Platelet-Mediated Lymphocyte Delivery to High Endothelial Venules

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Circulating lymphocytes gain access to lymph nodes owing to their ability to initiate rolling along specialized high endothelial venules (HEVs). One mechanism of rolling involves L-selectin binding to peripheral node addressin (PNAd) on HEVs. Activated platelets are shown to bind to circulating lymphocytes and to mediate rolling in HEVs, in vivo, through another molecule, P-selectin, which also interacts with PNAd. In vitro, activated platelets enhanced tethering of lymphocytes to PNAd and sustained lymphocyte rolling, even in the absence of functional L-selectin. Thus, a platelet pathway operating through P-selectin provides a second mechanism for lymphocyte delivery to HEVs.

Lymphocytes continuously migrate from the blood to peripheral lymph nodes (PLNs) and other lymphoid organs. This “homing” event is mediated by sequential engagement of tissue-specific adhesion and activation pathways (1). Homing to PLNs, for instance, is dependent on the lymphocyte homing receptor L-selectin (2), which interacts with a ligand on HEVs that is defined by the monoclonal antibody (mAb) MECA-79 (3) in mouse and human. Both MECA-79 and recombinant L-selectin-immunoglobulin chimeric protein precipitate sulfated and sialylated glycoproteins that are collectively known as the peripheral node addressin (PNAd) (4, 5). Affinity-purified PNAd glycoproteins mediate L-selectin–dependent lymphocyte rolling in vitro (6, 7). Except for its physiologic expression in HEVs of PLNs, venular PNAd expression is also induced in several chronic inflammatory disease states (8). Some of these pathologic conditions, such as inflammatory bowel disease, are also associated with an increase in activated platelets both in the circulation and at vascular sites of inflammation (9). Because activated platelets interact with both leukocytes and endothelial cells in vitro, we examined whether activated platelets in the bloodstream can alter lymphocyte behavior in HEVs.

We used an in vivo model to study lymphocytes in murine PLN HEVs by intravital microscopy (10). A mAb to L-selectin, Mel-14 (2), inhibited rolling of fluorescently labeled white blood cells (WBCs) by 80 to 90% [Fig. 1 and (11)], confirming the important role of this molecule in lymphocyte homing. A subsequent injection of resting human platelets (12) into the arterial bloodstream did not detectably alter WBC behavior in HEVs (13). In contrast, platelet activation before injection resulted in a marked reappearance of endogenous rolling WBCs despite the continued presence of mAb to L-selectin. These findings indicate a previously uncharacterized platelet-mediated mechanism for WBC adhesion to HEVs. Because the phenomenon was apparently L-selectin–independent, our observations further suggest that activated platelets may alter the composition of lymphocyte populations that are recruited to PLNs and other sites containing HEVs.

To define the molecular basis for platelet-dependent WBC rolling, we initially investigated the role of P-selectin (14). This molecule is expressed on the surface of activated, but not resting, platelets and endothelial cells and supports leukocyte rolling and accumulation in acutely inflamed nonlymphoid sites (15). It also mediates leukocyte interactions with activated platelets that are in circulation or bound to thrombogenic surfaces (16). When activated platelets were first incubated with mAb WAPS 12.2 directed against human, but not murine, P-selectin (12), injection of treated cells did not induce WBC rolling in anti-L-selectin–treated mice. Thus, P-selectin expressed on platelets, but not on the animals’ endothelial cells, was responsible for WBC rolling.

To identify the target cells and ligands for platelet P-selectin, we labeled human platelets fluorescently to directly observe their intravascular behavior. Resting platelets rarely bound to HEVs or other cells in the bloodstream (13). Activated platelets, in contrast, frequently interacted with both WBCs and the venular lining. Numerous rolling WBCs were detected with one or more brightly fluorescent platelets attached to their surface. Treatment of animals with mAb Mel-14 blocked rolling of WBCs free of surface-bound platelets. In contrast, the rosettes of WBCs and platelets persisted to roll in a cartwheeling fashion, suggesting an indirect mode of WBC adhesion to HEVs through bridging platelets. This mechanism is supported by the finding that a large number of activated platelets could bind directly to endothelial cells without WBC association. These interactions were characterized by slow rolling, occasionally followed by stationary adhesion (Fig. 2, A, C, and D), and were nearly abolished by mAb WAPS 12.2 (inhibition of 80 ± 9%, mean ± SD), indicating that platelet P-selectin was responsible. In contrast, endothelial P-selectin, implicated in platelet rolling in other vascular beds (17), was not required; activated human platelets rolled in HEVs of P-selectin–deficient mice (13). Furthermore, platelet rolling mediated by platelet P-selectin in HEVs was associated with the formation of stronger or a larger number of bonds (or both) than platelet rolling on endothelial P-selectin because the mean rolling velocities measured for the former were reduced by 80% compared with those reported for the latter (17).

To show that platelet rolling in HEVs was not the result of P-selectin interactions with ligands on endothelium-bound leukocytes such as P-selectin glycoprotein ligand 1 (PSGL-1; 18, 19), we first treated some

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First infusion Second infusion

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Fig. 1. Activated platelets mediate rolling of anti-L-selectin–treated mouse leukocytes in HEVs. Blood-borne nucleated cells (lymphocytes and granulocytes) were fluorescently labeled by iv injection of rhodamine 6-G and visualized in HEVs of a sublethal Ln by fluorescence microscopy. Treatment of mice with mAb Mel-14 (100 µg) significantly reduced the WBC rolling fraction (the percentage of rolling cells in the total flux). Injection of thrombin receptor activating peptide (TRAP)-stimulated human platelets markedly increased tethering and rolling of endogenous WBCs. Blockade of human P-selectin function by mAb WAPS 12.2 abolished this effect of activated platelets. Values shown are the mean ± SD of nine venules in three animals.
animals for 30 min with mAb Mel-14 to block leukocyte binding to HEVs. P-selectin–dependent platelet interactions persisted in the presence of anti-L-selectin and were equivalent to those seen in untreated animals (rolling fraction of 73 ± 8% versus 74 ± 18%, respectively; mean ± SD). Treatment of mice with mAb MECA-79, which reduces L-selectin–dependent homing of lymphocytes (3), also reduced adhesion of activated platelets by ~70%, suggesting that L- and P-selectin can share the same endothelial ligand, or ligands, on HEVs.

Because platelets express other adhesion molecules, we examined whether expression of cellular P-selectin alone was sufficient to promote rolling in HEVs. L1-2 lymphoma cells transfectcd with human P-selectin [L1-2P-selectin, (20)], but not vector control transfectants (L1-2vector), displayed slow rolling interactions in the identical segments of the lymph node (LN) microvasculature as platelets (Fig. 2B). As shown for activated platelets, mAb MECA-79 also reduced adhesion of L1-2P-selectin (inhibition of 59 ± 5%, mean ± SD), suggesting that P-selectin:PNAAd interactions per se are sufficient for tethering and rolling in vivo.

To show directly that human PNAAd contains a P-selectin ligand, we evaluated platelet and L1-2P-selectin–binding to affinity-purified PNAAd from human tonsils in vitro. Both activated platelets and L1-2P-selectin were tethered to PNAAd at a wall shear stress of 1.6 dyne cm⁻² (Fig. 3A). Once attached, >90% of the tethered platelets rolled continuously. Sticking without displacement (>30 s) or skipping rolling motions (repeated detachment and reattachment) occurred rarely (5.6 and 4.1%, respectively). Spontaneous detachment of rolling platelets was not observed. Interactions required Ca²⁺ and were completely inhibited by first treating platelets or L1-2 cells with antibody to P-selectin (mAb WAPS 12.2), but not with isotype-matched control antibody to CD31 (mAb PECAM 1.3) (anti-CD31). As observed in vivo, incubation with PNAAd with mAb MECA-79 also reduced P-selectin-mediated rolling (Fig. 3B). This antibody recognizes sialomucin–like glycoproteins that are decorated with its carbohydrate epitope. Members of the sialomucin family mediate L-selectin–dependent tethering of peripheral blood lymphocytes to purified PNAAd (21, 22) and are required for leukocyte adhesion to P-selectin (18, 19). Consistent with these earlier findings, sialylated, O-glycosylated structures were also essential for P-selectin:PNAAd interactions; O-glyco-protease or neuraminidase treatment of PNAAd abrogated activated platelet attachment in flow.

To date, PSGL-1, a sialomucin expressed on leukocytes (18, 19), is the only known human P-selectin ligand. It is unclear whether PSGL-1 is also expressed on endothelium and, in particular, whether PSGL-1 is a component of PNAAd, which consists of several glycoproteins (65 to 200 kD). To show that a glycoprotein component of PNAAd distinct from PSGL-1 can support P-selectin–mediated adhesion, we examined CD34, a major constituent of murine and human PNAAd (21, 22). Puriﬁed CD34 from human tonsil PNAAd supported P-selectin–mediated tethering and
rolling as observed for the entire mixture of PNAδ glycoproteins (Fig. 3C). However, CD34 is not a prerequisite scaffold for carbohydrate presentation to P-selectin, because its depletion from PNAδ (21) resulted in only a 20% decrease in tethering of platelets compared with nondepleted material under identical biophysical conditions. This result suggests that other constituents of PNAδ also present P-selectin ligand (or ligands), consistent with the concept of parallel contribution of multiple PNAδ components to L-selectin binding (4, 5).

These experiments support an important role for P-selectin in adhesive interactions of activated platelets with HEVs. They also suggest a mechanism for platelet-dependent lymphocyte recruitment to HEVs in which activated platelets bound to PNAδ can capture circulating lymphocytes through high-density expression of P-selectin. As many as 60% of peripheral blood T lymphocytes bear functional ligands for P-selectin (23). Thus, it is likely that platelet P-selectin and endothelial PNAδ act in synergy in supporting lymphocyte recruitment to HEVs through simultaneous interactions with PSGL-1 and L-selectin, respectively. In vitro evidence supports this hypothesis: low-density binding of activated platelets to purified PNAδ resulted in a threefold increase in the rolling flux of lymphocytes as compared with lymphocyte binding in the absence of platelets (Fig. 4). Furthermore, incubation of lymphocytes with mAb Dreg 200 to L-selectin (20 μg/ml) (24) abolished cell attachment to PNAδ in the absence of platelets. In contrast, in the presence of surface bound platelets, mAb Dreg 200 attenuated lymphocyte attachment only to the level found with lymphocytes with functional L-selectin. These experiments also demonstrate that rolling was exclusively mediated by platelets attached to the lymphocyte surface; antibodies to either platelet P-selectin or lymphocyte PSGL-1 (19) completely blocked lymphocyte rolling.

P-selectin on platelet monolayers that cover thrombogenic surfaces can recruit circulating leukocytes in vitro and in vivo (16). However, firmly adherent platelet monolayers are rarely observed on intact endothelial cells, even in chronic inflammation. Our findings suggest a more dynamic interplay between activated platelets and vascular endothelium, characterized by reversible, but continuous, interactions with much smaller amounts of platelet deposition. Such interactions may not be apparent in histologic studies of chronic inflammation. The observation that P- and L-selectin share PNAδ as a common endothelial ligand suggests a unifying mechanism for platelet and lymphocyte recruitment to lymphoid organs and inflamed tissues where PNAδ is expressed. Although it is not surprising that carbohydrate moieties such as PNAδ can bind to more than one selectin (25), this pathway may allow activated platelets to sup-

**Fig. 3.** Purified components of PNAδ from human tonsils support in vitro tethering and rolling of platelets and L-2-selectin cells. (A) Platelets (1 x 10^8 per milliliter) or L-2 transfectants (1 x 10^6 per milliliter) were incubated at 1.6 dynes/cm^2 over polystyrene plates that were coated with affinity-purified human tonsillar PNAδ as described (21). The number of adherent cells per field of view (0.69 mm^2) was quantitated by video analysis. C-type lectin dependence of interactions was confirmed by incubating assay medium containing 5 mM EDTA between consecutive data sets, which resulted in the release of all bound cells. Antibody inhibition studies were done in triplicate by preincubating platelets and L-2 cells with mAb (20 μg/ml; n = 3). (B) Tethering of thrombin-stimulated platelets is blocked by treatment of PNAδ with O-glycoprotease, neuraminidase, or mAb MECA-79, but not mAbs to CD34 (shear stress of 1.6 dynes/cm^2). Interacting cells were counted for 3 min within the identical field of view. Antibody inhibition studies were done by incubating cells or the PNAδ substrate with saturating concentrations of mAb MECA-79 (20 μg/ml) or anti-CD34 mAbs (mixture of 10 μg/ml of each of mAb 547, 563, 581, and My-10) for 15 min before each experiment. For some experiments, a pre-analyzed fraction of PNAδ was incubated with Vibrio cholerae neuraminidase (0.1 U/ml; Calbiochem) in 50 mM NaHPO₄ (pH 6.0), 2 mM CaCl₂, or O-sialoglycoprotease (0.2 mg/ml; Pasteurella hemolytica, Cederlane Lab) in assay medium for 1 hour at room temperature. The substrate was subsequently washed with assay medium, and activated platelets were perfused over the identical field of view. Values shown are the means ± SD of three duplicate experiments. (C) Distinct PNAδ glycoprotein constituents support tethering and rolling of platelets in flow. CD34 was purified from human tonsillar PNAδ (21), immobilized on plastic (200 ng/ml), and tested for the ability to support platelet tethering and rolling in flow (1.6 dynes/cm^2; n = 3). Undepleted material and the PNAδ fraction that was depleted of CD34 (~88% depletion) were used for comparison. Site density and protein concentration of this glycoprotein ligand was determined by saturation binding of iodinated mAb and capture enzyme-linked immunosorbent assay, respectively.

**Fig. 4.** Role of activated platelets in lymphocyte rolling on purified PNAδ. Peripheral blood lymphocytes (PBLs, ~90% T cells after plastic adherence) were incubated (1 x 10^8 per milliliter) at a shear stress of 2.1 dynes/cm^2 before (first infusion) or after (second infusion) activated platelets were allowed to accumulate on the PNAδ substrate (final density of 250 ± 20 platelets per square millimeter, corresponding to ~0.1% of the PNAδ surface area). Three minutes later, the rolling flux of PBLs in the identical field of view (0.12 mm^2) was measured as described (30). Some PBLs were first incubated with mAb Dreg 200 (20 μg/ml) and cell attachment to PNAδ evaluated in the absence and presence of surface bound platelets. Simultaneous engagement of L- and P-selectin on lymphocytes and platelets, respectively, resulted in a threefold increase in PBL rolling. Blockade of L-selectin function abolished PBL rolling in the absence of platelets. In contrast, the low number of adherent platelets maintained rolling of PBLs even without a contribution by L-selectin at levels that were comparable to rolling of untreated PBLs in the absence of platelets. The requirement for PSGL-1-P-selectin interactions in platelet-mediated recruitment of mAb Dreg 200-treated T lymphocytes is shown by the inhibitory effects of P-selectin mAb WAPS 12.2 and the PSGL-1 function blocking mAb PL1 (19), but not mAb PL2 (nonblocking), on T cell adhesion. Error bars represent the SD of three experiments performed in triplicate.
port a constant influx of WBCs into chronically inflamed lesions. This may provide an important synergistic mechanism to enhance the recruitment of pathophysiologically relevant lymphocyte subsets such as memory cells that express little or no L-selectin.

Delivery of WBCs by way of activated platelets may not be limited to venules that express PNAdh. P-selectin-mediated platelet adhesion to endothelium may also occur in acutely inflamed nonlymphoid vascular beds where immunologically distinct L-selectin ligands have been observed (26). Moreover, recent in vivo studies have shown that exposure to atherogenic stimuli such as oxidized low density lipoprotein or cigarette smoke induces rapid P-selectin-dependent aggregation and accumulation of leukocytes and platelets in arteries and arterioles (27). In light of our present results, it is reasonable to speculate that activated platelets may have the capacity to deliver leukocytes to vascular beds such as arteries that may not express selectins or selectin ligands but do have receptors for other platelet adhesion molecules (28).

REFERENCES AND NOTES
10. Mice were anesthetized by intraperitoneal injection (0.2 ml) of a mixture of ketamine (5 mg/ml) and xylazine (1 mg/ml), and catheters were inserted into the right jugular vein (for injection of anesthesia and mAb), the left carotid artery (for blood pressure monitoring), and the right femoral artery (for retrograde injection of fluorescently labeled cells). Subsequently, the left subscapular LN was microsurgically prepared for intravital microscopy as described (J. H. von Andrian, Microcirculation, in press). The preparation was transferred to a custom-built intravital microscope (IV-600; Micron Instruments, San Diego, CA). For microscopic observation of un SCE S SCP hyperemic microvascular WBC behavior, a bolus injection of sterile Ringer’s lactate (10 ml per kilogram of body weight) containing the fluorescent dye-6-dimodamine 6G (2.2 mg/ml; Molecular Probes) was given intravenously. Rhodamine 6G stains nuclei and mitochondria in living cells, thereby permitting visualization of interacting and freely flowing leukocytes in LN HEVs by fluorescent stroboscopic epillumination through a 20X Zeiss objective. Video scenes were recorded through a low-lag SIT camera (Dage MTI) and stored on HI 8 videotape for off-line analysis of rolling fractions, rolling velocities, and microvascular hemodynamics as described (J. H. von Andrian et al., Am. J. Physiol. 263, H1034 (1992)). After a 3-5 min control recording of HEVs, some animals were treated with mAb 0.9 μg per mouse, intravenously (i.v.), and leukocyte behavior in the same vascular bed was recorded 15 min later.
12. Human platelets were purified from the blood of healthy donors by standard procedures. Aliquots of platelets were stimulated with human thrombin (Sigma; 0.5 U/ml for 5 min) or TRAP (Bachem; 25 μM for 5 min) in the presence of 2 mM Arg-Gly-Asp-Ser (FGDS) (Peninsula Labs) to minimize aggregation. Excess thrombin was neutralized with hirudin (Sigma; 0.5 U/ml, 5 min, 37°C) before injection into the animal. Activation status of platelets was confirmed by flow cytometry of P-selectin expression. Aliquots of activated platelets were incubated with mAb WAPS 12.2 (20 μg/ml) [M. A. Jutila et al., J. Immunol. 153, 5917 (1994)] or control mAb PECA-1 [1.7.2-βis-(2-carboxethyl)-S-(and-6-carboxyfluorescein) (BCECF) Molecular Probes] as described (T. G. Diaco, A. R. de Fougerolles, D. A. Bainton, T. A. Springer, J. Clin. Invest. 94, 1243 (1994)]. Fluorescent platelets were injected through the femoral artery catheter into the arterial bloodstream supplying the node. In some experiments (n = 3), mAb MECA-79 (100 μg per mouse) was injected after assessment of rolling of un-treated HEVs. After allowing mAb binding to PNAH for 10 min, freshly activated platelets or transfectants were again injected to assess the effects of mAb treatment.
13. T. G. Diaco and U. H. von Andrian, data not shown.
20. A CDNA encoding full length P-selectin was isolat- ed by polymerase chain reaction from the human megakaryocyte cell line HEL 92.17 (American Type Culture Collection). Sequence fidelity was verified by restriction analysis and sequencing (14). The P-selectin cDNA was subcloned into pMFB101 to stably transfet murine L1-2 pre-B cells as described (40). High P-selectin-expressing subclones were grown in RPMI 1640 containing 10% fetal calf serum and standard supplements. For in vivo experiments, transfertants were labeled with BCECF, resuspended in RPMI (1 x 107 per milli- ter), and injected into mice as described (12).
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