Assessment of the mechanism of juxtacrine activation and adhesion of leukocytes in liver microcirculation

JULIANA CARVALHO-TAVARES, ALISON FOX-ROBICHAUD, AND PAUL KUBES
Immunology Research Group, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Leukotriene C4 (LTC4), histamine, and other mediators can induce expression of P-selectin and platelet-activating factor (PAF) on venular endothelium to recruit leukocytes in vivo and in vitro via a juxtacrine mechanism of adhesion. The objective of this study was to assess the effect of histamine and LTC4 on the leukocyte recruitment in the liver and to study the components and molecular mechanisms involved in this process. We visualized the hepatic microvasculature using intravital microscopy and determined that LTC4 (20 nM) but not histamine (0.1, 0.3, or 1 mM) induced leukocyte recruitment in the liver microcirculation. Histamine could induce leukocyte recruitment but only in the presence of an antihistaminase. The LTC4-induced leukocyte recruitment occurred primarily in sinusoids (not venules) and was not inhibitable by three different anti-P-selectin antibodies (SH1, RMP-1, and RB40). Leukocyte recruitment in P-selectin-deficient mice, intercellular adhesion molecule 1 (ICAM-1)-deficient mice, and mice treated with a PAF antagonist was of the same magnitude as in wild-type animals in response to LTC4. Although PAF alone could induce adhesion in both sinusoids and postsinusoidal venules, this chemotactic agent was not involved in LTC4-induced adhesion in the liver. Finally, an overlapping role for P-selectin and ICAM-1 was ruled out as LTC4 induced leukocyte recruitment in P-selectin and ICAM-1-deficient mice. These data demonstrate that LTC4 does not activate the known early mechanisms of leukocyte recruitment, including P-selectin, PAF, or ICAM-1 in the hepatic microvasculature.

The recruitment of leukocytes from the circulation to the endothelial interface is the hallmark feature of inflammation. It is well established that the recruitment of circulating leukocytes is dependent on a multistep cascade of events involving adhesion molecules. The initial step in this process, namely rolling, occurs when the leukocyte makes contact with the endothelial cells and rolls along the venular wall. The rolling event in very acute inflammatory conditions is principally mediated by P-selectin. P-selectin, a glycoprotein stored in Weibel-Palade bodies of vascular endothelial cells (15, 36), is rapidly translocated to the surface of these cells, on activation by histamine, leukotriene C4 (LTC4), and other mediators (1, 10, 33, 36). Once a cell is rolling on P-selectin, it can interact with activating molecules expressed on the endothelial surface, such as platelet-activating factor (PAF), leading to activation of the leukocyte integrins, which bind to intercellular adhesion molecule 1 (ICAM-1), a process termed juxtacrine activation. This paradigm for leukocyte recruitment appears to be critical in venules with brisk blood flow and/or with diameters larger than leukocytes such that tethering and rolling are an absolute requirement for adhesion. Indeed, this mechanism was first shown in the mesentery (20, 21, 24) and then in cremaster (8, 17, 23) and skin (30) but may not be relevant in tissues like the liver that support significant adhesion in the sinusoids (38). These vessels have diameters smaller than leukocytes and slow flow rates relative to postcapillary venules.

The hepatic microcirculatory unit consists of afferent vessels, represented by portal venules and hepatic arterioles, the capillary sinusoids, and the postsinusoidal venules (terminal hepatic venule or central venule). Previous work (38) has demonstrated leukocyte adhesion in response to bacterial by-products such as N-formylmethionyl-leucyl-phenylalanine (fMLP) or lipopolysaccharide (LPS) primarily in the sinusoids with less adhesion in postsinusoidal venules. Furthermore, mice lacking both P- and E-selectin that were treated with anti-L-selectin antibodies to inhibit all selectin function, recruited leukocytes in sinusoids to the same extent as wild-type mice in response to fMLP or LPS (38). These data suggest that selectins were not required for fMLP- or LPS-induced leukocyte recruitment in the liver. It is possible that fMLP and LPS directly activate leukocytes to trap in liver sinusoids, whereas mediators such as histamine and LTC4, which selectively target the endothelium to rapidly upregulate P-selectin and PAF, may use a similar recruitment paradigm to that found in other vascular beds.

Therefore, the aims of this study were to use intravital microscopy to directly visualize leukocyte trafficking in the hepatic microcirculation to systematically assess 1) whether histamine and LTC4 can induce leukocyte recruitment in liver, 2) which components of the hepatic microvasculature are involved in this process, and 3) what are the underlying molecular mechanisms. The latter was aimed at assessing specifically the role of P-selectin, PAF, and ICAM-1. Finally, new evidence suggests overlapping function for P-selectin and ICAM-1 in rolling, as the P-selectin- and ICAM-1-deficient mice have less rolling than P-selectin- or ICAM-1-deficient mice alone (22). Based on this observation, we tested whether at lower shear forces, such as that found in the sinusoids, there was not an overlapping role for P-selectin and ICAM-1 such that inhibition of either molecule alone was not sufficient to

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inhibit leukocyte recruitment. This was accomplished using P-selectin and ICAM-1 double-deficient mice.

MATERIALS AND METHODS

Intravital microscopy in mouse liver. Mice deficient in P-selectin were generated by gene targeting in embryonic stem cells as described previously (3, 4). All mice were initially from a mixed background of 129sv × C57BL/6. More recently, all animals were backcrossed six generations to a C57BL/6 background. Because both strains behaved similarly, all mice were grouped together. All the animals were maintained on purified laboratory diet in specific pathogen-free facilities. Animals were anesthetized with a mixture of ketamine and xylazine administered intraperitoneally (200 and 10 mg/kg, respectively) and prepared for intravital microscopy. Briefly, the right jugular vein was cannulated for maintenance of anesthesia during the experiments. A midline incision and a left subcostal incision were made to exteriorize the liver. The hepatic ligaments were dissected, and the intestine was positioned with moist gauze. Animals were placed in a left supine position, and the left liver lobe was fixed under the intravital microscope in position. A silicon-intensified fluorescent camera (model C-2400–08; Hamamatsu Photonics, Hamamatsu City, Japan) was used to observe the microcirculatory events on the surface of the liver. A silicon-intensified fluorescent camera (model C-2400–08; Hamamatsu Photonics, Hamamatsu City, Japan) was mounted on the microscope, and the images were recorded for playback analysis using a video cassette recorder.

Leukocyte parameters. Initially, animals received rhodamine 6G intravenously (0.3 mg/kg body wt) to label leukocytes as previously described (37). Rhodamine 6G-associated fluorescence was visualized by epi-illumination at 510–560 nm, using a 590-nm emission filter.

After the liver was isolated and placed under the intravital microscope, the centrilobular zones were located. Within each field of view (2.1 × 104 mm²) 8–10 centrilobular sinusoids were counted along with three or four terminal hepatic venules (postsinusoidal venules). In a single animal, 10–15 acinar zones were studied. Additionally, in a separate group of animals we examined whether leukocyte adhesion occurred in portal venules, which flow into the sinusoidal vessels. A microscope (Optiphot-2; Nikon, Mississauga, Canada) with a ×40 water immersion lens (40/0.55 WI; Nikon, Tokyo, Japan) was used to observe the microcirculatory events on the surface of the liver. A silicon-intensified fluorescent camera (model C-2400–08; Hamamatsu Photonics, Hamamatsu City, Japan) was mounted on the microscope, and the images were recorded for playback analysis using a video cassette recorder. The number of rolling and adherent leukocytes was determined offline during video playback analysis. Leukocytes are considered adherent to the venular endothelium if they remain stationary for 30 s. Rolling leukocytes were defined as those moving at a velocity less than that of erythrocytes within a given vessel.

Experimental protocol. Immediately after finding an appropriate site, the image was recorded for 5 min, followed by three additional 5-min recordings (10 min apart), during which the experimental parameters were assessed. In the first set of experiments, the liver of wild-type mice were superfused with histamine (0.1, 0.3, or 1.0 mM) for 30 min. The oxidation of histamine by the diamine oxidase pathway can be effectively inhibited by aminoguanidine treatment (32). We used 16 mg/kg of aminoguanidine injected intravenously as previously described (34). In the second group of experiments we studied the role of LTC4 (Cayman Chemical, Ann Arbor, MI) in the liver microcirculation. LTC4 (20 nM) was superfused onto the mouse liver for 30 min, and leukocyte rolling, leukocyte adhesion in postsinusoidal venule, and leukocyte adhesion in sinusoids were assessed.

RESULTS

LTC4 but not histamine induces leukocyte recruitment in liver microvasculature. Previous work has clearly demonstrated that histamine can induce leukocyte rolling and adhesion in vivo via a P-selectin-dependent event in rat mesenteric venules (21). Figure 1 demonstrated that histamine at 0.1, 0.3, and 1.0 mM has no effect on the adhesion of leukocytes inside the postcapillary sinusoids (terminal hepatic-central venules), as well as inside the capillary sinusoids. There was no difference in leukocyte adhesion (venules or sinusoids) over the course of the experiment (30 min) or compared with control animals superfused with buffer solution (data not shown). No increase in rolling was observed in any vessels studied. The number of stationary leukocytes was increased with histamine (300 µM) but only in sinusoids and only if animals were pretreated with aminoguanidine, an antihistaminase (Table 1). These data suggest that normally there is sufficient histaminase in the liver to prevent histamine-induced leukocyte recruitment.

Another known inducer of P-selectin, the biologically active lipid LTC4 (20 nM), was tested for proadhesive activity in the liver. Figure 1 shows that LTC4 induced leukocyte recruitment almost exclusively in sinusoids with almost no adhesion in postsinusoidal venules.
fact, leukocyte recruitment in the sinusoids accounted for 70–80% of the total number of stationary cells within the liver microcirculation. The peak level of leukocytes in sinusoids with LTC4 occurred at 30 min of the experiment and was significantly greater than even the highest concentration of histamine (50,000 times greater concentration than LTC4).

LTC4-induced leukocyte recruitment is not dependent on P-selectin. Previous work has shown (18) that LTC4 induces leukocyte rolling and adhesion in postcapillary venules via a P-selectin-dependent mechanism. Therefore, we examined the effect of three different P-selectin antibodies on the liver microcirculation. The anti-P-selectin antibodies 5H1, RMP-1, or RB40 were administered intravenously, 5 min before LTC4 superfusion. Figure 2 demonstrates that the pretreatment with P-selectin antibodies had no effect on leukocyte recruitment in the sinusoids or postsinusoidal venules (data not shown). This result suggests that LTC4-induced leukocyte recruitment was a P-selectin-independent mechanism. To further test whether P-selectin is involved in LTC4-induced leukocyte recruitment, we used P-selectin-deficient mice. Figure 3 shows that leukocyte recruitment with LTC4 in sinusoids of P-selectin-deficient mice was as efficient as in wild-type animals superfused with LTC4. In fact, at 10 min leukocytes appeared to trap more effectively in P-selectin-deficient mice.

Neither PAF nor ICAM-1 contributes to LTC4-induced leukocyte recruitment. PAF is synthesized by endothelial cells within minutes after stimulation by LTC4 and is expressed on the cell surface where it activates neutrophils and upregulates CD11b by binding to a specific cell-surface receptor (38). Figure 4 shows that the PAF receptor antagonist WEB-2086 (1 mg/animal) had no effect on LTC4-dependent leukocyte recruitment in sinusoidal vessels. Figure 4 also demonstrates that ICAM-1-deficient animals showed no decrease in leukocyte sequestration inside the sinusoids when compared with wild-type animals superfused with LTC4.

To ensure that PAF could induce cell adhesion we superfused PAF and observed very rapid accumulation of leukocytes (within 10 min) in the sinusoids (Fig. 5). Unlike LTC4 there was a more pronounced increase in leukocyte adhesion in postsinusoidal venules with PAF, but the value was still lower than in sinusoids. WEB-2086 inhibited 50% of PAF-induced accumulation within 5 min before LTC4 superfusion.
the sinusoids and 100% of adhesion in venules (Fig. 5), suggesting that the PAF receptor antagonist was able to at least partially reduce leukocyte recruitment in response to PAF. The lack of complete inhibition of PAF effects may be related to the rather high doses of PAF used. PAF-induced leukocyte recruitment in ICAM-1-deficient animals was reduced ~50% in sinusoids but not in the postsinusoidal venules (Fig. 5).

Previous work from our laboratory has demonstrated that the absence of both ICAM-1 and P-selectin was very effective at reducing leukocyte adhesion in response to fMLP (38). Moreover, this double mutant has a greater impairment in leukocyte recruitment than either the ICAM-1- or P-selectin-deficient animal alone (22). Therefore, in a final series of experiments, we examined leukocyte adhesion in the liver microvasculature of ICAM-1 and P-selectin double-mutant mice. The results demonstrate that, in the absence of P-selectin and ICAM-1, leukocyte recruitment in sinusoids was unaffected with LTC4 (Table 2). Leukocyte adhesion in postsinusoidal vessels was again minimal. In response to PAF, the P-selectin and ICAM-1 double-deficient mice still had a significant increase in leukocyte adhesion. More importantly the double deficiency did not reduce recruitment relative to the ICAM-1 deficiency alone (see Fig. 5).

In a final series of experiments we examined the possibility that leukocytes may adhere in presinusoidal, hepatic venules. There was absolutely no effect of PAF in these presinusoidal venules (data not shown).

**DISCUSSION**

Histamine and LTC4 have been commonly used as mediators to demonstrate the importance of P-selectin as an adhesion molecule that rapidly causes leukocyte rolling in vivo in mesenteric microvessels (14, 18, 21). P-selectin antibodies completely eliminate the P-selectin-dependent increase in leukocyte rolling. Moreover, in P-selectin-deficient mice there is a complete absence of leukocyte rolling in response to many of these mediators. Previous studies have proposed that leukocyte recruitment in liver inflammation occurs primarily in the sinusoids of the liver, vessels that are thought not to express P-selectin (6). Therefore, histamine, LTC4, and other P-selectin inducers either do not recruit leukocytes into the liver or else they induce leukocyte recruitment into compartments of the liver other than the sinusoids. Using fluorescence intravital microscopy, we report for the first time that histamine, a potent inducer of P-selectin in numerous tissues in animal models (2, 9, 21) and on human umbilical vein endothelium (16, 24), did not induce notable leukocyte-endothelium interactions in the liver microcirculation. At 0.1 mM histamine, rapid leukocyte rolling could be seen in the mesentery (21) but not in the liver. Even at 3 or 10 times greater concentrations of histamine, an increase in rolling was not noted in the postsinusoidal vessels or sinusoids and adhesion still did not increase significantly in either of these subsets of vessels, suggesting that histamine does not appear to recruit leukocytes into the liver. This observation may be related to the large amount of liver histaminase, inasmuch as animals administered an antihistaminase did respond to histamine.
In direct contrast, LTC$_4$ at a concentration (20 nM) that was previously shown to be optimal for P-selectin-dependent rolling in mesentery did enhance leukocyte-endothelium interactions, but primarily in the sinusoids where as already stated P-selectin is not expressed even with maximal endothelial activation (6). Interestingly, the interactions were manifested as firm adhesion rather than the transient interactions associated with P-selectin-dependent rolling. Systematic exploration of mechanisms revealed that P-selectin was not involved in this leukocyte-sinusoidal endothelium interaction as three different P-selectin antibodies had no effect on this interaction and the leukocyte recruitment occurred in P-selectin-deficient mice. These data for the first time demonstrate that a proinflammatory mediator such as LTC$_4$ has differential adhesion molecule profiles depending on the tissue (liver vs. mesentery).

Juxtacrine activation of leukocytes by endothelium was first coined by Zimmerman and colleagues (24) to describe the series of events leading to leukocyte adhesion. Leukocytes are initially tethered to endothelium by selectins and specifically P-selectin under acute conditions. This permits the tethered-rolling cells to encounter critically localized chemotactic agents on the surface of endothelium (including PAF), which rapidly activate the leukocytes to firmly bind to constitutive ICAM-1. In this study, LTC$_4$ did not induce any notable rolling but did induce leukocytes to come to a complete stop in sinusoids. Because LTC$_4$ does not directly activate leukocytes (39), a likely scenario is that LTC$_4$ activates endothelium to produce a proinflammatory mediator such as PAF (39) that could directly induce firm leukocyte adhesion. Indeed, LTC$_4$ significantly increased PAF production by hepatic sinusoidal endothelial cells in mice (27). However, in this study the PAF receptor antagonist WEB-2086, which has previously blocked leukocyte adhesion in the mesentery in response to LTC$_4$ (18), did not affect firm adhesion in the liver microvasculature. We believe that sufficient amounts of the PAF receptor antagonist was used inasmuch as it reduced adhesion in response to very high concentrations of PAF in sinusoids and completely inhibited adhesion in post-sinusoidal venules. Moreover, PAF did induce an abundance of adhesion in liver sinusoids, suggesting that PAF-dependent adhesion can occur in the liver microvasculature, but PAF does not contribute to LTC$_4$-induced leukocyte recruitment. Absence of ICAM-1 also did not affect LTC$_4$-induced leukocyte recruitment, suggestive of a CD18-ICAM-1-independent event. It is conceivable that either other adhesive pathways exist or simple trapping due to enhanced rigidity of leukocytes and swelling of endothelial and Kupffer cells is involved in the LTC$_4$-induced leukocyte recruitment.

**Table 2.** Mediator-induced sinusoidal adhesion in P-selectin- and ICAM-1-deficient mice

<table>
<thead>
<tr>
<th>Time of Exposure, min</th>
<th>PAF (100nM)</th>
<th>PAF (ICAM-1 KO)</th>
<th>PAF + WEB 2086 (1mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.05±0.11</td>
<td>2.3±0.10</td>
<td>1.94±0.09</td>
</tr>
<tr>
<td>10</td>
<td>5.28±0.21</td>
<td>7.15±0.84</td>
<td>5.43±0.28</td>
</tr>
<tr>
<td>20</td>
<td>6.96±0.31</td>
<td>5.53±0.46</td>
<td>5.43±0.69</td>
</tr>
<tr>
<td>30</td>
<td>7.36±0.18</td>
<td>5.88±1.07</td>
<td>6.63±0.39</td>
</tr>
</tbody>
</table>

Values are means ± SE; no. mice in parentheses. PAF, platelet-activating factor; LTC$_4$, leukotriene C$_4$; P/ICAM −/−, P-selectin- and ICAM-1-deficient mice. *P < 0.05 compared with 0 min.
proinflammatory mediators induce three adhesive profiles to each of the different stimuli in the liver. As already mentioned, there was dramatic reduction in leukocyte recruitment in P-selectin- and ICAM-1-deficient mice (38). The data clearly demonstrate that an overlapping role does not exist for these two molecules in the liver endothelium via both P-selectin and perhaps ICAM-1 (28, 35). Just like the LTC4 results, it is conceivable that the 50% ICAM-1-independent adhesion with PAF in the liver is due to simple trapping as a result of enhanced rigidity of leukocytes and swelling of endothelium.

Other work has also suggested adhesion molecule-independent recruitment in the liver. J. Kunkel and co-workers (6, 13) were unable to inhibit sepsis-induced leukocyte recruitment with either a P-selectin or ICAM-1 antibody. In this sepsis model, these colleagues were able to reduce neutrophil emigration out of the liver sinusoids with a vascular cell adhesion molecule 1 (VCAM-1) antibody, suggesting an unexpected alternative pathway of neutrophil recruitment in this liver inflammation model (5). In that study, however, the VCAM-1 antibody did not inhibit the neutrophil adhesion (only emigration) in the liver vasculature, suggesting no role for VCAM-1, ICAM-1, or P-selectin in leukocyte adhesion in the liver microvasculature in response to galactosamine and endotoxin (5, 6, 13). One consistent finding is that the leukocytes in that study were also accumulating primarily in the sinusoids rather than in other vascular sites. In a less severe model of sepsis where only endotoxin was given, P-selectin and ICAM-1 deficiency did suppress leukocyte recruitment into the liver sinusoids, suggesting that adhesion in that particular LPS model was dependent at least in part on adhesion molecules (38).

Based on the aforementioned effect in P-selectin and ICAM-1 deficiency in the endotoxin model, we wished to see whether there may not be overlapping function of P-selectin and ICAM-1 in the liver in response to either LTC4 or PAF. In addition to the positive results in the endotoxin model there were two other reasons to test this hypothesis. First, leukocyte rolling was far more impaired in P-selectin and ICAM-1 double-deficient mice than in P-selectin-deficient mice and the double mutation had a far greater impairment in leukocyte recruitment into the peritoneal cavity than the single mutation (4, 22). These data suggest an overlapping rather than a sequential role for these adhesion molecules. Second, at low flow rates as occurs in the sinusoids it is possible that leukocytes can tether to endothelium via both P-selectin and perhaps ICAM-1 (38). The data clearly demonstrate that an overlapping role does not exist for these two molecules in the liver microcirculation in response to PAF or LTC4. However, as already mentioned, there was dramatic reduction in recruitment in P-selectin- and ICAM-1-deficient mice in response to LPS, further highlighting the diverse adhesive profiles to each of the different stimuli in the liver.

Clearly, in this study we reveal that three different proinflammatory mediators induce three adhesive profiles that would not have been predictable from work in other vascular beds. Histamine alone caused no leukocyte recruitment, LTC4 induced P-selectin- and ICAM-1-independent adhesion but only in sinusoids, and PAF caused ICAM-1-dependent and -independent adhesion in sinusoids and in postsinusoidal venules. Increased understanding of these basic mechanisms of interaction between hepatic endothelial cells and infiltrating leukocytes will be absolutely necessary before one can begin to design therapies for liver diseases. It is noteworthy that a reduction in liver damage occurred following ICAM-1 inhibition in ischemia-reperfusion of the liver (7), and less leukocyte accumulation in liver following gut ischemia-reperfusion (11, 12) in P-selectin-deficient mice. This raises the possibility that P-selectin and ICAM-1 may play a role in ischemia-reperfusion and further underscores the diverse responses of the liver to inflammatory stimuli.

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Address for reprint requests and other correspondence: P. Kubes, Immunology Research Group, Univ. of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta, Canada T2N 4N1 (E-mail: pkubes@acs.ucalgary.ca).

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REFERENCES


