Impact of Delivery Mode on Fragment Size Effects of Hyaluronan on Vascular Endothelial Cell Function

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Statement of Purpose: Due to their thromboprotective functions and their positive impact on maintaining vascular homeostasis, it is recognized that a healthy, confluent lumenal layer of endothelial cells (ECs) is vital to ensure long-term patency of vascular grafts, especially those used for small vessel bypass. Our lab is currently investigating the potential use of hyaluronan (HA), a glycosaminoglycan, as a biomaterial to improve functional endothelialization of synthetic small diameter grafts, which does not occur spontaneously in humans. HA has been shown to exhibit a size-specificity in modulating EC proliferation, key to successful graft endothelialization. Exogenous HA oligomers have been shown to stimulate angiogenesis and proliferation of vascular ECs while high molecular weight (HMW) HA (MW > 1×10^6 Da) generally have the opposite effect. However, HA oligomers also have the potential to elicit exaggerated responses (e.g. inflammation, thrombogenesis) that may be damaging. Thus, from the perspective of developing HA biomaterials for vascular applications, we aim to elucidate the impact of HA, its large fragments, and oligomers on vascular EC phenotype and functionality. Our previous studies suggest that exogenous HA does not necessarily replicate cell responses to HA scaffolds. Therefore, we seek to determine size-specific responses of ECs to HA substrates of defined fragment sizes by creation of HA-tethered culture surfaces and compare these results to an exogenous model.

Methods: HA with MWs of 1000 KDa, 200 KDa, 20 KDa (HA 1000, HA 200, HA 20), and oligomer mixtures (size determined by Fluorescence Assisted Carbohydrate Electrophoresis; FACE) were separately immobilized onto a glass culture surface. To bind HA, the chamber slides were rendered reactive by amination with an amino-silane (3-aminopropyl-trimethoxysilane; APTMS). Successful immobilization of amines was confirmed with an amine reactive-fluorescent probe, SEM, AFM and XPS. The surface density of amine groups was quantified by spectrophotometric detection (λ= 498 nm) of the 4, 4'-dimethoxytrityl cation upon its release when the amine groups are reacted with sulfo-succinimidyl-4-O-(4,4'-dimethoxytrityl) butyrate (sulfo-SDTB). Using a carbodiimide/N-hydroxysulfosuccinimide (NHS) reaction chemistry, the bound amines were then reacted with carboxyl groups present on the HA chains. The presence of surface-bound HA was determined through immunofluorescence, SEM, AFM and XPS. Dissolution of HA from the surface during incubation in culture medium over 21 days was assessed by digestion of bound HA with *hyaluronidase SD*. The digests containing HA disaccharides was analyzed using FACE and the band intensity was compared to a standard to determine the amount of immobilized HA.

Rat aortic endothelial cells (RAECs) were cultured on immobilized HA and with exogenously supplemented HA. Cell proliferation was assessed by a DNA assay in the exogenous experiments and MTT assay on ECs cultured on HA surfaces. Fluorescent activated cell sorting (FACS) determined the EC expression of activation markers (PECAM-1, ICAM-1, VCAM-1) and functionality markers (LDL receptor, eNOS, VWF). The cytokine release (IL-1, IL-6, IL-8) and coagulation potential (anti-thrombin, tissue factor release) of the ECs were also measured through an enzyme-linked immunosobant assays (ELISA) to gauge the inflammatory and thrombogenic response under induced (TNF-α induced) and basal conditions.

Results/Discussion: The exogenous and surface tethered models appeared to have a similar effect on the ECs. The culture of ECs in contact with HA 1000, HA 200, HA 20 and oligomer mixtures (of varied 6mer content) resulted in a direct correlation between 6mer content and proliferation rate. The large HA fragments of defined length and mixtures with a low 6mer content (oligos) inhibited EC proliferation. ECs were unable to attach to surfaces of large HA fragments; a possible effect of smooth surface topography, high anionicity, and non-interaction with the primary HA cell-surface receptors (CD44). Alternatively, ECs were able to attach and proliferate on surfaces of oligomer mixtures with a high 6mer content (oligos6mer) to a greater degree than ECs cultured on fibronectin (control). Exogenously supplemented oligos6mer also resulted in enhanced proliferation to a greater degree than ECs cultured without HA (control). This is likely due to the interaction of HA 6mer with the EC surface receptor CD44, which is known to stimulate VEGF production and promote EC proliferation. Fluorescent activated cell sorting (FACS) determined oligomer6mer mixtures stimulated the expression of activation markers (PECAM-1, ICAM-1, VCAM-1) and inhibited EC functionality markers (LDL receptor, eNOS, VWF). HA oligomers have been shown to stimulate VEGF production and our results indicate VEGFR2 was also expressed to a greater degree in exogenous HA oligos6mer cultures, a possible reason for enhanced proliferation. Enzyme-linked immunosobant assays (ELISA) found oligomer6mer mixtures promoted cytokine (IL-1, IL-6, IL-8) and tissue factor release, and inhibited anti-thrombin production by ECs generating an inflammatory and pro-coagulation environment. Exogenous large fragment HA appeared to have the opposite effect of oligos6mer and EC cultured on large fragment HA surfaces did not attach and therefore the EC response could not be assessed.

Conclusions: The results show that coating of HA onto a surface through a carbodiimide reaction does not interfere with its biological function with regard to vascular endothelial cells. Large HA fragments prevent EC attachment and do not stimulate a cell response, while small HA fragments appear to be more biologically interactive. Future work will combine both bioinert HMW HA and bioactive oligos6mer into hydrogels and develop vascular graft coatings that can benefit recruitment and adherence of a healthy, normally functional, complete endothelium on the graft surface.