Platelet regulation of thrombin generation in cardiovascular disease

Beth A. Bouchard*, Paula B. Tracy*§

*Department of Biochemistry, §Department of Medicine, University of Vermont, College of Medicine, Burlington, VT, USA

Key words:
Cardiovascular disease; Platelets; Thrombin.

Platelets are intimately involved in the events leading to cardiac ischemia through their release of bioactive substances, aggregation, and support of procoagulant reactions at sites of atherosclerotic plaque formation and rupture. This review article will focus on what is currently known about the regulation of thrombin generation on the surface of activated platelets, and how it relates to thrombus formation.

(Ital Heart J 2001; 2 (11): 819-823)

This work was supported by grant P01 HL46703 (Project 4) (P.B.T.), and the Department of Biochemistry, University of Vermont, College of Medicine.

Address:

Paula B. Tracy, PhD

Department of Biochemistry
University of Vermont
College of Medicine
Given C409
89 Beaumont Avenue
Burlington, VT 05405-0068
USA
E-mail:
ptracy@salus.uvm.edu

Cardiac ischemic events, such as myocardial infarction, are characterized by thrombosis associated with atherosclerotic plaque rupture[1-5]. Exposure of subendothelial matrix proteins subsequent to plaque rupture initiates adhesion of, and consequently, activation of platelets. Platelet activation results in a myriad of events involved in atherosclerotic lesion formation[6]. Activated platelets release the contents of their α- and dense-granules, which contain cytokines and growth factors that contribute to the migration and proliferation of smooth muscle cells and monocytes[7]. Arachidonic acid is formed, which can be transformed into prostaglandins such as thromboxane A_2, a potent vasoconstricting and platelet-aggregating substance, or into leukotrienes, which can amplify the inflammatory response involved in atherosclerosis[6]. Activated platelets aggregate via the crosslinking of platelets through fibrinogen interactions with activated $\alpha IIb\beta_3$ that is expressed on the platelet surface. This important event results in recruitment of additional platelets to the growing, platelet-rich thrombus[8]. Activated platelets also express binding sites for the assembly of procoagulant enzyme complexes resulting in thrombin generation.

Thrombin is an important bioregulatory molecule, which, beyond its role in blood coagulation, promotes numerous cellular effects including chemotaxis, proliferation, cytokine release, and extracellular matrix turnover[9]. All of these cellular effects of thrombin have been implicated in inflammatory and fibroproliferative disorders such as atherosclerosis[9]. Typically, thrombin formation requires a series of zymogen to protease conversions catalyzed by select enzymatic complexes assembled on the surface of activated platelets or perhaps damaged endothelium, as well as thrombogenic surfaces exposed subsequent to atherosclerotic plaque rupture. The plasma serine protease factor VIIa binds to tissue factor expressed on subendothelial cells such as pericytes[10], fibroblasts[11], and smooth muscle cells[11]; or smooth muscle cells and macrophages within the atherosclerotic plaque[6,11,12]. The tissue factor/factor VIIa complex catalyzes the formation of factors IXa and Xa[13]. Factors IXa and Xa interact with their required cofactors resulting in thrombin formation.

Role of the activated platelet in regulation of thrombin generation

Activated platelets promote the assembly and function of the procoagulant enzyme complexes intrinsic tenase and prothrombinase to effect the generation of factor Xa and thrombin, respectively[14]. To function effectively, both complexes require the Ca^{2+}-dependent formation of a stoichiometric (1:1), platelet-bound complex of a cofactor protein (factor VIIIa or Va) and serine protease (factor IXa or Xa, respectively)[15]. Multiple studies suggest that specific platelet receptors for factors Va and Xa are expressed on the surface of activated platelets. Although factor Va is required, it is not sufficient to mediate binding of factor Xa. Instead, factors Va and Xa

bind to discrete binding sites on activated platelets whose expression is independently regulated as a function of agonist concentration. Assembly of functional prothrombinase is regulated further by anionic phospholipid made available subsequent to platelet activation[16-18]. Platelet activation also results in the expression of a prothrombin-binding site proposed by Walsh and colleagues[19]. Prothrombin's occupancy of this site has no effect on binding of factors Va and Xa. Thus, platelets modulate the assembly of prothrombinase at their surface through activation-dependent events that independently regulate both factors Va and Xa binding[20].

Several other studies support the notion that factors Va and Xa interact with specific receptors on the surface of activated platelets. For example, factor V does not bind to activated platelets until it is activated to the functional cofactor[21]. Factor X, factor IXa, and prothrombin do not compete with factor Xa for platelet binding[22]. Furthermore, various mutations in factor X or factor Xa have different effects on the ability of the protease to interact with factor Va on platelets as compared to factor Va on phospholipid vesicles[23,24]. We demonstrated that effector cell protease receptor-1 (EPR-1), a factor Xa receptor shown to be expressed by select leukocyte subpopulations[25,26], and vascular endothelial cells and smooth muscle cells[27], is important in mediating factor Xa binding to platelets using specific anti-EPR-1 MoAbs[20]. The presence of EPR-1 on collagen-adherent platelets, and fibrinogen-adherent platelets activated by thrombin was recently confirmed using a monoclonal antibody against a peptide[28] that corresponds to the factor Xa binding site on EPR-1 expressed by human umbilical vein endothelial cells[29]. However, this antibody, as well as the synthetic EPR-1 peptide it was directed against, had no effect on thrombin generation on the surface of thrombin-activated, fibrinogen-adherent platelets. We have made similar observations with this peptide using thrombin-activated, non-adherent platelets[30]. However, it is important to note that in contrast to other cell types, platelet activation is required for EPR-1 expression. Furthermore, factor Xa binding to activated platelets is absolutely dependent upon platelet-bound factor Va. Clearly, EPR-1 or an EPR-1-like molecule expressed by platelets is more complex than that expressed by human umbilical vein endothelial cells. Thus, based on all the data described to date, and the ability of antibodies that inhibit factor Xa binding to leukocytes to inhibit platelet prothrombinase, we hypothesize that the receptor for factor Xa assembly into prothrombinase at the activated platelet surface consists *minimally* of EPR-1 or an EPR-1-like molecule, factor Va and anionic phospholipids[20,30].

In contrast to what has been observed for factors Va and Xa, unactivated platelets appear to support the binding of prothrombin, via its RGD sequence, to $\alpha IIb\beta_3$ – an interaction that appears to accelerate prothrombinase-catalyzed thrombin generation[31]. The authors hypothesized that prothrombin bound to $\alpha IIb\beta_3$ on circulating platelets may play an important physiological role in the early events of thrombin generation. However, this point remains controversial as earlier studies were not able to demonstrate prothrombin binding to resting platelets[32,33].

Platelet activation may not be sufficient to define coagulation complex assembly and function. Discrete subpopulations of platelets have been identified, which differentially regulate the expression of prothrombinase[34]. When platelets are thrombin-activated to a level consistent with expression of maximal prothrombinase activity and saturating concentrations of factors Va and Xa are added, factor Va and factor Xa colocalize to a subset of activated platelets and, as expected, no factor Xa binding is observed in the absence of factor Va binding. These results extend those of Alberio et al.[35] who demonstrated that a fraction of platelets express very high levels of surface-bound, platelet-derived factor Va on simultaneous activation with thrombin and convulxin. These combined data clearly indicate that a population of platelets exists that are fully activated and express P-selectin at their surface, but cannot assemble a functional prothrombinase complex. This concept is supported by *post-mortem* examination of individuals subsequent to myocardial infarction[4]. Examination of coronary arteries from these individuals revealed the presence of two kinds of thrombi: those that were platelet-rich and lacked fibrin, and others that contained platelets and fibrin. These combined observations suggest that platelets may express different procoagulant phenotypes and, therefore, are under distinct hematopoietic regulation. Alternatively, the presence of fibrin-poor and fibrin-rich thrombi in the same individual may be a result of a lack of thrombin formation at some sites of thrombus formation because of: deficient tissue factor expression; inadequate factor Xa formation via the intrinsic tenase; or insufficient activation of platelets to effect procoagulant enzyme complex assembly and function.

Role of platelet-derived factor Va

The thrombin-activated, human platelet surface not only plays an important role in promoting and regulating the procoagulant response, but also in sustaining it by modulating the effects of activated protein C[36,37] and plasmin[38], potent proteolytic inactivators of factor Va. On the activated platelet surface, both platelet- and plasma-derived factor Va are inactivated by activated protein C at substantially slower rates than when bound to phospholipid vesicles[36,37]. Plasmin, on the other hand, effects a dramatic increase in the activity expressed by the thrombin-activated platelet-derived cofactor bound to activated platelets[38]. The increased cofactor activity can be sustained for several hours, fol-

lowed by its gradual decline. The plasmin-induced increase in cofactor activity is unique to both activated platelet membranes and the platelet-derived cofactor.

Like plasmin, platelet-derived factor Va also modulates the effects of activated protein C[36,37]. In contrast to observations made using plasma-derived factor Va, complete inactivation of the platelet-derived cofactor is never achieved, such that substantial cofactor activity remains at the platelet surface, suggesting that the plasma and platelet-derived cofactor pools represent different substrates for activated protein C[36,37].

The studies described above illustrate that platelet prothrombinase assembly and function is regulated by their release of factor V(a). The platelet-derived cofactor is stored in the α-granule in complex with multimerin[39] as a partially cleaved molecule[40]. In marked contrast to plasma-derived factor V, platelet-derived factor V expresses significant cofactor activity subsequent to its release from the platelet[40], which parallels expression of a putative factor Va receptor that is competent to bind either platelet- or plasma-derived factor Va[20]. Thus, at an injury site, the release of platelet-derived factor Va initiates prothrombinase assembly and function without the need for thrombin-catalyzed activation of the plasma-derived cofactor. Factor Xa generated at a site other than the platelet surface does not appear to contribute significantly to thrombin generation[41], thus, effective channeling of factor Xa, produced via intrinsic tenase, to platelet-bound factor Va to form prothrombinase, would result in thrombin generation at the activated platelet surface. Clinical observations support the notion that the platelet-derived cofactor plays a more important role in hemostasis[42,43].

Consequently, studies have been initiated to determine what physical differences make the two factor Va pools function differently in hemostasis. Purified plasma factor V can be readily phosphorylated at Ser692 by casein kinase 2[44] whereas the purified, platelet-derived molecule is resistant to phosphorylation (Silveira and Tracy, unpublished observations). Again, in contrast to plasma-derived factor V, the platelet-derived cofactor shows little intrinsic phosphorylation at that site. We hypothesize that platelet-derived factor V is *O*-linked glycosylated on Ser692 making it resistant to phosphorylation. How, and if, these physical differences contribute to the distinct functional phenotypes of the two molecules requires rigorous assessment.

The notion that plasma- and platelet-derived factor V are physically distinct is consistent with their purported sites of synthesis. Plasma-derived factor V is known to be synthesized by the liver[45,46], while early studies suggested that platelet-derived factor V was synthesized by megakaryocytes[47-49]. However, recent studies using two patients heterozygous for factor V Leiden, who underwent allogeneic transplantation from homozygous wild-type factor V donors (bone marrow or liver), demonstrated that the secretable platelet-derived factor V phenotype mirrored the plasma-derived factor V phenotype, and was independent of the megakaryocyte factor V gene[50]. These observations were confirmed by more recent analysis of the platelet-derived factor V from a patient homozygous for factor V Leiden who received a wild-type liver[51]. These combined studies demonstrate unequivocally that the *majority* of platelet-derived factor V is endocytosed by megakaryocytes from plasma. Thus, we hypothesize that the functional and physical differences in plasma- and platelet-derived factor V are a result of "retailoring" of the posttranslational modifications of the platelet-derived cofactor subsequent to its endocytosis from the plasma by megakaryocytes.

Implications for future therapy

The ability of anti-platelet agents, which inhibit platelet activation or aggregation, to prevent cardiac ischemic events such as myocardial infarction clearly demonstrates the importance of platelets in thrombus formation[12]. However, coronary thrombosis is a complex process involving the vessel wall, platelets, and the coagulant response culminating in a substantial increase in local concentrations of thrombin and formation of a platelet/fibrin plug at the injured site. These combined studies described above demonstrate that platelets are active participants in effecting thrombin generation. Platelets appear to perform these functions in part through the regulated "receptor"-mediated assembly of proteolytic activities at their membrane surface subsequent to platelet activation. Alternatively, proteins may be released or constitutively expressed that modulate platelet responsiveness and function. Thrombin, once formed, not only catalyzes fibrin formation, but also accelerates additional platelet activation therefore supporting a positive feedback mechanism. Furthermore, platelets sustain thrombin formation by protecting the proteases and cofactors from inactivation/inhibition by various constitutive plasma inhibitors or formed activators. Thus, regulation of platelet activity and thrombin generation are considered important goals in clinical therapy for cardiovascular diseases.

References

1. Davies MJ, Thomas AC. Plaque fissuring - the cause of acute myocardial infarction, sudden ischaemic death, and crescendo angina. Br Heart J 1985; 53: 363-73.
2. Badimon L, Chesebro JH, Badimon JJ. Thrombus formation on ruptured atherosclerotic plaques and rethrombosis on evolving thrombi. Circulation 1992; 86: III74-III85.
3. Mizuno K, Satomura K, Miyamoto A, et al. Angioscopic evaluation of coronary-artery thrombi in acute coronary syndromes. N Engl J Med 1992; 326: 287-91.
4. Farb A, Tang AL, Burke AP, Sessums L, Liang Y, Virmani

R. Sudden coronary death. Frequency of active coronary lesions, inactive coronary lesions, and myocardial infarction. Circulation 1995; 92: 1701-9.
5. Arbustini E, Dal Bello B, Morbini P, et al. Plaque erosion is a major substrate for coronary thrombosis in acute myocardial infarction. Heart 1999; 82: 269-72.
6. Ross R. Atherosclerosis - an inflammatory disease. N Engl J Med 1999; 340: 115-26.
7. Bombeli T, Schwartz BR, Harlan JM. Adhesion of activated platelets to endothelial cells: evidence for a GPIIbIIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), alphavbeta3 integrin, and GPIbalpha. J Exp Med 1998; 187: 329-39.
8. Plow EF, Shattil SJ. Integrin $\alpha_{IIb}\beta_3$ and platelet aggregation. In: Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN, eds. Hemostasis and thrombosis: basic principles and clinical practice. Philadelphia, PA: Lippincott Williams & Wilkins, 2001: 479-91.
9. Goldsack NR, Chambers RC, Dabbagh K, Laurent GJ. Thrombin. Int J Biochem Cell Biol 1998; 30: 641-6.
10. Bouchard BA, Shatos MA, Tracy PB. Human brain pericytes differentially regulate expression of procoagulant enzyme complexes comprising the extrinsic pathway of blood coagulation. Arterioscler Thromb Vasc Biol 1997; 17: 1-9.
11. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proc Natl Acad Sci USA 1989; 86: 2839-43.
12. Rauch U, Osende JI, Fuster V, Badimon JJ, Fayad Z, Chesebro JH. Thrombus formation on atherosclerotic plaques: pathogenesis and clinical consequences. Ann Intern Med 2001; 134: 224-38.
13. Morrissey JH. Tissue factor and factor VII initiation of coagulation. In: Colman RW, Hirsh J, Marder VJ, Clowes AW, George JN, eds. Hemostasis and thrombosis: basic principles and clinical practice. Philadelphia, PA: Lippincott Williams & Wilkins, 2001: 89-101.
14. Tracy PB, Nesheim ME, Mann KG. Platelet factor Xa receptor. Methods Enzymol 1992; 215: 329-60.
15. Mann KG, Nesheim ME, Church WR, Haley P, Krishnaswamy S. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. Blood 1990; 76: 1-16.
16. Comfurius P, Senden JM, Tilly RH, Schroit AJ, Bevers EM, Zwaal RF. Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase. Biochim Biophys Acta 1990; 1026: 153-60.
17. Thiagarajan P, Tait JF. Binding of annexin V/placental anticoagulant protein I to platelets. Evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. J Biol Chem 1990; 265: 17420-3.
18. Thiagarajan P, Tait JF. Collagen-induced exposure of anionic phospholipid in platelets and platelet-derived microparticles. J Biol Chem 1991; 266: 24302-7.
19. Scandura JM, Ahmad SS, Walsh PN. A binding site expressed on the surface of activated human platelets is shared by factor X and prothrombin. Biochemistry 1996; 35: 8890-902.
20. Bouchard BA, Catcher CS, Thrash BR, Adida C, Tracy PB. Effector cell protease receptor-1, a platelet activation-dependent membrane protein, regulates prothrombinase-catalyzed thrombin generation. J Biol Chem 1997; 272: 9244-51.
21. Tracy PB, Eide LL, Mann KG. Human prothrombinase complex assembly and function on isolated peripheral blood cell populations. J Biol Chem 1985; 260: 2119-24.
22. Miletich JP, Jackson CM, Majerus PW. Properties of the factor Xa binding site on human platelets. J Biol Chem 1978; 253: 6908-16.
23. Larson PJ, Camire RM, Wong D, et al. Structure/function analyses of recombinant variants of human factor Xa: factor Xa incorporation into prothrombinase on the thrombin-activated platelet surface is not mimicked by synthetic phospholipid vesicles. Biochemistry 1998; 37: 5029-38.
24. Rudolph AE, Porche-Sorbet R, Miletich JP. Substitution of asparagine for arginine 347 of recombinant factor Xa markedly reduces factor Va binding. Biochemistry 2000; 39: 2861-7.
25. Altieri DC, Edgington TS. Identification of effector cell protease receptor-1. A leukocyte-distributed receptor for the serine protease factor Xa. J Immunol 1990; 145: 246-53.
26. Altieri DC, Stamnes SJ. Protease-dependent T cell activation: ligation of effector cell protease receptor-1 (EPR-1) stimulates lymphocyte proliferation. Cell Immunol 1994; 155: 372-83.
27. Nicholson AC, Nachman RL, Altieri DC, et al. Effector cell protease receptor-1 is a vascular receptor for coagulation factor Xa. J Biol Chem 1996; 271: 28407-13.
28. Briedé JJ, Heemskerk JWM, van't Veer C, Hemker HC, Lindhout T. Contribution of platelet-derived factor Va to thrombin generation on immobilized collagen- and fibrinogen-adherent platelets. Thromb Haemost 2001; 85: 509-13.
29. Ambrosini G, Altieri DC. Molecular dissection of effector cell protease receptor-1 recognition of factor Xa. Assignment of critical residues involved in antibody reactivity and ligand binding. J Biol Chem 1996; 271: 1243-8.
30. Bouchard BA, Silveira JR, Tracy PB. On the role of EPR-1 or an EPR-1-like molecule in regulating factor Xa incorporation into platelet prothrombinase. Thromb Haemost 2001; 86: 1133-4.
31. Byzova TV, Plow EF. Networking in the hemostatic system. Integrin alphaIIbbeta3 binds prothrombin and influences its activation. J Biol Chem 1997; 272: 27183-8.
32. Scandura JM, Walsh PN. Factor X bound to the surface of activated human platelets is preferentially activated by platelet-bound factor IXa. Biochemistry 1996; 35: 8903-13.
33. Slonosky D, Nesheim ME. Prothrombin binding to human activated platelets. (abstr) FASEB J 1988; 2: 5038.
34. Feng P, Tracy PB. Not all platelets are equal procoagulants? (abstr) Blood 1998; 92: 1441.
35. Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. Blood 2000; 95: 1694-702.
36. Camire RM, Kalafatis M, Cushman M, Tracy RP, Mann KG, Tracy PB. The mechanism of inactivation of human platelet factor Va from normal and activated protein C-resistant individuals. J Biol Chem 1995; 270: 20794-800.
37. Camire RM, Kalafatis M, Simioni P, Girolami A, Tracy PB. Platelet-derived factor Va/Va Leiden cofactor activities are sustained on the surface of activated platelets despite the presence of activated protein C. Blood 1998; 91: 2818-29.
38. Conlon SJ, Camire RM, Kalafatis M, Tracy PB. Cleavage of platelet-derived factor Va by plasmin results in increased and sustained cofactor activity on the thrombin-activated platelet surface. (abstr) Thromb Haemost 1997; 77: 2507.
39. Hayward CP, Furmaniak-Kazmierczak E, Cieutat AM, et al. Factor V is complexed with multimerin in resting platelet lysates and colocalizes with multimerin in platelet alpha-granules. J Biol Chem 1995; 270: 19217-24.
40. Monkovic DD, Tracy PB. Functional characterization of human platelet-released factor V and its activation by factor Xa and thrombin. J Biol Chem 1990; 265: 17132-40.
41. Hoffman M, Monroe DM, Oliver JA, Roberts HR. Factors

IXa and Xa play distinct roles in tissue factor-dependent initiation of coagulation. Blood 1995; 86: 1794-801.
42. Tracy PB, Giles AR, Mann KG, Eide LL, Hoogendoorn H, Rivard GE. Factor V (Quebec): a bleeding diathesis associated with a qualitative platelet factor V deficiency. J Clin Invest 1984; 74: 1221-8.
43. Nesheim ME, Nichols WL, Cole TL, et al. Isolation and study of an acquired inhibitor of human coagulation factor V. J Clin Invest 1986; 77: 405-15.
44. Kalafatis M. Identification and partial characterization of factor Va heavy chain kinase from human platelets. J Biol Chem 1998; 273: 8459-66.
45. Wilson DB, Salem HH, Mruk JS, Maruyama I, Majerus PW. Biosynthesis of coagulation factor V by a human hepatocellular carcinoma cell line. J Clin Invest 1984; 73: 654-8.
46. Mazzorana M, Baffet G, Kneip B, Launois B, Guguen-Guillouzo C. Expression of coagulation factor V gene by normal adult human hepatocytes in primary culture. Br J Haematol 1991; 78: 229-35.
47. Chiu HC, Schick PK, Colman RW. Biosynthesis of factor V in isolated guinea pig megakaryocytes. J Clin Invest 1985; 75: 339-46.
48. Gewirtz AM, Keefer M, Doshi K, Annamalai AE, Chiu HC, Colman RW. Biology of human megakaryocyte factor V. Blood 1986; 67: 1639-48.
49. Gewirtz AM, Shapiro C, Shen YM, Boyd R, Colman RW. Cellular and molecular regulation of factor V expression in human megakaryocytes. J Cell Physiol 1992; 153: 277-87.
50. Camire RM, Pollak ES, Kaushansky K, Tracy PB. Secretable human platelet-derived factor V originates from the plasma pool. Blood 1998; 92: 3035-41.
51. Simioni P, Silveira JR, Kalafatis M, et al. An in vivo demonstration that megakaryocyte endocytosis of plasma factor V, rather than endogenous synthesis, defines the platelet-derived factor V pool. (abstr) Thromb Haemost 2001; 86 (Suppl): 541.