Biology and Pathogenesis of Thrombosis and Procoagulant Activity in Invasive Infections Caused by Group A Streptococci and Clostridium perfringens

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INTRODUCTION

Systemic activation of coagulation and dysregulation of the anticoagulation pathways contribute to the pathogenesis of many diverse disease entities of infectious etiology. For instance, the clinical manifestations of thrombotic thrombocytopenic purpura and hemolytic-uremic syndrome result from occlusive microthrombus formation in the arterioles and capillaries of the brain, gastrointestinal tract, kidneys, and other organs (reviewed in reference 56). Similarly, formation of microvascular thrombi contributes to multiple-organ failure in human cases of gram-negative bacteremia (reviewed in reference 101), to purpura fulminans associated with meningococcal sepsis (34), and to deep-vein thrombosis associated with Rocky Mountain and Mediterranean spotted fevers. Microvascular thrombus formation is also an integral part of the pathogenesis of cardiovascular disease following infection of the endothelium by Chlamydia pneumoniae (35), enterococci (30), or members of the herpesvirus group (103). Similarly, attachment of pathogenic organisms such as Staphylococcus aureus, Staphylococcus epidermidis, or Streptococcus anginosus to cardiac valvar endothelium initiates a local inflammatory reaction that triggers activation of the coagulation cascade and results in formation of platelet-fibrin vegetations characteristic of bacterial endocarditis. Lastly, as we shall see, occlusive microvascular thrombosis participates in the rapid destruction of viable tissue in gram-positive necrotizing infections such as invasive streptococcal and clostridial myonecrosis.

In each instance, the events that precipitate and sustain the
coagulopathy (e.g., activation, injury or infection of endothelial cells, leukocytes, or platelets; induction of cytokine synthesis; and direct activation or inhibition of coagulation or anticoagulation factors) are as unique as the host responses they evoke, with outcomes ranging from minor local activation to fulminant disseminated intravascular coagulation (DIC) with multiorgan failure. A thorough understanding of these mechanisms may suggest novel therapeutic targets for patients with these devastating infections.

**ROLE OF COAGULATION IN INVASIVE GRAM-POSITIVE INFECTIONS**

Group A streptococcal necrotizing fasciitis/myonecrosis and *Clostridium perfringens* gas gangrene are two of the most fulminant gram-positive infections in humans. Tissue destruction associated with streptococcal toxic shock syndrome (StrepTSS) progresses rapidly to involve an entire extremity (13, 88), and such patients require emergent amputation or extensive surgical debridement and prolonged hospitalization (13, 88). In fact, a recent article in the *American Journal of Surgery* recommended radical debridement to maximize limb salvage and survival in cases of severe soft tissue infection due to group A streptococcus (GAS) (79). Rapid destruction of viable, healthy tissue is also characteristic of gas gangrene due to *C. perfringens*. In this infection, the margin between healthy and necrotic tissue often advances several inches per hour despite appropriate antibiotic therapy (53, 55), and radical amputation remains the single best life-saving treatment.

Both GAS necrotizing fasciitis/myonecrosis and clostridial gas gangrene are also characterized by excruciating pain at the infection site (13, 53, 88). In StrepTSS, the onset of this pain occurs well before shock, renal impairment, or acute respiratory distress syndrome are manifest (88). Similarly, the onset of severe pain in gas gangrene is “sometimes so sudden as to suggest a vascular catastrophe” (53).

The mechanisms responsible for the early onset of severe pain and the rapid regional destruction of tissues in these infections have not been completely elucidated. It has been our hypothesis that these features are due to microvascular thrombosis leading to reduced tissue perfusion and to hypoxia and subsequent regional tissue necrosis. Clinical and experimental observations support this concept. First, the speed with which skin, subcutaneous tissue, fascia, and muscle are destroyed in these infections is similar to the rate of tissue death following acute arterial thrombosis. Second, intense pain is a prominent feature in clinical conditions that involve occlusion of the arterial blood supply, such as myocardial infarction. Third, tissues which are being rapidly destroyed in the progression of gas gangrene do not bleed. This age-old observation has become dictum for surgeons, who routinely remove necrotic tissue until bleeding is encountered. Indeed, histologic examination of necrotic tissues obtained from patients with StrepTSS at biopsy or amputation (13, 88) or experimental animals challenged with group A streptococcus (7, 92) reveals platelet thrombi and fibrin clots in capillaries, postcapillary venules, and arterioles of the affected musculature and soft tissues. Similarly, tissues from experimental animals in the early stages of gas gangrene demonstrate numerous occlusive thrombi throughout adjacent musculature (19, 20).

Taken together, these observations suggest that severe pain and rapid tissue destruction associated with both GAS myonecrosis and clostridial gas gangrene result from vascular occlusion that begins as a local ischemic process and expands regionally until an entire limb is destroyed. The following discussion reviews the coagulation-anticoagulation systems and examines the molecular mechanisms contributing to microvascular thrombosis and tissue destruction in these two fulminant infections.

**THE COAGULATION SYSTEM—A REVIEW**

**Procoagulants**

**Tissue factor.** Tissue factor (TF) is the principal activator of coagulation in vivo (reviewed in reference 64) (Fig. 1). When expressed by monocytes or endothelial cells following injury, viral infection, or exposure to lipopolysaccharide or cytokines, this membrane glycoprotein forms a proteolytically active complex with circulating factor VIIa. This complex initiates the downstream clotting events of both the intrinsic (via activation of factor IX) and extrinsic (via activation of factor X) coagulation pathways, culminating in the conversion of prothrombin to thrombin. Thrombin proteolytically cleaves fibrinogen, yielding fibrin molecules that rapidly polymerize into a stable clot. In the absence of factor VIIa, TF-initiated clotting does not occur. TF activity is controlled in vivo by several unique mechanisms. First, extravascular TF is physically separated from clotting factors in the blood until it is exposed following vessel injury. Second, TF-mediated procoagulant activity is inhibited by four main anticoagulant systems (see below). Lastly, TF expressed on cell surfaces is encrypted (8) and requires an activation step to manifest its procoagulant activity. Such activation in vitro is accomplished by mechanical disruption of cells or by calcium ionophore treatment (8), but the in vivo signal that releases encrypted TF from its dimerized state has not been elucidated.

**Contact system.** Interaction of cells that correspond to gran-negative and gram-positive bacteria can activate the contact system, resulting in the generation of activated factor XII (Fig. 1) and the potent vasoreactive moiety bradykinin. Bradykinin may contribute to the capillary leak, decreased vascular resistance, and hypotension characteristic of septic shock (reviewed in reference 48).

**Thrombin.** Thrombin generation can act as a feedback amplification pathway for coagulation through its ability to activate cofactors V and VIII.

**Anticoagulants**

The procoagulant response is counterbalanced by four main anticoagulant systems (Fig. 1): the antithrombin (AT)-heparan system, the thrombomodulin (TM)-protein C-protein S complex, tissue factor pathway inhibitor (TFPI), and the fibrinolytic system. Genetic or acquired defects in these systems contribute to the widespread coagulopathy and microvascular thrombosis associated with sepsis.

**Antithrombin-heparan system.** AT is a broad-spectrum serine protease inhibitor found in plasma and on the surface of microvascular endothelial cells (reviewed in reference 60). It is
best known as an inactivator of thrombin, but it also inactivates other serine proteases of the coagulation cascade (e.g., factors Xα, IXα, XIα, and VIIα). AT contains both a heparin-binding domain and a serine-binding domain at its active site. The binding of heparin (either from endogenous heparan sulfates or from exogenously administered heparin) enhances the inhibitory effects of AT 1,000- to 10,000-fold. On simultaneous binding of heparin and the target serine protease, AT undergoes a conformational change that facilitates covalent bonding between an arginine residue in AT and the critical active-site serine residue of the protease. This complexed form of the protease is inactive. Specifically in the case of thrombin, only high-molecular-weight heparin supports the thrombin-AT complex formation; low-molecular-weight heparin cannot facilitate AT-mediated inactivation of thrombin.

In the absence of heparin, AT also has potent anti-inflammatory properties. At high concentrations, AT stimulates increased endothelial cell production of prostaglandin I₂, reduces proinflammatory cytokine production by monocytes and endothelial cells (82), and dampens neutrophil activation responses.

**Thrombomodulin-protein C-protein S system.** TM is an integral membrane protein of endothelial cells. When thrombin binds to TM, it loses its capacity to generate fibrin but gains the ability to activate protein C. Activated protein C, together with cofactor protein S, inactivates factor Va on platelets, thereby shutting down thrombin synthesis. This complex also increases the activity of the fibrinolytic molecule tissue plasminogen activator (tPA). The procoagulant state during gram-negative bacterial sepsis is enhanced by the downregulation of the anticoagulant TM-protein C-protein S system (reviewed in reference 101). This downregulation is mediated by tumor necrosis factor alpha (TNF-α) (see “Cytokines” below) and occurs concomitantly with increased expression of TF and endothelial cell adherence molecules, such as E-selectin.

**Tissue factor pathway inhibitor.** TFPI is a multivalent plasma proteinase inhibitor with three tandem Kunitz-type domains (reviewed in reference 16). In the presence of factor Xa, TFPI forms a quaternary complex with TF and factor VIIa on the endothelial cell or platelet surface. In this state, TF is inactivated. The requirement for activated factor X suggests that TFPI is effective only after factor Xa has been generated, i.e., after initiation of coagulation. TFPI itself can be inactivated by elastase released from activated neutrophils (39), providing one link between inflammation and coagulation. Platelets and endothelial cells are the primary sources of TFPI. Platelets release TFPI on activation by thrombin or other agonists (reviewed in reference 16), and heparin administration...
increases circulating TFPI levels two- to fourfold, presumably by stimulating the release of TFPI from endothelial cells.

**Fibrinolytic system.** The enzymatic cleavage of polymerized fibrin is induced by tissue plasminogen activator (tPA) and urokinase (uPA) released from endothelial cells. These factors stimulate the conversion of plasminogen to plasmin, which hydrolyzes fibrin into soluble fragments. Activation of this system occurs transiently secondary to thrombin generation, which in turn induces the release of tPA from the vascular endothelium. The generation of fibrin accelerates tPA activation of plasminogen by serving as a binding surface for both tPA and plasminogen. This system can be rapidly curtailed by increased production of plasminogen activator inhibitor type 1 (PAI-1). Several studies have shown that this system is strongly activated in humans with sepsis and may be exhausted in patients with septic shock. Recently, novel posttranscriptional pathways regulating the expression of uPA, tPA, and plasminogen activator inhibitor-1 have been identified (reviewed in reference 40).

**The Cellular Players**

**Endothelial cells.** Under normal conditions, the vascular endothelium maintains a profoundly anticoagulant state. So critical is this function to hemostasis that this status is maintained by multiple, functionally discrete mechanisms. These include (i) production of prostaglandin I2, which inhibits activation of platelets and promotes vasodilation; (ii) the presence of large amounts of endogenous heparan sulfates, which act synergistically with AT to inactivate thrombin; (iii) the expression of thrombomodulin, TFPI, tPA, and nitric oxide (NO); and (iv) the limited expression of cellular adherence molecules. However, when a rapid and focal procoagulant response is required, the endothelium responds with immediate synthesis of platelet-activating factor and expression of the adherence molecule P-selectin; exposure of subendothelial matrix proteins also provides binding sites for platelets. Coagulation is sustained by synthesis of von Willebrand factor, PAI, and TF, by upregulation of cellular adherence molecules (e.g., E-selectins, intracellular cell adherence molecule type 1 [ICAM-1]), and by exposure of binding sites for coagulation factor complexes.

The precise physiologic mechanisms invoked following endothelial cell perturbation differ from one vascular bed and one organ to another (reviewed in reference 71). For instance, TM is a more important anticoagulant in vessels of the lungs and heart than in the liver (71). Plasminogen activators also contribute to the maintenance of blood fluidity in these organs, but neither TM or tPA plays a significant role in the brain (71). Thus, a delicate and tissue- or organ-specific balance between procoagulant and anticoagulant activities is maintained by a complex series of mechanisms designed to limit the formation of a hemostatic plug precisely to the site of injury. A shift in this balance to a prothrombotic, hypercoagulable state has devastating consequences as described for the above infections.

**Platelets.** Under normal conditions, platelets circulate in close proximity to the vascular endothelium but do not adhere to it. Activation of endothelial cells, exposure of subendothelial matrix proteins, or an increase in shear stress (74) stimulates the rapid adhesion of platelets at the site. The process of adhesion triggers a signal transduction pathway that culminates in activation of platelet receptors (e.g., the glycoprotein heterodimer gpIIbIIIa) and in exposure of phosphatidylserine on the outer plasma membrane surface. Once activated, gpIIbIIIa promotes fibrinogen-dependent platelet-platelet aggregation such that platelets from the circulating pool are recruited to the growing hemostatic plug. Exposure of phosphatidyl serine accelerates the binding and assembly of activated Factors VIII, V, and X into a functional prothrombinase enzyme complex for the generation of thrombin. Lastly, adhesion and aggregation cause platelets to release intracellular granule contents, including serotonin from the dense granules and fibrinogen, P-selectin, clotting factors, β-thromboglobulin, and platelet factor 4 from the alpha granules. The last two substances are used clinically as circulating markers of platelet activation. Thromboxane A2, also released from activated platelets, amplifies platelet aggregation and secretion responses, smooth muscle contraction, and vasocostriction and thus facilitates hemostasis.

**Monocytes/macrophages.** Originally, TF activity was thought to be limited to brain, lungs, and placenta and to sites in other organs that do not contact flowing blood. Exposure of this extravascular pool of TF following vessel injury, or its induction on fixed tissue macrophages or endothelial cells following viral infection, or exposure to endotoxin or cytokines was thought to be solely responsible for both the initiation and the propagation of thrombus formation (63). However, recent studies have demonstrated detectable TF procoagulant activity in whole-blood samples from healthy individuals (37, 45). This activity was associated principally with the mononuclear cell fraction (45) and was elevated in patients with sickle cell disease (45), severe meningococcal infection (65), or peritonitis (2). Current opinion suggests that this intravascular pool of TF may contribute significantly to the pathologic propagation of thrombus formation (46), as is observed in septic states associated with DIC.

In addition to the expression of surface-bound TF, monocytes bind factor X via CD11b/CD18 (5). This binding stimulates monocyte release of cathespin G, which proteolytically activates factor X. Thus, monocytes possess an alternative means of initiating procoagulant responses to inflammatory stimuli (69). Interestingly, this binding, as well as the binding of other ligands, such as fibrinogen, ICAM-1, or iC3b, to the I domain of the CD11b molecule can be inhibited by both unfractionated and low-molecular-weight heparin at therapeutically relevant concentrations (67). Thus, this feature is distinct from the anticoagulation effects of heparin and may provide additional clinical benefits (e.g., moderation of leukocyte function) (67).

**Polymorphonuclear leukocytes.** Polymorphonuclear leukocytes (PMNL) also contribute to the procoagulant state. Like monocytes, stimulated PMNL bind factor X and convert it to its activated form. The generation of thrombin then results from the interaction of bound factor Xa with the factor V-like effector cell protease receptor type 1 expressed on PMNL and endothelial cells (3). In addition, simultaneous engagement of PMNL ligands with the endothelial cell leukocyte adherence molecules E-selectin and ICAM-1 results in increased TF and decreased TM synthesis by the endothelium (78)—a process that can be inhibited by antibody to TNF-α or by the platelet-activating factor receptor antagonist WEB2086 (78). Further-
Platelet-leukocyte complexes. Recent studies have demonstrated both the physiologic and pathologic significance of the interaction of activated platelets and leukocytes. As part of the normal inflammatory response, this interaction facilitates the recruitment of PMNL to sites of vascular activation or injury, primes neutrophils for enhanced phagocytosis and killing of foreign microbes (68), enhances monocyte production of cytokines (57), and permits chemical cross talk and signaling between these cell types (33, 54). In contrast, these heterotypic cellular complexes also contribute to infectious (19, 20) and noninfectious (10, 66; P. A. Ward, G. O. Till, and K. J. Johnson Abstract, Fed. Proc. 1312a, 1986) ischemic-tissue injury. The balance between controlled hemostasis (physiologic) and widespread thrombosis (pathologic) is a fine one, since in both settings the events are mediated by increased cell adhesion, PMNL and platelet granule release, superoxide anion generation, and thromboxane and leukotriene synthesis (reviewed in reference 27). In a recent study of platelet function in septic patients, the numbers of circulating platelet-PMNL complexes were significantly reduced in individuals with evidence of multiorgan dysfunction compared to those with sepsis alone (36), suggesting that sequestration of these aggregates in the microvasculature contributes to organ failure.

Platelet-leukocyte interactions are mediated by several distinct contact mechanisms. Although platelet P-selectin binding of PMNL glycoproteins has been the paradigm for platelet-PMNL interactions, other investigators have demonstrated that gpIIbIIIa (CD41/CD61) also participates in the adhesion of thrombin-activated platelets to PMNL in vitro (Fig. 2) (73, 83, 107). These studies have shown that this interaction is fibrinogen dependent (73, 107), that CD11b/CD18 serves as the PMNL ligand for fibrinogen (4, 109), and that this interaction is further enhanced when the functionally active conformation of CD11b/CD18 is expressed (33). These findings have been assimilated into a multistep adhesion cascade model in which a platelet P-selectin-dependent recognition step is followed by a gpIIbIIIa/CD18-dependent stabilization step (reviewed in reference 27).

Cytokines

The role of cytokines in the regulation of procoagulant and anticoagulant activities is well established (reviewed in reference 98). TNF-α, when injected into human volunteers, exerts a procoagulant effect by increasing the synthesis of TF (9, 97) and the procoagulant cytokines interleukin-6 (IL-6) and IL-8 (100). Neutralization of IL-6 decreases the procoagulant responses induced by TNF-α (99). Interestingly, TNF-induced activation of the coagulation cascade is preceded by a transient activation of the fibrinolytic system (reviewed in reference 101); activation of coagulation then triggers a secondary activation of fibrinolysis, which is short-lived due to TNF-induced production of PAI (77). The net result is a shift to a procoagulant state. This hypocoagulable state is then sustained by TNF-α-induced suppression of TM synthesis (24, 76; E. Conway and D. Rosenberg, Abstract, Circulation 78: 0462 1998) and by decreased formation of the activated protein C-protein S complex on endothelial cells (24). TNF-α mediates the reduction in TM synthesis by a posttranslational mechanism (76)—a process that can be counteracted with IL-4 (43). In addition, TNF-α increases production of the acute-phase reactant, C4b-binding protein (C4BP), a molecule that complexes with and sequesters free protein S such that it is no longer available for TM complex formation. Infusion of C4BP with sublethal doses of Escherichia coli into experimental animals results in a consumptive coagulopathy, thrombocytopenia, and microvascular thrombosis (93) similar to that seen in hemolytic-uremic syndrome, suggesting that inactivation of free protein S may contribute to microvascular thrombosis in other diseases. Administration of anti-TNF-α in models of endotoxemia blocks the activation of fibrinolysis but not coagulation (101). In contrast, treatment with anti-IL-6 blocks coagulation but not fibrinolysis.

IL-1 induces TF activity in endothelial cells (12) and monocytes (62). Injection of IL-1 into experimental animals (ba-boons) also elicits an initial but transient activation of the fibrinolytic system, followed by a TNF-like shift to a procoagulant state (41, 101). Treatment with an IL-1 receptor antagonist inhibits the activation of both the fibrinolytic and coagulation systems in humans with gram-negative bacterial sepsis (15) and in animals with experimental endotoxemia (41). The anti-inflammatory cytokines IL-4 and IL-10 inhibit IL-1- and lipopolysaccharide-induced TF activity in monocytes (62), and pretreatment of animals with IL-10 limits the size of thrombi formed in response to stasis-induced venous thrombosis (29).

MECHANISMS OF COAGULOPATHY IN INVASIVE S. PYOGENES AND C. PERFRINGENS INFECTIONS

Invasive S. pyogenes Infections

In 1989, we reported a series of patients from the Rocky Mountain West with GAS infections associated with the sudden onset of shock, adult respiratory distress syndrome, renal
failure, bacteremia, and death (88). Similar cases emerged worldwide (reviewed in reference 86), prompting an official case definition of StrepTSS by the Centers for Disease Control Working Group on Streptococcal Infections (108a). Despite better clinical recognition of StrepTSS and intense research on streptococcal virulence factors, morbidity is high and mortality remains between 30 and 70% (13).

Clinical reports and epidemiologic studies have repeatedly shown an association between invasive infections (i.e., bacteremia, necrotizing fasciitis, myonecrosis, and StrepTSS) and GAS strains of M protein types 1, 3, 12, and 28. Among patients having a defined portal of entry, M type 1 and 3 GAS account for over 40% of all isolates (reviewed in reference 13). Further, among the 50% of all StrepTSS patients having no defined portal of entry, virtually all strains of GAS isolated are of M type 1 or 3. Thus, invasive infections due to M type 1 and 3 GAS are characterized by coagulopathy and rapid tissue destruction.

Numerous studies have investigated the interactions of GAS with the coagulation system (Fig. 1). In vitro work has demonstrated that M protein binds fibrinogen and that streptokinase forms a high-affinity complex with plasminogen. When this latter complex interacts with M-protein-bound fibrinogen on the surface of GAS, it acquires potent plasminogen activator activity that is not inhibitable by antiproteases such as α2-antiplasmin (Fig. 1) (51, 104). Further, in a murine model of M type 1 GAS necrotizing fasciitis, Sriskandan et al. have recently shown that the activated partial thromboplastin time (aPTT) was prolonged and associated with reduced levels of factor XII and prekallikrein (Fig. 1) (85). Similarly, in a small study of humans with StrepTSS, seven of seven patients had a significantly elevated aPTT compared to that of patients with GAS infections not associated with shock and organ failure (84). From these findings, it might be predicted that patients with severe GAS infections would develop a largely fibrinolytic clinical picture and perhaps a bleeding diathesis.

However, in the murine model mentioned above, the prothrombin time (PT) was normal and mice actually demonstrated a hypercoagulable state despite prolongation of the aPTT (85). Further, our recent studies of the responses of experimental animals (baboons) to GAS infection have demonstrated an intense, systemic activation of the coagulation system. Specifically, we have shown that bacteremia due to M type 3 GAS was associated with a profound drop in the plasma fibrinogen concentration (to 1% of baseline control values), a 50% reduction in platelet count, and marked increases in the levels of fibrin degradation products (>640 μg/dl) and thrombin-anthithrombin and plasmin-antiplasmin complexes (87). Lastly, the marked DIC observed in the lungs (60%), adrenals (100%), and kidneys (80%) of primates with streptococcal bacteremia (87) and in tissues from humans with StrepTSS also suggests that the hemostatic balance is shifted toward a pathologic coagulant state in severe GAS infections. Indeed, coagulopathy is part of the case definition of StrepTSS (108a).

In the experimental nonhuman primate model of GAS bacteremia, pretreatment of animals with neutralizing monoclonal antibody against TNF improved survival but did not reverse the observed coagulopathy (87). This suggested that (i) the coagulopathy associated with GAS bacteremia was not strictly an epiphenomenon of cytokine generation in the septic state and (ii) specific bacterium-host cell interactions drive the coagulation response to GAS.

Our recent investigations of this latter hypothesis demonstrated that killed, washed M type 1 and 3 strains of GAS isolated from patients with invasive infection stimulated the in vitro production of TF (21). Interestingly, GAS appeared to have an M-type-specific predilection for the TF-producing cells they stimulated. Specifically, M type 3 GAS elicited high levels of TF from endothelial cells but not monocytes, whereas with M type 1 strains, the converse was observed (21). This disparity existed although both M types elicited high levels of TNF-α and IL-8 from cultured monocytes (21) and enhanced leukocyte adherence molecule expression in endothelial cells (my unpublished observations). Such tissue-specific tropism among different GAS strains is not unique to this setting. For instance, it has long been recognized that some M types of GAS are primarily skin associated whereas others are throat strains. Recently, Kalia et al. have demonstrated that this type of tissue tropism and niche separation is associated with specific patterns found in the gene locus for M protein (42). Together, these findings support the concept that specific bacterium-host cell interactions contribute to the coagulopathy associated with StrepTSS.

That these responses are unique to GAS is suggested by the fact that other gram-positive cocci do not stimulate these critical endothelial cells responses controlling inflammation and hemostasis. Specifically, Noel et al. (59) have shown that S. aureus, Enterococcus faecium, and Streptococcus pneumoniae could not induce E-selectin expression in cultured endothelial cells. Similarly, Veltrop et al. demonstrated that S. sanguis and S. epidermidis could not elicit TF from human vascular endothelial cells (102). In addition, clinical isolates of S. aureus did not elicit the expression of endothelial cell E-selectin or ICAM-1 (90) or induce TF synthesis (102).

TF-mediated coagulation by GAS could also be initiated by direct injury of the endothelium by soluble streptococcal virulence factors such as streptolysin O (SLO) or the cysteine protease. For instance, Kapur et al. have shown that the GAS cysteine protease induces the detachment of endothelial cells in culture (44). Shanley et al. have shown that this protease, when instilled directly into the lungs of rats, augments lung endothelial cell injury induced by instillation of either streptococcal cell wall antigen or SLO (80). Thus, in some settings, SLO or the streptococcal cysteine protease could augment coagulation by damaging the endothelium and exposing subendothelial TF and matrix proteins. However, it should be noted that only 50 to 70% of isolates of GAS from StrepTSS patients produce the cysteine protease precursor streptococcal pyrogenic exotoxin B (23, 38, 88).

Sublytic concentrations of SLO and a related toxin, perfringolysin O, from C. perfringens each stimulate the functional upregulation of PMNL CD11b/CD18 (18, 22) and prime neutrophils for enhanced respiratory burst activity (18). Thus, it is likely that these thiol-activated toxins may indirectly contribute to coagulation by stimulating CD11b/CD18-dependent activation of factor X (69) (see above section on platelet-leukocyte complexes) and by stimulating premature degranulation of hyperadherent PMNL. In addition, toxin-induced functional upregulation of CD11b/CD18 may serve to stabilize gpIIbIIIa-mediated intravascular aggregates of platelets and PMNL.
Such aggregates appear to occlude vessels at the site of GAS infection in experimental animals (92) and may contribute to the local intense pain and rapid destruction of adjacent healthy tissue characteristic of human cases of streptococcal myositis and necrotizing fasciitis.

For a hypercoagulable state to progress to clinical DIC in invasive S. pyogenes infections, the opposing anticoagulant systems (e.g., TFPI, TM-protein C-protein S) must also be functionally downregulated (Fig. 1). Interestingly, certain GAS strains bind protein S in its plasma and endothelial cell-bound forms (96). In addition, some S. pyogenes strains bind C4BP (95), a serum protein that complexes with free protein S. In terms of the coagulation system, we suggest that streptococcal binding of protein S would effectively remove this molecule from participation in the anticoagulant system and thereby promote coagulation. However, the role of protein S binding in the pathogenesis of invasive streptococcal disease remains unclear since GAS serotypes that are commonly associated with invasive infections (i.e., M type 1 or 3) do not bind protein S (49) or C4BP (95). Lastly, novel anticoagulant strategies such as those involving activated protein C and TFPI may hold promise for the treatment of invasive streptococcal infections by limiting both the local and systemic manifestations of coagulopathy. However, these modalities remain to be evaluated in animal models of GAS bacteremia and soft tissue infection.

In summary, GAS possess unique cell-associated components and soluble factors that elicit important functional responses in cells of the coagulation-hemostatic system. In vivo, these responses probably contribute to intravascular thrombosis and leukostasis, multiple-organ failure, and rapid tissue destruction characteristic of StrepTSS.

**C. perfringens Gas Gangrene**

*C. perfringens* type A is the most common organism isolated from patients with trauma-induced gas gangrene (53). The local and systemic manifestations of gas gangrene are related to the elaboration of potent extracellular protein toxins, especially alpha-toxin, a phospholipase C (PLC), and theta-toxin, a thiol-activated cytolysin (53, 81, 89). We have recently demonstrated that intramuscular injection of PLC induced a rapid and irreversible decline in skeletal muscle perfusion (Fig. 3) that occurred concomitantly with the formation of intravascular aggregates of activated platelets, leukocytes, and fibrin (19). These aggregates were freely moving (Fig. 4A) until achieving sufficient size to completely and irreversibly occlude capillaries, venules, and arterioles (Fig. 4B). Only at the later stages did these heterotypic aggregates appear fixed to the endothelium (19, 20).

This sequence of events was strikingly different from that which occurs following traumatic vessel injury. Following the initial insult to the endothelium, tethering of platelets at precisely the injured site is mediated by platelet gpIb/9251 binding to von Willebrand factor (74). This adhesion then stimulates “outside-in” signaling events that result in activation of platelet gpIIbIIIa (47, 50) and in redistribution of platelet membrane phospholipids. These events promote the adherence of additional platelets and interactions with elements of the clotting cascade to generate a localized clot.

However, the appearance of nonadherent, freely mobile, and growing platelet-leukocyte aggregates suggested that PLC stimulated cellular aggregation in a different manner. For platelets in circulation to coaggregate and to bind other cell types, a conformational change in platelet gpIIbIIIa is neces-
FIG. 4. Histopathology of skeletal muscle following injection of PLC. Routine hematoxylin-eosin staining of rat abdominal muscles 2 min (A) or 20 min (B) after injection with PLC is shown. Reprinted from reference 19 with permission.
sary to permit fibrinogen binding (reviewed in reference 14). Indeed, flow cytometric analyses of PLC-treated whole blood demonstrated that the formation of both platelet-platelet and platelet-leukocyte aggregates was mediated by the activation of platelet gpIIbIIIa (20).

Because several pathways are known to contribute to the activation of this adherence molecule, we next investigated the intracellular signaling events leading to functional upregulation of gpIIbIIIa induced by PLC. Classically, increases in cytosolic calcium levels and activation of protein kinase C (PKC) independently regulate many cellular functions including activation of platelet gpIIbIIIa. Further, calcium mobilization and PKC activation act synergistically to elicit the full physiological responses of platelets (reviewed in reference 58). Our studies of these signaling pathways demonstrated that PLC-induced activation of gpIIbIIIa is highly calcium dependent but, surprisingly, PKC independent even though PKC is strongly activated by PLC treatment (17). This finding was unexpected since most agonists that directly or indirectly activate PKC also activate gpIIbIIIa. However, Tapley and Murray have previously demonstrated that treatment of platelets with C. perfringens PLC results in the proteolytic cleavage of soluble PKC into a calcium- and phospholipid-independent but enzymatically active low-molecular-weight form of the kinase (91). Since PKC-mediated signal transduction requires relocation of the enzyme from subcellular sites to membrane regions (reviewed in reference 28), it is possible that PLC-induced cleavage of PKC prevents translocation of the truncated kinase to the plasma membrane, where it would normally contribute to gpIIbIIIa activation. Definitive resolution of this apparent paradox requires further studies.

Increases in intracellular calcium levels in platelets occur primarily by two mechanisms: receptor-mediated opening of plasma membrane calcium channels (75) and store-operated calcium entry (reviewed in references 32 and 70). In store-operated calcium entry, agonist-induced depletion of calcium from intracellular stores triggers the opening of plasma membrane calcium channels and the influx of extracellular calcium. Pharmacologic inhibitors of intracellular calcium release and of store-operated plasma membrane calcium channels blocked both gpIIbIIIa activation and calcium mobilization in PLC-treated platelets (17).

Thus, one can conclude that PLC initiates an “inside-out” signaling cascade that begins with the depletion of internal calcium stores, is sustained by an influx of calcium through store-sensitive channels, and culminates in functional activation in gpIIbIIIa. Further, the lack of involvement of PKC suggests that the response to PLC does not follow receptor-linked, G-protein-mediated signaling with inositol trisphosphate generation. This conclusion is further supported by the fact that prostaglandin E₁, a known inhibitor of Gαi-mediated signaling, had no effect on PLC-induced activation of gpIIbIIIa. Instead, neutralization studies with monoclonal antibody against PLC suggest that activation of gpIIbIIIa is a direct consequence of the PLC and/or sphingomyelinase activities of the toxin and not receptor occupancy. This model does not, however, exclude a potentiating role of other mediators (e.g., thromboxane production) in the PLC-induced effects.

In summary, these findings suggest that calcium channel blockade and/or therapeutic strategies targeting gpIIbIIIa, such as those currently used to treat acute myocardial infarction, may prevent vascular occlusion, maintain tissue viability, and provide an alternative to radical amputation for patients with clostridial gas gangrene. Experimental studies to evaluate these therapeutic approaches are under way in my laboratory.

**PROMISING ANTITHROMBOTIC AGENTS IN THE TREATMENT OF SEPSIS AND SEPTIC SHOCK**

**Antithrombin**

A meta-analysis of four small, randomized, placebo-controlled studies of AT in severe sepsis demonstrated a trend toward reduction of 30-day all-cause mortality in the group receiving AT compared to placebo. This trend was also associated with shortened stays in the intensive care unit (31). Recently, a larger study (2,314 patients) examined the efficacy of high-dose AT alone and in combination with heparin for the treatment of severe sepsis (106). The 28-day all-cause mortality was not different between the treatment and placebo groups, and increased bleeding was associated with combination AT-heparin therapy. However, the difference in survival between those receiving AT alone versus placebo became significant after 90 days (106). Over this extended period, patients who received AT without heparin also demonstrated increased improvements in their quality of life (72).

**Tissue Factor Pathway Inhibitor**

In patients with disease conditions associated with DIC, a massive production and continuous exposure of excess TF is thought to exhaust the available TFPI (reviewed in reference 64). This concept formed the basis for several efficacy studies of TFPI in both humans and experimental animals with gram-negative bacterial sepsis. Administration of TFPI limited the development of acute lung and renal injury (108), DIC, and mortality (26) in baboons with E. coli bacteremia and reduced mortality in mice with polymicrobial intra-abdominal infection (61). A safety study of TFPI in humans with sepsis has recently been completed (1). Although not geared to demonstrate efficacy, this study noted improvements in pulmonary, cardiovascular, and coagulation scores, a reduction of the circulating levels of IL-6 and thrombin-AT complexes, and a trend toward increased survival in patients receiving recombinant TFPI (1).

**Activated protein C**

A recent large clinical trial (1,690 patients) investigated the efficacy and safety of activated protein C (APC) replacement therapy in patients with severe sepsis (11). Causes of infection in patients with severe sepsis included S. aureus (~14%) and other staphylococci (~6%), S. pneumoniae (~11%) and other streptococcal (~9%) and enterococcal (~7%) species, multiple gram-negative organisms (~48%), and a few fungal species. The incidence of infections due to gram-positive versus gram-negative organisms was similar between the placebo and treatment groups. Administration of APC resulted in a 6.1% reduction in the risk of death compared to the placebo group (11) but was associated with an increased risk of severe bleeding.
CONCLUSIONS

The severe pain and rapid tissue destruction characteristic of invasive streptococcal and clostridial infections probably result from hypercoagulation and vascular occlusion mediated by unique interactions of the organisms and their toxins with the human coagulation system. Understanding the specific mechanisms by which bacterial virulence factors interact with human cells may offer new insights into the pathogenesis of these infections while providing novel therapeutic targets to limit the severity of these invasive diseases.

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REFERENCES


94. Reference deleted.


