Hepatic sinusoidal endothelium avidly binds platelets in an integrin-dependent manner, leading to platelet and endothelial activation and leukocyte recruitment

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Lalor PF, Herbert J, Bicknell R, Adams DH. Hepatic sinusoidal endothelium avidly binds platelets in an integrin-dependent manner, leading to platelet and endothelial activation and leukocyte recruitment. Am J Physiol Gastrointest Liver Physiol 304: G469–G478, 2013. First published December 20, 2012; doi:10.1152/ajpgi.00407.2012.—Platelets have recently been shown to drive liver injury in murine models of viral hepatitis and promote liver regeneration through the release of serotonin. Despite their emerging role in inflammatory liver disease, little is known about the mechanisms by which platelets bind to the hepatic vasculature. Therefore, we referenced public expression data to determine the profile of potential adhesive receptors expressed by hepatic endothelium. We then used a combination of tissue-binding and flow-based endothelial-binding adhesion assays to show that resting platelets bind to human hepatic sinusoidal endothelial cells and that the magnitude of adhesion is greatly enhanced by thrombin-induced platelet activation. Adhesion was mediated by the integrins GpIb, α6β1, and α4β3, as well as immobilized fibrinogen. Platelet binding to hepatic endothelial cells resulted in NF-κB activation and increased chemokine secretion. The functional relevance of platelet binding was confirmed by experiments that showed markedly increased binding of neutrophils and lymphocytes to hepatic endothelial cells under shear conditions replicating those found in the hepatic sinusoid, which was in part dependent on P-selectin expression. Thus the ability of platelets to activate endothelium and promote leukocyte adhesion may reflect an additional mechanism through which they promote liver injury.

Hepatic sinusoidal; human integrin; liver

HEPATIC INFLAMMATION IN RESPONSE TO liver injury drives chronic hepatitis, fibrogenesis, and cirrhosis (8, 13, 17, 24, 49). Persistent inflammation at any site, including the liver, is the result of an accumulation of leukocytes, which become organized into stable inflammatory infiltrates (2). For this to occur, leukocytes must be recruited into tissue from the circulation, following adhesion to endothelium from flow and transmigration through the endothelial barrier. The complex microvascular anatomy of the liver allows leukocytes to bind endothelium at three anatomical sites, the portal tract, the sinusoids, or the terminal hepatic veins (63) although the majority of recruitment occurs through the sinusoids (71). In response to injury, recruitment increases as a consequence of endothelial activation (9, 25).

Platelet adhesion to the vascular wall occurs at sites of endothelial cell loss to prevent bleeding and vascular leak.

However, recently it has become apparent that platelets can, under some circumstances, bind to endothelial cells, where they can support leukocyte recruitment to the vessel wall (28, 30) (reviewed in Ref. 62). In L-selectin-deficient mice, activated platelets can reconstitute P-selectin-dependent lymphocyte homing to lymph node high endothelial venules (14). Hepatic endothelial cells fail to express P-selectin even when inflamed, and a similar mechanism could provide a P-selectin substrate within hepatic sinusoids in the absence of endothelial P-selectin (1, 68). Platelets are sequestered in the liver following experimental transplantation (12, 73) and correlate with graft survival (12). Similarly increased binding of platelets to sinusoidal endothelium and enhanced neutrophil recruitment is observed in mice exposed to LPS (27) and following I/R injury (49), and serum from cirrhotic patients contains elevated levels of von Willebrand factor (vWF) and promotes platelet binding to collagen (41). Despite the recent evidence implicating platelets in liver injury and inflammation, little is known about the molecular regulation of platelet adhesion to hepatic endothelium within the low-perfusion sinusoidal bed. We now report the molecular regulation of platelet adhesion to human hepatic endothelium and demonstrate that adherent platelets activateNF-κB-dependent endothelial chemokine and adhesion molecule expression and act as a bridge to enhance leukocyte recruitment.

MATERIALS AND METHODS

Platelet isolation. Platelets were isolated using standard methods from whole blood of healthy volunteers who had not taken aspirin or other platelet-modulating agents in the previous 10 days (28).Platelet-rich plasma (PRP) was prepared by centrifuging EDTA anticoagulated blood at room temperature for 5 min at 290 g, and platelets were pelleted by centrifuging the PRP at room temperature for 5 min at 1,800 g. The platelet-rich pellet was washed in PBS without calcium chloride and magnesium chloride (Sigma) containing 5 mM glucose and 6 mM theophylline (both Sigma) and resuspended to a final concentration of 1 × 10^5 platelets/ml in Ca/Mg-free PBS for static adhesion assays or serum-free and protein-free hybridoma medium, containing 0.15% bovine serum albumin (both Sigma) for flow-based adhesion assays.

Human liver tissue. All tissue samples used were obtained from The Liver Unit, Queen Elizabeth Hospital in Birmingham with informed consent and approval from the Birmingham Ethics Committee. Normal liver samples were surplus to surgical requirements, and diseased livers were explanted during transplantation or regraft surgery for alcoholic liver disease (ALD) or primary biliary cirrhosis (PBC).

Endothelial cell isolation and culture. Primary cultures of human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained from Birmingham Women’s Hospital and pre-
pared according to previously described methodology (26). Human hepatic sinusoidal endothelial cells (HSEC) were isolated in house from ~50 g of human liver tissue. Isolation was performed as previously described (33, 34). Briefly, nonparenchymal cells were collected after collagenase digestion of mechanically disaggregated liver and were further purified by density gradient centrifugation over Percoll. Endothelial cells were isolated from the resultant heterogeneous cell mixture by positive immunomagnetic selection using antibodies raised against CD31 (DAKO) and magnetic beads (Dyna) conjugated with goat anti-mouse antibody according to the manufacturer’s protocol. All endothelial cells were maintained in complete media comprising Human Endothelial-SFM basal growth medium (Invitrogen) containing 10^8 U/ml penicillin and 10 μl/ml streptomycin, 10 ng/ml epidermal growth factor (R & D Systems), 10 μg/ml hydrocortisone (Sigma), and either 10% heat-inactivated human serum (TCS Biologicals, for HSEC) or 10% fetal calf serum (Invitrogen, for HUVEC). All endothelial cells were plated out into collagen-coated culture flasks (Sigma) and maintained at 37°C in a humidified 3% CO_2 incubator until confluent. The endothelial cells were used only up to passage 6, and phenotypic identity and purity were confirmed by staining for endothelial markers (35).

SAGE analysis of sinusoidal endothelial cell phenotype. To find genes that might be used by platelets to bind liver endothelium in the absence of vWF, and that are differentially expressed in liver endothelium vs. other types of endothelium, we searched SAGE libraries (55, 65) from the Gene Expression Omnibus database (GEO, Ref. 45). One library from liver containing 77,759 tags was contrasted with five libraries from other sources of endothelium, totaling 241,804 tags. Table 1 lists the SAGE libraries used. To analyze the data, the source code algorithm for the SAGEmap xProfiler tool was downloaded and run locally from the NCBI (ftp://ftp.ncbi.nih.gov/pub/sage/obsolete/source/pp). All the default settings were used except a posterior probability of 0.8 cutoff was used, and the c statistic was set at 3 as previously (29). All SAGE libraries were short 10-bp tags, and tag-to-gene mappings were made using the latest file available from the SAGE download site (46).

Investigation of platelet adhesion to cultured endothelial cells in a static assay. Endothelial cells were grown to confluence on gelatin-coated 2.5-mm-diameter coverslips (Thermanox, Fisher Scientific). The coverslips were fixed in ethanol, and endothelial cells were placed uppermost on a microscopic slide and treated with platelet suspension as described above (1 × 10^6 platelets/ml, 100 μl per coverslip). The adherent platelets were then visualized and photographed using fluorescently labeled CD41 antibody as described above. However, this method only permitted qualitative assessment of the degree of platelet binding. Therefore, for a more quantitative assessment of binding, the number of platelets bound to the luminal side of the endothelial cells was counted using phase-contrast microscopy. Then, a platelet/endothelial cell ratio was calculated using the average of the number of adherent platelets binding to the endothelial cells in a high-power microscope field of view.

Investigation of platelet adhesion to cultured endothelial cells in a flow-based assay. To investigate platelet adhesion to hepatic endothelial cells under conditions of physiological blood flow, we used a flow-based adhesion assay system (36). Briefly, platelet solution (1 × 10^6/ml freshly isolated neutrophils or peripheral blood lymphocytes isolated as described previously (30). Leukocytes were perfused for 5 min before quantification of adherent cells/mm^2 per 10^9 cells perfused (36). Where indicated, we also used a specific inhibitor of P-selectin (KF 38789, 10 μM, Tocris Bioscience) to determine selectivity of neutrophil binding.

Blocking antibodies and peptides. To investigate which molecules were responsible for platelet adhesion to endothelial cells, we employed antibodies and blocking peptides in the assay systems described above. Reagents directed against platelet receptors were added

<table>
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<tr>
<th>Liver libraries</th>
<th>Final GEO Accession</th>
<th>Final Count</th>
<th>SAGE Library</th>
<th>Origin</th>
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<tr>
<td>Anchoring Enzyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaIII</td>
<td>GSM384018</td>
<td>77759</td>
<td>SAGE Vascular endothelium normal liver associated AP NLEC1</td>
<td>Normal liver</td>
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<td>Other endothelial libraries</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>68987</td>
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<td>Glomerular</td>
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<td>GSM384019</td>
<td>51642</td>
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<tr>
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<td>Primary human pulmonary artery endothelial cells</td>
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<tr>
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<td>GSM62240</td>
<td>38446</td>
<td>Human Aortic Endothelial Cell Exposure (Control)</td>
<td>Heart</td>
</tr>
</tbody>
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Table 1. SAGE libraries used
to the platelet suspension 20 min before incubation with the endothelial cells. Antibodies raised against human CD41 (Glycoprotein Ibα, Pharmingen) and CD42a (GPⅠbα, Pharmingen) were used at final concentrations of 5 and 10 μg/ml. Arg-gly-asp (RGD) peptide (A8052, Sigma) was used at 50 and 100 μg/ml and thrombin (Sigma, UK) at a concentration of 0.5 U/ml. Antibodies and blocking peptides directed against endothelial cell receptors were added to cultured endothelium or liver sections 20 min before addition of platelets. Antibody raised against human αvβ3 (Chemicon International) was used at concentrations of 5, 10, and 15 μg/ml, whereas fibrinogen binding peptide (Sigma) was added at concentrations of 50 and 100 μg/ml. To activate platelets in vitro, samples of freshly prepared platelets were treated with 0.5 U/ml thrombin (Sigma) for 20 min, washed, and then incubated on tissue sections as normal.

ELISA to detect chemokine expression. Production of CCL2 (monocyte chemotactant protein-1) and CXCL8 (IL-8) by HSEC in response to binding of platelets was determined using a capture ELISA (R&D Systems) according to the manufacturer’s instructions. Confluent monolayers of HSEC grown in 24-well plates were incubated with control serum-free media or platelets (0.05 × 10^6-0.1 × 10^6/ml) for 4 h. Cell-free supernatants were collected and stored at −80°C until ELISA development.

Measurement of endothelial NF-κB activation. To determine whether binding of platelets to HSEC induced activation of NF-κB, we used a commercially available ELISA-based reporter assay (NF-κB p50 Transfactor Kit, Clontech) according to the manufacturer’s instructions. HSEC were exposed to 1 × 10^6 platelets/ml or media alone for 10 min before trypsinization and generation of nuclear and cytoplasmic extracts. Protein concentration of extracts was calculated using a micro Lowry assay (Sigma) according to manufacturer’s instructions. Binding of NF-κB in nuclear extracts to consensus sequences in DNA immobilized to the ELISA plate was quantified colorimetrically, and signals were compared with binding to scrambled DNA sequences. Stimulation of HSEC with TNF-α was used as a positive control.

Statistical analysis. For functional data, standard error was used to express variation about the mean, and an unpaired Student’s t-test was used to assess significance. A value of P < 0.05 was classified as significant for all investigations. To measure statistical significance of differentially expressed genes between the endothelial sources in our SAGE analysis, two methods were employed. First, the SAGEmap algorithm was employed that computes a Bayesian posterior distribution for a fold ratio changed in gene expression between two groups of SAGE libraries (38). Second, a recently published likelihood ratio test was also used with a False Discovery Rate procedure to find significantly differentially expressed genes between the two groups of endothelial SAGE libraries (23).

**RESULTS**

**SAGE analysis of sinusoidal endothelium shows reduced expression of vWF, CD31, and αv integrin by hepatic endothelium.** To compare the expression of potential platelet-binding receptors on hepatic and other endothelial cells, genes differentially expressed in liver endothelium compared with pooled data from kidney, breast, lung, and aortic endothelium, SAGE libraries (55, 65) (Table 1) were analyzed from the Gene Expression Omnibus database (GEO, Ref. 45). This analysis generated a list of 1,535 genes with significant differential expression using a FDR Q value ≤0.05 and a posterior probability of ≥0.8. Expression of characteristic sinusoidal endothelial phenotypic indicators CD32b (FCGR2B), LYVE-1 (LYVE1), CLEVER-1 (STAB1), and the mannose receptor (MRC1) (35) was increased in hepatic compared with nonhepatic endothelium (Table 2), confirming the sinusoidal origin of the hepatic endothelium. We then focused on genes implicated in platelet adhesion and showed significantly reduced expression of vWF, the αv integrin subunit, and CD31 by sinusoidal endothelium (Table 2) compared with the nonhepatic cells. ICAM-1, E-, and P-selectin were not differentially expressed between unstimulated liver endothelium and other endothelium in the SAGE library analyses. Supplemental Table S1 shows the full results of the SAGE analyses, listing all genes that were significantly differentially expressed; 864 genes were enriched in liver endothelium vs. other endothelial cell types, and 671 genes were enriched in other endothelial cells vs. liver endothelium. Supplemental material is available online at the *American Journal of Physiology Gastrointestinal and Liver Physiology* website.

Unstimulated platelets bind to human liver endothelial cells. Unstimulated human platelets bound to vessels within fresh snap-frozen liver tissue. Figure 1, A and B, shows binding of fluorescently labeled platelets to liver sinusoids and vessels with minimal adhesion to epithelial cells or stroma. The adherent platelets were localized to vascular endothelial cells in portal and central vessels and were observed on the majority of such vessels in the liver sections studied. Platelets also bound to hepatic sinusoids (see Fig. 1). Additional immunohistochemical detection methods confirmed that platelet binding localized to vascular structures in the portal tracts and to sinusoidal endothelium in both normal and disease liver sec-

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**Table 2. Differentially expressed genes of interest**

<table>
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<tr>
<th>Gene</th>
<th>Product</th>
<th>Q-value</th>
<th>Up/Down regulated in Liver</th>
<th>Posterior Probability</th>
<th>Liver Count</th>
<th>Other Count</th>
<th>Tag</th>
<th>Score of Mapping</th>
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<td>up</td>
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<td>0</td>
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<tr>
<td>MRC1</td>
<td>Mannose receptor, C type 1</td>
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<td>Stabilin 1</td>
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<td>1</td>
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<td>down</td>
<td>1</td>
<td>2</td>
<td>77</td>
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<td>4060121</td>
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<tr>
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<td>Platelet/endothelial cell adhesion molecule (CD31 antigen)</td>
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<td>down</td>
<td>0.84</td>
<td>4</td>
<td>57</td>
<td>CCTCTAGATAT</td>
<td>3015037</td>
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<tr>
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<td>von Willebrand factor</td>
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tions (see Fig. 1C) with increased numbers of platelets binding to vessels in inflamed portal areas in disease. To show that the staining was due to the addition of exogenous platelets, we stained the sections with an antibody against the platelet receptor CD41. No staining for CD41 was detected on normal sinusoidal or vascular endothelium in the absence of exogenous platelets (not shown). We compared platelet binding to liver tissue from patients with inflammatory liver disease including PBC, ALD, and chronic allograft rejection using sections of tonsil as controls (Fig. 2A). The pattern of binding to chronically diseased liver was not significantly different to that seen in normal tissue (Fig. 2B) although adherent platelets tended to aggregate into clumps on diseased tissue (see arrows Fig. 2A), suggesting that components of the diseased liver sections are acting as a stimulus for platelet activation. We also noted that there was a tendency for reduced platelet binding to acutely injured liver in patients with graft failure (regrafted tissue in Fig. 2B).

Adhesion of platelets to liver endothelial cells is integrin dependent. Incubation of platelets for 20 min with RGD-containing peptides resulted in a greater than 50% dose-dependent reduction in binding to both vascular and sinusoidal endothelium, demonstrating the involvement of integrin-mediated adhesion (Fig. 3, A and C). We next used specific monoclonal antibodies raised against either the CD41 α chain or the αIIIβ3 integrin or glycoprotein Ibα (GP1bα, CD42) in the tissue-binding assay. Antibody raised against CD41 had a small inhibitory effect on platelet binding that was less than that observed with RGD peptide; CD42 antibody had no detectable effect (not shown). Because platelets can bind intact endothelium via fibrinogen (44) on the endothelial surface (6), we tested the effect of a fibrinogen-binding inhibitory peptide in the tissue-binding assay. This peptide markedly reduced platelet binding, particularly to the vascular endothelium to a similar level as that seen with RGD (Fig. 3, B and C). A blocking antibody against integrin αvβ3, which is expressed by platelets and can bind fibrinogen (19), had no effect in the tissue-binding assay.
Thus the adhesion experiments using human liver tissue sections suggested that CD41 and fibrinogen were the main factors involved in the adhesion of resting platelets to hepatic endothelial cells. Because of the difficulty in quantifying adhesion to endothelium within tissue sections and the fact that adhesion in vivo occurs under conditions of blood flow, we used flow-based binding assays to isolated human hepatic endothelial cells to pursue these studies further.

Resting and activated platelets adhere to cultured human hepatic endothelial cells under static conditions. We compared the binding seen to HSEC with that observed with HUVEC, which are a frequently used as a model endothelium in experimental studies. Phase-contrast video microscopy was used to calculate the number of platelets binding to each endothelial cell (platelet/EC ratio). Figure 4A shows that platelet adhesion to both endothelial cell lines examined increased with time. Platelet binding was observed after 5-min coincubation with endothelial cells and doubled over the subsequent 75-min incubation. Human liver endothelial cells bound significantly more platelets at all time points studied per endothelial cell compared with HUVEC (Fig. 4). We then used thrombin to activate the platelets and found that this significant activation doubled the number of adherent platelets (Fig. 4B). However, it is likely that we underestimated the number of adherent platelets that bound following thrombin treatment because activation induced platelet aggregation, making the counting of individual platelets difficult.

Adhesion of platelets to endothelial cells under physiological shear stress. We next set up experiments to model platelet interactions with liver endothelium under conditions of shear stress. This is important because, in the static assays, platelets settled on endothelial cells in the absence of blood flow, and this may have permitted interactions, which would not occur under flow in vivo. We used flow rates that correspond to those observed in hepatic sinusoids and post capillary venules. When $1 \times 10^9/\text{ml}$ platelets were perfused over HSEC at 0.1 Pa, binding events per endothelial cell were equivalent to those observed in static assays (Fig. 5A). Use of predetermined optimal concentrations of RGD peptide and blocking antibodies revealed that the adhesive event was mediated at least in part by the integrins $\alpha I I b \beta 3$ and Gp1b (Fig. 5B). An important difference compared with the static assays was the role we found for the integrin $\alpha I b \beta 3$ under flow.

Binding of platelets to HSEC induces activation of NF-$\kappa B$ and release of CCL2 and CXCL8 and promotes adhesion of neutrophils and lymphocytes. Finally, we sought to determine the proinflammatory consequences of platelet binding to...
Finally, we used the flow-based adhesion assay to show which was dependent on the numbers of platelets applied (Fig. 6C). Use of a selective inhibitor of P-selectin confirmed that, at least in part, the enhanced adhesion of neutrophils was dependent on P-selectin expression by the adherent platelets.

DISCUSSION

Platelets promote liver injury in models of hepatitis, and antiplatelet therapy is under investigation for treatment of chronic diseases such as nonalcoholic steatohepatitis (18). However, platelets also serve key hemostatic roles (51) and help neutrophils clear pathogens during sepsis (43). In addition, they modulate fibrogenesis (66) and secrete serotonin and other mediators that support liver regeneration (10, 39). Despite their important multiple roles in liver injury and inflammation, little is known about the mechanisms by which platelets bind to the hepatic vasculature.

Sinusoidal endothelial cells have a unique phenotype, characterized by a lack of Wiebel Palade bodies and consequent failure to express P-selectin and low levels of vWF even in response to inflammation (1, 20, 35, 68, 70) in their mature differentiated state. This combined with the relatively modest shear rate within the sinusoids provides a unique environment for platelet and leukocyte adhesion. Studies suggest that platelet activation is important for adhesion to both sinusoidal and postsinusoidal vessels and that platelet aggregation and perfusion failure can be improved by inhibiting Rho kinase (37). In most vascular beds expression of P-selectin is important for leukocyte adhesion, but the lack of P-selectin expression by HSEC. Figure 6A shows that brief interactions between platelets and HSEC from two independent donors are sufficient to cause activation of NF-κB and translocation of p50 to the endothelial cell nucleus, as detected by increased binding to target DNA. Signal was comparable to that generated following activation of cells with TNF-α, and specificity was confirmed by decreased signal in the presence of a competitive inhibitor of NF-κB binding and to a mutated consensus sequence. Endothelial activation resulted in an increase in secretion of CCL2 and CXCL8 in response to binding of platelets, which was dependent on the numbers of platelets applied (Fig. 6B). Finally, we used the flow-based adhesion assay to show that when platelets were prebound to HSEC monolayers, increased numbers of lymphocytes, and particularly neutrophils were recruited by the endothelial cells (Fig. 6C). Use of a selective inhibitor of P-selectin confirmed that, at least in part, the enhanced adhesion of neutrophils was dependent on P-selectin expression by the adherent platelets.

**Fig. 4.** Resting and activated platelets bind to isolated, cultured endothelial cells under static conditions. Platelet binding was assessed using phase-contrast microscopy. At least 10 fields of view were counted to calculate the average number of platelets binding per endothelial cell (Platelet/EC ratio). A: data generated experiments where resting platelets were allowed to bind to hepatic sinusoidal endothelial cells (HSEC) or human umbilical vein endothelial cells (HUVEC) for up to 75 min. ANOVA revealed significantly increased binding to HSEC vs. HUVEC (**P < 0.001). B: adhesion of both resting and thrombin activated (0.5 U/ml thrombin pretreatment) platelets to HSEC and ANOVA indicated a significant stimulatory effect (***P < 0.0001). All data are means ± SE of 3 replicate experiments.

**Fig. 5.** Integrin-dependent binding of platelets bind to resting endothelial cells under conditions of shear stress. Platelets were perfused over endothelial cells for 5 min at a wall shear stress of 0.1 Pa. Nonadherent platelets were then washed free of the system before assessment of the platelet/EC ratio as before. A: binding of resting platelets from matched donors under static or flow conditions. Data represent means ± SD of 2 replicate experiments with different platelet donors. B: binding of platelets per endothelial cell expressed as a percentage of control binding. Platelets were perfused over endothelial cells for 5 min at a wall shear stress of 0.1 Pa in the absence or presence of fibrinogen-binding peptide (100 μg/ml, Fg peptide) or blocking monoclonal antibodies raised against αIibβ3 integrin (10 μg/ml), Gp1b (10 μg/ml), or αvβ3 integrin (av Block, 10 μg/ml). Data represent means ± SE of 4 replicate experiments. All reagents significantly inhibited adhesion compared with untreated controls (ANOVA, *P < 0.05 or **P < 0.01).
hepatic sinusoids (68) means it is less likely to be important within the liver. Thus we used human hepatic sinusoidal endothelial cells and ex vivo human tissue to determine the molecular regulation of platelet:HSEC binding and the possible proinflammatory consequences of this interaction.

We found that resting platelets can bind to both vascular and sinusoidal vessels in normal liver tissue. We observed more binding to vascular endothelium in the portal tract compared with binding to sinusoids. Previous studies in liver ischemia/reperfusion (58, 59, 73) have reported platelet sequestration on periportal and midzonal sinusoidal endothelium (11, 57), but other studies in cholestatic liver injury report adhesion within both sinusoids and postsinusoidal vessels (37) consistent with the binding we observed to PBC tissue (Figs. 1 and 2). The absence of physiological shear stress in the tissue-binding experiments allows cells to settle on the vessels without the requirement for capture and removes the effect of shear stress on subsequent adhesive interactions with endothelium, leading us to study interactions using cultured human sinusoidal endothelial cells under physiological shear stress.

Resting platelets bound readily to primary hepatic sinusoidal endothelial cells under flow, confirming that platelets are able to bind to endothelium in the microvascular sinusoidal circulation despite low expression of some important adhesion receptors. The molecular basis of binding involves integrins because we could block it with RGD peptides or anti-integrin antibodies. RGD peptides have previously been shown to block platelet adhesion to human saphenous vein cells and HUVEC in vitro (6, 40, 53). However, in these investigations, either the platelets or the endothelial cells were activated before the adhesive event. To study interactions between resting platelets and endothelium, we used theophylline to maintain platelets in a quiescent state (28) and then used thrombin to study activated cells. Thrombin activation led to increased adhesion to endothelial cells and to a degree of platelet aggregation comparable to the aggregated platelet phenotype seen when platelets bound inflamed liver tissue. Activation of adherent platelets has also been observed in vivo following liver transplantation (52), suggesting that factors in inflamed liver can activate platelets upon contact (16). This may also happen in liver disease because, although circulating platelets in cirrhotic patients are functionally normal, serum from such patients contains elevated levels of vWF and promotes platelet binding to collagen (41). Our studies on liver tissue binding suggest that fibrinogen within the liver microenvironment may promote platelet activation and adhesion in situ. Fibrinogen has roles in both platelet adhesion and aggregation, and it is transiently released from activated platelets from where it can bind to the platelet surface via interactions with αIIbβ3 integrin (50, 67). Fibrinogen can also accumulate on the surface of endothelial cells

Fig. 6. Binding of platelets to HSEC induces activation of NF-κB and release of CCL2 and CXCL8 and promotes P-selectin-dependent adhesion of neutrophils and lymphocytes. A: colorimetric quantification of NF-κB activation was performed using a commercially available ELISA (NF-κB p50 Transfactor Kit, Clontech) according to manufacturer’s instructions. Nuclear extracts were generated from endothelial cells (2 independent donors) following stimulation with platelets (1 × 108/ml, 10 min) or TNF-α (10 ng/ml, 30 min) or unstimulated controls. Binding of NF-κB p50 to consensus or scrambled (mut) DNA was quantified. A competitive inhibitor supplied in the kit (platelets+inhibitor) was used to confirm specificity of signal. Data represent means ± SD absorbance at 655 nm. Statistical analysis was not performed, as samples from only 2 donors were tested. B: production of CXCL8 and CCL2 was determined using commercially available ELISA kits (R&D Systems HIS800 and DCP00, respectively) according to manufacturer’s instructions. Supernatants were collected from unstimulated EC or cells incubated with indicated numbers of platelets for 4 h. Data represent means ± SD chemokine production (pg/ml) in triplicate samples with 2 HSEC donors. *p < 0.05, **p < 0.01. C and D: platelets were perfused over endothelial cells for 5 min at a wall shear stress of 0.1 Pa. Nonadherent platelets were then washed free of the system before perfusion of 1 × 108/ml freshly isolated neutrophils (PMN) or peripheral blood lymphocytes (PBL) for 5 min (C). Leukocyte adhesion was counted and expressed as adherent cells/mm2/106 cells perfused on EC alone (EC+) or EC with preimmobilized platelets (EC+Plat). Data represent means ± SE chemokine production (pg/ml) in triplicate samples with 2 HSEC donors. *p < 0.05, **p < 0.01. C and D: platelets were perfused over endothelial cells for 5 min at a wall shear stress of 0.1 Pa. Nonadherent platelets were then washed free of the system before perfusion of 1 × 108/ml freshly isolated neutrophils (PMN) or peripheral blood lymphocytes (PBL) for 5 min (C). Leukocyte adhesion was counted and expressed as adherent cells/mm2/106 cells perfused on EC alone (EC+) or EC with preimmobilized platelets (EC+Plat). Data represent means ± SE from 4 replicate experiments with independent blood donors, and paired r-test indicated significant enhancement of neutrophil adhesion to HSEC in the presence of platelets **p < 0.01. D: binding of neutrophils to endothelium alone (control) endothelium and immobilized platelets (platelets) or endothelium and immobilized platelets pretreated with P-selectin inhibitor (P-selectin block, KF 38789, 10 μM). Paired r-test indicated significant enhancement of neutrophil adhesion to HSEC in the presence of platelets and significant inhibition by KF38789, ***p < 0.001 for both.
following reperfusion injury where it can act as a focus for the adhesion of platelets (44), consistent with our findings that adhesion of platelets to vessels in liver sections was significantly reduced in the presence of a fibrinogen blocking peptide. The peptide also blocked adhesion under flow to HSEC but had little effect on binding to HUVEC. Endothelial cells within tissue will have been exposed to fibrinogen in circulating blood and exposure is increased during inflammation and liver disease (42, 64); thus fibrinogen may have been deposited on the hepatic endothelial cells in contrast to those cultured in normal serum in vitro.

Although the results of the static adhesion assays yield important insights into the mechanisms of platelet binding to endothelium, interactions in vivo occur under the constraints of blood flow, and we thus established a more physiological shear stress-dependent assay to model the in vivo environment and used it to demonstrate that resting platelets can indeed bind to unstimulated endothelium from flow. Approximately six platelets bound to each endothelial cell, and, although this appears to be a relatively low level of coverage, it has been demonstrated that coverage of less than 1% of the endothelial cell surface with adherent platelets significantly enhances neutrophil adhesion (28). Thus equivalent levels of platelet binding in the liver in vivo could precipitate leukocyte recruitment into grafted or injured tissue. Use of RGD peptide and blocking antibodies against the integrins GPIbα, αIIbβ3, and endothelial αvβ3 revealed that these receptors all contributed to platelet adhesion under shear stress. Platelet GPIbα binds vWF and can operate at high levels of shear stress such as those found in larger arteries (54). In addition, signaling through this receptor can also activate platelet β3 integrins. The platelet receptor GPIbα is composed of four different polypeptide chains (GPIbα, GpIbβ, GpIIX, and GpV); GPIbα contains the binding sites for vWF (54, 69), as does the integrin αIIbβ3 (21) although this interaction requires platelet activation. Thus the GPIbα-dependent platelet adhesion to HSEC was interesting in the context of low levels of vWF expression (21). The expression of vWF by HSEC increases as cells dedifferentiate in culture (35), and deposition on endothelial cells (5) may provide sufficient protein to support platelet interactions. However, the low shear stress used for our experiments and evidence that knockout mice deficient in both fibrinogen and vWF still demonstrate normal platelet deposition and thrombus formation in response to experimental vessel injury (47) suggest minimal contribution to initial adhesion. In addition, it has been demonstrated that some endothelial cells can express the GPIbα receptor (69), which can interact with activated platelets. It is possible, therefore, that unbound GPIbα antibody perfused with the platelet solution may interact with endothelial GPIbα.

We also demonstrated roles for the integrins αIIbβ3 and αvβ3 in platelet adhesion to hepatic endothelial cells under low shear conditions consistent with evidence from other microvascular beds (reviewed in Ref. 48). Previous studies using human saphenous vein endothelial cells have shown that thrombin-treated platelets use αIIbβ3 to interact with activated endothelial cells (40), but ours is the first demonstration of a role for this receptor in the adherence of platelets to unstimulated hepatic endothelium. αvβ3 integrin is expressed on platelets and endothelial cells (3, 15), where it mediates RGD-dependent adhesion to fibrinogen, fibronectin, vitronectin, and vWF (4). Our SAGE analysis and previous reports (7, 56) suggest that αvβ3 is expressed at low levels on sinusoidal endothelial cells, but it is clearly present at a sufficient concentration to support platelet adhesion in vitro.

Finally, we studied the functional consequences of platelet: HSEC binding for leukocyte recruitment by studying lymphocyte and neutrophil adhesion to platelet-bound endothelial cells under flow. We found that platelet binding triggered NF-κB-dependent secretion of the chemokines CXCL8 and CCL2, which could promote neutrophil and lymphocyte adhesion, respectively (22). Parallel hepatic NF-κB p50 and p65 activation occurs in liver injury (74), and sinusoidal endothelial cell p65 nuclear translocation leads to chemokine secretion (75). Our enhanced activation of NF-κB p50 in nuclear extracts from HSEC stimulated by platelet binding supports the role of this transcription factor in sinusoidal chemokine release and inflammation. Hence, platelet binding enhanced leukocyte adhesion to unstimulated hepatic sinusoidal endothelium, which express basal levels of VCAM-1 and ICAM-1 but no P-selectin in culture (Table 2). Our inhibitor data supports the idea that adherent platelets may provide a P-selectin bridge for leukocyte adhesion (11, 52, 72) although other adhesion receptors likely also play a role. Release of chemokines by activated endothelium or presentation of platelet-derived chemokine then permits immune cell integrin activation and binding to VCAM-1 and ICAM-1.

In conclusion, we have demonstrated that resting platelets can adhere to human hepatic endothelial cells using the integrins αIIbβ3 and αvβ3 under physiological shear stress. Our findings explain how platelets bind to hepatic vessels and suggest that such interactions promote immune cell recruitment by triggering chemokine secretion and providing an adhesive substrate for circulating leukocytes. These findings provide further evidence that antiplatelet therapy might modify liver injury in inflammatory liver disease (18, 61).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: P.F.L. and D.H.A. conception and design of research; P.F.L. and J.M.H. performed experiments; P.F.L. and J.M.H. analyzed data; P.F.L. prepared figures; P.F.L. drafted manuscript; P.F.L. and D.H.A. edited and revised manuscript; P.F.L., J.M.H., R.B., and D.H.A. approved final version of manuscript.

REFERENCES


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