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Research Paper

Complement Activation in Arterial and Venous Thrombosis is Mediated by Plasmin

Jonathan H. Foley [a,b,c], Bethany L. Walton [d], Maria M. Aleman [d], Alice M. O'Byrne [a], Victor Lei [a], Micaela Harrasser [b], Kimberley A. Foley [e], Alisa S. Wolberg [d], Edward M. Conway [a,*]

[a] Centre for Blood Research, Department of Medicine, Life Sciences Institute, University of British Columbia, 2350 Health Sciences Mall, LSC4306, Vancouver V6T 1Z3, Canada
[b] Department of Haematology, UCL Cancer Institute, University College London, London, United Kingdom
[c] Katharine Dormandy Haemophilia Centre and Thrombosis Unit, Royal Free NHS Trust, London, United Kingdom
[d] Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, 819 Brinkhous-Bullitt Building, CB# 7525, Chapel Hill, NC 27599-7525, USA
[e] Cancer Care and Epidemiology, Queen's Cancer Research Institute, Queen's University, Kingston, Canada

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Thrombus formation leading to vaso-occlusive events is a major cause of death, and involves complex interactions between coagulation, fibrinolytic and innate immune systems. Leukocyte recruitment is a key step, mediated partly by chemotactic complement activation factors C3a and C5a. However, mechanisms mediating C3a/C5a generation during thrombosis have not been studied. In a murine venous thrombosis model, levels of thrombin–antithrombin complexes poorly correlated with C3a and C5a, excluding a central role for thrombin in C3a/C5a production. However, clot weight strongly correlated with C5a, suggesting processes triggered during thrombosis promote C5a generation. Since thrombosis elicits fibrinolysis, we hypothesized that plasmin activates C5 during thrombosis. *In vitro*, the catalytic efficiency of plasmin-mediated C5a generation greatly exceeded that of thrombin or factor Xa, but was similar to the recognized complement C5 convertases. Plasmin-activated C5 yielded a functional membrane attack complex (MAC). In an arterial thrombosis model, plasminogen activator administration increased C5a levels. Overall, these findings suggest plasmin bridges thrombosis and the immune response by liberating C5a and inducing MAC assembly. These new insights may lead to the development of strategies to limit thrombus formation and/or enhance resolution.

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1. Introduction

The coagulation system and innate immunity are coordinately activated and highly integrated during venous and arterial thrombus formation and progression (von Bruhl et al., 2012; Engelmann and Massberg, 2013; Fuchs et al., 2012). Vascular endothelial activation or damage causes release of ultralarge von Willebrand factor (VWF) and P-selectin from Weibel-Palade bodies, and local activation of complement with liberation of anaphylatoxic and chemotactic factors C3a and C5a. These pathways cooperate to trigger platelet, neutrophil, and monocyte recruitment and activation (von Bruhl et al., 2012). The locally accumulated cells release proteases, reactive oxygen species, and nucleosomes, which provide a scaffold for aggregating platelets and red blood cells and further promote coagulation and fibrin formation (Fuchs et al., 2012). Several complement factors, including C3, C4, C3a, C5a and factor H are incorporated into the thrombus, where they modulate thrombus stability and the inflammatory process (Distelmaier et al., 2009; Howes et al., 2012). The fibrinolytic system and plasmin-mediated proteolysis are also intimately coupled to the axis of thrombus development and inflammation by controlling fibrin degradation, activation of matrix metalloproteinases, infiltration of monocytes/macrophages and other immune mediators, vessel wall remodeling, and ultimately thrombus resolution (Wakefield et al., 2008).

The mechanisms by which leukocytes are recruited early in thrombus formation and later during thrombus extension or resolution, are poorly understood. However, C5a, the most potent chemotactic complement activation fragment, is released following proteolytic cleavage of C5 and is considered a critical determinant of neutrophil recruitment and activation in thrombosis (Distelmaier et al., 2009; Salmon et al., 2002; Pierangeli et al., 2005). Moreover, terminal complement pathway complexes formed as C5 is activated, have multiple procoagulant properties (Langer et al., 2013; Hamilton et al., 1990). Thus, there is interest in understanding how C5a and the other major complement-derived

Abbreviations: MAC, membrane attack complex; VWF, von Willebrand factor; R751, arginine 751; TAT, thrombin antithrombin; IVC, inferior vena cava; VFKck, Val-Phe-Lys-chloromethylketone; PPACK, Phe-Pro-Arg-chloromethylketone; $FeCl_3$, ferric chloride; tPA, tissue-type plasminogen activator; NETs, neutrophil extracellular traps; PAR1, protease activated receptor 1; MCP1-1, monocyte chemoattracant protein-1; IL-8, interleukin-8; FDP, fibrin degradation product.

* Corresponding author at: Centre for Blood Research, 4306-2350 Health Sciences Mall, University of British Columbia, Vancouver, BC V6T 1Z3, Canada.
E-mail address: ed.conway@ubc.ca (E.M. Conway).

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chemotactic factor, C3a, are generated, so that novel therapeutic strategies may be designed to prevent thrombosis.

Complement activation typically proceeds via three pathways — classical, lectin and alternative — which converge to form C3 convertases that proteolyse C3 into C3b with release of C3a (Ricklin et al., 2010). As complement activation exceeds a threshold, and the density of C3b increases, the specificity of the convertase shifts from C3 to C5. The resultant C5 convertases — C3bBbC3b for the alternative pathway and C4b2aC3b for the classical/lectin pathway — efficiently cleave C5 at arginine 751 (R751), liberating C5a and generating C5b, the initiating factor for assembly of the lytic C5b-9 membrane attack complex (MAC). Although the C3/C5 convertases are well-recognized for their capacity to cleave C3 and C5, other serine proteases reportedly also exhibit convertase activity (Huber-Lang et al., 2006; Amara et al., 2010; Wiggins et al., 1981). Notably, thrombin was implicated in providing a "new pathway" to activate complement by cleaving C5 in a C3-independent manner, thereby bypassing the bona fide C5 convertases (Huber-Lang et al., 2006). However, C5 is a relatively poor substrate for thrombin cleavage at R751 (Krisinger et al., 2012), raising questions as to its importance in contributing to C5a generation during thrombus formation *in vivo*. We therefore explored the mechanisms by which C3a and C5a are generated using biochemical approaches and *in vivo* models of venous and arterial thrombosis.

2. Materials and Methods

2.1. Materials

Human complement C3a and C5a were measured using Quidel MicroVue C3a Plus or C5a ELISA kits (Cedarlane Laboratories, Burlington, Ontario). Murine thrombin–antithrombin (TAT) levels were measured using Enzygnost TAT micro ELISA (Siemens, Munich, Germany). Human complement proteins C3 and C5 were obtained from Complement Technology, Inc. (Tyler, TX) and human hemostatic enzymes (plasmin, factor Xa and thrombin) were from Haematologic Technologies, Inc. (Essex Junction, VT).

2.2. ELISAs to Measure C3a and C5a

Murine complement C5a levels were determined using the mouse complement component C5a duoset and accompanying standard from R&D systems (catalog #DY2150; Minneapolis, MN). An ELISA for murine complement C3a was established using a murine C3a standard and antibodies from BD Biosciences (Mississauga, Canada). The capture rat monoclonal anti-mouse C3a antibody (catalog # 55820, clone: I87-1162) was coated overnight onto 96-well plates in 100 μL of PBS at a concentration of 2 μg/mL. Wells were washed ×3 with wash buffer (R&D catalog #WA126) followed by blocking for 2 h with 300 μl of reagent diluent (R&D systems catalog #DY995). Plasma samples in duplicate were diluted 1/50 and 1/125 in sample diluent to a final volume of 100 μl and incubated for 2 h. After 3 washes, 100 μl of the biotinylated detection monoclonal rat anti-mouse C3a antibody (clone I87-419, catalog #55821) 0.5 μg/mL in reagent diluent was incubated for 1 h. Wells were washed ×4 and incubated for 20 min at room temperature with 100 μl of streptavidin-biotinylated horseradish peroxidase (1:3000), followed by 2 washes, and development with the substrate solution containing o-phenylenediamine using a plate reader set to 450 nm. A standard curve was generated with purified murine C3a (catalog # 558618). The sensitivity range of the assay was 0.1 nM to 2.5 nM. Intra-assay and inter-assay precision was 8–10%.

2.3. Animal Models

All experiments with animals were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committees. The mice (C57Bl/6) were male and between 6 and 8 weeks of age. The number of animals for each model was determined based on previous work that showed a broad range of TAT levels in the respective models (Machlus et al., 2011a, 2011b). On each day of the experiments, animals were randomly assigned to receive the stated treatment or to be used for baseline measurements. Quantification of biomarkers was performed in a blinded fashion wherein an experimenter, different from the one who performed the procedures on the animals, carried out the ELISAs on coded samples that were only de-coded after results had been generated.

2.4. Murine model of Venous Thrombosis

The inferior vena cava (IVC) stasis model was performed as previously described (Aleman et al., 2013). Briefly, mice were anesthetized with 1·5–2% isoflurane in oxygen and human prothrombin (to 300%, final, mouse plus human prothrombin) or vehicle was infused via tail vein injection. Prothrombin was infused to give a broader range of thrombin generation and clot weight. Following sterile laparotomy, the intestines were exteriorized, the IVC was dissected bluntly, and side branches were ligated with 8–0 prolene suture and lumbar branches closed by cautery. The IVC was separated from the aorta by blunt dissection and completely ligated with 8–0 prolene suture. After replacing the intestines, the muscle layer was closed with 5–0 vicryl suture and skin closed with 8–0 prolene suture and skin glue. Mice recovered with analgesia (buprenorphine, 0·05 mg/kg subcutaneous). After 12 h, blood was drawn from the IVC above the ligation site into 3.2% sodium citrate and processed to platelet-poor plasma by centrifugation at 5000 ×*g* for 10 min. Thrombi were collected and weighed. Plasmas were stored at −80 °C for analysis of TAT, C3a and C5a levels by a person that was blinded to the treatment group. Two samples showing hemolysis were excluded.

2.5. In Vitro Generation of C3a or C5a by Hemostatic Enzymes

The relative efficiency, time course, and rate of complement cleavage by plasmin, factor Xa or thrombin were determined using a series of assays. Briefly, complement C3 (20 μM) or C5 (2 μM) was incubated with 100 nM plasmin or 250 nM factor Xa or thrombin. Reactions were quenched at various time points and C3a or C5a levels were quantified by ELISA. To determine the relative efficiency of cleavage, 2 μM of C5 was incubated with 100 nM of plasmin, factor Xa or thrombin at 37 °C. After 10 min the reaction was quenched with the appropriate chloromethylketone (Val-Phe-Lys-chloromethylketone (VFKck) for plasmin and Phe-Pro-Arg-chloromethylketone (PPAck) for factor Xa and thrombin). In similar experiments, aliquots of the reaction mixture were sub-sampled into chloromethylketones at various time points to determine the time courses of C3 and C5 cleavages by plasmin, factor Xa and thrombin. The kinetics of C5a generation were determined by incubating 0–3 μM of C5 with 100 nM of plasmin at 37 °C. Reactions were quenched after 1 min and C5a levels were quantified by ELISA. Similar experiments substituting factor Xa or thrombin for plasmin were conducted, but the amount of C5a generated in these assays over 30 min (for factor Xa) or 1 h (for thrombin) was below the limit of detection for the assay.

C5a generation occurring during clot formation and degradation was assessed *in vitro* by incubating physiological concentrations of fibrinogen (9 μM), plasminogen (2 μM), antiplasmin (1 μM) and C5 (400 nM) at 37 °C. High (1 μM) or low (10 nM) concentrations of thrombin were added to induce clot formation and 10 nM of tPA was used to induce plasminogen activation and clot lysis. The tPA concentration chosen resulted in complete clot lysis in 30 min. At the end of the 30-minute incubation period, enzymes were quenched with PPAck and VFKck, the sample centrifuged and supernatant or sample stored at −80 °C for quantification of C5a levels. Background C5a signal when PPAck/VFKck was added at t = 0 were subtracted.

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