Modifying Venous Valve Biomaterial for Protein C Activation

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Introduction: The development of an artificial venous valve has been limited by the thrombotic nature of the venous system. An artificial venous valve constructed with leaflets of decellularized, porcine small intestinal submucosa (SIS) performed well in a long-term sheep model, and reached human clinical trials where it promoted the healing of venous ulcers.[1] However, the performance of the valves was significantly compromised by thrombus formation, and all 15 of the implanted valves were unable to fully close 12 months post-implantation. Strategies to reduce thrombus formation on the SIS leaflets may prolong SIS venous valve function.

Thrombomodulin, an endothelial surface protein, regulates thrombus formation by binding free thrombin to form the thrombin-thrombomodulin complex and generate activated protein C (APC). APC directly inhibits coagulation and also inhibits endothelial apoptosis to sustain endothelium-dependent regulation of thrombosis. This work modified SIS with thrombomodulin and characterized APC generation and the effect on the intrinsic coagulation pathway.

Methods: *Thrombomodulin modification:* Free carboxyl groups on SIS (Cook Biotech) were activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHS). Soluble thrombomodulin (500 ng) was immobilized via an amide bond to the EDC/NHS-activated SIS. Alternatively, thrombomodulin was adsorbed to SIS. To determine if drying affects thrombomodulin activity, modified SIS was dried overnight and compared to freshly-modified SIS. *Thrombomodulin activity:* Thrombin and protein C were added to SIS and incubated for 1 hour at 37°C. The chromogenic substrate S-2366 (Chromogenix) was added and the rate of absorbance increase at 405 nm was used to calculate APC concentration. The background activity of thrombin and protein C only (no thrombomodulin) was subtracted from all measurements.

Anticoagulant activity: Prolonging the activated partial thromboplastin time (aPTT) indicates inhibition of the intrinsic coagulation pathway. SIS samples with and without thrombomodulin modification were placed into cuvettes with baboon plasma anticoagulated with citrate. HemosIL® aPTT reagent (Instrumentation Laboratory) was added followed by exogenous calcium to initiate coagulation. Time to coagulation was automatically recorded by the KC1 Delta (Tcoag). Additionally, thrombomodulin or APC were added to plasma to determine the sensitivity of the aPTT to these factors.

Results: Modifying SIS with thrombomodulin, either by adsorption or by EDC/NHS-mediated binding, enabled APC generation on SIS (**Figure 1**). Drying the modified SIS reduced thrombomodulin activity by approximately 50%. However, the APC generation of thrombomodulin-modified, dried SIS was still ~25-fold greater than unmodified SIS. Adsorption and EDC/NHS-mediated thrombomodulin binding enabled similar APC generation.

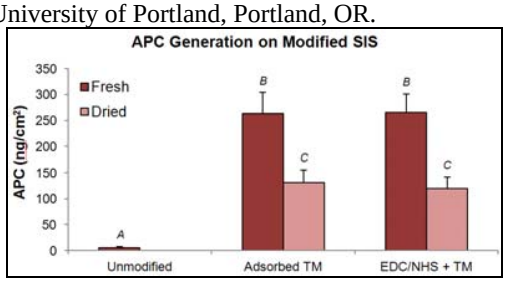

Figure 1: APC generation on thrombomodulin (TM)-modified SIS. Letters indicate homogenous subsets, $p<0.05$ by one-way ANOVA and Tukey's post-hoc, $n = 4$.

Despite similar APC generation, adsorbed thrombomodulin prolonged the aPTT more than EDC/NHS immobilized thrombomodulin **(Figure 2**). Unmodified SIS prolonged the aPTT, an effect negated by EDC/NHS treatment, more than thrombomodulin-modified SIS. Shortening of the aPTT may be due to thrombin-thrombomodulin complexes on the modified SIS exerting anti-fibrinolytic effects via thrombin-activatable fibrinolysis inhibitor (TAFI), as thrombomodulin at low concentrations preferentially promotes hemostatic effects via TAFI.[2] Increasing the concentration of thrombomodulin may favor APC generation by the thrombin-thrombomodulin complex over TAFI activation. Assays that measure the extrinsic coagulation pathway, such as the prothrombin time, or fibrinolysis will also be investigated to better characterize coagulation on SIS.

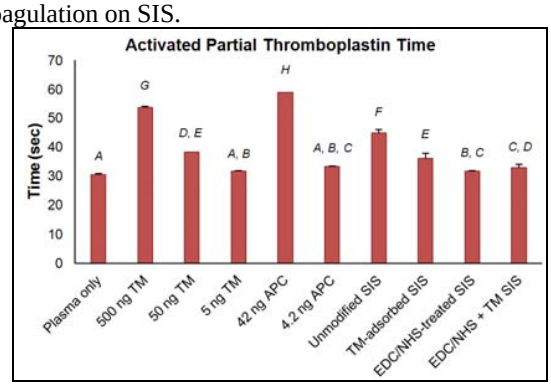

Figure 2: Activated partial thromboplastin times of plasma with thrombomodulin (TM), APC, unmodified or thrombomodulin-modified SIS, charted as the means + S.D. Letters indicate homogenous subsets, $p<0.05$ with a one-way ANOVA and Tukey's post-hoc, $n >3$.

Conclusions: Modifying SIS with thrombomodulin, either by adsorption or by EDC/NHS-mediated covalent binding, enabled generation of the anticoagulant and cytoprotective APC. As blood flow conditions dictate the transport of coagulation factors and cells to the material surface, future work will utilize a baboon *ex vivo* shunt model to characterize thrombus formation on modified SIS venous valves at venous shear rates.

References:

1. Pavcnik D, et al. Vasc Med. 2008:13;75-84.
2. Foley JH, et al. Blood. 2012:119;3622-3628.