Role of PECAM-1 (CD31) in neutrophil transmigration in murine models of liver and peritoneal inflammation

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Chosay, John G., Michael A. Fisher, Anwar Farhood, Kathleen A. Ready, Colin J. Dunn, and Hartmut Jaeschke. Role of PECAM-1 (CD31) in neutrophil transmigration in murine models of liver and peritoneal inflammation. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G776-G782, 1998.—Platelet endothelial cell adhesion molecule-1 (PECAM-1) is thought to be critical for transendothelial migration of leukocytes, including neutrophils. Because neutrophil-mediated liver injury during endotoxemia is dependent on transmigration, we investigated the role of PECAM-1 in the pathophysiology of endotoxin-induced liver injury. Male C3Heb/Fej mice were treated with galactosamine (Gal) and endotoxin (ET) (700 mg/kg Gal/100 µg/kg ET), and liver sections were stained for PECAM-1 expression. Control livers showed the presence of PECAM-1 on endothelial cells of large vessels but not in sinusoids. Gal/ET treatment did not change the expression pattern of PECAM-1. Gal/ET-induced liver injury (area of necrosis: 36 ± 3%) was not attenuated by treatment with 3 mg/kg of the antimurine PECAM-1 antibody 2H8. The antibody had no effect on sequestration and transmigration of neutrophils in sinusoids or the margination of neutrophils in large vessels. In contrast, 2H8 inhibited glycogen-induced neutrophil migration into the peritoneum by 74%; this effect correlated with PECAM-1 expression in the intestinal vasculature. Thus PECAM-1 is neither expressed nor inducible in hepatic sinusoids and is consequently not involved in neutrophil transmigration in the liver during endotoxemia. On the other hand, expression of PECAM-1 in mesenteric veins is critical for peritoneal neutrophil accumulation.

Endotoxin; sepsis; peritonitis; integrins; adhesion molecules

PLATELET-ENDOTHELIAL CELL ADHESION MOLECULE-1 (PECAM-1; CD31) is a member of the immunoglobulin gene superfamily. PECAM-1 is expressed on leukocytes, i.e., platelets, neutrophils, monocytes, and selected T cell subsets. On endothelial cells, PECAM-1 is concentrated at the intercellular junction. Pretreatment of monocytes or neutrophils with an antibody against PECAM-1 prevented leukocyte transmigration across an endothelial cell monolayer without inhibition of adherence. On the other hand, treatment of endothelial cells with the PECAM-1 antibody was also effective in inhibiting leukocyte transmigration. These data suggest that PECAM-1 on leukocytes and on endothelial cells is critically involved in the transendothelial migration process. Recently, these in vitro findings have been substantiated in a variety of in vivo models. Anti-PECAM-1 antibodies attenuated neutrophil accumulation in the alveolar space in an IgG immune complex model of lung inflammation and in the peritoneum after glycogen or thioglycollate administration. Furthermore, neutrophil transmigration and tissue necrosis was inhibited by anti-PECAM-1 antibodies without affecting overall neutrophil accumulation in a feline model of myocardial ischemia-reperfusion (I/R) injury. In a related study using a rat model of myocardial infarction, Fab fragments of PECAM-1 antibodies attenuated tissue necrosis and reduced neutrophil infiltration into the necrotic tissue. However, the role of PECAM-1 has not been investigated in any experimental model of liver inflammation.

Neutrophils contribute to liver injury during hepatic I/R, endotoxemia, alcoholic intoxication, hepatic chemokine overexpression, partial hepatectomy, or intestinal I/R. General mechanisms of neutrophil-induced liver injury include the sequestration of these cells in the hepatic vasculature, transmigration, and adherence to parenchymal cells. Similar to many other organs, e.g., intestine, heart, skeletal muscle, and skin, neutrophil margination takes place in hepatic postsinusoidal venules. However, most neutrophils sequestered in the liver vasculature are located in sinusoids; neutrophils that migrate out of the sinusoids have not been shown to be responsible for parenchymal cell injury in an endotoxin (ET) shock model. The importance of the transendothelial migration step for the pathophysiology was further demonstrated by antibodies against intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These antibodies protected against liver injury by inhibiting the extravasation of sinusoidal neutrophils. Because of the critical role of PECAM-1 in leukocyte transendothelial migration, as demonstrated in the lung, intestine, skin, and heart, we evaluated the expression of PECAM-1 in the hepatic vasculature and characterized the effect of an anti-PECAM-1 antibody on hepatic neutrophil sequestration, transendothelial migration, and parenchymal cell injury in a well-established model of ET-induced liver failure. For com-
comparison, we investigated PECAM-1 expression in mesenteric vessels and the effect of the anti-PECAM-1 antibody in the glycogen peritonitis model.

MATERIALS AND METHODS

Animals. Male mice (strain C3Heb/FeJ; 20–25 g body wt) were purchased from Jackson Laboratories (Bar Harbor, ME). The animals had free access to food (Certified Rodent Diet #5002C; PMI Feeds, Richmond, IN) and water. The experimental protocols followed the criteria of Pharmacaa & Upjohn, Inc. and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were treated intraperitoneally with 700 mg/kg D-galactosamine (Gal; Sigma, St. Louis, MO) and 100 µg/kg Salmonella abortus equi-ET (Sigma) dissolved in sterile PBS (pH 7.0). Some animals were treated with 3 mg/kg anti-mouse PECAM-1 antibody (clone 2H8) (Endogen, Cambridge, MA), control IgG (Sigma), or anti-mouse P-selectin antibody (clone RB40.34) (PharMingen, San Diego, CA) intravenously at the time of Gal/ET injection.

Experimental protocols. The animals were killed by cervical dislocation 4 or 7 h after administration of Gal/ET. Blood was collected from the right ventricle into a heparinized syringe and centrifuged, and plasma was used for determination of alanine aminotransferase (ALT) activity with Sigma test kit DG 159-UV. Pieces of the liver or the mesentery were fixed in phosphate-buffered Formalin for histological analysis or embedded in Tissue-Tek O.C.T./Sakura Finetek, Torrance, CA) embedding medium and snap-frozen in liquid nitrogen-chilled methylbutane for immunohistochemistry. Histology. Formalin-fixed portions of the liver were embedded in paraffin, and sections 5 µm thick were cut. Neutrophils were stained with the use of the AS-D chloroacetate esterase technique as described in detail previously (18). Neutrophils were identified by positive staining and morphology and were counted in 50 high-power fields (magnification, ×400), using a Nikon Labophot microscope. Only neutrophils present within sinusoids or extravasated into the tissue were counted; the number of neutrophils marginated within large vessels, e.g., hepatic veins, was evaluated separately. Cell damage was evaluated in parallel sections stained with hematoxylin and eosin. We estimated the percentage of cell necrosis by evaluating the number of microscopic fields with necrosis compared with the entire histological section. The pathologist (A. Farhood) performing the histological evaluation (number of neutrophils, area of necrosis) was blinded as to the treatment of animals.

Immunohistochemistry. Cryostat sections (8 µm) of livers and mesentery were dehydrated in acetone, air-dried, and fixed in 2% buffered Formalin (2 min). For localization of PECAM-1 (CD31) or ICAM-1 (CD54), slides were washed in PBS buffer, labeled with rat anti-murine CD31 antibody (MEC 13.3, PharMingen), rat anti-murine CD54 (YN1/1.7.4, American Type Culture Collection, Rockville, MD), or an appropriate negative antibody control (R35–95, PharMingen) at 3.0 µg/ml for 30 min. Sections were washed in buffer, and endogenous peroxidase activity was reduced by dehydrating the tissues with methanol containing 3% hydrogen peroxide for 20 min. Slides were rehydrated with buffer and labeled with 25 µg/ml of peroxidase-conjugated mouse anti-rat IgG secondary antibody Fab2 fragments (Jackson Immunoresearch, West Grove, PA) for 30 min. Secondary antibody

Fig. 2. Hepatic neutrophil sequestration was evaluated in C3Heb/FeJ mice 4 and 7 h after combined administration of 700 mg/kg Gal and 100 µg/kg Salmonella abortus ET. Animals were pretreated with 3 mg/kg murine anti-PECAM-1 antibody (clone 2H8) or isotype-matched control IgG. Neutrophils were counted in 50 high-power fields (HPF). PMN, polymorphonuclear leukocytes (neutrophils). Data are means ± SE of n = 5 animals (4 h) or n = 8 animals (7 h) per group. *P < 0.05 compared with untreated controls (C).

Fig. 3. Neutrophil margination in large hepatic vessels, i.e., portal and postsinusoidal venules, was evaluated 4 h after combined administration of 700 mg/kg Gal and 100 µg/kg Salmonella abortus ET. Animals were treated with 3 mg/kg murine anti-PECAM-1 antibody (2H8), control IgG, or anti-P-selectin antibody (clone RB40.34). No marginalized PMN were found in these vessels in control livers 4 h after Gal/ET. Neutrophils were counted in 10 large vessels of approximately similar size. Data are means ± SE of n = 5 animals per group. *P < 0.05 compared with untreated controls (C). #P < 0.05 compared with IgG.
was detected by using 3,3'-diaminobenzidine tetrahydrochloride tablets (Sigma) in 50 mM Tris·HCl and 150 mM sodium chloride (pH 7.6) for 10 min. Sections were washed and counterstained with Mayer's hematoxylin solution (Sigma) for 3 min.

Peritonitis experiments. To test the efficacy of the anti-PECAM-1 antibody to inhibit adhesion to vascular endothelium and transendothelial migration, animals were intravenously injected with 250 µl of saline, 3 mg/kg of clone 2H8, or control immunoglobulin. At the same time, the animals received an intraperitoneal injection of saline (500 µl) or glycogen (1 g/kg body wt) in sterile saline. After 4 h, the animals were killed and their peritoneal cavities were lavaged twice with 2 ml of PBS. The lavage fluids were centrifuged (1,000 g) for 10 min to sediment the neutrophils. The pellets were resuspended in Tris-buffered 0.75% NH₄Cl for 10 min to lyse erythrocytes. After centrifugation, the pellets were resuspended in detergent buffer (50 mM phosphate buffer containing 0.5% cetyltrimethylammonium bromide), briefly sonicated, and freeze-thawed twice. Myeloperoxidase (MPO) activity as an index for neutrophil accumulation was determined spectrophotometrically in 50 mM phosphate buffer (pH 6.0) containing 0.165 mg/ml o-dianisidine hydrochloride and 0.15 mM hydrogen peroxide (23). The change in absorbance was determined at 460 nm. In a separate experiment, the cell pellet was resuspended in HEPES buffer and stained with modified Giemsa-Wright stain (Neat Stain; Midlantic Biomedical, Paulsboro, NJ) for differential cell count.

Statistics. All data are means ± SE. Statistical significance between the control group and a treated group was determined with the unpaired Student's t-test or Wilcoxon's rank-
sum test. Comparisons between multiple groups were performed with one-way ANOVA followed by Bonferroni t-test. P < 0.05 was considered significant.

RESULTS

Administration of Gal/ET caused severe liver injury at 7 h as indicated by high plasma ALT values and substantial hepatocellular necrosis (Fig. 1). Preceding liver injury (4 h), large numbers of neutrophils accumulated in sinusoids (Fig. 2). At the time of injury (7 h), there was a moderate further increase in the number of neutrophils (Fig. 2); ~30–35% of these leukocytes had transmigrated by that time. In addition to sinusoidal sequestration of neutrophils, there was margination of neutrophils in postsinusoidal venules at 4 h (Fig. 3). However, at 7 h no neutrophils were observed in the lumen of larger vessels or in the surrounding tissue (data not shown). Treatment with an anti-mouse PECAM-1 antibody (3 mg/kg) did not significantly change plasma ALT activity or the area of necrosis after Gal/ET administration compared with control IgG-treated or untreated animals (Fig. 1). The anti-PECAM-1 antibody had no effect on hepatic neutrophil sequestration in sinusoids before liver injury (4 h) or at the time of injury (7 h) (Fig. 2). Furthermore, the PECAM-1 antibody did not affect neutrophil margination in postsinusoidal venules at 4 h (Fig. 3). As a control experiment, the anti-P-selectin antibody RB40.34 attenuated neutrophil margination in these large vessels by 76% at 4 h after Gal/ET (Fig. 3) but had no effect on sinusoidal neutrophil sequestration (data not shown).

To assess the expression of PECAM-1 in the hepatic vasculature, immunohistochemical analysis for PECAM-1 was performed. In control animals, there was clear PECAM-1 expression on large vessel endothelial cells; however, no PECAM-1 could be detected on sinusoidal lining cells (Fig. 4). The expression pattern of PECAM-1 in ET-treated animals (4 h) was identical to that observed in control livers. Thus PECAM-1 appears to be constitutively expressed selectively on large-vessel endothelial cells. For comparison, sections from the same livers were stained with an anti-ICAM-1 antibody (Fig. 4). In control animals, ICAM-1 was moderately expressed on large vessel endothelium and lightly expressed on sinusoidal lining cells. However, 4 h after ET, ICAM-1 expression was substantially increased on all endothelial cells, particularly in sinusoids.

To test the efficacy of the anti-PECAM-1 antibody batch and the dose used, glycogen peritonitis experi-

Fig. 6. Normal mouse mesenteric vasculature was stained histochemically by hematoxylin and eosin (H&E) or immunohistochemically for granulocytes (GR-1), ICAM-1, and PECAM-1. a: H&E staining of a normal mesenteric vein showing no leukocyte accumulation within the vessel. b: Anti-granulocyte staining, showing no granulocyte involvement within mesenteric vein (left) and artery (right). c: Parallel section showing ICAM-1 staining of normal mesenteric vein with weaker staining of the artery. d: Another parallel section showing good PECAM-1 staining of normal mouse mesenteric vasculature. Original magnification, ×400.
ments were performed. This model is useful to assess the effect of therapeutic interventions on neutrophil adhesion and transmigration in mesenteric vessels in vivo (18). Intraperitoneal injection of glycogen induced substantial neutrophil infiltration within 4 h, as indicated by high MPO activity in the peritoneal lavage fluid (Fig. 5). The anti-PECAM-1 antibody (3 mg/kg) inhibited MPO activity by 74% compared with control animals or control IgG-treated animals. Immunohistochemical evaluation of PECAM-1 expression showed strong staining in both intestinal arteries and veins in control animals (Fig. 6). This expression pattern and its intensity did not change during glycogen peritonitis (Fig. 7). However, numerous leukocytes could be detected in the mesenteric veins during the inflammatory response (Fig. 7) and staining with the GR-1 antibody (Fig. 7b) identified these leukocytes as granulocytes. For comparison, parallel sections were stained for ICAM-1 (Figs. 6c and 7c); in contrast to PECAM-1, ICAM-1 was predominantly expressed on venular endothelium.

DISCUSSION

The principal objective of this investigation was to study the expression of PECAM-1 in murine liver and to evaluate its role in the pathophysiology of a neutrophil-induced injury mechanism. Our data showed that PECAM-1 is constitutively expressed on endothelial cells of large hepatic vessels, i.e., portal veins and arteries and hepatic veins, but not on sinusoidal endothelium. The distribution of PECAM-1 in murine liver is similar to its distribution in human liver (32). PECAM-1 expression was not increased by ET treatment. These findings are consistent with previous reports that PECAM-1 is not inducible by cytokines in vitro (5). The PECAM-1 expression pattern in the liver and its lack of inducibility is in contrast to most other adhesion molecules investigated in the murine ET shock model. The most abundant adhesion molecule in the liver is ICAM-1, which is constitutively present on endothelial cells of the entire hepatic vasculature and on Kupffer cells; ET further increases the expression of ICAM-1 on these cells and additionally induces moderate ICAM-1 expression on hepatocytes (9, 10, 16). VCAM-1 can only be found on large-vessel endothelial cells in control animals, but it is upregulated on the entire hepatic endothelium, including sinusoidal lining cells (6, 35). P-selectin is not constitutively expressed on any cell type in the liver, but it can be temporarily induced on large-vessel endothelial cells (8). Although

Fig. 7. Mouse mesenteric vasculature 2 h after intraperitoneal glycogen administration. Mesentery was stained histochemically by H&E or immunohistochemically for GR-1, ICAM-1, and PECAM-1. a: H&E staining of mesentery vein showing intravascular accumulation of leukocytes. b: Anti-granulocyte staining of mesentery vein (right) and artery (left) showing strong granulocyte accumulation within vein but not in artery. c: Parallel section showing strong ICAM-1 staining of mouse mesentery vein but weaker staining in artery. d: Another parallel section showing PECAM-1 staining of artery and vein. Original magnification, ×400.
there is also no E-selectin expression in the normal liver, it is inducible on all endothelial cells during sepsis (31). The function of these adhesion molecules in the pathophysiology of a neutrophil-mediated injury correlates with their expression pattern and inducibility. None of these molecules appear to be involved in the initial sequestration of neutrophils in the sinusoids (6, 7, 10, 18, 38, 39). However, P-selectin and ICAM-1 are necessary for neutrophil margination in hepatic venules (8, 38). ICAM-1 and VCAM-1 are necessary for transendothelial migration (6, 7), and ICAM-1 supports adherence of neutrophils to parenchymal cells (28). On the basis of these results it appears that neutrophil adhesion in hepatic veins follows the classical scheme of selectin-mediated rolling followed by integrin/ICAM-1-mediated firm adhesion. Thus it can be expected that PECAM-1 would be involved only in neutrophil transmigration. However, as recently shown, at least in the Gal/ET model, neutrophil transmigration occurs only from sinusoids (3). One reason for this special behavior is that large numbers of primed neutrophils are located in sinusoids when a significant number of parenchymal cells undergo apoptosis. Selectively preventing apoptosis with an inhibitor of caspases prevented transmigration and injury in this model; thus it is likely that parenchymal cell apoptosis represents a signal for neutrophil transmigration (21). On the other hand, maximal neutrophil margination in venules correlates with P-selectin expression, and both events are declining at 6 h (8). Thus the lack of transmigration from venules may be caused by the fading P-selectin expres-
sion, which no longer supports neutrophil margination in these vessels, and the lack of a chemotactic stimulus that induces neutrophil transmigration from this location. Nevertheless, the fact that adhesion molecules, including PECAM-1, are expressed in these larger vessels suggests that under different pathophysiological conditions P-selectin, ICAM-1, and PECAM-1 may facilitate neutrophil adhesion and transmigration.

In contrast to the observations in the liver, PECAM-1 is critical for transmigration of neutrophils in the mesenteric vasculature. These findings confirm previous reports (26, 36). Interestingly, PECAM-1 is expressed similarly on endothelial cells of arterioles as well as venules. As expected for an inflammatory response, neutrophil adhesion and transmigration occurred only in venules and correlated with the expression of ICAM-1 on venular endothelial cells. In postcapillary venules of the intestine, neutrophil transmigration requires first selectin-mediated rolling and then ICAM-1/integrin-mediated firm adhesion (11). Thus PECAM-1 is critical for transmigration but appears not to be the determining factor for neutrophil adhesion in the intestinal vasculature. These observations further support the conclusion that PECAM-1 may be able to facilitate transmigration in the postsinusoidal venules of the liver if the expression of other adhesion molecules, i.e., selectins and ICAM-1, supports the adhesion of neutrophils and a chemotactic factor provides the necessary stimulation.

In summary, PECAM-1 was exclusively expressed on large-vessel endothelial cells and was not expressed on sinusoidal endothelium in murine liver. PECAM-1 expression was not inducible by ET. In the liver, transmigration of neutrophils during endotoxemia did not require PECAM-1. These observations are fundamentally different from observations of other organs such as the intestine, where PECAM-1 expression is critical for neutrophil transmigration. However, if under different pathophysiological conditions the expression of other adhesion molecules, e.g., P-selectin and ICAM-1, can support adhesion of neutrophils in postsinusoidal venules and appropriate chemotactic stimuli are generated, PECAM-1 may also be involved in neutrophil transmigration in these hepatic vessels. We conclude that PECAM-1 expression is necessary but is not the determining factor of neutrophil transendothelial migration in venular beds of most organs during inflammation. Transmigration of neutrophils in sinusoids is independent of PECAM-1 expression.

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PECAM-1 and Hepatic Neutrophil Transmigration


