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Endothelial retention and phenotype on carbonized cardiovascular implant surfaces

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ABSTRACT

Heart valve disease is an increasing clinical burden for which there is no effective treatment outside of prosthetic replacement. Over the last 20 years, clinicians have increasingly preferred the use of biological prosthetics to mechanical valves despite their superior durability because of the lifelong anticoagulation therapy that is required. Mechanical valve surface engineering has largely focused on being as non-thrombogenic as possible, but despite decades of iteration has had insufficient impact on the anti-coagulation burden. In this study, we systematically evaluate the potential for endothelialization of the pyrolytic carbon surface used in mechanical valves. We compared adsorbed adhesion ligand type (collagen I, fibronectin, laminin, and purified adhesion domain fragments GFOGER and $FN_{7\text{-}10}$) and concentration on endothelial adhesion rates and adhesion strength on Medtronic-Hall prosthetic valve surfaces. Regardless of ligand type or concentration, endothelial adhesion strengthening was insufficient for their intended ultra-high shear stress environment. We then hypothesized that microfabricated trenches would reduce shear stress to tolerable levels while maintaining endothelial access to the flow stream, thereby promoting a confluent and anticoagulant endothelial monolayer. Computational fluid dynamics simulations predicted an empirical relationship of channel width, depth, and spacing that would maintain interior surface shear stress within tolerable levels. Endothelial cells seeded to confluence in these channels retained a confluent monolayer when exposed to 600 dyn/cm^2 shear stress for 48 h regardless of applied adhesive ligand. Furthermore, sheared EC expressed a mature anti-coagulant profile, including endothelial nitric oxide synthase (eNOS), VE-cadherin, and significantly downregulated plasminogen activator inhibitor-1 (PAI-1). As a final test, channeled pyrolytic carbon surfaces with confluent EC reduced human platelet adhesion 1000-fold over pyrolytic carbon alone. These results advance a promising biohybrid approach to enable active moderation of local coagulative response in mechanical heart valves, which could significantly extend the utility of this important treatment for heart valve disease.

1. Introduction

Prosthetic implantable devices remain an essential component of healthcare as the clinician's last line of defense for the treatment of serious cardiovascular disease. These devices include ventricular assist devices, total artificial hearts, mechanical heart valve replacements, and vascular stents. Each design is optimized to generate and/or maintain blood flow, which requires mechanical durability but also necessitates that the surface of the materials from which they are comprised to be in direct and continuous contact with blood. Research and clinical findings over 40 years of the so-called "first generation" of biomaterial design established that only a small set of biomaterials were suitable for implantation, including cobalt–chromium, titanium, silicone, expanded poly(-tetrafluoroethylene) (ePTFE), and some poly(ethylene) polymers [1]. The primary quality of each of these materials was that they did not elicit an inflammatory or immune response from the host. The fundamental clinical drawback from using these devices remains

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its hemocompatibility, or ability to function in contact with blood without inducing inappropriate clotting response. Biomaterial contact with blood can induce spontaneous coagulation through both extrinsic and intrinsic pathway cascades [2,3]. The formation of a clot can lead to local occlusion of the device, or a component can break off and occlude a distal vessel, most often leading to a stroke. As these thromboembolic events are highly unpredictable and very serious, clinicians administer anti-coagulant and anti-thrombotic cocktails to reduce these risks. Warfarin is most often prescribed for its multifaceted inhibitory properties, but anti-platelet, anti-thrombin (e.g. Dabigatran etexilate), and heparin-like molecules, and aspirin are also prescribed depending on the circumstances [4,5]. While reducing the risk of clot formation, these drugs also cause an increase in the risk of internal bleeding events, such as gastrointestinal bleeding and brain hemorrhages. Maintaining this tight balance of pro- and anti- coagulation behavior is further compounded by the fact that the coagulability of blood is dependent on the demographics and activity levels of the patient [6,7]. Despite the significant occupational and lifestyle limitations to which these patients are subjected, a 2–4% annually cumulative risk of major bleeding event is expected [8]. The superior mechanical durability of these devices, especially in the case of mechanical heart valves, has led some to propose that there would be no need for bioprosthetics if coagulation could be better controlled [1].

The first step of surface contact-induced coagulation is through platelet adhesion and activation. While hemodynamic conditions created by some devices can induce platelet activation in the blood stream, circulating platelets will readily adhere to a biomaterial surface if there are any adsorbed proteins [9]. Many attempts have been made to render prosthetic surfaces non-fouling through polymer coatings, but in vivo results to date suggest that these coatings are susceptible to cracking and wear [1,10–12]. Similarly, Milner and colleagues implemented a nano-pillar array to reduce the contact area for platelet adhesion [13]. Etching and surface texturing has also shown to modulate platelet adhesion in vitro, but results in vivo have been lacking [14,15]. Given that these devices are intended to last the rest of the patient's lifetime once implanted, it is likely impossible to ensure no protein adhesion to these surfaces with a passive process. In contrast, immobilizing anti-coagulant and/or anti-thrombotic molecules on biomaterial surfaces (e.g. thrombomodulin) has also suggested an improvement in hemocompatibility [16], but it is likely difficult to control coagulation using only one species in the cascade.

It is well known that endothelial cells, which line all natural blood contacting surfaces, are the ideal anti-coagulant and anti-thrombotic agent [17–19]. Endothelial cells secrete nitric oxide (NO), produced through the enzyme endothelial nitric oxide synthase (eNOS), which inhibits clot formation through a number of effects, including vasodilation, inhibiting the thromboxane receptor A2 (TXA2), and downregulation of adhesion receptors such as P-selectin and GPIIb-IIIa [20–22]. Endothelium also secretes tissue plasminogen activator to dissolve fibrin clots and thrombomodulin to inhibit the coagulation cascade [23,24]. Conversely, endothelium can induce clot formation as an injury response through the expression of von Willebrand Factor (vWF), Tissue Factor (TF), and/or plasminogen activator inhibitor-1 (PAI-1) [25]. Endothelial cells ensure a confluent surface layer by the formation of tight adherens junctions comprised of a number of molecules, including vascular endothelial cadherin (VE-Cad) [26]. Disruption of these junctions and/or exposure to the subendothelial tissue matrix can also induce platelet activation and aggregation. Endothelial function is directly modulated by hemodynamic signaling, namely wall shear stress, which permits natural real-time tuning of the coagulation response [27,28]. Recently, Douglas and colleagues enhanced NO production in endothelium in mice using GTP-cyclohydrolase 1 overexpression, and showed that robust healthy endothelial activity was better at reducing in-stent restenosis and clot formation than drug eluting stents regardless of strut design [29].

Endothelialization of cardiovascular biomaterial surfaces has been studied for over 30 years, most often for synthetic vascular grafts. Many studies have shown that grafts with an intact endothelial monolayer perform better and last longer than those without endothelium. Achieving confluent endothelial coverage however has been extremely challenging, in particular for long grafts where natural endothelial migration across anastomoses is of little benefit while ex vivo seeding is incomplete [30]. Strategies used to enhance endothelial coverage include enhancing bulk material porosity, surface texturing, and coating with cell adhesive peptides [31,32]. The operating range of shear stress in blood vessels is generally <50 dyn/cm^2, which suggests that once adhered an endothelial cell will not likely be detached mechanically [33]. This is not the case with mechanical prosthetic heart valves, where surface shear stresses regularly exceed 500 dyn/cm^2, far greater than what endothelium on tissue culture polystyrene can withstand (~80 dyn/cm^2) [34,35]. Only one study to date has tested whether endothelial cells could adhere to the mechanical valve surface. While a confluent monolayer was present on all the components after 1 week in culture, virtually all the cells were lost after only 1-h implantation in the mitral position of a pig [36]. The authors concluded that pyrolytic carbon was not a suitable substrate for endothelial retention under high shear stress, but no quantitative analysis was made nor complementary adhesion strategies attempted.

Recent studies have identified specific motifs on extracellular matrix ligands that increase cell adhesion strength and also modulate adherent cell phenotype [37–39]. The objective of this study therefore was to determine the effects of ligand-mediated adhesion strengthening on endothelial coverage on pyrolytic carbon surfaces exposed to fluid flow. In addition, we tested the effect of local surface shear stress reduction via microfabricated trenches on endothelial adhesion, retention under high shear stress in vitro, and hemostatic phenotype.

2. Materials and methods

2.1. Endothelial cell isolation and culture

Aortic valve endothelial cells (EC) were isolated from healthy porcine aortic valves were collagenase digestion as previously described [40, 41]. ECs were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 3.7 g/L sodium bicarbonate (Cell Grow), 1% penicillin-streptomycin (Gibco), and 50 mg/L heparin salts (Sigma Aldrich). Cells were grown at 37 °C and 5% CO_2 and used between passage number 3 and 5.

2.2. Cell adhesion experiments

Medtronic-Hall prosthetic valve discs (25 mm diameter, kind gift from Medtronic, Inc.) were chosen because of their flat, large surface-area profile. Disc surfaces were temporarily divided into equal geometric regions with silicone isolation gaskets, and regions coated for 1 h with either type I collagen (Coll I, BD Biosciences), human fibronectin (FN, BD Biosciences), recombinant human laminin (Sigma), a recombinant fibronectin 7-10 fragment (FN_{7-10})[39, 42], or collagen-mimetic GFOGER triple helical synthetic peptide [43]at 10 μg/mL in PBS with untreated controls. This was followed by blocking of nonspecific binding with incubation in 5% nonfat dry milk in PBS for 1 h. EC were first fluorescently labeled with CellTracker RED (CTMX, Molecular Probes) according to the manufacturer's instructions. After rinsing the surfaces in PBS, 50 μL suspensions containing 10,000 labeled cells in PBS were inoculated in each well and adhesion assessed at 10, 20, 30, 40, and 50 min. Non or poorly adherent cells were dislodged by rotary shaker for 1 min at 60 rpm and then aspirated. Each region was then imaged via fluorescence microscopy and adherent cells counted via color thresholded particle image analysis using ImageJ (NIH). Next, disc regions were incubated with single proteins or peptides as above at concentrations ranging from 0.1 to 20 μg/mL in PBS for 1 h followed by blocking in 5% dry milk as before. After rinsing, similar suspensions of cells were inoculated and adhesion quantified after 10 min as before.