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Post-translational oxidative modification of fibrinogen is associated with coagulopathy after traumatic injury

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ABSTRACT

Victims of trauma often develop impaired blood clot formation (coagulopathy) that contributes to bleeding and mortality. Fibrin polymerization is one critical component of clot formation that can be impacted by post-translational oxidative modifications of fibrinogen after exposure to oxidants. *In vitro* evidence suggests that Aα-C domain methionine sulfoxide formation, in particular, can induce conformational changes that prevent lateral aggregation of fibrin protofibrils during polymerization. We used mass spectrometry of plasma from trauma patients to find that fibrinogen Aα-C domain methionine sulfoxide content was selectively-increased in patients with coagulopathy *vs.* those without coagulopathy. This evidence supports a novel linkage between oxidative stress, coagulopathy, and bleeding after injury.

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1. Introduction

Hemostasis is often impaired after severe traumatic injury leading to pathological bleeding that is known as trauma-induced coagulopathy (TIC). TIC is present in one-quarter to one-third of severely injured patients almost immediately after injury, carries a 4–6 times increased risk of mortality, and is associated with increased incidence of multi-organ failure, intensive care utilization, and increased need for blood transfusion [1–4]. Early hallmarks of TIC include extrinsic coagulation pathway dysfunction identified by a prolongation of prothrombin time (PT) ratio and/or International normalized ratio (INR) and decreased viscoelastic clot strength [1–3]. Those patients with abnormally elevated PT ratio's recorded in the Emergency Department are twice as likely to die compared to those with normal PT ratios [4]. The cause of TIC is felt to be multifactorial as a result tissue hypoperfusion and hypoxia, activation of the anticoagulant Protein C (aPC), thrombin deficiency, and fibrinogen proteolysis and dysfunction [5].

Perhaps the most significant component of TIC is increased enzymatic clot breakdown, or hyperfibrinolysis. After injury with blood loss producing hemorrhagic shock, the concentration of tissue plasminogen activator (tPA) is increased in blood, which increases activation of plasminogen to plasmin [5–7]. Plasmin is the primary proteolytic enzyme for fibrin, causing rapid degradation of fibrin clots by cleaving fibrin strands at lysine residues. Impaired fibrin polymerization can also directly contribute to hyperfibrinolysis by increasing susceptibility to enzymatic degradation [8,9]. Hyperfibrinolysis is strongly associated with trauma patient mortality and when significant, often predicts imminent death [10]. In addition, antifibrinolytic therapy using the lysine analogue tranexamic acid is the only therapy found to reduce mortality when given early after injury in a large randomized controlled trial [11].

The hemostatic plasma protein fibrinogen is a key determinant of bleeding after injury. Fibrinogen becomes fibrin clot by cleavage of short peptides by thrombin from the Aα (FpA) and Bβ (FpB) chains. Fibrin polymerization and subsequent fibrin clot structure

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is then governed by specific interaction of complementary binding sites exposed after fibrinopeptide cleavage. Loss of fibrinogen to hemorrhage, consumption, and hyperfibrinolysis are associated with decreased survival after trauma [12]. Fibrinogen is also the first hemostatic protein to decrease to critically low functional concentrations in surgical patients with significant blood loss [13]. Therefore, fibrinogen is an important and labile hemostatic protein that is affected by trauma.

Fibrinogen is also extremely sensitive to oxidation relative to other plasma proteins [14]. We have previously demonstrated *in vitro* that exposure of fibrinogen to hypochlorous acid is associated with preferential oxidation of Methionine 476 in the Aα-C domain to methionine sulfoxide (Aα-Met476(SO), inhibition of lateral aggregation of fibrin protofibrils during fibrin polymerization, and formation of thin-fibered fibrin clots that are mechanically weak [15]. *In silico* modelling has also revealed that specific Aα-Met476 oxidation to Aα-Met476(SO) plausibly mediates the inhibition of fibrin lateral aggregation by disruption of a key beta hairpin structure involved in the fibrin lateral aggregation mechanism [16]. HOCl is predominantly generated in the plasma as a product of neutrophil lysozomal myeloperoxidase, chloride, and hydrogen peroxide and is a key leukocyte-specific host-defense mechanism mediating bacterial killing by the formation of methionine sulfoxide in bacterial membrane proteins [17]. Leukocytes upregulate the same oxidative enzymes after blunt trauma and oxidation has been implicated in vascular responses to hemorrhagic shock [18–20].

Despite evidence that oxidants are generated after trauma and fibrinogen is highly sensitive to these oxidants, fibrinogen oxidation has not been detected in trauma patients with coagulopathy. Having established a plausible *in vitro* link between Aα-Met476 (SO) content and altered fibrin clot formation, the purpose of this study was to test the hypothesis that the same methionine residue of fibrinogen is selectively-oxidized in trauma patients with coagulopathy.

2. Materials and methods

2.1. Trauma patients

Plasma was obtained from three de-identified human Emergency Department trauma repositories. Human subjects approvals were obtained from local institutional review boards for repository and medical records access according to the guidelines set forth by the declaration of Helsinki. The first cohort was used to identify selectively-vulnerable methionine residues on fibrinogen that were oxidized to methionine sulfoxide in coagulopathic trauma patients. Nano-liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) approach was used to identify these residues in this cohort from glycine-purified trauma patient fibrinogen For the second cohort, we used an ultra-performance LC (UPLC)-MS/MS with Multiple Reaction Monitoring (MRM) mode to confirm increased fibrinogen Aα-Met476(SO) content directly in plasma without purification. We then used LC (UPLC)-MS/MS and viscoelastic clotting measurements to examine associations between fibrinogen Aα-Met476(SO)% and clot formation after injury in a third cohort.

2.2. Trauma derivation cohort

Samples of platelet-poor plasma were obtained directly from a trauma plasma biorepository. Blood samples were drawn from trauma patients at Harborview Medical Center, a U.S. Level-I trauma center in Seattle WA, on arrival to the Emergency Department. Leftover citrated plasma was obtained from these samples after all clinical tests were performed. Leftover plasma was immediately frozen at $-80\,^{\circ}C$ and given a unique study number by the clinical laboratory for inclusion in the biorepository. Patient medical records were then reviewed and vital signs, laboratory data, injury patterns, and clinical outcomes were abstracted and matched to plasma samples after removal of all protected health information. To detect fibrinogen oxidation, fibrinogen was first purified from plasma by 4 rounds of glycine purification yielding highly-purified fibrinogen. Purified fibrinogen was then subjected to nanoLC-MS/MS to identify sites of increased methionine sulfoxide content. Samples were grouped for comparison by the presence or absence of coagulopathy as defined by an INR >1.2 reported by the hospital laboratory [4].

2.3. Trauma confirmation cohort

The specific MS signatures identified in the first cohort were then used to quantify fibrinogen methionine sulfoxide content directly in plasma without purification using UPLC-MS/MS-MRM. Blood was sampled from trauma patients presenting to Virginia Commonwealth University Medical Center, a U.S. Level I trauma center in Richmond VA. Blood was obtained in the Emergency Department and any subjects known to have received blood product transfusions prior to the blood sample were excluded. A subset of N=25 de-identified plasma samples with matched clinical laboratory data were submitted for UPLC-MS/MS-MRM for specific AαMet476(SO) signatures as detected directly in plasma without purification. MS personnel were blinded during measurement and analysis. Samples were again grouped for comparison by the presence or absence of coagulopathy as defined by an INR >1.2 reported by the hospital laboratory. To examine for selectivity of oxidation of fibrinogen over other plasma proteins, the M353(SO) content of albumin, a similar solvent-exposed methionine residue, was simultaneously measured in the same plasma.

2.4. Clot formation cohort

Given the importance of clot formation and fibrinolysis to trauma patient outcomes, we then examined for a direct association between fibrinogen Aα-M476(SO) content and viscoelastic clot formation in a third cohort of trauma patients. This cohort was obtained from a trauma biorepository of citrated plasma from Emergency Department trauma patients sampled on arrival to Memorial-Hermann Medical Center, a U.S. Level I trauma center in Houston TX. These patients also underwent Emergency Department point of care rapid thrombelastography of whole blood (rTEG, Haemonetics, Braintree MA, USA) at the time of initial blood draw as part of their clinical care. rTEG is a viscoelastic measurement of clot formation using tissue factor is useful to guide bleeding management after trauma [21]. rTEG reports the activated clotting time (ACT) and onset time (R), or time required to achieve detectable clot formation, representing coagulation factor activity; clot formation time (K) and alpha angle which represent fibrin polymerization and platelet activity; maximal amplitude of deflection (MA) which represents fibrin clot integrity and platelet-induced clot contraction; and clot durability (LY30%) reported as percent clot breakdown, or lysis, at 30 min after MA, representing the activity of fibrinolytic enzymes. We examined n=50 citrated plasma samples, half of which demonstrated deficient clot formation by rTEG as indicated by increased clot breakdown of at least 3% LY30% to ensure a range of coagulopathy and clot formation phenotypes in the cohort [22,23]. Fibrinogen Aα-M476(SO) content was again measured directly in these plasma samples using UPLC-MS/MS- MRM. Corresponding clinical data regarding injury severity, shock severity, and relevant outcomes were also abstracted from the repository.

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