Elucidating Endothelial Cell Hemostatic Regulation by Isolating Integrin-mediated Adhesion to Bioactive Hydrogels

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Statement of Purpose: A major limitation of early blood-contacting medical devices was their propensity to fail due to thrombosis. Significant research effort has been made to develop antithrombotic coatings for blood-contacting devices to address this limitation. The native cardiovascular system produces anti-thrombitic surfaces through the utilization of an endothelial cell (EC) monolayer covering all blood-contacting surfaces in the body. The EC monolayer prevents platelet activation, provides a protective and selective barrier to underlying tissues, responds to injury, and activates clotting when necessary by releasing pro- and anti-thrombotic factors. Studies have demonstrated that EC phenotype and expression of hemostatic regulators is dependent on the ECM substrate, suggesting that integrin and syndecan binding affects regulation of hemostasis. However, the individual and synergistic roles of integrin binding on intracellular signaling leading to different expression of pro- and anti-thrombotic factors is not well understood. We hypothesize that elucidation of integrin-mediated hemostatic function of ECs will allow for the design of biomaterials with sustained thromboresistance. In this study, we present the first phase of isolating specific EC integrin interactions using a combination of ECM surfaces and antibody blocking. These systems will then be used to examine EC hemostatic function and finally design materials with targeted integrin interactions as thromboresistant coatings for blood contacting devices.

Methods: *Protein hydrogels*: To generate bioactive hydrogels with different ECM components, rat tail collagen I and bovine gelatin type B were first functionalized with acrylate-PEG-N-hydroxysuccinimide (Acr-PEG-NHS) at a ratio of 0.1:1 of the protein lysines. Functionalization was confirmed with FTIR spectroscopy. Functionalized protein was then added at 4 mg/ml to a hydrogel precursor solution (poly(ethylene glycol) diacrylate (PEGDA), 10%) and crosslinked under UV light with 1% Irgacure. *Integrin expression with flow cytometry:* HUVECs cultured in tissue-culture treated poly(styrene) T-75 flasks to 90% confluence and released from flasks using TryplE Express 1:10 in sterile PBS. Cells were spun down and resuspended in flow buffer (PBS with 2% FBS) at a concentration of 10^6cells/mL with 1mL samples. Cells were then stained with fixable viability dye 450 for 20 minutes on ice then washed with 3mL flow buffer. Cells were then incubated with the respective integrin antibody (anti-α1 and anti-α2 at 5µl/mL; anti-αV and anti-α5 at 2.5µl/mL) on ice for 30 minutes. Cells were again washed with flow buffer and resuspended in 400µL 4% paraformaldehyde to fix the cells. Surface expression was then analyzed using the BD LSR II Flow Cytometer. Relative fluorescence was used to compare levels of expression. *Integrin-specific adhesion*: Antibodies for integrins were added to HUVECs and allowed to incubate at room temperature for 15 min before adding cells to the substrates and incubating at 37°C for 30 min: anti-integrin α1 (13.3µg/mL); anti-integrin α2 (6.65µg/mL); anti-integrinαV (2.5µg/mL); anti-integrin α5 (3.33µg/mL); anti-integrin β1 (3.33 µg/mL). Samples were washed 2X with PBS then fixed with 3.7% glutaraldehyde and stained with rhodamine phalloidin and SYBR green for imaging.

Results: HUVEC integrin expression with flow cytometry indicated that α2β1 was the most highly expressed and integrins α5 β1 and αV β3 were both expressed at levels approximately 50% compared to α2β1. The expression of integrin α1β1 was only about 20% compared to α2β1 and antibody blocking of α1 displayed little difference in HUVEC attachment on collagen hydrogel compared to the non-blocked control. Similarly, there was little reduction in attachment when blocking αv, as expected as there are no available binding sites for αvβ3 or α5β1 integrins on collagen. In contrast, a significant reduction in attachment was observed when blocking α2. These findings indicate that α2β1 was the primary integrin involved in HUVEC attachment to the collagen hydrogel. Of note, a further reduction was observed by combining α1 and α2 integrin blocking, suggesting that there is some compensatory mechanism of α1β1 interactions when α2β1 is unavailable. On gelatin hydrogels, blocking α2 resulted in very little difference in HUVEC attachment as expected given the loss of α2β1 affinity with loss of triple helix conformation. Significant reduction in attachment was observed when blocking αv and α5 with a further reduction observed by combining αv and α5 blocking. This indicates that integrins αvβ3 and α5β1 contribute somewhat equally to HUVEC attachment to gelatin, and each interaction can be isolated with selective antibody blocking.

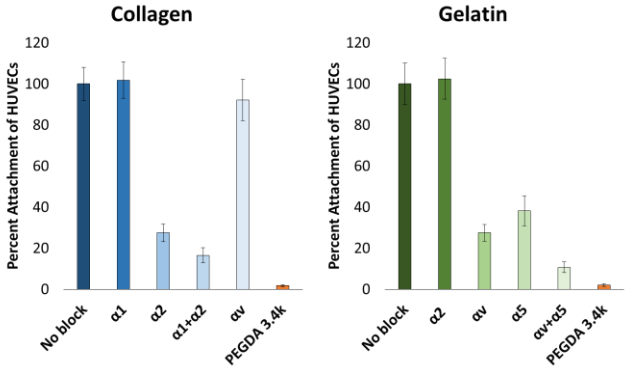

Figure 1*:* Attachment of HUVECs on protein hydrogels with integrin blocking using integrin antibodies.

Conclusions: We have demonstrated a method to isolate individual integrin interactions of HUVECs on ECM hydrogels. This will allow us to then correlate changes in hemostatic regulator protein gene expression, specifically TF, TFPI, ADAMTS-13, VWF, tPA, eNOS, and PAI-1, to specific integrin-mediated attachment and elucidate individual and synergistic effects. Integrin-targeting proteins can then be utilized in hydrogel coatings to promote not only EC attachment and migration, but also a thromboresistant phenotype.