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A novel assay for the measurement of activated thrombin activatable fibrinolysis inhibitor (TAFIa) activity

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Importance of Project

The proper functioning of the circulatory system relies on a balance between the coagulation and fibrinolysis cascades. Blood clot formation at a site of vascular injury prevents excessive blood loss and allows cellular repair, while subsequent clot dissolution ensures that blood circulation is restored. Thrombin activatable fibrinolysis inhibitor (TAFI) is a human plasma zymogen that plays an important role in maintaining this balance. Activated TAFI (TAFIa), a basic carboxypeptidase enzyme, attenuates the fibrinolytic positive feedback cascade by cleaving carboxyl-terminal lysine residues in partially-degraded fibrin. TAFI is activated by proteolytic cleavage in response to activation of coagulation. Since excessive blood loss, characteristic of hemophilia, as well as thrombotic events, as seen in heart attacks and strokes, are related to an imbalance in these cascades, the study of TAFI is of great importance to the medical field. The role of TAFI in wound healing, inflammation and cancer cell metastasis are additional areas of current research.

Existing State of Knowledge

The ability to accurately measure TAFIa activity is pivotal to understanding the function and regulation of TAFI in biological systems. Various methods are available for the detection of TAFIa, including the immunological method ELISA, enzymological assays and functional assays. Enzymological assays exploit the carboxypeptidase activity of TAFIa for cleavage of carboxyl-terminal arginine and lysine residues from small substrates containing a chromophore. This is advantageous in comparison to ELISA, since only TAFIa fragments are measured, not the zymogen nor inactive fragments of TAFIa. Two current classes of substrates for the enzymatic study of TAFIa include the hippuryl-arginine/lysine and anisylazoformyl-arginine/lysine assays, which possess various pitfalls. For instance, turbidity resulting from blood clot formation interferes with reading the absorbance of a chromogenic substrate or product in the assay. Additionally, low concentrations of TAFIa cannot be detected as the assays are not particularly sensitive and the activity of TAFIa cannot be monitored in real time. In comparison, a

functional TAFIa assay designed by Kim and coworkers is very sensitive, allowing measurement of picomolar concentrations of TAFIa in plasma. It also utilizes fluorescence detection, which avoids the issue of turbidity interference for absorbance measurements. Despite these advantages, the numerous purified protein components that must be prepared for this assay may lead to inconsistent results. Moreover, TAFIa activity cannot be measured in real time using this assay.

Research Question

Thus, the focus of my project is to design and optimize a novel fluorescence assay for the measurement of TAFIa activity in real time, with increased sensitivity compared to current methods.

Methodology

Baby hamster kidney (BHK) cells expressing recombinant poly-histidine tagged wild type TAFI have been cultured, followed by protein purification, to acquire pure wild type TAFI protein. Complete activation of TAFI to TAFIa is achieved with a serine protease enzyme, thrombin (IIa), in complex with its cofactor thrombomodulin (TM). The synthetic substrate designed for the assay is a peptide composed of four amino acids (GAGR) modified with an amino-terminal tetramethylrhodamine (TAMRA) fluorophore. An anionic quencher, Evan's Blue dye, interacts electrostatically with the positively charged carboxyl-terminal arginine in the substrate, quenching TAMRA's fluorescence. Arginine cleavage by TAFIa releases the associated quencher resulting in a measurable increase in fluorescence, indicative of TAFIa activity.

Findings

Thus far, my research has shown that the cleavage of the TAMRA-GAGR peptide by TAFIa is measurable as an increase in fluorescence intensity and thus, in principle, the assay is a promising means of measuring TAFIa activity. Optimization of the assay will be conducted in terms of the relative concentrations of fluorescent substrate and quencher, as will development of a TAFIa standard curve. The sensitivity of the assay will be explored further, which is important due to the low TAFIa concentration (20 – 100 pmol/L) present in human plasma. This assay will be applied to measuring TAFIa

activity natively present in plasma samples and total TAFI concentrations (after activation by IIa-TM) present in plasma, tissue or secreted by cells. It will also be applied to routine characterization of TAFI, such as after purification of recombinant variants. The ability to study TAFIa generation in real time within clots will allow further insight into the regulation of TAFIa elaboration in response to thrombogenic stimuli. There is also potential to identify individuals at risk for hematological or vascular diseases based on variation in plasma TAFI levels or altered TAFIa generation and function.

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