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Role of Inflammatory Mediators in Thrombogenesis

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ABSTRACT
The role of inflammation in cardiovascular disease and especially in thrombogenesis has become increasingly recognized as an important component of the overall disease process. Plaque rupture promotes activation of the inflammatory response and increased expression of tissue factor (TF), which in turn acts as one of the major initiators of extrinsic coagulation. It is becoming apparent that the expression of TF on endothelial cells, underlying smooth muscle cells and monocytes is regulated, in part, by proinflammatory cytokines including tumor necrosis factor and IL-1. In addition to initiating coagulation, interaction of TF with the adhesion molecule, P-selectin, has been demonstrated to accelerate the rate and extent of fibrin formation and deposition. P-selectin is expressed on activated platelets and endothelium and serves as the receptor for the endogenous ligand, P-selectin glycoprotein-1 (PSGL-1), expressed on various leukocytic cell types. In addition to mediating transient interactions between endothelial cells and leukocytes, P-selectin has been reported to mediate adherence of platelets to monocytes and neutrophils via specific interaction with PSGL-1. P-selectin is rapidly cleaved off the surface of the platelet membrane and appears in the circulation as a soluble form, which has been reported to be elevated in patients with acute coronary syndromes including unstable angina and non-Q-wave myocardial infarction. This review will focus on the role of cytokines in mediating TF expression and also explore the significance of the relationship between P-selectin and tissue factor in thrombus generation. In addition, possible pharmacological mechanisms to interrupt this disease process will be discussed.

Role of Cytokines in Promotion of Thrombosis

The expression of proinflammatory cytokines has been implicated in mediating the pathogenesis of a number of cardiovascular diseases including ischemia/reperfusion injury, heart failure, and atherosclerosis (Zhou et al., 1999). However, the role of these inflammatory mediators in unrestrained coagulation remains to be fully understood. Several lines of evidence derived from both preclinical and clinical studies demonstrate a link between inflammation and coagulation. Foremost is the increased coagulation in Gram-negative sepsis/endotoxemia and increased circulating levels of thrombin following infusion of IL-6 in cancer patients (Stouthard et al., 1996; Grignani and Maiolo, 2000). While the involvement of cytokines in mediating thrombosis has focused primarily on sepsis, it is becoming increasingly apparent that the expression of these mediators can shift the intravascular environment from hemodynamically stable to a procoagulative state, even in nonseptic conditions.

Proinflammatory cytokines, IL-1β, IL-6, MCP-1, and TNFβ, have been shown to be up-regulated in the setting of thrombosis and may be involved in maintaining the balance between coagulation and fibrinolysis. However, during progression of the inflammatory response the balance between anticoagulant and prothrombotic activity is shifted toward the procoagulant state by the ability of these molecules to down-regulate antithrombotic proteins (i.e., thrombomodulin/protein C pathway) while up-regulating prothrombotic proteins (ten Cate et al., 1997). One of the primary consequences of increased proinflammatory cytokines in the vasculature is the increased expression of a number of proteins that serve to regulate coagulation. Foremost among these proteins is tissue factor (TF, coagulation factor III, CD142), which acts to regulate the activation state of the extrinsic pathway of coagulation. Tissue factor is a 46-kDa transmembrane glycoprotein that serves as one of the primary initiators of blood coagulation (Giesen and Nemerson, 2000). Cell-anchored TF interacts with soluble factor VIIa (FVIIa) to

ABBREVIATIONS: IL, interleukin; TNF, tumor necrosis factor; TF, tissue factor; PSGL-1, P-selectin glycoprotein-1; rPSGL-1, recombinant PSGL-1.

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induce factor Xa (FXa) activation, leading to cleavage of prothrombin to form proteolytically active thrombin. Thrombin in turn is responsible for conversion of plasma fibrinogen to fibrin, which envelopes and stabilizes developing thrombi/blood clots. Thrombin also has other diverse biological actions such as inducing platelet aggregation and influencing cell growth and migration signaling pathways. With respect to procoagulant activity of TF, the majority of cell surface TF activity is normally “encrypted” in which state it is capable of binding FVIIa but does not express full activity as it does not bind FXa. To become fully activated, TF must be “de-encrypted” by some form of cellular perturbation, which may involve plasma membrane phosphatidylserine-dependent and independent mechanisms (Bach and Moldow, 1997). Agents such as Annexin V, which binds to and inhibits phosphatidylserine, may block the de-encryption process or selectively inhibit the de-encrypted form of TF.

Identification of the primary cell types expressing TF is critical to understanding the role of the inflammatory response in mediating thrombosis. TF is constitutively expressed by a variety of cell types including fibroblasts, glomerular epithelial cells, and tumor cells (Hair et al., 1996; Mackman, 1997). TF is also constantly present in the adventitia of blood vessels and is thought to provide a protective barrier that serves an important role in maintaining hemo- stasis of the vascular system. TF also has recently been described to circulate as TF-rich microparticles (Giesen et al., 1999) that may interact with blood-borne FVIIa to initiate coagulation. Although expressed constitutively by a number of nonvasculature-associated cell types (i.e., fibroblasts), TF activity is rapidly up-regulated by monocytes and endothelial cells in response to various chemical and mechanical stimuli (Grabowski and Lam, 1995; Lorenzet et al., 1998). It is becoming evident that the cytokine-mediated expression of TF by endothelial cells and inflammatory cells acts as one of the primary initiators of thrombosis (Dosquet et al., 1995). The initiation of the inflammatory response and the subsequent stimulation of the vascular endothelium by TNF and/or IL-1 result in increased TF expression, thereby shifting the vascular environment to the prothrombotic state.

Monocytes and smooth muscle cells in atherosclerotic plaques strongly express TF. Presumably, these cells are responding to inflammatory signals within the plaque. Rupture of plaques exposes active TF directly to blood in the lumen of the vessel and is thought to be the triggering event that causes myocardial infarction and ischemic stroke. Recent studies suggest the accumulation of TF in atherosclerotic plaques plays a major role in determining plaque thrombogenicity (Taubman et al., 1997). However, the classical view that active, constitutively expressed TF present on the atherosclerotic plaque itself was the primary initiator of thrombosis has been contested (Libby, 2000). It is becoming increasingly apparent that the inappropriate expression of TF by circulating monocytes plays an important role in pathological conditions characterized by hypercoagulation such as that noted in acute thrombotic episodes (Esmann, 2001). In cell culture, monocytes and endothelial cells can be induced by TNF, IL-1, MCP-1, or IL-6 to strongly express tissue factor on their cell surfaces (Grabowski and Lam, 1995; Ernofsson and Siegbahn, 1996). In addition to the other thrombotic events associated with the molecule, TF expression on the monocyte surface facilitates the interaction of the monocyte with activated platelets and endothelial cells via binding of P-selectin (Fig. 1). The end result of the ability of inflammatory mediators to increase the expression of TF on monocytes and endothelial cells is the acceleration of the rate and extent of fibrin formation and deposition in thrombus. It is apparent that circulating monocytes may represent one of the principle players in the cross-talk between the inflammatory and coagulative pathways (Napoleone et al., 1997).

In addition to the evidence derived from in vitro studies, there is substantial in vivo data supporting the important role of cytokines in mediating thrombogenesis. In sepsis, TF is strongly expressed by circulating monocytes. In contrast, expression of TF on endothelium in vivo is very rare, even in fulminant sepsis. So, it is suspected that the coagulopathy seen in sepsis is driven largely by the de novo expression of tissue factor on circulating monocytes. In animal models of sepsis, specific antagonists of tissue factor or factor VII block the coagulopathy and lead to survival of animals that would have died without intervention (Taylor et al., 1991; Uchiba et al., 1997). These antagonists include inhibitory antibodies to tissue factor and factor VII, and active site-blocked factor VIIa (VIIai).
Inhibitors of cytokine action have also been demonstrated to regulate the prothrombotic actions of TF in the setting of sepsis. For example, administration of an anti-IL-6 antibody has been shown to prevent abnormal coagulation during systemic infection (Stouthard et al., 1996). Similar protective effects against hypercoagulation have been noted in monkeys with the use of anti-TNF and anti-TF antibodies following infusion of endotoxin or live bacteria (Salat et al., 1996; Levi et al., 1997). Attenuation of IL-1 activity via administration of recombinant IL-1 receptor antagonist (IL-1ra) has also been shown to decrease coagulation in patients with sepsis and in septic baboons (Boermeester et al., 1995; Jansen et al., 1995). The anticoagulative effects of anti-TNF and anti-IL-6 antibodies coupled with the positive effects of recombinant IL-1ra in primate models, supports anticytokine therapies as potential pharmacologic approaches in the setting of thrombogenesis.

**Attenuation of TF-Mediated Coagulation by Cytokine Inhibitors**

It is becoming increasingly apparent that the inflammatory response, as defined by generation of cytokines and activation of proinflammatory cell types, plays an important role in mediating TF expression and subsequent activation of the coagulation cascade in pathophysiologic settings. The dynamic role of cytokines and inflammatory cell types in thrombogenesis has opened up potential new avenues for therapeutic intervention. While a great deal of effort has been placed on identifying direct inhibitors of the TF pathway, the inflammatory cascade represents another attractive, yet feasible, means to prevent TF up-regulation and subsequent thrombosis. Multiple pharmacologic avenues including biology (i.e., antibodies, peptides) and low-molecule weight inhibitors exist by which to attenuate actions of the cytokines that are involved in up-regulating TF.

While direct cytokine inhibitors (antibodies) have been previously demonstrated to be effective against untoward thrombosis, other, more pharmacologically attractive approaches, should also be considered. A great deal of effort has been expended into inhibitors of the second messenger systems responsible for transducing intracellular signals following binding of cytokines to their respective receptors. For example, selective and potent inhibitors of nuclear factor-κB activity and the mitogen-activated protein kinase pathway have been proven to be efficacious in animal models of inflammation via their inhibitory actions on cytokine production/action (Lee et al., 2000; Yamamoto and Gaynor, 2001). However, as of yet, these novel anti-inflammatory approaches have yet to be investigated in the setting of thrombosis. The emergent role of inflammation in thrombogenesis suggests that therapeutic strategies designed to attenuate the inflammatory response hold immense therapeutic potential and merit further study.

**Pharmacological Approaches to Interruption of TF-Mediated Coagulation**

Various approaches to intervention in TF-induced extrinsic coagulation have recently emerged as investigators continue to move up higher in the coagulation cascade to intervene pharmacologically in an attempt to interrupt the well appre-
studies have demonstrated that inflammatory reactions and platelet accumulation occur following vascular injury. This “response to injury” is noted most readily following balloon angioplasty/PTCA in which endothelial disruption is followed by platelet adherence and local inflammatory reactions, which often predispose the injured vessel segment to a phenomenon known as “restenosis”. Restenosis is the process that refers to vessel narrowing at a previously balloon-dilated site due to smooth muscle and inflammatory cell accumulation to form a “neointima” and is the largest clinical drawback to prolonged success following PTCA. In fact, experimental studies have clearly demonstrated that P-selectin-dependent, platelet-leukocyte-induced luminal TF expression and fibrin deposition, is required for neointimal formation to occur following vascular injury (Hayashi et al., 2000; Kawasaki et al., 2001; Singh et al., 2001).

Following platelet activation, P-selectin and GPIIb/IIIa are rapidly expressed on the platelet surface. Whereas GPIIb/IIIa serves as a receptor for dimeric plasma fibrinogen and is necessary for platelet-platelet interaction in thrombus formation, P-selectin does not mediate platelet-platelet interactions. However, P-selectin mediates platelet-leukocyte interaction in the developing thrombus and may play a very important role in determining the size and stability of the platelet aggregates in the developing thrombus (Merten and Thiagarajan, 2000).

Subsequent to expression of P-selectin on the platelet surface, P-selectin is rapidly cleaved off the surface by a yet unidentified mechanism and appears in the circulation as the soluble form (Dunlop et al., 1992). This cleavage occurs within 2 h of platelet surface expression and the platelets, from which the P-selectin has been cleaved, continue to circulate and function normally (Michelson et al., 1996). Interestingly, it appears that P-selectin expressed on the plasma membrane of activated endothelial cells may be preferentially recycled back into the cell whereas the soluble form of P-selectin present in the circulation may be principally derived from the platelet source (Subramaniam et al., 1993). Interestingly, the enzyme responsible for the cleavage of P-selectin from the platelet surface has not been identified.

Surface expression of platelet P-selectin and appearance of the soluble form in the circulation has been utilized as a potential marker for platelet activation in acute coronary syndromes (unstable angina and non-Q-wave myocardial infarction) that are known to be platelet-mediated events. Soluble P-selectin levels have been measured in patients with acute myocardial infarction and start to rise 1 to 2 h following the onset of chest pain and may remain elevated for prolonged periods of time (Sakurai et al., 1997; Shimomura et al., 1998; Gurbel et al., 2001; Serebruany et al., 2001). Membrane P-selectin also increases rapidly in patients suffering from acute coronary syndromes (Becker et al., 1994; Gurbel et al., 1998; Serebruany et al., 1998; Ault et al., 1999), and retrospective studies utilizing flow cytometry to assess platelet membrane P-selectin have suggested that this marker may be useful for its “negative” predictive ability in “ruling-out” acute coronary syndromes when used in combination with other markers of myocardial necrosis such as TnI and creatinine kinase MB isozymes (Hollander et al., 1999). In fact, membrane and soluble P-selectin may serve as “early” markers of platelet activation and thrombosis-induced impending acute myocardial infarction as cardiac enzymes indicative of ischemia and myocyte degradation and necrosis due to thrombus formation, such as TnI and creatinine kinase MB isozymes, appear much later in the circulation and may take up to 24 h to peak following the onset of chest pain (Fig. 2). Furthermore, soluble P-selectin has also been recently proposed to act as a circulating pro-coagulant protein and may serve dual roles in promoting platelet and coagulation activation (Andre et al., 2000). Pro-coagulant, soluble P-selectin microparticle activity could be reversed in genetically altered mice by PSGL-Ig, a P-selectin inhibitor.

**Interaction of TF and P-selectin in Promotion of Fibrin Deposition**

A role for platelet-leukocyte interaction in promotion of fibrin deposition was first demonstrated by Palabrica et al. (1992). The accumulation of leukocytes, platelets, and fibrin was measured in thrombogenic Dacron grafts implanted in
arteriovenous shunts in baboons. Although the P-selectin-blocking monoclonal antibody utilized (GA6) did not prevent thrombus formation in the graft, the occlusive thrombus that formed was markedly different from that formed in the control, nontreated animals. Although the extent of platelet deposition was equal between the two groups, thrombi that formed in the GA6-treated animals contained approximately one-half the number of indium-111-labeled leukocytes and one-third the quantity of fibrin (as assessed by anti-fibrin antibodies) that were present in thrombi from the control group. This interesting and unexpected observation was followed by work demonstrating enhanced pharmacological arterial thrombolysis in the presence of either GA6 (Toombs et al., 1995) or IgG1-Fc modified recombinant PSGL-1 (rPSGL-1) (Kumar et al., 1999) in experimental studies. Furthermore, endogenous fibrinolysis was enhanced on the venous side of the circulation against preformed thrombi in nonhuman primates by the P-selectin directed antibodies GA6 (Downing et al., 1997) and CY1748 (Shebuski et al., 1997) or with IgG1-Fc-modified rPSGL-1 (Myers et al., 2001). Clearly, enhancement of pharmacological thrombolysis or endogenous fibrinolysis in all of the aforementioned studies, in which animals were pretreated with agents directed against P-selectin, was primarily due to the decreased presence of fibrin, which allowed plasmin to perform more efficiently and, thus, lysis to occur more readily. Additionally, P-selectin antagonism conferred a significant degree of anti-inflammatory action in the venous blood vessel wall as less infiltrates were noted in segments containing preformed thrombus.

The anti-inflammatory actions of P-selectin antagonism were confirmed in P-selectin knock-out mice in which not only were less inflammatory cells present in the developing neointima following carotid artery ligation, but the overall neointimal response was decreased by 76% compared with control mice (Kumar et al., 1997). Additionally, pharmacological attenuation of P-selectin binding with IgG1-Fc-modified rPSGL-1 also reduced the restenotic response in swine subjected to balloon angioplasty-induced injury (Bienvenu et al., 2001). Recently, an in vitro study has confirmed that rPSGL-1 inhibits circulating activated platelet binding to neutrophils following blood perfusion over damaged arterial vasculature (Theoret et al., 2001). Besides being expressed on circulating leukocytes, PSGL-1 has also recently been demonstrated to be expressed on platelets and can mediate platelet-endothelial interactions as well (Frenette et al., 2000). Thus, interruption of the interaction of P-selectin on platelets or endothelial cells with its natural ligand PSGL-1 on circulating leukocytes has important pharmacological ramifications to not only suppress fibrin deposition in developing thrombi but also to attenuate smooth muscle cell proliferation at the sites of vascular injury.

The mechanism of fibrin deposition by the interaction of platelets and leukocytes has been investigated. Specifically, it has been suggested that monocytes that express TF on the cell surface and also possess the ligand for P-selectin, PSGL-1, are involved in decreased fibrin deposition if they are inhibited from binding to the site of thrombus initiation and vascular damage by therapies directed against P-selectin (monoclonal antibodies or rPSGL-1). Furie and Furie (1997) have suggested that activated platelets trapped in the developing thrombus provide a nidus where leukocytes (monocytes) accumulate via a P-selectin dependent mechanism. Additional results suggest that P-selectin can directly increase TF expression on monocytes (Celi et al., 1994).

### Pharmacological Approaches to Antagonism of P-selectin

Antagonism of P-selectin may be accomplished by either monoclonal antibodies directed against P-selectin or with rPSGL-1, which competes with the natural ligand PSGL-1 present on circulating leukocytes for binding (Yang et al., 1998). Recently, rPSGL-1 has been engineered to possess a longer half-life in the circulation and is currently in phase I/II clinical trials (Khor et al., 2000). This dimeric molecule consists of the first 47 amino acids of native PSGL-1 fused to human IgG1-Fc, which has been mutated to reduce complement activation and Fc receptor binding.

### Summary

The etiology of acute coronary syndromes have more recently come to be appreciated as a combination of factors involving inflammation and thrombosis. Acute plaque rupture leads to elaboration of cytokines and chemokines that are important in the induction of cell adhesion molecules, which in turn allow cells to anchor at sites of vascular damage or cellular activation to initiate the repair process. Inflammatory cells interact with other cellular components of the blood such as platelets, and a synergy results in which excessive fibrin deposition may occur with resulting formation of thrombus. It is apparent that both P-selectin and TF are important in venous and arterial thrombogenesis. However, there may be a relative difference in the extent of involvement of these molecules. One may speculate that platelet-derived P-selectin may play a greater role in arterial thrombosis due to the increased presence of platelets known to exist histologically in arterial thrombi; however, the role of TF-induced fibrin deposition in stabilizing platelet-rich thrombi cannot be overlooked. Venous thrombosis, on the other hand, may involve less platelet recruitment; however, the role of endothelial P-selectin may be enhanced as leukocytes expressing PSGL-1 hone to sites of vascular damage and initiate local inflammation and eventual TF-induced thrombus/clot generation. The role of both P-selectin and TF in developing thrombi on either side of the circulation suggests that pharmacological approaches may be designed to target not only soluble coagulation factors and platelet aggregation targets but also pro-inflammatory mediators such as cytokines, chemokines, adhesion molecules, and soluble (microparticle) and cell-anchored TF. Recognition of the importance that inflammation plays in the etiology of thrombosis and thrombogenesis will undoubtedly lead to the development of additional safe and effective therapeutic agents, which will be available to a broader context of patients with cardiovascular disease.

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