P-Selectin–Dependent Inhibition of Thrombosis During Venous Stasis

Michael J. Eppihimer, Robert G. Schaub

Abstract—Leukocyte adhesion, transendothelial migration, and stasis are important components in the pathogenesis of deep vein thrombosis. Anesthetized cats were treated with saline, a recombinant soluble form of P-selectin glycoprotein ligand-1 (rPSGL-Ig), or an E- and L-selectin antibody (EL-246) before exposure and occlusion of a jugular vein. After 2 or 6 hours of occlusion, jugular veins were perfused with buffer, fixed, and prepared for scanning electron microscopy. In cats receiving saline, 2 and 6 hours of occlusion produced moderate levels of leukocyte and platelet adhesion and endothelial cell injury. Treatment of cats with rPSGL-Ig or EL-246 had no apparent effect on the magnitude of cell adhesion and endothelial cell injury compared with no treatment. After 6 hours of occlusion, the presence of a mural thrombus in untreated veins was observed and confirmed by scanning electron microscopy. Pretreatment of cats with rPSGL-Ig completely (4.0 mg/kg) or partially (1.0 mg/kg) prevented the occurrence of thrombi in the jugular veins. The reduction in thrombosis by rPSGL-Ig treatment after 6 hours of venous stasis, in the absence of any effect on leukocyte-mediated endothelial cell injury, suggests an antithrombotic mechanism of action for this protein.

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Key Words: leukocytes • platelets • endothelial cells • thrombosis • selectin

Thrombosis in the deep veins has been associated with remote tissue injury, combined with a period of vascular stasis.1–4 Leukocyte adhesion and transmigration have been demonstrated as early events in the initiation of deep vein thrombosis (DVT), presumably because of disruption of the endothelial cell (EC) barrier and exposure of the underlying basement membrane, providing a surface for the activation of coagulation.1,3 Leukocyte adhesion to ECs is regulated though a variety of cell surface adhesion molecules, including the selectins and members of the immunoglobulin superfamily. The selectins represent a family of 3 structurally similar carbohydrates.5 L-selectin is constitutively expressed on the surface of all leukocytes and is shed by proteolysis after cell activation.6 E- and P-selectins are expressed on the surface of stimulated ECs, with the latter also expressed on activated platelets. E- and P-selectins can be transcriptionally induced, with maximal presentation of the glycoproteins to the EC surface occurring 3 to 4 hours after stimulation by cytokines.7 P-selectin is also stored in Weibel-Palade bodies of ECs and can be rapidly translocated to the cell surface after stimulation with agonists such as thrombin and histamine.8,9 In addition to mediating leukocyte-EC interactions, L- and P-selectins are capable of mediating leukocyte-leukocyte and leukocyte-platelet interactions, respectively, through binding to P-selectin glycoprotein ligand-1 (PSGL-1), located on the surface of leukocytes.10,11

Although studies have implicated leukocytes in the pathogenesis of DVT, there are relatively few studies that have attempted to delineate the cellular adhesion mechanisms responsible for leukocyte adhesion and transmigration in this disease. Leukotrienes and tumor necrosis factor-α have been implicated as important mediators of leukocyte adhesion and transmigration during venous stasis12,13 and have been demonstrated to induce selectin expression in several inflammatory models.14

Several studies have suggested roles for P-selectin in the pathogenesis of DVT.15–17 In a baboon model of DVT, a P-selectin monoclonal antibody (mAb) was found to reduce the incidence of thrombus, venous wall cytokine levels, and gadolinium enhancement, a measure of vascular injury, 6 days after venous stasis.15 In addition, blocking P-selectin function with a soluble recombinant form of PSGL-1 (rPSGL-Ig), which has a high affinity for P-selectin, had a similar effect in this model.16 However, in both studies, compared with saline administration, the administration of P-selectin mAbs and rPSGL-Ig had no effect on the number of leukocytes in the vein wall 6 days after venous stasis.15,16 Furthermore, soluble levels of P-selectin have been observed to increase in patients who have undergone venous surgery, suggesting that platelets and/or ECs were activated.17

The involvement of P-selectin in leukocyte binding to endothelium would suggest that the primary effect of a P-selectin mAb would be the reduction of leukocyte adhesion, migration, and EC injury. The purpose of the present study was to examine the early vascular response in a model

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of vein stasis and to compare the effect of P-selectin–specific rPSGL-Ig and an E- and L-selectin mAb.

Methods

Selectin Antagonists

The mAb used for the in vivo evaluation of E- and L-selectin was EL-246 (kindly provided by Dr Robert Bargatze, Montana Immunotech, Bozeman, Mont), a mouse IgG, that recognizes a common epitope presented on the short consensus repeat domains of human E- and L-selectin.18 EL-246 mAb has been shown to bind to L- and E-selectin on feline neutrophils and cytokine-stimulated feline ECs, respectively. Furthermore, EL-246 mAb is effective at blocking neutrophils rolling in E- and L-selectin–dependent in vitro systems.19 Recombinant human PSGL-1 (rPSGL-Ig) was used to evaluate the role of P-selectin in this model. rPSGL-Ig was produced by truncating the NH2 47 amino acids, thereby maintaining a high affinity for P-selectin but dramatically reducing binding to L- and E-selectin. In addition, the NH2 47 amino acids were linked to an Fc portion of human IgG1. This restored the bivalent presentation observed in the native PSGL-1 molecule. Finally, 2 amino acids of the IgG-Fc region have been mutated to disable Fc receptor binding and complement fixation effector functions. In cats, rPSGL-Ig has been shown to bind to thrombin-activated platelets and inhibit leukocyte adhesion to thrombin-stimulated ECs.20 In addition, rPSGL-Ig at doses as high as 10 mg/kg was observed to have an insignificant effect on standard coagulation parameters in cynomolgus monkeys, as evidenced by an invariance in the duration of prothrombin and activated partial thromboplastin times between saline-treated and rPSGL-Ig–treated animals.21 A lack of effect of rPSGL-Ig on coagulation was also observed in baboons. In the present study, activated partial thromboplastin, thrombin clotting time, and template bleeding time were not significantly different between saline-treated and rPSGL-Ig (4 mg/kg)–treated animals.16

Surgical Procedures

Male domestic shorthair cats (n=45, body weight 1.8 to 3.2 kg, Harlan, Madison, Wis) were injected intramuscularly with ketamine at a dose of 35 mg/kg. Animals were intubated with a 3.0-gauge endotracheal tube and anesthetized with 1% to 2% isoflurane at a flow rate of 1 L/min. Before surgery, animals were injected intravenously with one of the following: 3 mL saline (vehicle; n=7 at 2 hours, n=5 at 6 hours), rPSGL-Ig (0.1, 1.0, and 4.0 mg/kg; n=3 at 2 hours, n=5 at 6 hours), or EL-246 mAb (2 mg/kg, n=3 at 2 hours) in 3 mL saline. Saline, although not an ideal control, was selected because of the difficulty in selecting the appropriate nonactive protein to represent the mutated human Fc rPSGL-Ig chimera and the intact murine IgG, anti–E selectin antibody. Studies with a low activity form of rPSGL-Ig, which was available for limited animal studies, have shown results similar to our saline control and the saline controls used in baboon DVT studies.16,20 Systemic leukocyte counts from venous blood samples were obtained in control animals and in animals exposed to 6 hours of occlusion before treating the animals with rPSGL-Ig (initial) and after 6 hours of occlusion (final). After exposure, jugular veins were occluded with a vascular clamp for 2 or 6 hours. The contralateral jugular vein was not surgically manipulated. Control veins were obtained from animals (n=5) that were anesthetized for 2 hours but not surgically manipulated. After 6 hours of occlusion, veins were visualized for the presence of thrombi within the lumen. After venous stasis, side branches and the distal end of the vein were tied off with silk sutures, and veins were perfused with Ca2+-Mg2+-free Tyrode’s buffer to remove nonadherent blood cells. Subsequently, the vein was reclamped to prevent the reentry of blood cells. Immediately thereafter, the vein was perfused with 1% glutaraldehyde (in Ca2+-Mg2+-free Tyrode’s buffer) and tied off under physiological pressure. Veins were harvested, tied down to wooden tongue blades, and placed in 1% glutaraldehyde at 4°C for 24 hours.

Preparation of Veins for Scanning Electron Microscopy

After 24 hours, venous segments were cut longitudinally, and vessels were tied down to glass coverslips (EC side up) and placed in 1% glutaraldehyde at 4°C for 24 hours. Venous segments were washed 3 times with Ca2+-Mg2+-free Tyrode’s buffer and placed in 1% osmium tetroxide (in Ca2+-Mg2+-free Tyrode’s buffer) at 4°C for 15 hours. Samples were washed 5 times with distilled water, 4 times with 100% ethyl alcohol, and 3 times with amyl acetate. Samples were placed in amyl acetate for 24 hours, removed, and allowed to air-dry in a desiccator. Venous segments were mounted on stubs, gold-coated (model SC-4, Pelco), and examined under a scanning electron microscope (model 3200-C ECO-SEM, Amray). Thirty to 50 regions of a venous segment were observed and given a histological score of inflammation ranging from 0 to 5 and defined as follows: 0, intact endothelium with no adherent leukocytes and/or platelets; 1, intact endothelium with some adherent leukocytes and/or platelets; 2 focal EC damage with adherent leukocytes and/or platelets; 3, focal EC damage with surface thrombosis and/or migrating leukocytes; 4, focal EC damage with migrating leukocytes, adherent leukocytes, and/or platelets and/or fibrin; and 5, extensive EC damage with migrating leukocytes, adherent leukocytes, and/or platelets and/or fibrin.

Statistical Analysis

All treatments were analyzed and determined to be normally distributed and have equal variances. Means of treatment groups were

Figure 1. SEM micrographs from control (not occluded) veins. A and C, Low-magnification view demonstrates nominal adherence of leukocytes and platelets on venous endothelium and around venous valves. Original magnification ×143 (A) and ×61 (C). B and D, At higher magnifications, although some leukocyte adhesion exists, it is evident that the venous endothelium remains intact. Original magnification ×1010 (B) and ×1270 (D).
compared by ANOVA with multiple comparisons with use of the Student-Newman-Keuls test. Values of the incidence of thrombosis were compared by Student's t test. All values shown are mean ± SE. Statistical significance was set at <i>P</i> < 0.05.

**Results**

Initial and final leukocyte counts for control animals were found to be 13.8 ± 2.4 × 10³ and 9.6 ± 1.5 × 10³ leukocytes per microliter, respectively. In vehicle-treated animals exposed to 6 hours of venous occlusion, initial and final leukocyte counts were 9.8 ± 2.3 × 10³ and 10.8 ± 2.0 × 10³ leukocytes per microliter, respectively. In animals treated with 0.1, 1.0, and 4.0 mg/kg rPSGL-Ig, initial leukocyte counts were observed to be 11.1 ± 2.3 × 10³, 12.7 ± 1.2 × 10³, and 12.0 ± 3.2 × 10³ leukocytes per microliter, respectively, and final leukocyte counts were observed to be 16.6 ± 3.8 × 10³, 15.5 ± 2.3 × 10³, and 12.1 ± 1.5 × 10³ leukocytes per microliter, respectively. Initial and final leukocyte counts were not significantly different within each treatment group, as assessed by a paired t test. Furthermore, leukocyte counts were observed to be insignificantly different among treatment groups.

Scanning electron microscopic (SEM) analysis of control veins revealed an intact endothelium with sparsely distributed adherent leukocytes (Figure 1 and Table), representing an inflammation score of ≈ 1. In addition, areas in proximity of valves and side branches had intact endothelium and were devoid of leukocytes and platelets. At higher magnification, raised EC nuclei and gap junctions were observed. The cells that were attached to endothelium did not appear to be activated, as evidenced by a spherical shape and an absence of pseudopodia.

In veins subjected to 2 hours of stasis, an increase in the level of leukocyte and platelet adherence was observed, as evidenced by a 2-fold elevation in the inflammation score (Table). Substantial zones of injured endothelium, defined by the presence adherent leukocytes and platelets and sloughing ECs, were found immediately adjacent to intact endothelium (Figure 2). Injured regions of endothelium were readily apparent around valves and side branches. Localized areas of injured endothelium with exposed basement membrane were observed at higher magnification, as were platelets bound to the leukocyte surface. SEM analysis revealed that pretreatment of cats with rPSGL-Ig and EL-246 mAb did not reduce the venous inflammation and injury compared with saline treatment in cats occluded for 2 hours (<i>P</i> > 0.05, Table). Furthermore, there was no apparent difference in the location of cell accumulation and injury with pharmacological treat-
ment; ie, valve regions and side branches in rPSGL-Ig– and EL-246 mAb–treated veins exhibited levels of inflammation similar to the levels in untreated veins.

Figure 3 demonstrates the extent of inflammation and vascular injury in veins subjected to 6 hours of occlusion. In the Table, the level of venous inflammation and injury observed at 6 hours of occlusion was 50% greater compared with that observed in veins occluded for 2 hours ($P<0.05$). At lower magnification, it can be seen that areas of injured endothelium are immediately adjacent to intact endothelium and that valves are a common site for cell accumulation (Figure 3A and 3C). SEM analysis demonstrates the subendothelial accumulation of leukocytes and the associated EC detachment that results from transmigration. Leukocytes and platelets expressed pseudopodia, suggesting that these cells were activated. Figure 3C shows a large thrombus at the orifice of the valve. Large mural thrombi were observed in 80% of the saline-treated animals (Table). Although the larger mural thrombi embolized when the vascular clamp was removed, SEM analysis of the vein revealed remnants of a thrombus, which suggest that the valve was the nidus for clot formation. A layer of adherent and apparently activated leukocytes and platelets was also found in the region around the thrombus (Figure 3D). After 6 hours of occlusion, cell aggregates consisting of large numbers of leukocytes and platelets can be observed tethered to the injured endothelium. Treatment of cats with rPSGL-Ig at any dose was not effective in reducing the level of venous inflammation after 6 hours of occlusion compared with saline treatment in cats after 6 hours of occlusion ($P>0.05$, Table). Examination of cats treated with 4 mg/kg rPSGL-Ig revealed large areas of the EC injury, resulting in EC sloughing and exposure of the basement membrane but no incidence of thrombosis (Figure 4). Comparison of venous inflammation between rPSGL-Ig–treated veins presenting a thrombus and those veins not containing a thrombus was $2.9\pm0.14$ and $2.9\pm0.17$ ($P>0.05$), suggesting that thrombus formation is independent of venous inflammation in rPSGL-Ig–treated animals. However, the incidence of thrombus formation in veins was dependent on the administered dose of rPSGL-Ig. With saline...
administration, 80% of veins exhibited a thrombus during occlusion, whereas complete inhibition of thrombus formation was observed in veins treated with 4 mg/kg rPSGL-Ig (Table). Treatment of cats with 1.0 and 0.1 mg/kg rPSGL-Ig resulted in an incidence of thrombus formation of 40% and 60%, respectively (Table).

Discussion

Studies have demonstrated that leukocyte adhesion and transmigration contribute to the initiation of DVT by disrupting the EC barrier and exposing the underlying basement membrane, providing a surface for the activation of coagulation. It has been suggested that the stimuli for the initiation of leukocyte adhesion in the pathogenesis of DVT are an elevation in circulating leukocyte-EC adhesion by elevating the expression of cell adhesion molecules and by reducing the hemodynamic forces acting to remove the leukocytes from the ECs, respectively. In the present study, we and by reducing the hemodynamic forces acting to remove the leukocytes from the ECs, respectively.

In the present study, we used SEM analysis to delineate the relative role of selectins in mediating leukocyte adhesion, EC damage, and thrombosis during venous stasis. Although studies have demonstrated that immunoneutralization of P-selectin reduces the vein wall cytokine elevation, edema, and thrombosis several days after stasis, relatively little is known regarding the role of cell adhesion molecules in mediating leukocyte adhesion and transmigration during the pathogenesis of DVT. In the baboon, Downing et al demonstrated that P-selectin mAbs were ineffective at reducing leukocyte accumulation in the vein wall 48 hours after occlusion. However, these observations coincided with decreases in vein wall gadolinium enhancement and thrombus formation, suggesting that these factors are independent of neutrophil accumulation. In the same animal model, immunoneutralization of P-selectin with rPSGL-Ig, compared with vehicle, also resulted in a reduction in gadolinium enhancement and thrombus formation, with no effect on leukocyte accumulation. These results agree favorably with the present study, in which early leukocyte adhesion and transmigration were not reduced with treatment of rPSGL-Ig, but thrombosis was inhibited.

Although blocking selectin function has been demonstrated to reduce leukocyte adhesion and transmigration and EC damage in other models of inflammation, the present study reveals that antagonism of E-, P-, and L-selectin does not reduce leukocyte adhesion during venous stasis. This is in contrast to in vitro studies that found that P-selectin mAbs abolish hypoxia-induced leukocyte adhesion to ECs. A possible explanation for this discrepancy may be due to the differences in EC adhesion molecule expression between in vitro and in vivo conditions and the absence of platelets in vitro. Panes et al showed that the expression of adhesion molecules on human umbilical vein ECs in vitro was significantly different from their expression on ECs in animals. Leukocytes can initiate contact with ECs without using the selectin molecules by interactions between β1 and β2 integrins on the leukocyte surface and vascular cell adhesion molecule-1 and intercellular adhesion molecule (ICAM)-1 on the surface of ECs, respectively. Administration of a mAb to ICAM-1 but not CD18 was effective at reducing the total number of leukocytes accumulating in the vein wall after 6 hours of stasis. Given that leukocyte accumulation was not completely inhibited with an ICAM-1 mAb suggests that some leukocytes are capable of adhering and extravasating independent of this adhesion pathway. Although these data demonstrate that leukocytes can adhere to ECs and transmigrate without using the selectins, CD18, or ICAM-1 alone, it cannot be discounted that leukocytes use selectins and integrins interchangeably to adhere to veins under static conditions. Future studies examining these pathways as well as novel pathways are warranted to understand the trafficking of leukocytes at sites of venous stasis.

Although selectin blockade had no apparent effect on the level of leukocyte-mediated vascular injury, rPSGL-Ig was effective at reducing the incidence of thrombosis after 6 hours of stasis. SEM analysis demonstrates an absence of thrombosis in rPSGL-Ig-treated animals compared with saline-treated animals, although severe EC damage is readily observed in both treatment groups. This disparity suggests that rPSGL-Ig may affect a cellular or biochemical event in the formation of a thrombus. As mentioned previously, P-selectin is also located in abundance on the surface of activated platelets and has been demonstrated to mediate adhesive interactions with neutrophils through binding to PSGL-1. Studies have shown that leukocytes roll and adhere to immobilized platelets and are attenuated with an antibody directed against P-selectin. Because of the limitations of SEM analysis, we were unable to discriminate changes in the level of leukocyte-platelet interactions in the present study; however, in vitro analysis showed that rPSGL-Ig was effective in inhibiting the formation of neutrophil-platelet aggregates resulting from flow over a damaged artery. In addition, rPSGL-Ig attenuates the detrimental effect of leukocyte-platelet suspensions during their perfusion through ischemic hearts by reducing leukocyte accumulation and improving cardiac function, presumably by inhibiting leukocyte-platelet interactions.

SEM analysis of Dacron grafts demonstrated that P-selectin blockade attenuated the number of leukocytes bound and the amount of fibrin deposited within a thrombus, suggesting that platelets offer an adhesive surface whereby leukocytes can be recruited to the thrombus. Because activated platelets, as opposed to ECs, have a 7-fold greater expression of P-selectin and at sites of thrombus a 3D structure, it seems reasonable that rPSGL-Ig may be inhibiting leukocyte-platelet interactions and the subsequent formation of a thrombus in the present study.

For platelets and leukocytes, each can modulate the reactivity of the other by virtue of their physical contact or proximity. Platelet adhesion to neutrophils facilitates the production of superoxide, thromboxane, thromkriene C5, and platelet activating factor. Inhibition of leukocyte-platelet adhesion with a P-selectin mAb significantly reduced the production of thromboxane and leukotriene C5. Several studies have demonstrated that neutrophils can inhibit platelet aggregation and function by the release of products such as elastase and NO-like endothelium-derived relaxing factor-like factors. Furthermore, this ability of leukocytes to affect platelet function is exacerbated with the treatment of P-selectin mAbs. Thus, it is possible that rPSGL-Ig may inhibit leukocyte-platelet interactions and reduce the potential of thrombus formation by reducing the reactivity of leukocytes and platelets to produce prothrombotic mediators.

In conclusion, the results of the present study indicate that the level of leukocyte adhesion and transmigration in the vein
is not dependent on E-, L-, and P-selectin and that the function of thrombi is not dependent on the level of venous inflammation. In addition, the development and/or dissolution of thrombi in veins can be mediated through P-selectin immunoneutralization; thus, rPSGL-Ig may be an effective treatment for patients at risk of adverse thrombotic events.

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