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Generation of complement molecular complex C5b-9 (C5b-9) in response to poly-traumatic hemorrhagic shock and evaluation of C5 cleavage inhibitors in non-human primates

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A R T I C L E I N F O

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A B S T R A C T

Severe trauma initiates a systemic inflammatory cascade and that involves early activation of complement and cleavage of C5 into C5a (anaphylatoxin) and C5b (C5b-9 membrane attack complex). We examined activation of C5 in non-human primate (NHP) models of hemorrhagic shock.

Blood plasma concentrations of C5b-9 were significantly increased in NHPs in response to hemorrhage alone and were further increased with the addition of tissue trauma. The onset of increased C5 cleavage was accelerated in NHPs that experienced decompensated poly-traumatic hemorrhagic shock. Next, to identify an effective inhibitor of NHP C5 cleavage *in vitro*, as a first step in the development of a potential therapy, three inhibitors of human C5 cleavage and hemolysis were tested *in vitro*. NHP C5 cleavage and complement-mediated hemolysis were successfully inhibited by pre-treatment of serum samples with a small, inhibitory peptide RA101348. Commercially-available C5 inhibitory antibodies were found to exhibit species-specific efficacy *in vitro*. Quidel's A217 antibody demonstrated dose-dependent inhibition of C5 cleavage and hemolysis in NHP samples, whereas LGM-Eculizumab only inhibited complement-mediated hemolysis in human samples.

This study shows that complement activation in NHPs following experimental poly-traumatic hemorrhagic shock is consistent with clinical reports, and that cleavage of C5 and complement-mediated hemolysis can be effectively inhibited *in vitro* using a small peptide inhibitor. Taken together, these findings offer a clinically-relevant vehicle and a potential strategy for treatment of hemorrhagic shock with poly-traumatic injury.

1. Introduction

Poly-traumatic hemorrhagic shock is associated with systemic inflammation, dysregulation of the coagulation system and activation of endothelial cells [1]. As a result, patients who survive the initial traumatic injury are at acute risk of multiple organ dysfunction and/or failure, and mortality. There is evidence that this post-injury pathogenesis is attributable, at least in part, to complement activation [2,3]. Early activation of the complement system, and the degree of activation, are known to directly correlate with the severity of hemorrhagic shock and mortality rate [4]. In animal models of hemorrhagic shock and poly-trauma, serum hemolysis data show increased concentrations of circulating complement components and functional complement activity [5]. The expression of membrane attack complex (MAC) proteins (C5b-9) is also reported to increase proportionally with the severity of clinical trauma [5]. Consequently, the development of strategies to diminish complement activation following poly-traumatic hemorrhagic shock has become a priority in research efforts to improve morbidity and mortality outcomes.

The complement system is an important, upstream component of the inflammatory response to both exogenous and endogenous stimuli. It is comprised of > 50 proteins organized into three distinct pathways

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that are activated by different stimuli (the classical, alternative and lectin pathways) but converge at a central C3 convertase prior to activation of the common C3 and C5 effector molecules [6]. Selective interference with complement activation using monoclonal antibodies or peptide fragments presents an attractive therapeutic option, but the effects vary according to pathway and/or the specific protein(s) that is targeted. Targeting of the classical pathway *via* inhibition of C1 has been shown to improve metabolic acidosis, reduce edema and circulating Tumor Necrosis Factor-α, and attenuate tissue damage in animal models of hemorrhagic shock [7] and ischemia/reperfusion injury [8]; however, the alternative and lectin pathways remain unaffected. Conversely, global inhibition of complement activation can be achieved by selective inhibition of C3, the convergence point for all three pathways. C3 deficiency or inhibition has been shown to provide a protective effect in the acute phase following ischemic stroke [9,10], but prolonged C3 deficiency can lead to infection and worse long term outcomes [11].

Selective inhibition of C5 provides a unique therapeutic opportunity as it is located in the final stage of the common complement cascade, but its activation is an upstream step in the development of inflammatory responses [12]. Local C5a produces a regional inflammatory response *via* activation of endothelium, mast cells, and phagocytes and recruits neutrophils to the injury micro-environment. Excessive systemic C5a generation results in a shock-like state with systemic pro-inflammatory cytokine production, upregulation of leukocyte adhesion molecules and disseminated intravascular coagulation. Systemic blockade of C5 activation could provide an approach for reducing the formation of the more deleterious complement proteins, C5a and C5b-9, while preserving the normal, disease-preventing functions of the upstream portions of the complement system necessary for the opsonization of microbes. An anti-C5 monoclonal antibody has been approved for treatment of paroxysmal nocturnal hemoglobinurea (PNH) and atypical hemolytic-uremic syndrome (aHUS) [6], but systemic use of anti-C5 antibodies is associated with an increased risk of infection with encapsulated microorganism, particularly *Neisseria meningitidis* [13]. C5 blockade has also demonstrated benefit in animal models for the attenuation of shock [14], prevention of ischemia/reperfusion injury [15], and improved responsiveness to fluid resuscitation [16].

Our group is interested in evaluating the potential therapeutic benefits of C5 blockade in poly-traumatic hemorrhagic shock. Anti-C5 antibodies and C5 small inhibitory peptides are available; however, effective preclinical models for their evaluation remain to be developed and characterized [17]. Non-human primates (NHP) offer a useful platform for pre-clinical evaluation of C5 cleavage inhibition in the setting of hemorrhagic shock. In this study we measured complement activation in response to poly-traumatic hemorrhagic shock and examined the effectiveness of anti-human C5 monoclonal antibodies and a small molecule inhibitor in blockade of NHP C5 cleavage *ex vivo*. We report increased production of C5b-9 *in vivo* in response to injury involving hemorrhagic shock of increasing severity, and effective inhibition of C5b-9 production and function (hemolysis) *in vitro* following pre-treatment with a small-peptide inhibitory molecule.

2. Materials and methods

2.1. Non-human primates

Male, NHPs (*Macaca mulatta*, n = 43) weighing 7–14 kg were used in accordance with the protocol approved by the Institutional Animal Care and Use Committee at the 711th Human Performance Wing, Joint Base San Antonio-Fort Sam Houston, TX and the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 2011 in facilities accredited by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International).

2.2. Hemorrhagic shock and poly-trauma injury

Anesthetized NHPs were randomly assigned to one of five models of hemorrhagic shock of increasing severity: pressure-targeted controlled hemorrhagic shock (PTHS) for 30 min (PTHS-30, n = 8), 60 min (PTHS-60, n = 8), 60 min with soft-tissue injury (PTHS-60 + ST, n = 8), 60 min with soft tissue plus musculoskeletal injury (PTHS-60 + ST + MST, n = 8), or soft-tissue plus musculoskeletal injury and cardiovascular decompensation (PTHS-D, n = 8) as previously described [16]. Sham animals (n = 3) were anesthetized but received neither injury nor resuscitation. Blood samples were collected at baseline (BSLN), end of shock (EOS), end of resuscitation (EOR), T = 360 min (T360) and end of the experiment (END) in EDTA vacutainers. The end time was T = 1440 min (24 h) for all groups, except for the decompensation group whose average END time (D-END) was T = 653.25 min (close to 11 h).

2.3. Complement C5b-9 ELISA

Plasma and/or serum samples were analyzed by enzyme-linked immunosorbent assay (ELISA) to detect C5 cleavage products C5a and C5b-9 according to the manufacturer's instructions (MicroVue Complement C5a EIA and MicroVue SC5b-9 Plus EIA, Quidel Corp., San Diego, CA). Plasma samples from PTHS models were diluted 1:25, NHP and human serum samples were diluted 1:50 and 1:750, respectively, in the ELISA diluent buffer provided by the manufacturer. Absorbance was read at 450 nm on a plate reader (BioTek Instruments Inc., VT).

2.4. Inhibition of complement C5 cleavage

Normal NHP (*Macaca mulatta*) and human sera were purchased (Bioreclamation IVT, Westbury, NY) and diluted in 1 × phosphate buffered saline (PBS) to a final concentration of 40% v/v and kept on ice. Three commercially available C5 cleavage inhibitors were diluted in PBS and added as follows: RA101348 (0.5 μM–17.5 nM, Ra Pharmaceuticals Inc.; Cambridge, MA); LGM-Eculizumab, a humanized, mouse anti-human C5 monoclonal antibody (1.02 μM–1.63 nM, LGM-Pharma; Nashville, TN); and Quidel's A217 mouse anti-human C5 monoclonal antibody (3.29 μM–21.9 nM, Quidel Corp., San Diego, CA). To activate complement cleavage, Cobra Venom Factor (CVF, Quidel Corp., San Diego, CA) was diluted in PBS and added to each well to a final concentration of 8 units/mL. Reagents and samples were placed in the wells of a 96-well, flat-bottom plate to a final volume of 200 μL and incubated for 1 h at 37 °C. Supernatants were diluted as described in Section 2.3 and cleavage of C5 to C5b-9 was measured by ELISA.

2.5. CH50 hemolysis assay

The assay for complement-mediated lysis was carried out according to a protocol supplied by Complement Technology Inc. (Tyler, TX). In brief, sheep erythrocytes coated with rabbit anti-sheep IgM antibodies were suspended at 5×10^8 cells/mL in gelatin veronal buffered saline (GVB + +), both purchased from Complement Technology, Inc. NHP and human sera, pre-incubated with each C5-cleavage inhibitor (as described above), were cooled on ice and diluted in GVB + + to achieve a final concentration of 1% v/v serum. The ratio of serum to sheep erythrocytes was optimized separately to achieve approximately 85% hemolysis. Serum, GVB + + and erythrocytes were placed in 4 mL tubes to a final volume of 675 μL and incubated for 1 h at 37 °C with moderate mixing every 10 min. Supernatants were aspirated after centrifugation for 3 min at 500 × *g* and absorbance was read at 541 nm. The mean absorbance of duplicate samples was then calculated as a percentage of untreated (no inhibitor) samples (normalized to 100% hemolysis).

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