Introduction

Blood coagulation is part of the host defense system that maintains the vascular integrity of the high pressure circulatory system after blood vessel injury. Multiple factors and events must occur in a highly regulated fashion in order for a blood clot to form. Upon injury to the blood vessel wall, platelets adhere and aggregate to the site of injury. These platelets then become activated, undergo morphologic change, and release the contents of alpha granules (e.g. von Willebrand factor, factor V, fibrinogen) and dense granules (e.g. calcium, ADP). These released components lead to further platelet activation. Also, according to the classical model of coagulation, upon blood vessel injury, subendothelial tissue factor is exposed (1). This tissue factor then interacts with activated factor VII to initiate a series of coordinated enzymatic reactions leading to the formation of thrombin. Thrombin then converts fibrinogen to fibrin to stabilize the initial platelet plug and to form a fibrin clot. Activation of platelets also leads to the exposure of P-selectin on the surface of platelets. Accumulating lines of evidence now invoke a role for P-selectin and its major ligand, P-selectin glycoprotein ligand-1 (PSGL-1) in the accumulation of tissue factor and subsequent generation of fibrin within the platelet thrombus.

Structure and function of P-selectin

P-selectin is a 140,000 molecular weight transmembrane glycoprotein, which was identified using monoclonal antibodies specific for thrombin-activated platelets (2, 3). It is a member of the selectin family of adhesion molecules, a family which also includes L-selectin and E-selectin (4-6). The selectins all share a common domain structure: a lectin domain, an EGF domain, a variable number of consensus repeats, a transmembrane domain, and a cytoplasmic tail. The common domain structure of these proteins is reflected in their function as adhesion molecules supporting interactions of platelets or endothelial cells with leukocytes during thrombosis and inflammation. P-selectin is expressed in platelets, stored on the membrane of alpha granules. Upon cell activation, it is translocated to the surface of...
platelets (7, 8). P-selectin is also stored on the Weibel-Palade bodies of endothelial cells (9, 10). Upon stimulation, these cells degranulate with the rapid translocation of P-selectin to the plasma membrane. P-selectin expression in endothelial cells is also stimulated de novo when induced by inflammatory cytokines such as tumor necrosis factor-α (TNF-α). P-selectin mediates the adhesion of leukocytes to stimulated endothelial cells and to activated platelets (11). This interaction can be inhibited using EDTA, blocking antibodies directed against P-selectin, or excess soluble P-selectin. Further insight into P-selectin’s function was obtained using a transgenic mouse deficient in P-selectin. P-selectin null mice have reduced leukocyte rolling on post-capillary venules, an increased number of circulating neutrophils, and delayed recruitment of leukocytes to sites of inflammation in a variety of mouse models of inflammation (12-14). These in vivo studies showed that P-selectin is important for the initial rolling interactions between leukocytes and the blood vessel wall and that this initial rolling is required for the subsequent recruitment of leukocytes to sites of inflammation or infection.

**Structure and function of PSGL-1**

P-selectin glycoprotein ligand-1 (PSGL-1) is an adhesion molecule originally identified on HL60 cells (15) and cloned from a human HL-60 cDNA library by virtue of its ability bind to P-selectin when coexpressed in COS cells with an α1,3/1,4 fucosyltransferase (16). The mouse homolog was subsequently cloned based upon sequence similarity (17). The α1,3/1,4 fucosyltransferase is necessary for the correct glycosylation of the PSGL-1 molecule. Post-translational modification of the PSGL-1 molecule to form tyrosine sulfates and sialylated, fucosylated, O-glycans is necessary for optimal binding to P-selectin (18-20). PSGL-1 is primarily expressed as a homodimer with a molecular weight of 210,000, on myeloid cells and T cells (21-22). It has been shown to mediate interactions between myeloid cells and endothelial cells as well as between myeloid cells and platelets. Antibodies which block the function of PSGL-1, also block the interactions of myeloid cells with immobilized P-selectin under flow conditions (21). In vivo studies in a PSGL-1 deficient mouse have shown that PSGL-1 functions as the major P-selectin ligand and is important for many of the same functions as P-selectin. PSGL-1 is required for normal leukocyte rolling on post-capillary venules and is required for the normal recruitment of T cells and myeloid cells to sites of inflammation (23-25).

**A P-selectin dependent pathway in blood coagulation**

The first suggestion that P-selectin was important in thrombosis came from in vivo studies in the baboon. In some of these initial experiments, thrombosis was caused by inflation of a balloon catheter in the baboon femoral vein, and P-selectin expression was visualized in thrombi using radiolabeled anti-P-selectin antibodies (26). The kinetics of thrombus formation was then studied in a Dacron graft placed in an external arteriovenous shunt in a baboon. Radiolabeled anti-P-selectin antibodies rapidly accumulated in the developing Dacron graft thrombus, and antibodies that blocked P-selectin function had no effect on thrombus size when infused into baboons. However, when compared to nonblocking antibodies, blocking anti-P-selectin antibodies inhibited the accumulation of fibrin in the developing thrombi by 50 to 70% (27). This was the first demonstration that P-selectin was required for the normal generation of fibrin in thrombi and that P-selectin had a role in blood coagulation in vivo.

The initial experiments using anti-P-selectin antibodies in the ex vivo model of thrombosis in a baboon arteriovenous shunt showed that P-selectin is important for fibrin accumulation. However, the mechanism by which P-selectin contributes to fibrin generation was not understood. In the classical model of coagulation, fibrin generation requires tissue factor exposure to flowing blood in order to initiate a series of enzymatic reactions leading to fibrin generation. Historically, this tissue factor was thought to originate from subendothelial cells (28). However, in vitro, endothelial cells and monocytes can be induced to express tissue factor (29, 30). For example, incubation of monocytes with platelets induces tissue factor expression on monocytes (31, 32). Celi and colleagues then showed that soluble P-selectin as well as CHO cells expressing P-selectin can induce monocytes to express tissue factor (33). However, this induction of tissue factor expression requires transcriptional activation of the tissue factor gene, and the protein is only detected 4 hours after incubation of the monocytes with P-selectin or platelets. Thus, the recruitment of monocytes to the developing thrombus may lead to activation of monocytes, followed by delayed tissue factor expression and fibrin generation. In the baboon model of thrombosis, leukocytes were recruited to the thrombus, and anti-P-selectin antibodies block this recruitment. Therefore, although the recruitment of monocytes to thrombi result in increased fibrin generation, this process cannot explain the immediate P-selectin-dependent fibrin generation seen in the baboon model of thrombosis, measurable within the first 20 minutes of thrombosis (27).

**Blood-borne tissue factor**

Tissue factor has been measured in peripheral blood at concentrations of 100-150 pg/ml (34-36). Elevated levels of circulating tissue factor have also been noted in certain pro-thrombotic states, such as after a myocardial infarction and in diabetes mellitus (34, 37). Giesen and colleagues showed the importance of blood borne tissue factor by perfusing human blood over
collagen-coated slides or over pig arterial media (38). Thrombi consisting of activated platelets, tissue factor, and fibrin formed on the collagen-coated surfaces. Adding anti-tissue factor antibodies to the perfused blood reduced the number of thrombi formed, suggesting that circulating tissue factor accumulated in the developing thrombi and led to fibrin generation in the absence of a vessel wall. Immunoelectron microscopy of these thrombi revealed the presence of tissue factor-positive membrane vesicles. Because leukocytes expressing tissue factor were observed in these perfusion chamber experiments, the authors speculated that the circulating tissue factor might be derived from activated leukocytes. Rauch and colleagues showed that an activated monocytoid cell line can transfer human tissue factor to activated platelets via microparticles (39). This transfer can be inhibited by anti-CD15 antibodies and anti-tissue factor antibodies (39). The above studies show that multiple sources of tissue factor exist and can contribute to thrombus formation. Consideration of these varied sources of tissue factor is important in determining the mechanism by which P-selectin could affect fibrin generation in thrombi.

The role of P-selectin and PSGL-1 in thrombosis

The use of mouse models of thrombosis has allowed further investigation of the role of P-selectin and PSGL-1 in fibrin generation. Several different mouse models have been used to study P-selectin and PSGL-1 in thrombosis: 1) the local Schwartzman reaction in which lipopolysaccharide and TNFα are injected into a defined site to induce hemorrhagic inflammation and fibrosis (40), 2) a venous thrombosis model in which thrombosis and venous vessel wall inflammation is induced by causing venous stasis in the inferior vena cava (41), 3) a thrombosis model of the mouse cremaster muscle microcirculation in which thrombosis can be observed in real-time by intravital microscopy (42). In the mouse microvasculature, thrombosis has been induced by exposure of the microcirculation to ferric chloride (43) or by a more localized injury induced by laser (44).

P-selectin and PSGL-1 function in these models have been tested by the use of antibodies which block P-selectin function, chimeric proteins (such as a P-selectin-immunoglobulin or a PSGL-1-immunoglobulin chimeric protein) which also interfere with P-selectin or PSGL-1 function, and by the use of knock-out mice deficient in P-selectin or PSGL-1.

Sullivan and colleagues used a mouse model of venous thrombosis to study the importance of the selectins in fibrin generation (41). In their model, both E-selectin and P-selectin play a role in the accumulation of inflammatory cells and fibrin generation after venous thrombosis. Venous thrombi were generated by venous stasis after ligation of the infrarenal inferior vena cava. Thrombi were then examined and stained with an anti-fibrin/fibrinogen antibody after sacrificing the animals at day 2 and day 6 post-ligation. P-selectin null mice were shown to have less fibrin and decreased thrombus mass when compared to their wild-type controls. E-selectin deficient mice and E-selectin/P-selectin double knock-out mice also formed thrombi with reduced mass and fibrin. This study provided some of the first evidence for the role of the selectins in fibrin generation in an in vivo mouse model of venous thrombosis. However, in this model, only relatively late time points after thrombus formation (2 days and 6 days) were studied. At these latter time points, inflammatory cells have accumulated in the venous wall. In this model system, the P/E-selectin double knock-out mice appeared to have lower numbers of extravasated inflammatory cells in the IVC venous walls (45). Therefore, P-selectin and E-selectin appear to be critical in the recruitment of leukocytes to the thrombi. P-selectin is expressed in platelets and endothelial cells, while E-selectin is expressed only in endothelial cells, suggesting that in venous thrombosis, both endothelial and platelet selectins may cooperate in the recruitment of inflammatory cells. Based on previous work showing that activated mononuclear cells can express tissue factor, it seems likely that the recruited inflammatory cells may deliver tissue factor to the venous thrombi, thus contributing to fibrin generation. This study, however, did not analyze the effects of P-selectin on thrombus formation or fibrin generation prior to the recruitment of inflammatory cells.

This venous thrombosis model was also studied in mice overexpressing a soluble form of P-selectin (46). These mice have been genetically engineered to express P-selectin without its cytoplasmic domain and have 3-4 times the amount of circulating P-selectin compared to wild-type mice (47). These mice also appear to have increased numbers of circulating microparticles that contain tissue factor (40). After ligation of the inferior vena cava, the mice overexpressing soluble P-selectin were compared to wild-type mice as well as to mice deficient in P-selectin and/or E-selectin at 2 and 6 days after ligation. The mice overexpressing soluble P-selectin had larger thrombi and increased numbers of inflammatory cells in the affected vein walls when compared to wild-type mice. The E-selectin, P-selectin doubly deficient mice had the smallest thrombi and the fewest infiltrating inflammatory cells. These results further confirmed the importance of both E-selectin and P-selectin in the recruitment of inflammatory cells and in the evolution of venous thrombi. Furthermore, the larger thrombi in the P-selectin overexpressing mice suggest that the increased number of circulating microparticles in these mice may be responsible for their larger thrombi. Presumably, the tissue factor bearing microparticles are recruited to the site of venous stasis and inflammation; however, it is not known how the ligation of inferior vena cava may affect the subsequent recruitment of these microparticles.

To investigate the role of P-selectin and PSGL-1 in early thrombogenesis, Falati and colleagues used widefield intravital
microscopy to image real-time thrombus formation after laser-induced injury in the cremaster microcirculation (42). In this model, the cremaster muscle is exteriorized in a living anesthetized animal. Thrombi are formed by laser-induced injury of the endothelium as described by Rosen and colleagues (44). Thrombi are then observed using brightfield microscopy and using fluorescently labeled antibodies directed against components of the developing thrombus. Antibodies against CD41 (glycoprotein αIIb), a platelet specific marker, are used to quantitate platelet accumulation and thrombus size. Anti-tissue factor antibodies and anti-fibrin antibodies are used to quantify tissue factor accumulation and fibrin generation, respectively. Successive images of the thrombi are digitally recorded and the fluorescence per thrombi quantitated. This model of thrombus formation permits study of the early phase of thrombus formation in real-time in a living mouse. Falati et al. demonstrated platelet accumulation, tissue factor expression, fibrin generation, and P-selectin expression in developing mouse thrombi after laser induced injury of the endothelium (Fig. 1). In P-selectin null mice and in PSGL-1 null mice, the thrombi which formed had markedly reduced tissue factor accumulation and fibrin generation when compared to wild-type mice (48) (Fig. 2). Similar results were seen when blocking anti-P-selectin antibodies were injected into a wild-type mouse prior to injury. This result supports a critical role for P-selectin as well as its major ligand, PSGL-1, in the recruitment of tissue factor to thrombi and for the subsequent generation of fibrin. This difference in tissue factor expression and fibrin generation in wild-type versus P-selectin or PSGL-1 null mice is apparent within the first 20 seconds after injury, prior to the recruitment of leukocytes. Leukocyte recruitment and leukocyte rolling on thrombi in cremaster arterioles is not seen until approximately 3
minutes after vessel wall injury (49). Hence, the recruitment of leukocytes to thrombi cannot be responsible for the P-selectin dependent tissue factor accumulation and fibrin generation. To determine whether leukocyte microparticles could be responsible for the accumulation of tissue factor and fibrin, Falati et al. showed that microparticles containing both functional PSGL-1 and tissue factor activity can be isolated from monocytes stimulated with LPS and a calcium ionophore. In addition, the thrombi in P-selectin deficient mice had significantly decreased recruitment of mouse leukocyte microparticles compared to thrombi formed in wild-type mice (48) (Fig. 3). These results suggest that P-selectin and PSGL-1 have a crucial role in the recruitment of tissue factor bearing microparticles in early thrombi, and that a P-selectin/PSGL-1 interaction was likely to be responsible for this recruitment of tissue factor positive microparticles and for the subsequent generation of fibrin (Fig. 4).

This novel role for P-selectin and PSGL-1 in blood coagulation also supports the hypothesis that the tissue factor in thrombi not only originates from the blood vessel wall, but also originates from blood-borne tissue factor in the circulation. To further test this hypothesis, chimeric mice were constructed using mice that express low levels of tissue factor (50). These low tissue factor-expressing mice produce smaller thrombi with significantly reduced levels of tissue factor and fibrin in thrombi after laser induced injury in the cremaster muscle microcirculation. Wild-type mice were lethally irradiated to destroy bone marrow cells and subsequently reconstituted with bone marrow from low tissue factor mice to produce chimeric mice with wild-type arterial walls but
with blood derived from low-tissue factor expressing hematopoietic cells. Thrombi were then generated by laser-induced injury in the cremaster arterioles of these chimeric mice. The chimeric mice with reduced levels of bone marrow-derived tissue factor had smaller thrombi and reduced levels of both tissue factor and fibrin in thrombi when compared to thrombi in wild-type mice transplanted with wild-type hematopoietic cells. Similarly, low tissue factor expressing mice were transplanted with wild-type bone marrow to produce chimeric mice with low levels of subendothelial tissue factor but with wild-type hematopoietic cells. These low tissue factor expressing chimeric mice with wild-type bone marrow had small thrombi, but normal levels of tissue factor and fibrin expression in thrombi when adjusted for thrombus size. Therefore, both vessel wall tissue factor and blood borne hematopoietically derived tissue factor appear to contribute to initial thrombus formation. However, the hematopoietically derived tissue factor appeared to have the predominant role in the subsequent tissue factor and fibrin accumulation.

The above studies support a role for P-selectin and PSGL-1 in the recruitment of circulating leukocyte derived microparticles expressing PSGL-1 and tissue factor to early thrombi. P-selectin is expressed on activated endothelial cells and activated platelets. The P-selectin expressed on activated platelets is likely to be important in this recruitment process. However, in addition to its expression on activated platelets and endothelial cells, P-selectin is also found in human and in mouse blood in a soluble form (51, 52). At least some of this soluble P-selectin is derived from proteolytic cleavage from activated platelets, with its subsequent release into the circulation (53, 54). Hartwell et al. (1998) genetically engineered mice to express P-selectin without its cytoplasmic domain, resulting in 3-4 fold increased levels of circulating soluble P-selectin when compared to wild-type mice (47). These mice were shown to be prothrombotic when compared to wild-type mice (40). These mutant P-selectin expressing mice had increased fibrin generation in an in vitro model of thrombosis, increased fibrin generation in a local Schwartzman reaction, shortened clotting times, and an increase in leukocyte microparticles expressing tissue factor. It was hypothesized that the increased levels of soluble P-selectin resulted in an increase in tissue factor bearing microparticles, which then led to a pro-coagulant state with increased fibrin generation. Infusion of soluble P-selectin immunoglobulin chimeric protein (P-selectin-Ig) into wild-type mice produced a similar state. The P-selectin ligand, PSGL-1, also appears to be important in the generation of tissue factor positive microparticles. PSGL-1 null mice infused with soluble P-selectin-Ig had fewer microparticles and a smaller percentage of those microparticles contained tissue factor by flow cytometry when compared to wild-type mice infused with soluble P-selectin-Ig (55). Therefore, both P-selectin and PSGL-1 appear to have a role in the generation of procoagulant microparticles. However, neither PSGL-1 nor P-selectin appear to be essential for microparticle generation.

P-selectin and PSGL-1 were originally characterized as adhesion molecules important for the recruitment of leukocytes to sites of inflammation. However, the above studies have also revealed a new role(s) for P-selectin and PSGL-1 in thrombosis (Fig. 4). Mice deficient in P-selectin or PSGL-1 form thrombi in the cremaster microcirculation with markedly reduced levels of fibrin compared to wild-type mice. This fibrin seems to result from blood borne tissue factor recruited to thrombi on microparticles in a P-selectin/PSGL-1 dependent manner. P-selectin is also important for the subsequent recruitment of leukocytes to thrombi, and this recruitment may have effects on thrombus size and fibrin levels, at least in venous thrombi. The data obtained in mice expressing increased levels of soluble P-selectin also suggest that P-selectin and PSGL-1 may play a role in microparticle generation. However, many questions remain unanswered. Is the residual tissue factor and fibrin accumulation in P-selectin and PSGL-1 deficient mice secondary to blood vessel wall tissue factor, or are there other adhesion molecules involved in the recruitment of tissue factor bearing microparticles to thrombi? Furthermore, the mechanism by which tissue factor bearing microparticles are formed remains to be elucidated. Is microparticle formation regulated, as is suggested by the observation of increased circulating microparticles and increased tissue factor in certain prothrombotic states? Furthermore, only a subset of microparticles appears to express tissue factor. What determines tissue factor expression on microparticles? And what are the roles of endothelial and platelet microparticles? Ultimately, the answers to these questions will lead to a greater understanding of thrombosis and potentially to new targets for anti-thrombotic therapy.
References


13. Vandendries, et al.: Role of P-selectin and PSGL-1 in coagulation and thrombosis


