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Capture of endothelial cells under flow using immobilized vascular endothelial growth factor

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ABSTRACT

We demonstrate the ability of immobilized vascular endothelial growth factor (VEGF) to capture endothelial cells (EC) with high specificity under fluid flow. To this end, we engineered a surface consisting of heparin bound to poly-L-lysine to permit immobilization of VEGF through the C-terminal heparin-binding domain. The immobilized growth factor retained its biological activity as shown by proliferation of EC and prolonged activation of KDR signaling. Using a microfluidic device we assessed the ability to capture EC under a range of shear stresses from low (0.5 dyne/cm^2) to physiological (15 dyne/cm^2). Capture was significant for all shear stresses tested. Immobilized VEGF was highly selective for EC as evidenced by significant capture of human umbilical vein and ovine pulmonary artery EC but no capture of human dermal fibroblasts, human hair follicle derived mesenchymal stem cells, or mouse fibroblasts. Further, VEGF could capture EC from mixtures with non-EC under low and high shear conditions as well as from complex fluids like whole human blood under high shear. Our findings may have far reaching implications, as they suggest that VEGF could be used to promote endothelialization of vascular grafts or neovascularization of implanted tissues by rare but continuously circulating EC.

1. Introduction

Coronary artery disease (CAD) is the leading cause of mortality in the United States, necessitating approximately half million coronary artery bypass graft (CABG) surgeries annually [1]. While autologous venous or arterial grafts have been the gold standard for many years, up to 30% of patients requiring venous grafts lack transplantable veins [2,3]. Limited availability of suitable autologous vessels, morbidity at the donor site, and the high rate of long-term failure of venous grafts necessitate the search for alternative strategies [4]. Various groups have demonstrated patent tissue engineered vessels (TEVs) comprised of various biomaterial scaffolds seeded with cells in the lumen and medial layers and further preconditioned in various bioreactors exposing tissues to shear stress and/or pulsatile pressure [5–12]. Alternatively, other groups demonstrated success with scaffold-free TEV's comprised of cell sheets that were rolled and preconditioned prior to implantation and such TEVs have even progressed to human clinical trials [13–17]. However, both types of TEVs require an autologous cell source and weeks to months of culture before implantation. Consequently, development of cell-free TEVs has re-emerged recently as several laboratories demonstrated successful approaches to decellularize cell-containing TEVs and provide "off-the-shelf" transplantable grafts [18–21]. However, a functional endothelium was still necessary to maintain graft patency [22]. One group reported engineering of a completely acellular TEV that exhibited patency and remodeling in a rat animal model [23], but evidence that this approach can also work in a larger, clinically relevant animal model is still lacking. For cell-free vascular grafts, a functional lumen is necessary for successful transplantation. Methods to capture cells after implantation are emerging as an alternative strategy that aims at capturing rare circulating

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endothelial progenitor cells (EPCs) to repopulate the lumen and maintain graft patency.

EPCs are rare, highly proliferative, circulating cells capable of endothelializing vascular grafts, and have been a consistent target of cell capture technologies, such as microfluidic devices with appropriate surface modifications [24–27]. Of note is the capture of EPCs using surface immobilized antibodies against cell surface molecules including VEGFR2, vWF, CD31, and CD34, under low shear stress [26,27]. In these studies cell capture occurred at shear stress lower than 4 dyne/cm^2, whereas physiological shear in arterial vessels, such as the carotids, ranges from 10 to 14 dyne/cm^2 [28].

While antibodies can capture cells, they may not promote and in fact, may even block cell proliferation [27]. On the other hand, growth factors are well-established inducers of cell proliferation and/or migration and may be a viable alternative to antibodies to capture rare cells. Moreover, surface immobilization of several growth factors such as NGF, EGF, GM-CSF, VEGF, and KGF has been shown to induce prolonged biological activity over their soluble counterparts [29–37]. Interestingly, the presentation of immobilized growth factors to the cells is directly related to their bioactivity and varies with the method of immobilization [29,38,39]. Recently, we showed that engineered TGF-β1 fused to the fibrin binding peptide, NQEQVSP, prolonged the phosphorylation of Smad2/3 from a few hours to several days, thereby increasing deposition of collagen and elastin and improving the contractility of vascular grafts [40]. Similarly, immobilization of heparin binding growth factors such as bFGF, TGF-β2, or VEGF to heparin via their heparin-binding domain (HBD) was shown to prolong their biological activity [41–44].

VEGF is a well-known potent mitogen for endothelial cells [45–55] and a major inducer of angiogenesis - the development of new blood vessels in the body [56–62]. Binding of VEGF to heparin through the C-terminal HBD was shown to increase its biological activity, possibly by enhancing presentation of the N-terminal VEGFR binding domain [53,63,64] and as a result this strategy has been employed in various biomaterial applications [29,38,39,65,66]. Interestingly, VEGF bound to extracellular matrix proteins such as collagen, fibronectin, vitronectin, laminin, and matrix-associated heparin was shown to prolong VEGF receptor 2 (VEGFR2) phosphorylation and subsequent signaling events [41,67–71]. In addition, binding of cells to matrix immobilized VEGF promoted association of VEGFR2 with β1-integrin, which in turn associated with focal adhesions, a process that did not occur with soluble VEGF [30]. Therefore, binding to heparin allows for optimal presentation of VEGF and subsequently, an enhanced cellular response.

Here we hypothesized that surface immobilized VEGF may capture endothelial cells under flow and subsequently support the proliferation and expansion of captured cells. Indeed, VEGF immobilized onto heparin could capture EC under low and high shear stress in a highly selective manner, even from complex biological fluids such as blood. Our findings suggest that this strategy may be useful in capturing rare endothelial cells for diagnostic or regenerative medicine applications.

2. Materials and methods

2.1. VEGF cloning and protein production

The pGEX-VEGF plasmid was graciously provided by Dr. Te-Chung Lee of the University at Buffalo, SUNY. This plasmid encodes for a thrombin cleavable glutathione-S-transferase (GST) tag followed by the *VEGF-165* gene. For protein production, bacteria strain *Escherichia coli* BL21-DE3-pLysis was kindly provided by Dr. Sriram Neelamegham of the University at Buffalo, SUNY. Bacteria was then expanded until O.D. = 0.8, then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for protein production for 4–6 h at 37 °C and 300 rpm. The bacteria was pelleted at 20,000 g for 30 min. Bacterial pellets were re-suspended in lysis buffer (50 mM Tris, 500 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.5, 1 mg/mL lysozyme, and protease inhibitors) and Triton X-100 was added at 1% prior to sonication. Sonication consisted of 10 cycles with 70% intensity, 30 s on/30 s off. Sonicated lysates were clarified by ultracentrifugation at 50,000 g for 30 min. Insoluble material consisting mostly of inclusion bodies was subjected to numerous rounds of washing and sonication. The final, washed, inclusion body pellet was re-suspended in solubilization buffer (50 mM Tris, 500 mM NaCl, 7 M Urea, 1 M Guanidine-HCl, 1 mM EDTA, 100 mM dithiothreitol (DTT), pH of 8.5) prior to refolding by dialysis. Briefly, solubilized GST-VEGF was immediately added to a dialysis membrane (SpectraPor-1 6–8 kDa cut-off) and dialyzed in Refolding Buffer-1 (50 mM Tris, 500 mM NaCl, 10 mM KCl, 1 mM EDTA, 2 M Urea, 500 mM L-Arginine, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, pH 8.5) for 24 h. The volume of the refolding buffer was 100× the volume of solubilized GST-VEGF. Each subsequent day the refolding buffer was replaced with half the urea concentration of the previous day for 3 days. The final dialysis step was performed in PBS. Refolding success was determined by homodimer formation as analyzed by 10% SDS-Page with and without reducing agent DTT. Properly refolded GST-VEGF has an apparent MW of 95–110 kDa, which reduces to 55 kDa upon DTT treatment. Refolded GST-VEGF was then subjected to sequential purification using GST agarose beads (Sigma,St. Lous, MO), thrombin cleavage of GST from VEGF, and a final purification step by passing cleaved VEGF through a Hitrap Heparin Column (GE Healthcare, Pittsburg, PA) according to the manufacturer's instructions.

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza as a pooled donor isolation, maintained in EGM2 complete media (Lonza; Allendale, NJ) and used between passage 2 and 6 and maintained below 75% confluence. Hair follicle derived mesenchymal stem cells (HF-MSC) were isolated as described and maintained in DMEM (Life Technologies) supplemented with 10% MSC-FBS (Invitrogen) and 1 ng/mL bFGF [72]. NIH-3T3 fibroblasts were purchased from American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% BS (Invitrogen). Ovine pulmonary artery endothelial cells (OPAECs) were isolated as previously described [73] and were maintained in DMEM supplemented with 20% FBS. Human dermal fibroblasts (h-dFB) were isolated as described previously from neonatal foreskin and maintained in DMEM supplemented with 10% FBS [37]. All media supplemented with 1% Pen/Strep AA cocktail (Invitrogen). All cells were maintained in a humidified incubator with 10% CO_2 at 37 °C.

2.3. Biological activity of recombinant VEGF

The biological activity of recombinant VEGF was assessed using a standard cell proliferation assay. To this end, HUVECs were seeded onto a 96 well plate at a density of 2×10^3 cells per well in M199 medium (Life Technologies, Grand Island, NY) supplemented with 2% heat inactivated FBS and varying concentrations of recombinant VEGF or commercial VEGF (Cell Signaling) that was used as control. Concentrations ranged from 0.05 ng/mL to 100 ng/mL. Cells were allowed to proliferate for 72 h prior to treatment with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Life Technologies) for 4 h. Then, the medium was carefully removed and replaced with 100 μl of DMSO to solubilize the purple formazan crystals. Absorbance was read at 570 nm using a Biotek Synergy 4 Spectrophotometer (with background absorbance at 650 nm subtracted).

2.4. Immobilization of VEGF

Poly-L-Lysine, MW 75,000–150,000 (Sigma) was used at the manufacturer's recommended concentration of 0.05 mg/mL to coat tissue culture treated polystyrene surfaces (TC). Coating was performed by adding sterile PLL solution for 2 h with rocking at RT. After coating with PLL, the surface was washed repeatedly with sterile water to remove unbound PLL. Heparin (17–19 kDa) from porcine submucosa (Sigma) was dissolved in sterile water at a concentration of 5 mg/mL and then applied directly to the PLL treated surfaces overnight at RT. Then the surface was washed with sterile water to remove unbound heparin and heparin binding to PLL was determined using the toluidine blue binding assay as described previously [74]. Heparin concentration on the surface was determined by comparing the unbound heparin concentration remaining in solution to the initial concentration. Briefly, unbound heparin was added to toluidine blue buffer (0.1 mg toluidine blue dissolved in 50 mM TBS buffer, pH 4.5) at a ratio of 1 volume toluidine blue to 12 volumes heparin. The reaction proceeded for 30 min prior to centrifugation and absorbance was measured at 631 nm using a Biotek Synergy 4 Spectrophotometer (background absorbance of an empty well was subtracted from each value).

Finally, recombinant VEGF was then added to the PLL-Heparin surface. Binding of VEGF was optimal at 37 °C without rocking, for 2 h. VEGF binding was determined by ELISA using biotin-conjugated goat anti-VEGF antibody (100 ng/mL, 2 h, RT, R&D Systems), followed by incubation with streptavidin-HRP (1:200, 30 min, RT) and addition of substrate (TMB, Sigma). Absorbance was read at 450 nm using a Biotek Synergy 4 Spectrophotometer (with subtraction of background absorbance of 570 nm).