1-5]. Approaches involve the use of either primary endothelial cells from biopsies or the differentiation of adult stem cells into endothelial-like cells to cover the scaffold [6]. One potential source of mature endothelial cells is adult stem cells found in circulating peripheral blood. Endothelial progenitor cells (EPC) are released into circulating peripheral blood where they contribute to the process of adult vasculogenesis and vascular homeostasis [7]. EPCs have been found in canine [8], ovine [5], and human [9] peripheral blood and mostly studied in the context of cardiac tissue repair. However, there are few studies on the interactions of these cells with biomaterials for potential application in vascular tissue engineering. Our laboratory has been developing elastomeric and biodegradable scaffolds that are based on poly(diol citrates) and investigating their interaction with vascular cells [10, 11]. Herein, we describe the isolation and characterization of EPCs from porcine peripheral blood and their potential to create a functional endothelium on poly (diol citrates). The pig was chosen as the model system because it is commonly used for vascular research due to the size, physiological, and mechanical similarities to humans.

Materials and Methods

Synthesis of poly(1,8-octanediol-co-citrate) (POC)

All chemicals were purchased from Sigma Aldrich (Milwaukee, WI). The synthesis of POC and the fabrication of POC scaffolds for vascular grafts are described elsewhere [10, 12]. The pre-polymer was post-polymerized in wells of multi-well tissue culture polystyrene (TCP) plates at 60° under vacuum (2Pa) for 7-10 days.

In vitro culture and differentiation of endothelial progenitor cells (EPC)

Peripheral blood was collected from the exterior jugular vein of domestic pigs in the presence of EDTA K_2 anti-coagulant. All procedures involving blood collection were performed in accordance with the regulations of the Northwestern University Animal Care and Use Committee (NU-ACUC). Peripheral blood mononuclear cells (PB-MNC) were isolated from whole blood via histopaque density gradient centrifugation using Accuspin tubes (Sigma Aldrich, Milwaukee, WI). The isolated PB-MNC were suspended in endothelial cell basal medium-2 (Clonetics, Baltimore, MD) supplemented with 17% FBS, and human fibroblast growth factor (rhFGF-B), epidermal growth factor (rhEGF), vascular endothelial growth factor (VEGF), human recombinant insulin-like growth factor (R^3-IGF-1), ascorbic acid, gentamicin

sulphate , amphotericin-B, and heparin. The cells were seeded onto fibronectin-coated plates (BD Biosciences; Bedford, MA). After 4 days, non-adherent cells were removed by complete media change and thereafter the media was changed every 3 days. Confluent cultures were passaged for all subsequent analysis onto uncoated tissue culture flasks and onto uncoated POC films.

Characterization of differentiated PE-like cells

Immunohistochemistry and uptake of low density lipoprotein (LDL): PB-MNC cultured for 8 days and differentiated PE-like cells at passage 8 were probed with primary antibodies to EC specific markers von Willebrand Factor (vWF) (DakoCytomation, Carpenteria, CA) and Vascular Endothelial-Cadherin (VE-Cadherin) (Sigma Aldrich, Milwaukee, WI). Cellular uptake of fluorescently labeled acetylated-LDL was evaluated in PE-like cells and PAEC cultured on TCP.

Growth kinetics: Differentiated PE-like cells were cultured on TCP and POC in 12 well plates. Cells were lysed with 0.1% triton-X-100 from 6 different wells each at 5, 24, 48, 72 and 96 hours. The total DNA content in each well was determined from cell lysates at each time point. The percent increase in DNA content over baseline (5 hours post seeding) was compared for each time point to obtain the growth kinetics of PE-like cells on TCP and POC.

Strength of adhesion: PE-like cells were seeded onto POC-coated or uncoated glass slides. Seeded PE-like cells were cultured for 48 hours to reach confluency. The CytoShear Flow Chamber System (Cytodyne, La Jolla, CA), and Masterflex pump (Cole Parmer, Vernon Hills, IL) were used to expose the cultured PE-like cells to 20dynes/cm^2 fluid shear stress for approximately 1 hour. Following fluid shear exposure, percent cell retention was calculated as the quantity of DNA from cells remaining attached on the surface after fluid shear stress divided by the quantity of DNA from attached cells under static conditions.

Nitric oxide (NO) and prostacyclin (PGI_2) synthesis and secretion: NO and PGI_2 secretion (baseline and stimulated) was evaluated in PE-like cells, PAEC, and fibroblasts cultured on POC. Stimulation of NO and PGI_2 was achieved by the addition of 100 μg/ml vascular endothelial growth factor (VEGF) and 100 ng/ml arachidonic acid (AA), respectively. The amount of total NO and PGI_2 secreted was determined by using the total NO assay kit and 6-keto-Prostaglandin $F_{1\alpha}$ enzyme immunoassay kit, respectively (Assay Design Inc., Ann Arbor, MI).

Clotting kinetics of recalcified plasma: Platelet poor plasma (PPP) was prepared by centrifuging porcine whole blood at 2000 g and collecting the supernatant, which was devoid of platelets. PAEC and PE-like cells were cultured to confluence on both TCP and POC-coated wells of a 48-well plate. 200μl of PPP was added to each well followed by 200 μl of 0.025 M $CaCl_2$, immediately thereafter. To record the kinetics of clot formation in real time, the absorbance at 405nm was measured every 30 seconds for 45 minutes, with an increase in absorbance indicating clot formation.

Quantitation of platelet adhesion: Platelet rich plasma (PRP) was prepared by centrifuging porcine whole blood at 250 g and collecting the supernatant, containing platelets. PRP was incubated on a confluent monolayer of PAEC, PE-like cells, and fibroblasts for 1 hour. TCP

serves as a positive control for maximum platelet adhesion. The number of adherent platelets was determined by detecting the amount of lactate dehydrogenase (LDH).

Statistical Analysis

Numerical data are reported as a mean ± standard deviation. A student's 2-sample t-test was used to compare means. Analysis of variance (ANOVA) with Newman-Keuls multiple comparison test post-hoc analysis was used to determine significant differences among 3 or more means. A P-value of 0.05 or less was required for significance.

Results

In vitro culture, differentiation, growth kinetics, and force of attachment of PE-like cells

The porcine PB-MNC population that attached to the fibronectin-coated plate became endothelial-like in as early as 8 days **(Figure 1A)**, and formed a confluent monolayer by 14 days of culture **(Figure 1B)**. At this time, the EC cobblestone morphology became apparent. Once differentiated the PE-like cells can be passed onto POC and based on the growth kinetic data, are highly proliferative on both TCP and our POC **(Figure 1C)**. When exposed to 20dynes/cm^2 of fluid shear detachment force, there was no difference in the amount of PE-like cell retention on both POC and glass **(Figure 1D)**. In fact, there was no significant difference between the percentage of cell retained to both POC and glass.

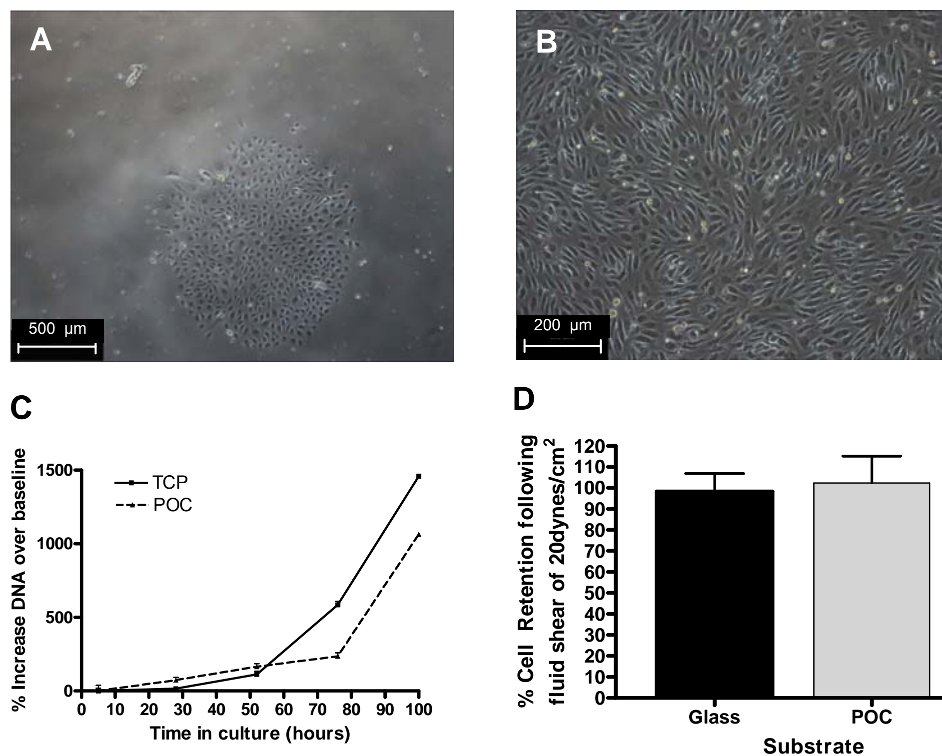

Figure 1. Characterization of PE-like cells
(A) Phase contrast image of PE-like cell colony formation present at 8 days on culture; (B) Phase contrast image of PE-like confluent monolayer formation; (C) Growth kinetics of PE-like cells on TCP and POC; (D) Assessment of attachment strength of PE-like cells to glass and POC.

Determination of endothelial cell phenotype and function

Immunohistochemistry: PE-like cells showed positive staining for the endothelial cell markers, vWF and VE-Cadherin as early as 8 days after initial isolation. This positive staining was maintained once the cells were passaged onto uncoated POC. Further, acetylated LDL uptake was seen in PE-like cells supporting the existence of the SREC-1 receptor on the differentiated cells.

NO and PGI_2 synthesis and secretion: PE-like cells cultured on POC secreted NO and PGI_2 under static culture conditions. There was no difference in the baseline amount of NO secreted by PE-like cells and PAEC. Stimulation with VEGF resulted in significant increases in NO production in both PAEC and PE-like cells **(Figure 2A)**. Conversely, baseline PGI_2 secretion was significantly lower in PE-like cells than in PAEC. However, treatment with AA resulted in a 4 fold and 21 fold increase in PGI_2 secretion relative to baseline amounts for PE-like cells and PAEC, respectively **(Figure 2B)**.

Clotting kinetics of recalcified plasma: Recalcification clotting profiles and clotting rates for PE-like cells and PAEC cultured on TCP and POC were obtained**.** These data show that when compared to TCP, there is a significant decrease in the rate of clot formation with the presence of either a PE-like cell or PAEC cell monolayer. Additionally, there was no difference in the rate of clotting of PPP on PAEC and PE-like cells grown on TCP and POC.

Quantitation of platelet adhesion: The number of platelets attached to PAEC cultured on TCP was significantly lower relative to all the other cell types and TCP alone. PE-like cells showed a significantly lower number of attached platelets than the positive controls, fibroblast and TCP ($P<.001$). Additionally, the two positive controls for platelet adhesion, fibroblasts and TCP showed no significant difference in the number of platelets attached.

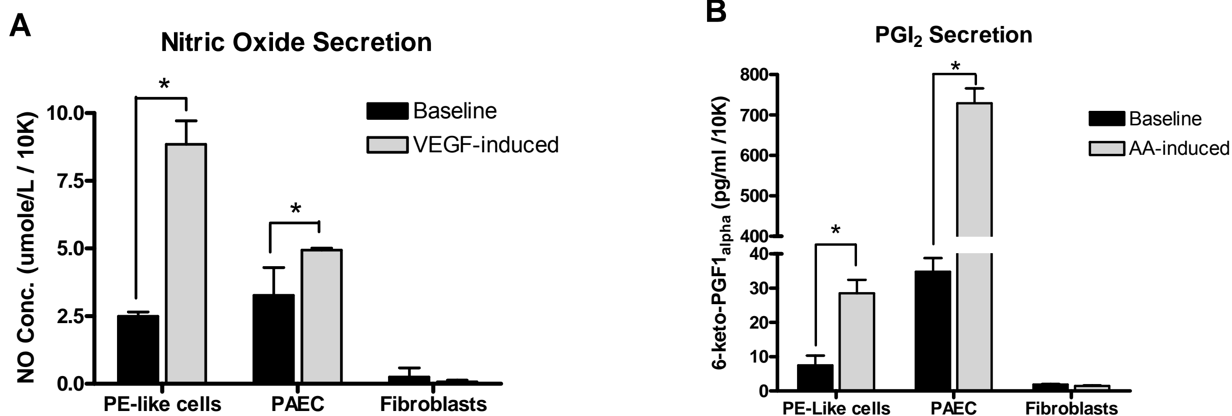

Figure 2. Secretion of anti-thrombogenic factors Nitric Oxide and PGI_2

(A) Baseline and VEGF-induced nitric oxide secretion by PE-like cells, PAEC and fibroblasts; (B) Baseline and AA induced secretion of PGI_2.

Discussion

The use of EPC as a cell source in tissue engineering was proposed soon after their discovery in peripheral blood. There is increasing evidence through clinical trials confirming the ability of these cells to promote postnatal vasculogenesis [2]. However, the utility of EPCs for vascular tissue engineering will depend on their successful isolation and *in vitro* expansion, as they only constitute 0.01% of the total circulating mononuclear cells [2]. EPC should also interact favorably with the biomaterial used as a vascular scaffold. Specifically, they should maintain their differentiated state, antithrombotic function, and adhesion characteristics on the biomaterial surface.

Porcine EPCs were isolated and differentiated into endothelial-like cells *in vitro*. *In-vitro* differentiated porcine endothelial-like cells are highly proliferative and display morphologic, phenotypic, and functional features of mature endothelial cells. These properties are retained on the synthetic polymer scaffold, POC, and will be useful to improve the biological functions of an engineered vessel. The integration of this endothelial-like cell source with a biocompatible and inexpensive biodegradable material such as POC is promising and warrants further evaluations *in vivo*. The use of POC vessels seeded with PE-like cells could potentially have a major impact on vascular tissue engineering by providing a suitable autologous cell source for vascular graft studies in the pig animal model.

References

1. Higgins, S.P., A.K. Solan, and L.E. Niklason, *Effects of polyglycolic acid on porcine smooth muscle cell growth and differentiation.* J Biomed Mater Res A, 2003. **67**(1): p. 295-302.
2. Nerem, R.M. and A.E. Ensley, *The tissue engineering of blood vessels and the heart.* Am J Transplant, 2004. **4 Suppl 6**: p. 36-42.
3. Schmedlen, R.H., et al., *Tissue engineered small-diameter vascular grafts.* Clin Plast Surg, 2003. **30**(4): p. 507-17.
4. Ross, J.J. and R.T. Tranquillo, *ECM gene expression correlates with in vitro tissue growth and development in fibrin gel remodeled by neonatal smooth muscle cells.* Matrix Biol, 2003. **22**(6): p. 477-90.
5. Kaushal, S., et al., *Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo.* Nat Med, 2001. **7**(9): p. 1035-40.
6. Zilla, P., M. Deutsch, and J. Meinhart, *Endothelial cell transplantation.* Semin Vasc Surg, 1999. **12**(1): p. 52-63.
7. Khakoo, A.Y. and T. Finkel, *Endothelial progenitor cells.* Annu Rev Med, 2005. **56**: p. 79-101.
8. He, H., et al., *Canine endothelial progenitor cell-lined hybrid vascular graft with nonthrombogenic potential.* J Thorac Cardiovasc Surg, 2003. **126**(2): p. 455-64.
9. Asahara, T., et al., *Isolation of putative progenitor endothelial cells for angiogenesis.* Science, 1997. **275**(5302): p. 964-7.
10. Webb, A.R., J. Yang, and G.A. Ameer, *Biodegradable polyester elastomers in tissue engineering.* Expert Opin Biol Ther, 2004. **4**(6): p. 801-12.
11. Yang, J., et al., *Modulating ePTFE vascular graft host response via citric acid-based biodegradable elastomers.* Advanced Materials, 2006. **18**(12): p. 1493-1498.
12. Yang, J., A.R. Webb, and G.A. Ameer, *Novel citric acid-based biodegradable elastomers for tissue engineering.* Advanced Materials, 2004. **16**(6): p. 511-+.