Decreased venous thrombosis with an oral inhibitor of P selectin

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Background: P-selectin inhibition with protein therapeutics such as antibodies or soluble ligands given intravenously can decrease thrombosis in a mouse ligation model of venous thrombosis. In this study, we hypothesized that oral inhibition of P selectin with a novel oral nonprotein inhibitor (PSI-697) would decrease thrombosis and circulating microparticle populations. This study evaluated the effects on thrombosis and circulating microparticle populations in this murine venous thrombosis model.

Methods: Mice underwent inferior vena cava ligation to induce thrombosis. Mice with high circulating level of P selectin, Delta Cytoplasmic Tail (^CT), mice gene-deleted for both E- and P-selectin knockout (EPKO), and wild-type C57BL/6 mice (WT) were studied without and with administration of PSI-697 in food (100 mg/kg daily) from 2 days before thrombosis until the end of the study. Animals were killed 2 and 6 days later. Evaluations included thrombus weight (TW), vein wall morphometrics, microparticle quantification by using fluorescence-activated cell sorter analysis, and vein wall enzyme-linked immunosorbent assays for interleukin (IL)-10, P selectin, and monocyte chemotactic protein 1.

Results: PSI-697 significantly decreased TW in WT and ^CT mice, with a treated vs nontreated TW of 132 ± 24 vs $228 \pm 29 \times 10^{-4}$ g ($P = .014$) and 166 ± 19 vs $281 \pm 16 \times 10^{-4}$ g ($P = .001$), respectively. At day 6, the effect was significant only in the ^CT group ($P < .05$). Drug therapy at day 2 significantly increased vein wall monocytes in WT mice and increased monocytes and total inflammatory cells in ^CT animals. A significant decrease in neutrophils and total inflammatory cells was seen in EPKO mice at day 2 with therapy. Therapy significantly increased platelet-derived microparticles and total microparticles in ^CT mice on day 2. Changes in treated WT and treated EPKO animals were not significant compared with respective vehicle treatments at day 2. On day 6, therapy significantly decreased total microparticles in EPKO animals. Vein wall expression of IL-10 increased in all groups with therapy at day 2 (n = 18) and was significantly increased in WT (2687.5 ± 903 pg/mL vs 636 ± 108 pg/mL total protein; $P = .038$) and ^CT (2078 ± 295 pg/mL vs 432 ± 62 pg/mL total protein; $P = .001$) mice. Therapy significantly decreased vein wall P selectin, monocyte chemotactic protein 1, and IL-10 levels at day 6.

Conclusions: PSI-697 decreased thrombosis. P-selectin inhibition allowed vein wall inflammatory cell extravasation in this model of complete ligation. Circulating microparticles (platelet-derived microparticles and total microparticles) increased with P-selectin inhibition, possibly because of decreased consumption into the thrombus. In summary, the oral administration of an inhibitor to P selectin provides significant TW reduction. (J Vasc Surg 2005;42:329-36.)

Clinical Relevance: Deep venous thrombosis is a significant national health problem in the general population. The average annual incidence of deep venous thrombosis is approximately 250,000 cases per year. The selectin family of adhesion molecules is thought to be largely responsible for the initial attachment and rolling of leukocytes on stimulated vascular endothelium. Recent studies have explored the possible therapeutic implications of P-selectin inhibition to modulate venous thrombosis. For example, prophylactic dosing of a recombinant P-selectin ligand decreases venous thrombosis in a dose-dependent fashion in both feline and nonhuman primate animal models. Additionally, treatment of 2-day iliac thrombi with a recombinant protein, P-selectin inhibitor, significantly improves vein reopening in nonhuman primates. It is interesting to note that P-selectin inhibition decreases thrombosis without adverse anticoagulation. On the basis of the results from these previous studies, the use of P-selectin antagonism is a logical therapeutic approach to treat venous thrombosis. All inhibitors developed to date are either proteins or small molecules with low oral bioavailability that require intravenous or subcutaneous injection. This study evaluates, for the first time, a novel orally bioavailable inhibitor of P-selectin (PSI-697).

Deep venous thrombosis is a significant national health problem in the general population. The average annual incidence of deep venous thrombosis is approximately 250,000 cases per year.[1-3] The selectin family of adhesion molecules is thought to be largely responsible for the initial attachment and rolling of leukocytes on stimulated vascular endothelium. This family consists of P selectin expressed on activated platelets and activated endothelium, E selectin

expressed on activated vascular endothelium, and L selectin expressed on the surface of neutrophils. All selectins have been shown to recognize the sialyl Lewis[x] carbohydrate ligands. The adhesion molecule P selectin, which is present in platelet α-granules and endothelial cell Weible-Palade bodies,[4] is expressed on the surface of these cells early during venous thrombosis and promotes vein wall inflammation in multiple animal models.[5]

The ligand for P selectin is a glycoprotein expressed on the surfaces of most hematopoietic cells termed P-selectin glycoprotein ligand (PSGL)-1. PSGL-1 is a homodimeric mucin associated with the adhesion interactions that are responsible for the initial rolling of neutrophils along stimulated vascular endothelium and the formation of platelet/leukocyte complexes on the vascular surface and in the circulation.[6] This ligand has a high affinity for P selectin and a lesser affinity for E and L selectin. P selectin is upregulated rapidly and downregulated within minutes to hours.

We have confirmed the importance of inflammation and selectins in venous thrombosis by using rodent models. In mice, vein wall neutrophils are significantly increased above sham control animals at day 2 after thrombosis, monocytes are significantly increased above sham controls at day 6 after thrombosis, and total inflammatory cell counts are significantly increased at both time points. Vein wall protein determination showed that P selectin was upregulated as early as 6 hours after thrombus induction, whereas marked protein increases of E selectin occurred 6 days after thrombosis. P selectin remained increased through day 6 after thrombosis, and messenger RNA activity preceded protein increases.[5] Also, mice gene-deleted for both E and P selectin have significant decreases in thrombus fibrin staining and weight when compared with wild-type (WT) control animals at both 2 and 6 days after thrombosis.[7] Recent studies have explored the possible therapeutic implications of P-selectin inhibition to modulate venous thrombosis. For example, the administration of a recombinant P-selectin ligand before the thrombogenic event decreased venous thrombosis in a dose-dependent fashion in both feline and nonhuman primate animal models.[8,9] Additionally, treatment of 2-day-old iliac thrombi with the same recombinant P-selectin ligand significantly improved vein reopening in nonhuman primates without adverse anticoagulation.[10]

Research attention has been focused on the roles P selectin may have in influencing the formation of circulating microparticles (MPs). MPs are described as fragments ($\leq$1 μm) of phospholipids from cell membranes that have hypercoagulable properties. Previous studies suggest that MPs are important in early venous thrombogenesis and that these MPs (with membrane-bound tissue factor) are incorporated into the developing thrombus.[11-13] In a recent study, we noted that mice with high circulating levels of soluble P selectin formed thrombi, whereas animals with gene deletions of both E selectin and P selectin (EPKO) formed smaller thrombi, and that MPs (derived more from leukocytes than platelets) were associated with early thrombogenesis.[14]

On the basis of the results from these previous studies, the use of P-selectin antagonism is a logical therapeutic approach to treat venous thrombosis. All inhibitors developed to date are either proteins or small molecules with low oral bioavailability that require intravenous or subcutaneous injection. This study evaluates, for the first time, a novel orally bioavailable inhibitor of P selectin (PSI-697). To evaluate this novel oral compound, mice underwent complete stasis-induced venous thrombosis. In this study, we hypothesized that blocking P selectin with this new orally available compound would decrease thrombosis and inflammation while modulating the effects of prothombotic MPs.

METHODS

Mouse inferior vena cava stasis model. Male mice (*Mus musculus*; n = 432) weighing 20 to 30 g made up the study population. All but 10 mice were anesthetized with isoflurane gas, a midline laparotomy was performed, and the inferior vena cava (IVC) was directly approached and ligated with 7-0 Prolene (Ethicon, Inc, Somerville, NJ) sutures just below the renal veins, with ligation of large venous side branches.[5] The 10 nonoperated animals were used to assess the effectiveness of the P-selectin inhibitor. Mice were evaluated at selected time points after IVC ligation, including day 2 and day 6. This study used mouse groups treated with and without PSI-697 in food: WT C57BL/6 controls (Harlan, Indianapolis, IN), hypercoagulable mice with high levels of soluble P selectin Delta Cytoplasmic Tail (^CT); (Denisa Wagner, Cambridge, MA),[11,15] and EPKO mice (Daniel Bullard, University of Alabama, AL).[16,17]

P-selectin inhibition. The small molecule inhibitor of P-selectin (PSI-697; Wyeth Research, Cambridge, MA) is an orally available noncarbohydrate and nonantibody inhibitor of P selectin. This compound has been demonstrated in vitro to disrupt the binding of P selectin to its ligand PSGL-1 and to reduce white cell rolling in the microvasculature of mice. From studies in mice, this drug seems to enter the bloodstream within 1 hour after consumption in chow (unpublished data, Wyeth Research).

Mice (five per cage) were fed either a powdered vehicle control rodent chow or rodent chow formulated with PSI-697 (drug-enhanced chow, 100 mg · kg^{-1} · d^{-1} orally) ad libitum. The PSI-697 animal groups were administered vehicle control chow for 5 days of chow acclimation and were then switched to PSI-697–containing chow (1.0 mg of PSI-697 per gram of chow) 2 days before thrombosis until the end of each experiment. Vehicle control chow animals remained on this diet for the full length of the study. Control animals consumed 126 ± 12 g compared with 136 ± 8 g consumed by mice with PSI-697 therapy over the duration of the study. Additionally, we measured weight changes in experimental animals. On average, WT mice, ^CT mice, and EPKO mice weighed 24.5 ± 0.6 g, 22.4 ± 0.7 g, and 22.5 ± 1.0 g, respectively, before thrombosis. Six days after thrombosis, the change of animal weight in experimental groups was recorded as follows: WT

mice, 25.0 ± 0.6 g (2.0% increase); ^CT mice, 22.7 ± 0.3 g (1.8% increase); and EPKO mice, 21.5 ± 0.4 g (0.5% decrease) with PSI-697 therapy. All powdered chow formulations were made available to mice in a 16-ounce powdered food feeder (model PFF16D; Allentown Caging Equipment Co, Inc, Allentown, NJ).

Thrombus weight. Groups of mice were analyzed for thrombus weight (TW) at each time point. This technique is an indirect measure of thrombus content. At death, the IVC and its associated thrombus were removed and weighed.

Vein wall morphometrics. Veins were examined under high-power oil-immersion light microscopy. Sections were stained with hematoxylin and eosin from paraffin-embedded tissues. Five representative high-power fields (HPFs; oil immersion; magnification, $\times 1000$) were examined around the vein wall, and the cell count of the vein wall was analyzed manually by a study-blinded veterinary pathologist. Cells were identified as neutrophils, monocyte/macrophages, or lymphocytes according to standard histologic criteria, including nuclear size, cytoplasmic content, and total cell size for the inflammatory cell types.[5,18] Results from the five HPFs were added together for each section studied, and the mean $\pm$ SEM were calculated for each group.

Murine platelet and leukocyte MP analysis. Mouse groups were evaluated for MP populations at days 2 and 6 after thrombosis. Whole blood (0.8 mL) was removed from the mice by intracardiac puncture with a syringe primed with 10% acid citrate dextrose. The whole blood was then centrifuged to produce platelet poor plasma (PPP). PPP samples were incubated with antibodies that stained leukocytes and platelets. These included rat anti-murine phycoerythrin (red; CD11b; stains leukocytes) 10 μg/mL (Chemicon International, Temecula, CA) and rat anti-murine fluorescein isothiocyanate (green; CD41; stains platelets) 5 μg/mL (BD Pharmingen, San Diego, CA).[14]

Flow cytometry analysis was performed to identify MP populations. For MP quantification, a known quantity (250,000) of 3.0- to 3.4-μm-diameter beads (SPHERO; BD Pharmingen) was added to each fixed sample before fluorescence-activated cell sorter analysis, and acquisition was stopped after 50,000 beads were counted in the R-1 bead gate. The diameter of these beads allowed for discrimination from the MP population on light scatter and was counted with a separate bead gate. The total MP number per 200 μL of PPP was then calculated. The quantitative MP data were divided into the following groups: leukocyte-derived MPs (LMPs), platelet-derived MPs (PMPs), and total MPs (TMPs).

Vein wall enzyme-linked immunosorbent assay. Protein levels of interleukin (IL)-10, P selectin, and the cytokine monocyte chemotactic protein 1 were measured from vein wall homogenates at 2 and 6 days after thrombosis. All enzyme-linked immunosorbent assay (ELISA) samples were run in duplicate according to the manufacturer's suggestions. ELISA plates were read on a Plate Reader Elx808 (Biotek, Winooski, VT) at a wavelength of 450 nm, and levels of total protein were reported in picograms per milliliter.

Statistical evaluation and animal use. Statistical analysis included analysis of variance, Mann-Whitney rank sum tests, and unpaired Student t tests for parametric data (SPSS SigmaStat 2.0; Leesburg, Va). Significance was defined as $P \leq .05$. The health status of all animals was monitored by use of a sentinel program, and all animals were free of pathogens. All mice were housed and cared for by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is an American Association for Accreditation of Laboratory Animal Care–accredited facility under the direction of a veterinarian according to the Principles of Laboratory Animal Care (formulated by the National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996). The University of Michigan Committee on Use and Care of Animals approved this research protocol.

RESULTS

PSI-697 decreases thrombosis. In this study, PSI-697 significantly decreased TW in both WT (n = 20) and ^CT (n = 19) mice compared with untreated animals 2 days after thrombosis (WT: 132 ± 24 vs $228 \pm 29 \times 10^{-4}$ g; $P = .014$; ^CT: 166 ± 19 vs $281 \pm 16 \times 10^{-4}$ g; $P = .001$; Fig 1, A). At day 6, the significant decrease in TW was noted only in the ^CT (n = 20) mice (204 ± 17 vs $274 \pm 21 \times 10^{-4}$ g; $P = .012$; Fig 1, B). Conversely, the EPKO (n = 14) mouse group with drug showed a slight decrease in TW at day 2, but this was not significant (Fig 1, A). Additionally, the evaluation of plasma samples from experimental mice showed that mice did receive therapeutic concentrations of the compound during the study (≥ 1000 ng/mL). It is important to note that no obvious bleeding or wound healing abnormality was noted in any of the animals, either treated or control.

The percentage decrease in TW between animals with drug therapy vs without drug therapy was also evaluated (n = 232; total number of animals with and without drug). WT animals (n = 20) with drug showed a significant 42% decrease in TW at day 2 when compared with WT animals (n = 20) without drug therapy ($P = .014$). Mice with high circulating levels of P selectin (^CT) with drug therapy (n = 19) showed a 41% decrease in TW vs without drug (n = 18) at day 2 ($P = .001$) and showed a 26% decrease in TW with drug (n = 20) vs without drug (n = 19) at day 6 ($P = .012$; Table I).

To elucidate the effects of the small molecule inhibitor of P selectin, we evaluated only the subset of experimental animals that were positive for thrombosis (n = 215; total number of animals with and without drug evaluated). Both the WT (n = 20) and EPKO (n = 20) groups showed significant decreases in TW in animals administered drug vs without drug therapy at day 2 only (34% and 42%, $P = .042$ and $P = .035$, respectively). The ^CT mice had significant decreases in TW noted at both day 2 (n = 17) and day 6 (n
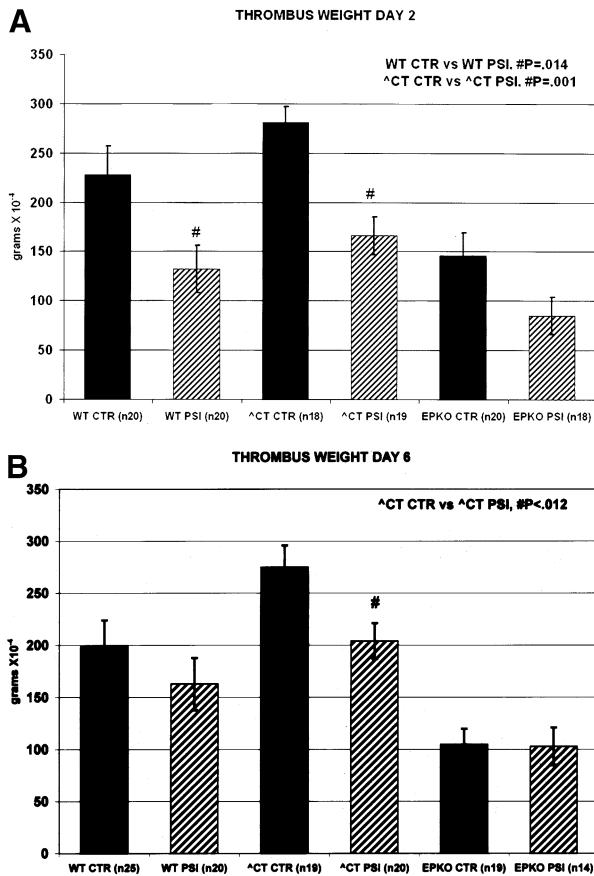
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B

Fig 1. A. Small molecule inhibition of P selectin (*PSI*-697) significantly decreased thrombus weight in the wild-type *(WT)* and Delta Cytoplasmic Tail *(^CT)* groups at day 2. *CTR,* Control without drug; *EPKO,* mice gene-deleted for both E and P selectin. **B,** Only the ^CT mouse group with drug therapy showed a significant decrease in thrombosis at day 6.

Table I. Percentage decrease in thrombus weight with drug versus without drug in all experimental animals

Mouse group	Day 2	Day 6
WT (n = 85)	42% (*P* = .014)	18%
^CT (n = 76)	41% (*P* = .001)	26% (*P* = .012)
EPKO (n = 71)	42%	2%

WT, Wild-type; *^CT,* Delta Cytoplasmic Tail; *EPKO,* mice gene-deleted for both E and P selectin.

= 19) with drug vs without drug (35% and 22%, *P* = .001 and *P* = .021, respectively; Table II).

PSI-697 increases vein wall inflammatory cell populations. Drug treatment at day 2 significantly increased vein wall monocytes in WT mice (30 ± 1 vs 11 ± 1 cells per 5 HPFs; *P* = .001) and increased monocytes (44 ± 6 vs 11 ± 3 cells per 5 HPFs; *P* = .001) and total inflammatory cells in ^CT animals (144 ± 14 vs 54 ± 13 cells per 5 HPFs; *P* = .002) when compared with animals

Table II. Percentage decrease in thrombus weight with drug versus without drug (only animals with inferior vena cava thrombus)

Mouse group	Day 2	Day 6
WT (n = 76)	34% (*P* = .042)	14%
^CT (n = 73)	35% (*P* = .001)	22% (*P* = .021)
EPKO (n = 66)	42% (*P* = .035)	4% (increase)

WT, Wild-type; *^CT,* Delta Cytoplasmic Tail; *EPKO,* mice gene-deleted for both E and P selectin.
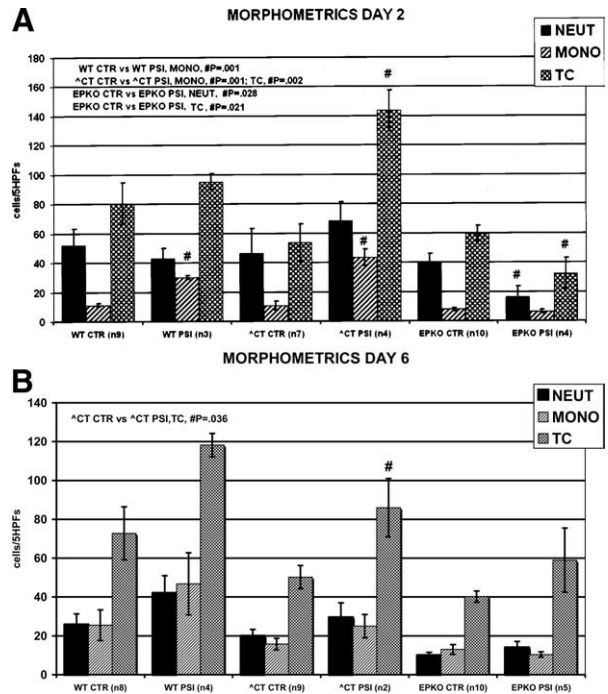
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Fig 2. A. Drug treatment at day 2 significantly increased vein wall inflammatory cell extravasation in wild-type *(WT)* and Delta Cytoplasmic Tail *(^CT)* animals, with increased thrombus burden. Animals deficient in selectins had significantly decreased vein wall inflammatory cell extravasation at day 2. *NEUT,* Neutrophils; *MONO,* mononuclear cells; *TC,* total inflammatory cells; *CTR,* control without drug; *PSI,* with PSI-697; *EPKO,* mice gene-deleted for both E and P selectin; *HPF,* high-power field. **B,** Only the ^CT group with PSI-697 therapy showed significant increases in vein wall inflammatory cells at day 6.

given vehicle control. A significant decrease in neutrophils (16.5 ± 7 vs 41 ± 5 cells per 5 HPFs; *P* = .028) and total inflammatory cells (32.5 ± 10 vs 60 ± 5 cells per 5 HPFs; *P* = .021) was seen in EPKO mice at day 2 with drug therapy vs vehicle control animals (Fig 2, A). For example, hematoxylin and eosin staining showed decreased inflammatory cells in vein endothelium and adventitial layers 2 days after thrombosis in the ^CT vehicle control mouse group, whereas with drug therapy, a significant increase in vein wall inflammatory cells was noted (Fig 3). On day 6, only
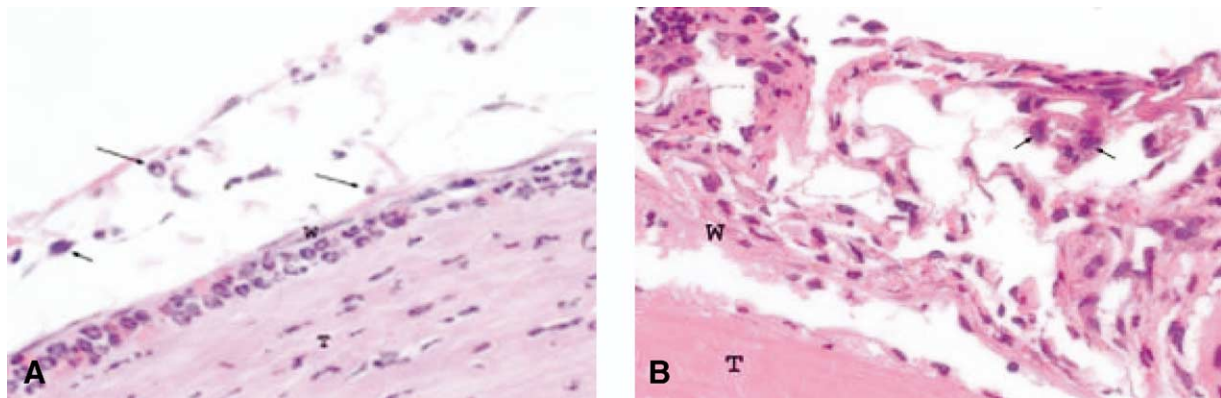
Fig 3. Hematoxylin and eosin staining showed decreased inflammatory cells in vein wall endothelium and adventitial layers 2 days after thrombosis in a Delta Cytoplasmic Tail (^CT) mouse without drug. Note that this animal had prominent inflammatory cells aligned at the vein wall/thrombus interface. (A) With drug treatment, a significant increase in vein wall inflammatory cells was noted in the ^CT mouse at day 2 (original magnification, ×80; short arrows depict mononuclear cells; long arrows depict neutrophils). (B) *CTR*, Control without drug; *PSI*, with PSI-697.

Table III. Day 2 microparticles

Group	PMP CTR	PMP PSI	LMP CTR	LMP PSI	TMP CTR	TMP PSI
WT (n = 9)	88,987	153,461	1810	1735	484,954	591,353
^CT (n = 10)	72,451	444,248 (P = .008)	2026	2326	425,050	838,162 (P = .02)
EPKO (n = 10)	234,607	195,315	3636	4752	683,078	577,855

Data are microparticles per 200 μL.

PMP, Platelet-derived microparticle; *CTR,* control without drug; *PSI,* with PSI-697; LMP, leukocyte-derived microparticle; *TMP,* total microparticles; *WT,* wild-type; *^CT,* Delta Cytoplasmic Tail; *EPKO,* mice gene-deleted for both E and P selectin.

Table IV. Day 6 microparticles

Group	PMP CTR	PMP PSI	LMP CTR	LMP PSI	TMP CTR	TMP PSI
WT (n = 11)	578,415	284,089	7200	17,326	1,008,502	793,217
^CT (n = 9)	331,508	657,923	23,633	4028	733,765	1,165,112
EPKO (n = 10)	362,322	250,393	21,509	6721	970,156	643,822 (P = .018)

Data are microparticles per 200 μL.

PMP, Platelet-derived microparticle; *CTR,* control without drug; *LMP,* leukocyte-derived microparticle; *TMP,* total microparticles; *WT,* wild-type; *^CT,* Delta Cytoplasmic Tail; *EPKO,* mice gene-deleted for both E and P selectin.

^CT mice showed a significant increase in total inflammatory cells with drug therapy vs vehicle controls (Fig 2, B).

PSI-697 influences MP populations during thrombosis. The MP data involve quantitative assessment of MP numbers and are divided into LMPs, PMPs, and TMPs. In control nonthrombosed animals, PSI-697 effectively decreased PMPs and TMPs (data not shown). This attests to the effectiveness of the drug.

In animals that had undergone thrombosis, we evaluated the effect of the drug on MP subtypes. At day 2, P-selectin inhibition caused a significant increase in PMPs with drug vs without drug in ^CT mice (444,248 ± 137,594 vs 72,451 ± 17,313 MPs per 200 μL of PPP; P = .008; Table III). PSI-697 therapy also caused a significant increase in TMPs in ^CT mice with drug compared with ^CT mice without drug therapy (838,162 ± 153,556 vs 425,050 ± 24,621 MPs per 200 μL of PPP; P = .02). A similar nonsignificant increase in PMPs and TMPs was noted in WT animals, whereas animals with the lowest TW (EPKO) revealed an opposite effect. The increase in MPs in those animals with the greatest TW is consistent with a proposed decrease in MP consumption into the thrombus with drug therapy.

At day 6, drug therapy significantly decreased TMPs in EPKO (643,822 ± 72,303 vs 970,156 ± 82,748 MPs per 200 μL of PPP; P = .018; Table IV). Regarding LMPs, drug therapy had little effect on day 2 and had no consistent effect on day 6.

PSI-697 increases vessel wall IL-10. Drug therapy increased vein wall expression of the anti-inflammatory IL-10 in all groups treated with the small molecule inhibitor to P selectin at day 2 (n = 18). IL-10 measured in the
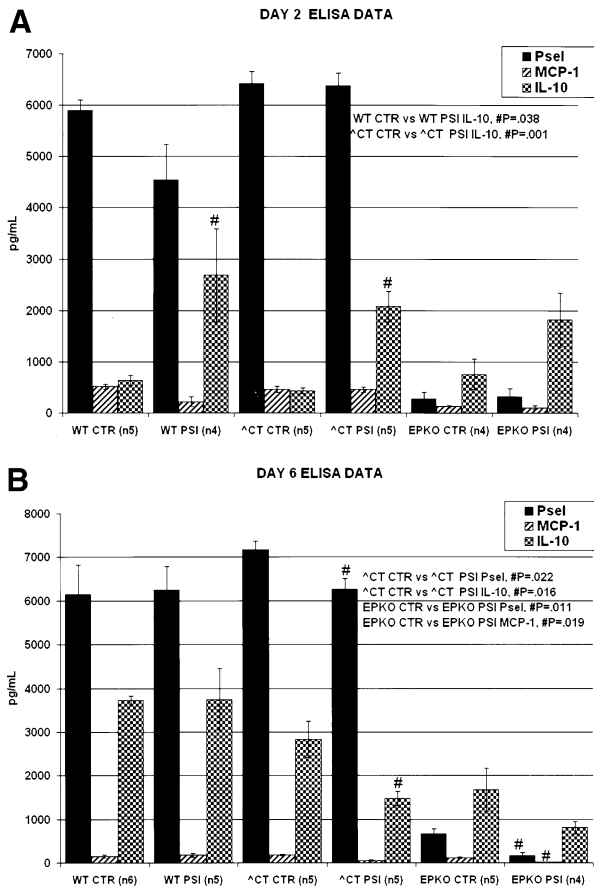
Fig 4. A. The anti-inflammatory cytokine IL-10 was significantly upregulated in wild-type (*WT*) and Delta Cytoplasmic Tail (*^CT*) animals at day 2 treated with the small molecule inhibitor of P selectin vs animals without drug. P selectin *(Psel)*, monocyte chemotactic protein 1 *(MCP-1)*, and interleukin 10 (IL-10) were measured from vein wall homogenates at 2 and 6 days after thrombosis. *CTR,* Control without drug; *PSI,* with PSI-697; *EPKO,* mice gene-deleted for both E and P selectin; *ELISA,* enzyme-linked immunosorbent assay. **B,** Significant decreases in protein expression were noted between the groups at day 6. Psel, MCP-1, and IL-10 were measured from vein wall homogenates at 2 and 6 days after thrombosis.

vessel wall was significantly increased in WT mice (2688 ± 903 pg/mL vs 636 ± 108 pg/mL; $P = .038$) and ^CT mice (2078 ± 295 pg/mL vs 432 ± 62 pg/mL; $P = .001$) administered drug compared with animals not given drug. Drug therapy significantly decreased vein wall protein levels of P selectin (7167 ± 202 pg/mL vs 6258 ± 248 pg/mL; $P = .022$) and IL-10 (2834 ± 413 pg/mL vs 1473 ± 160 pg/mL; $P = .016$) in ^CT mice and decreased P selectin (670 ± 107 pg/mL vs 168 ± 65 pg/mL; $P = .011$) and monocyte chemotactic protein 1 (116 ± 22 pg/mL vs 16 ± 8 pg/mL; $P = .019$) in EPKO mice at day 6 (Fig 4).

DISCUSSION

The need for the development of new therapies that decrease thrombosis without adverse effects on hemostasis

that increase bleeding potential has become a major research focus. The purpose of this study was to determine the effects PSI-697, a novel oral inhibitor of P selectin.

Prophylaxis with an oral inhibitor of P selectin can significantly decrease thrombosis (TW) at both early (day 2) and later (day 6) time points in our mouse model of stasis-induced complete venous thrombosis. To evaluate this oral compound, mice with high plasma levels of soluble P selectin,[11,15] EPKO mice,[16,17] and WT C57BL/6 mice were compared by using a complete stasis model of venous thrombosis. The small molecule inhibitor in this study significantly decreased TW in the ^CT and WT groups at the early day 2 time point. At day 6, the effect was less pronounced in WT mice but was still significant in the ^CT mouse group. Of interest, mice deficient in selectins (EPKO) demonstrated a decrease in thrombosis at day 2. We would expect no effect in EPKO mice if the mechanism of the small molecule inhibitor were only P selectin mediated. The finding of a decrease in thrombosis in animals devoid of E and P selectin at day 2 suggests that the drug has other effects, yet to be identified, at this early time point. We have shown in a previous study that mice deficient in both E and P selectin have a decreased thrombus burden and inflammatory cells 2 days after total blood stasis, but that these gradually increase over time.[5] This suggests that E and P selectin–independent receptor/ligand interactions are active during chronic thrombosis.[17]

Vein wall inflammatory cell populations (morphometrics) showed an inverse relationship when compared with TW. WT and ^CT mice during thrombosis showed an increase in vein wall neutrophils, monocytes, and total inflammatory cells extravasating through the vein wall in mice treated with PSI-697. This same population of mice showed decreases in thrombosis when compared with mice of the same strain given vehicle chow only, without drug. Recent investigations have indicated that venous thrombi in rats after induced neutropenia were larger and more fibrotic and showed decreased resolution compared with controls.[19] Although one of the major roles of inflammatory cells is the initiation of thrombus formation, neutrophils also possess cellular mediators, such as plasminogen activator and elastase, that may prove to be thrombolytic after the thrombus has formed.[19,20] Additionally, monocytes have been implicated in thrombus recanalization.[21-24] In contrast, experimental mice that were deficient in both E and P selectin had the least thrombus burden both with and without drug and vein wall inflammatory cell populations. Past studies have indicated that mice deficient in endothelial selectin activity (EPKO) have diminished leukocyte rolling and extravasation.[25]

The adhesion molecule P selectin, once activated by the stimulation of vascular endothelium, promotes P selectin/PSGL-1 binding interactions, which lead to endothelial cell/leukocyte, leukocyte/leukocyte, leukocyte/platelet, and circulating MP formation. The interaction between selectins and their leukocyte receptors stimulates fibrin formation.[26] Procoagulant MPs are recruited to the area of thrombosis,[27] where they amplify coagulation via tissue

factor and factor VIIa.[11,28-32] The colocalization of fibrin, platelets, and leukocytes in the developing thrombus[27] and the contribution of P selectin to leukocyte/platelet interactions that generate tissue factor[33,34] support this scheme of thrombogenesis.[35]

Notably, a recent investigation using intravital microscopy in a mouse model of arterial thrombosis showed that circulating MPs with membrane-bound tissue factor do in fact become incorporated into the developing thrombus immediately after thrombus induction.[13] We believe that the incorporation of MPs is aided by the large amount of IVC thrombosis in our experimental animals compared with their overall body size.

In nonthrombosed animals, the small molecule inhibitor effectively decreased MPs, thus indicating that the small molecule inhibitor of P selectin was effective in blocking P selectin/PSGL-1 binding. The consumption of MPs noted with thrombosis was effectively reversed by PSI-697. In mice with the largest thrombi (^CT and WT), these MPs were increased with drug therapy. This is likely due to a reversal of the consumption of these MPs in the clot by the drug, as noted in thrombosed animals without the benefit of the compound. In the animals with fewer thrombi (EPKO), the opposite effect was noted—that is, the MPs were decreased in mice given the small molecule inhibitor of P selectin. MPs from leukocyte origin were minimally affected at day 2.

Finally, we evaluated the effect the small molecule inhibitor of P selectin had on IL-10 in vein wall after thrombosis in mice with and without the drug. The amount of anti-inflammatory cytokine IL-10 measured in the vein wall was significantly increased in WT and ^CT animals at day 2 compared with nontreated animals. This increase in IL-10 may suggest another possible mechanism for the effectiveness of the therapy, because IL-10 has been found to decrease thrombosis in our rodent model and has been associated with decreased E- and P-selectin expression.[36] The ELISA data showed a trend toward high P-selectin upregulation at the early time points during thrombosis in this study. Notably, the EPKO animals had minimal P-selectin expression in this study, and this was expected.

In this study, we did not formally evaluate coagulation tests. However, there were no obvious bleeding complications in the animals treated with PSI-697. We will evaluate coagulation tests in further studies in other models. The potential advantages of PSI-697 include its ability to be administered orally and its reduction in thrombosis without any adverse effects on the coagulation system and associated bleeding.

CONCLUSION

In this study, the orally available small molecule inhibitor of P selectin, PSI-697, significantly decreased thrombosis at the early time point in a mouse model of venous thrombosis. P-selectin inhibition did allow for vein wall inflammatory cell extravasation. Circulating MPs (PMPs and TMPs) increased with P-selectin receptor inhibition,

possibly because of decreased consumption into the thrombus. Finally, with treatment, the anti-inflammatory cytokine IL-10 was significantly increased in the vein wall. In summary, we have demonstrated for the first time that the oral administration of a P-selectin inhibitor decreases thrombosis in an animal model of stasis-induced venous thrombosis formation. Such oral administration has potential significant advantages over intravenous or subcutaneous administration.

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