Vascular Adhesion Protein-1 Mediates Adhesion and Transmigration of Lymphocytes on Human Hepatic Endothelial Cells

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Vascular adhesion protein-1 (VAP-1) is an amine oxidase and adhesion receptor that is expressed by endothelium in the human liver. The hepatic sinusoids are perfused by blood at low flow rates, and sinusoidal endothelium lacks selectin expression and has low levels of CD31, suggesting that VAP-1 may play a specific role in lymphocyte recruitment to the liver. In support of this we now report the constitutive expression of VAP-1 on human hepatic sinusoidal endothelial cells (HSEC) in vitro and demonstrate that VAP-1 supports adhesion and transmigration of lymphocytes across these cells under physiological shear stress. These are the first studies to report the function of VAP-1 on primary human endothelial cells. Under static conditions lymphocyte adhesion to unstimulated HSEC was dependent on VAP-1 and ICAM-2, whereas adhesion to TNF-α-stimulated HSEC was dependent on ICAM-1, VCAM-1, and VAP-1. Under conditions of flow, blocking VAP-1 reduced lymphocyte adhesion to TNF-α-treated HSEC by 50% and significantly reduced the proportion of adherent lymphocytes that transmigrated across cytokine or LPS-activated endothelium. In addition, inhibition of the amine oxidase activity of VAP-1 reduced both adhesion and transmigration of lymphocytes to a level similar to that seen with VAP-1 Ab. Thus, VAP-1 can support transendothelial migration as well as adhesion, and both functions are dependent on its enzymatic activity. In the absence of selectins and CD31, VAP-1 may play a specific role in lymphocyte recruitment via hepatic sinusoidal endothelium. Moreover, since VAP-1 is induced on nonhepatic endothelium in response to inflammation, its ability to support lymphocyte transendothelial migration may be an important systemic function of VAP-1. The Journal of Immunology, 2002, 169: 983–992.

Lymphocyte recruitment from the circulation into tissue is dependent on the ability of the lymphocyte to recognize and bind to molecules on the endothelial cell surface that promote transendothelial migration. A multistep model of leukocyte adhesion to vascular endothelium has been described and is broadly applicable, although the details of the signals involved differ between tissues. In the generally accepted model, tethering or rolling receptors expressed on endothelial cells capture free-flowing leukocytes (1, 2). These receptors may be either selectins or members of the Ig superfamily (3–5). Once captured, the leukocyte can receive activating messages presented by endothelial cells in the form of chemokines that activate specific G protein-coupled receptors on the leukocyte surface (6, 7). Occupancy of these receptors triggers a cascade of intracellular signals that results in the presentation of high affinity integrin receptors on the leukocyte surface that bind to Ig family of counter-receptors on the endothelium to promote leukocyte arrest on the vessel wall (8–10). In the presence of the appropriate migratory signals the leukocyte will migrate across the endothelium into tissue, where it follows a hierarchy of chemotactic signals toward the focus of inflammation.

Lymphocytes recirculate continuously between blood and tissues as part of the process of immune surveillance, and recent evidence suggests that specialized subsets of T cells exhibit distinct tissue-specific patterns of recirculation in vivo (11, 12). These cells are directed to particular tissues by combinations of adhesion molecules and chemokines that control lymphocyte recognition of and adhesion to endothelium. For example, memory T cells that recirculate to the gut lamina propria express the integrin α4β7, and bind to an endothelial ligand, mucosal cell adhesion molecule-1, found mainly in gut endothelium (13), whereas T cells that migrate to the skin do not express α4β7, but do express high levels of the cutaneous lymphocyte Ag that binds to E-selectin on dermal endothelium (14). The liver is a major site of Ag exposure and contains large numbers of lymphocytes even under normal conditions. Although some of these are terminally differentiated lymphocytes that are removed by apoptosis, the liver also contains functional lymphocytes recruited as part of the process of immune surveillance (15–18). It is thus possible that tissue-specific signals will regulate lymphocyte recruitment to the liver (19).

The hepatic sinusoids are lined by specialized endothelium that supports lymphocyte adhesion and recruitment in a unique low shear environment (20). Hepatic sinusoidal endothelium has a distinct phenotype compared with endothelium from other vascular beds. In vivo sinusoidal endothelial cells express low levels of CD31 and show little, if any, expression of selectins, which are the
most common capture receptors in other tissues (21, 22). Moreover, functional studies in selectin-deficient animals suggest a minimal role for selectins in leukocyte recruitment via the sinusoids (23). However, human hepatic sinusoids constitutively express the endothelial adhesion molecule vascular adhesion protein-1 (VAP-1), which is largely absent from noninflamed vessels in extralymphoid organs (24, 25). The ability of VAP-1 to mediate sialic acid-dependent adhesion suggests that it could have a particular function in the liver by mediating shear-dependent adhesion in the absence of selectins (23). To date, the sequence of events governing lymphocyte recruitment by human liver endothelial cells under conditions of shear stress is unknown. We have previously shown (25, 26) that VAP-1 supports T cell adhesion to human hepatic endothelium in tissue sections, but in the present study we have determined in vitro the detailed nature of VAP-1-mediated adhesion of lymphocytes to human hepatic endothelium.

The molecular characterization and cloning of VAP-1 show it to be a 170-kDa homodimeric sialoglycoprotein consisting of two 90-kDa subunits with close sequence homology to the copper-dependent semicarbazide-sensitive amine oxidases (SSAO). Both the transmembrane and soluble forms of VAP-1 exhibit monoamine oxidase activity (27–29), and two of us (M. Salmi and S. Jalkanen) have recently proposed that VAP-1 on endothelium supports lymphocyte adhesion by binding to and oxidatively deaminating a primary amino group presented on the lymphocyte surface, resulting in the formation of a transient covalent bond between the two cell types (30).

In the present paper we show that human hepatic sinusoidal endothelial cells maintain a unique morphology and phenotype in culture and use VAP-1 in conjunction with ICAM-1 to support lymphocyte adhesion under flow. VAP-1 was not expressed by non-hepatic endothelial cells and could not be induced on these cells by cytokine treatment. Under physiologically relevant levels of shear stress, VAP-1 made a minor contribution to rolling adhesion of lymphocytes on hepatic endothelium, but mediated levels of stable adhesion equivalent to those supported by ICAM-1. In addition, blockade of the adhesive or enzymic capacity of VAP-1 consistently inhibited lymphocyte transendothelial migration through hepatic endothelial monolayers. Thus, we suggest that VAP-1 on hepatic endothelium can mediate adhesion and transendothelial migration of lymphocytes and thereby play an important role in directing T cell recirculation to the liver.

Materials and Methods

Isolation and culture of human hepatic sinusoidal endothelial cells (HSEC) and HUVEC

Liver endothelial cells were isolated from ~150 g human liver tissue obtained from donor tissue surplus to surgical requirements as described previously (16) using a modified collagenase perfusion technique. Nonparenchymal cells were separated by density gradient centrifugation over metrizamide (Sigma, Poole, U.K.), and endothelial cells were isolated from the resultant heterogeneous cell mixture by positive immunomagnetic selection using Abs against CD31 (Dako, Ely, U.K.; MB23, 10 μg/ml) and Dynabeads conjugated with goat-anti mouse mAb (Dynal Biotech, Wirral, U.K.) according to the manufacturer’s protocol. HUVEC, isolated using standard methods, were used as a control endothelial cell line.

Maintenance and culture of endothelial cells

Following isolation, hepatic endothelial cells were cultured in complete medium composed of human endothelial basal growth medium (Life Technologies, Paisley, U.K.), 10% AB human serum (HD Supplies, Glasgow, U.K.), 10 ng/ml vascular endothelial growth factor (VEGF) and 10 ng/ml HGF (R&D Systems, Abingdon, U.K.). The cells were placed in collagen-coated culture flasks and were maintained at 37°C in a humidified 5% CO2 incubator until confluent. HUVEC were maintained in the same medium, except the HGF and VEGF were replaced by 10 ng/ml EGF (R&D Systems) and 1 μg/ml hydrocortisone (Sigma). These cells were grown to confluence in culture flasks coated with 1% type B bovine skin gelatin dissolved in PBS (Sigma).

Expression of cell surface adhesion molecules on endothelial cells

Endothelial cells were grown to confluence in collagen-coated 96-well plates and fixed with methanol before performing ELISA. On some occasions endothelial cells were stimulated with cytokines (10 ng/ml recombinant human TNF-α, 10–50 ng/ml recombinant human TNF-β, 10–100 ng/ml recombinant human IFN-γ, 10 ng/ml recombinant human IL-1β), all from PeproTech, London, U.K.), LPS (1–10 μg/ml; Sigma), bile salts (sodiumobenzyldioxyacid and chenodeoxyacid acid, 50–200 μM; Sigma), or 50% heat-inactivated serum from patients with alcoholic hepatitis for 24 h before ELISA development. Non-specific binding of mAb to endothelial cells was prevented by preincubation of the cells with 4% goat serum (Sigma) for 1 h. The endothelial cells were then incubated with mouse anti-human primary mAb (ICAM-1: BBA3, 1 μg/ml; VCAM-1: BBA5, 1 μg/ml; E-selectin: M7105, 3 μg/ml; all from R&D Systems); and VAP-1: 1B2, 1 μg/ml for 45 min at 37°C. The cells were washed thoroughly and incubated with a peroxidase-conjugated goat anti-mouse secondary Ab (PO447, 1/5000; Dako) for 45 min at 37°C. The ELISA was developed using O-phenylenediamine substrate (Dako) according to the manufacturer’s instructions, and the absorbance values were determined at 490 nm. All treatments were performed in triplicate for each experiment.

Static adhesion assays

Peripheral blood lymphocytes were isolated from EDTA anti-coagulated venous blood by centrifugation over Ficoll (Histopaque; Sigma) as previously described (5). HSEC and HUVEC were grown to confluence in 48-well tissue culture plates either without stimulation or with 10 ng/ml TNF-α for 24 h before use. Lymphocytes at a final concentration of 1 × 10⁶ cells/ml were incubated with confluent monolayers of endothelial cells for 30 min at 37°C. The wells were then washed and fixed in methanol, and the number of adherent lymphocytes was determined by counting 10 replicate fields (×100 magnification) of cells in each of two replicate wells per experiment; adhesion was expressed as adherent cells per field.

Flow-based adhesion assays

To determine the effects of physiological blood flow on lymphocyte adhesion, cytokine-stimulated HSEC were cultured to confluence in glass capillary tubes and connected to the flow system previously described (5). Lymphocytes (1 × 10⁶ cells/ml) were perfused through the microslide over the endothelial cells within physiologically relevant ranges of shear stress between 0.05 and 0.2 Pa. Adherent lymphocytes were observed microscopically or via video monitor. Adhesion was converted to cells per square millimeter and corrected for the number of lymphocytes perfused (i.e., adherent cells per square millimeter per 10⁶ perfused). Phase contrast video recordings made during lymphocyte perfusion were analyzed off-line to determine the percentage of rolling cells, statically adherent cells, and transmigrated cells. Rolling cells moved slowly, but steadily, over the endothelial surface during ~5–10 s of observation, while stationary adherent cells did not make any detectable movement over the same period. Transmigrated cells appeared phase dark and were clearly distinguishable from polarized cells migrating across the luminal endothelial surface, which remained phase bright.

Ab interventions

To determine which molecules were involved in lymphocyte adhesion to HSEC, confluent monolayers of cytokine-treated endothelial cells or peripheral blood lymphocytes were treated with function-blocking mAb for 20 min before perfusion of lymphocytes. The mAb used were against CD11a (R3.1.E2; gift from A. Wayne, Boehringer Ingleheim, Hartford, CT), CD18 (R15.7/H4; gift from A. Wayne), P-selectin (Mab2154; Chemicon, Hartford, CT), VCAM-1 (BBA5; R&D Systems), ICAM-1 (BBA3; R&D Systems), VAP-1 (1B2), E-selectin (BBA2; R&D Systems), and...
CD31 (mAb 1398z; Chemicon). All mAb were used at saturating concentrations.

Inhibition of the enzymic capacity of VAP-1

To elucidate whether the monoamine oxidase activity of VAP-1 had a role in the adhesive function of this molecule when presented by hepatic endothelia we pretreated endothelial cells with semicarbazide (200–500 μM; Sigma) or hydroxylamine (1–10 μM; Sigma) for 20 min before perfusion of lymphocytes.

Statistical analysis

Comparisons between treatments were analyzed by ANOVA with MiniTab software.

Results

HSEC express VAP-1 in culture

Cultured HSEC demonstrated a typical endothelial cobblestone morphology after 1 wk in culture, bound Ulex europaeus lectin, and displayed characteristics of sinusoidal endothelium, in that they took up labeled acetylated low density lipoprotein with a cytoplasmic staining pattern (31) (data not shown). In addition, although TNF-α treatment stimulated substantial P-selectin expression on HUVEC we were unable to induce HSEC to express similar levels of P-selectin under the same conditions. Lack of P-selectin expression is another characteristic feature of sinusoidal endothelium (21). ELISA revealed constitutive expression of ICAM-1, CD31, and low levels of VAP-1 (Fig. 1A), induction of VCAM-1 and E-selectin, and increased levels of ICAM-1 following treatment with TNF-α. CD31 expression was consistently lower than that seen on HUVEC in keeping with the reported reduced expression of CD31 on hepatic sinusoidal endothelium in vivo. Compared with HSEC, HUVEC expressed similar profiles of most endothelial adhesion molecules (Fig. 1B), but could not be induced to express VAP-1. It was noted that basal VAP-1 expression on HSEC varied between donors and tended to be lost after five or six passages in culture (for example, the VAP-1 ELISA absorbance values for two typical isolate of cells treated identically were reduced from 0.214 and 0.115 to 0.093 and 0.058, respectively, after one passage in culture).

Cell surface VAP-1 is not increased by treatment with cytokines or bile acids

We attempted to increase cell surface VAP-1 expression on HSEC by treating the cells with a variety of factors, including TNF-α, IL-1β, IFN-γ, bile acids, and LPS. None of these factors consistently increased the expression of VAP-1, although levels of ICAM-1 were elevated appropriately (Fig. 2). Interestingly, serum from patients with alcoholic hepatitis who demonstrated high levels of circulating soluble VAP-1 (32) did appear to cause a modest, but significant, elevation of VAP-1. However, serum had no effect on the level of ICAM-1 expression by HSEC.

VAP-1 on HSEC mediates lymphocyte adhesion

The detection of constitutive expression of VAP-1 on the cell surface of HSEC allowed us to investigate the adhesive function of VAP-1 in detail. In static adhesion assays using peripheral blood lymphocytes, adhesion to unstimulated HSEC was inhibited by mAb against CD11a, CD18, or VAP-1, whereas inhibiting VCAM-1, ICAM-1, or the selectins alone had little effect. This suggests that both VAP-1 and ICAM-2 have a role in adhesion in this system. When TNF-α-activated HSEC were used, adhesion was inhibited by mAb against CD11a, CD18, ICAM-1, VCAM-1, and VAP-1. Anti-E-selectin and anti-P-selectin had no effect. VAP-1 Abs had no effect on lymphocyte adhesion to HUVEC (Fig. 3B) or the dermal endothelial cell line HMEC-1 (not shown).

HSEC support the adhesion of lymphocytes under conditions of flow.

We investigated the role of VAP-1 under conditions of shear stress using a flow-based adhesion assay to model physiological flow in the hepatic sinusoids. The level of lymphocyte adhesion to HSEC was dependent on the wall shear stress, and when lymphocytes were perfused over unstimulated HSEC at shear stresses between 0.05 and 0.1 Pa to reproduce the flow rates detected in sinusoids, very few cells adhered (less than one cell per field of view), and those that did arrested immediately without a prior rolling phase (data not shown). Treatment of the monolayers with the cytokines TNF-α, TNF-β, and LPS for 24 h before lymphocyte perfusion greatly increased the number of adherent cells (Fig. 4A), and the level of this adhesion was shear stress dependent (Fig. 5 shows representative data for TNFα-treated endothelial cells (EC)). Offline frame-by-frame analysis of the experimental video record was performed to quantify the number of adherent lymphocytes that exhibited sustained, rolling adhesion. The percentage of lymphocytes that underwent sustained rolling adhesion on HSEC varied according to which cytokine was used to stimulate the endothelial cells (Fig. 4B). However, on average, <10% of the total number of adherent lymphocytes rolled persistently over the HSEC, and the majority immediately arrested upon contact with EC. This finding was particularly interesting and suggested that in the unique rheological environment present within the hepatic sinusoid, classical sustained rolling adhesion is not a prerequisite for lymphocyte capture and arrest.
VAP-1 mediates lymphocyte adhesion to TNFα-stimulated HSEC. Since TNF-α treatment of HSEC resulted in the highest levels of lymphocyte adhesion, we decided to use this cytokine for subsequent experiments designed to elucidate whether VAP-1 has a role in lymphocyte adhesion to hepatic endothelium under conditions of shear stress. Treatment of TNF-α-stimulated HSEC with anti-VAP-1 mAb consistently reduced the total number of lymphocytes adhering by ~50% (Fig. 6A). The degree of adhesion blockade varied between different cell preparations from different livers, but was related to the level of VAP-1 expression seen on each cell isolate. Treatment of TNF-α-stimulated HUVEC with anti-VAP-1 had no effect on lymphocyte adhesion (for example, in one representative experiment adhesion of lymphocytes to HUVEC at 0.05 Pa was 818 and 850 cells/mm²/10⁶ cells perfused for control and VAP-1 Ab-treated cells, respectively). A blocking mAb raised against ICAM-1 reduced lymphocyte adhesion to both HSEC (Fig. 6A) and HUVEC. Combinations of both VAP-1 and ICAM-1 Abs reduced adhesion to HSEC more than either Ab alone, but no additional inhibitory effect on rolling adhesion was observed under these circumstances (Fig. 6). An isotype-matched control Ab had no effect (not shown). To totally inhibit lymphocyte adhesion to HSEC under shear, it was necessary to treat the endothelium with a combination of VAP-1, E-selectin, VCAM-1, and ICAM-1 Abs (not shown).

Previous intravital microscopy experiments in rabbits and in vitro studies with cell lines transfected with VAP-1 have raised the possibility that under certain conditions VAP-1 acts as a rolling receptor or molecular brake (33, 34). We therefore analyzed our experimental videos to investigate this possibility. Anti-VAP-1 Ab reduced the proportion of lymphocytes that rolled on TNF-α-stimulated HSEC (Fig. 6B), but the effect was small and was not statistically significant. However, treatment of cytokine-activated HSEC with anti-E-selectin mAb significantly reduced the percentage of adherent cells that rolled (Fig. 7). Although little if any E-selectin is expressed on hepatic sinusoidal endothelium in vivo, isolated cultured HSEC expressed E-selectin following TNF-α treatment (see Fig. 1A). Therefore, to reproduce the adhesive environment that exists in vivo, we repeated our adhesion experiments in the continuous presence of anti-E-selectin mAb. Under these conditions the addition of anti-VAP-1 mAb-1 reduced the total adhesion further, but did not reduce the number of rolling cells (Fig. 7). These data suggest that even in the absence of selectins, VAP-1 does not act as a classical rolling receptor.

VAP-1 mediates transmigration of lymphocytes after firm adhesion under conditions of flow. The proportion of adherent lymphocytes that migrated through the HSEC monolayers varied with the cytokine used to stimulate the endothelial cells. About 25% of adherent lymphocytes migrated through LPS- or TNF-α-stimulated endothelial cells, but only 10% migrated through TNF-α-stimulated HSEC (Fig. 8). However, in each experiment, regardless of which cytokine was used to stimulate the endothelium, treatment of HSEC with anti-VAP-1 mAb reduced the proportion of transmigrating cells. Anti-ICAM-1 also had a significant effect on lymphocyte transendothelial migration, whereas isotype-matched control Ab, anti-VCAM-1, and anti-E-selectin Abs had no effect.
VAP-1 enzyme activity is involved in the adhesion and transmigration of lymphocytes on HSEC under conditions of flow. Because VAP-1 is a member of the monoamine oxidase family of enzymes (classified as a semicarbazide-sensitive amine oxidase) (12, 29), we were interested to determine whether the enzymatic activity of VAP-1 is involved in the adhesive effects of the molecule. We performed flow-based adhesion assays in the presence of specific inhibitors of amine oxidase activity. Hydroxylamine was used to inhibit all monoamine oxidase activity. However, the effects of these inhibitors were rapidly reversible. If the monoamine oxidase inhibitors were not maintained in our wash solution, adhesion and transmigration of lymphocytes returned to those levels seen in the absence of inhibitor.

When similar experiments were conducted using HUVEC, the inhibitors of enzyme activity did not decrease the adhesion of lymphocytes (not shown).

Discussion

We have described for the first time the culture of primary human endothelial cells that express functional VAP-1 and have used these cells to determine the nature of the adhesive interactions supported by VAP-1. We have previously used tissue binding assays to demonstrate that VAP-1 supports lymphocyte adhesion to liver endothelium (25, 26). However, the information gained from such assays is limited; they do not operate under physiological shear stress, which in itself can affect the adhesion and migration of lymphocytes (35), and they do not allow analysis of the individual components of the adhesion cascade. In the present study we have used primary cultures of HSEC in a flow-based adhesion assay and shown that 1) VAP-1 on liver endothelium supports lymphocyte adhesion under laminar shear stress comparable with that in the hepatic circulation in vivo; 2) VAP-1 is an important mediator of lymphocyte transendothelial migration; and 3) the ability of VAP-1 to support adhesion and transendothelial migration is blocked by specific inhibitors of its enzyme activity.

Functional characterization of VAP-1 has been difficult because it is not expressed on the cell surface of previously studied human endothelial cell lines (36). Our finding of significant levels of cell surface VAP-1 on liver endothelial cells in vitro allowed us to report for the first time the detailed function of VAP-1 using primary endothelial cells. VAP-1 is constitutively expressed on human liver endothelium in vivo, and by isolating endothelial cells from human liver and culturing them in a combination of HGF and VEGF we were able to establish primary cultures that had morphological and functional characteristics of sinusoidal endothelium. We refer to these cells as human HSEC. Although rabbit cardiac endothelial cells have been shown to express VAP-1 in culture, we have failed to induce VAP-1 expression on non-hepatic human endothelial cells in vitro, suggesting that microenvironmental factors are critical for VAP-1 expression (34, 36). Compared with HUVEC cultured under the same conditions, HSEC had a distinctive phenotype-expressing cell surface VAP-1, low levels of CD31, and little or no P-selectin even under conditions in which P-selectin was detected on HUVEC. This is consistent with the phenotype in vivo where sinusoidal endothelial cells lack Weibel-Palade bodies, express little CD31 or P-selectin, but constitutively express VAP-1 (37–39), demonstrating that these cells maintain tissue-specific characteristics in vitro. HSEC could be induced to express functional E-selectin by treatment with pro-inflammatory cytokines, which contrasts with the situation in vivo where little or no E-selectin expression is detected even on inflamed sinusoidal endothelium, suggesting that E-selectin up-regulation is suppressed in vivo (21, 22).

We attempted to increase cell surface expression of VAP-1 with a variety of factors, including pro-inflammatory cytokines, LPS, and bile acids. None of these factors consistently increased cell surface VAP-1, suggesting that VAP-1 expression is tightly regulated and is not responsive to factors that increase the expression of broadly expressed adhesion molecules such as ICAM-1, VCAM-1, and the selectins. However, we observed that serum from patients with alcoholic liver disease who had high circulating soluble levels of VAP-1 modestly elevated endothelial expression of VAP-1 (Fig. 2A). Since we did not see a concurrent elevation in ICAM-1 levels (Fig. 2B) on the same endothelial isolates, we suspect that the VAP-1 response is probably not due to a classical proinflammatory cytokine, but as yet we have not identified the
factor responsible. Thus, VAP-1 shows restricted tissue expression in vivo and is up-regulated by a limited number of signals in vitro, suggesting that it might play a specific role in lymphocyte recruitment to the liver. This is supported by our previous observations that the circulating form of VAP-1 is derived from the hepatic vascular bed and is elevated in chronic inflammatory liver diseases (32, 40).

The constitutive expression of VAP-1 and the minimal role for selectins in the sinusoids (23) together with the distinct rheological features of this vascular bed (41) suggest that some of the elements of the adhesion cascade will differ from those operating in post-capillary venules. To assess the role of VAP-1 under conditions comparable to those seen within the liver in vivo, we established a flow-based adhesion system in which we replicated the low levels of shear stress detected within hepatic sinusoids. This model allowed us to study the nature of the adhesion mediated by hepatic endothelial VAP-1 under flow for the first time and thereby to elucidate the adhesive function of this unique receptor.

Under conditions of low shear stress that reflect those found in sinusoids in vivo, only a small proportion of lymphocytes exhibited sustained rolling, and most of the adherent cells underwent rapid arrest without prior rolling. This may reflect the comparatively low levels of E-selectin detected on these cells in vitro as well as a reduced dependency on rolling interactions to capture lymphocytes within the low shear environment of the sinusoids. Inhibition of VAP-1 had a consistent inhibitory effect on the adhesion of lymphocytes to HSEC under flow, although the magnitude of the effect varied between cell isolations and with the activating stimulus. When TNF-α-activated HSEC were used, the inhibition seen with anti-VAP-1 was comparable to that seen with an Ab to ICAM-1, but the ability of VAP-1 Ab to block adhesion to LPS- or TNF-β-activated endothelium was less marked despite similar levels of VAP-1 on all preparations. This may reflect increased expression of other molecules, because TNF-α-activated endothelium consistently supported higher total adhesion than TNF-β- or LPS-activated endothelium.

A role for VAP-1-mediated adhesion under shear stress is consistent with previous studies showing VAP-1-dependent lymphocyte adhesion to rat peripheral lymph node endothelial cells transfected with human VAP-1 and recent intravital studies where it has been shown to act as a brake for neutrophils on rabbit mesenteric vessels (34). The precise nature of the adhesion mediated by

**FIGURE 4.** The effects of different cytokines on the adhesion of peripheral blood lymphocytes to HSEC. A, Total adhesion of lymphocytes to HSEC stimulated with TNF-α, TNF-β, or LPS for 24 h before assay. Adherent lymphocytes were observed microscopically or via a video monitor. Adhesion was converted to cells per square millimeter and was corrected for the number of lymphocytes perfused (i.e., adherent cells per square millimeter per 10⁶ perfused). Data represent the mean ± SEM of at least four experiments performed at 0.05 Pa. B, Percentage of adherent lymphocytes that rolled on HSEC stimulated with TNF-α, TNF-β, or LPS for 24 h before assay. Phase contrast video recordings made during lymphocyte perfusion were analyzed off-line to determine the percentages of rolling cells and statically adherent cells. Rolling cells moved slowly but steadily over the endothelial surface during 5–10 s of observation, while stationary adherent cells did not make any detectable movement over the same period. Data represent the mean ± SEM of at least four experiments performed at 0.05 Pa.

**FIGURE 5.** Adhesion of lymphocytes to HSEC under conditions of shear stress. Data are from two experiments performed at a variety of different shear stresses using HSEC that were treated with 10 ng/ml TNF-α for 24 h before the experiment.
FIGURE 6. The effect of Abs raised against VAP-1 and ICAM-1 on the adhesion of PBL to TNF-α-stimulated HSEC. A, Inhibition of VAP-1 and ICAM-1 reduces total adhesion of lymphocytes to TNF-α-stimulated HSEC. The addition of individual blocking mAb against VAP-1 and ICAM or a combination of the two significantly reduced lymphocyte adhesion (by ANOVA, \( p < 0.05 \) for both). Data represent adhesion as a percentage of that seen in the absence of blocking Ab and are the mean ± SEM of three experiments performed at 0.05 Pa. B, The effect of VAP-1 and ICAM-1 blockade on the percentage of adherent lymphocytes that roll on TNF-α-stimulated HSEC. Data represent rolling as a percentage of that seen in the absence of blocking Ab and are the mean ± SEM of five experiments performed at 0.05 Pa. Neither Ab had a significant effect on rolling adhesion (by ANOVA, \( p > 0.1 \) for both).

FIGURE 7. The effect of blocking anti-VAP-1 and E-selectin on lymphocyte adhesion to TNF-α-activated HSEC. A, The total number of adherent lymphocytes binding to TNF-α-stimulated HSEC is reduced by Ab raised against VAP-1 and E-selectin. Data represent the mean ± SD of two experiments performed at 0.05 Pa. B, Ab raised against E-selectin significantly reduces the number of adherent lymphocytes that roll on HSEC, and this adhesion is not further reduced by treatment with VAP-1 Ab.
VAP-1 is unclear. It did not act as a classic rolling receptor because the proportion of rolling cells was unaffected by VAP-1 blockade in most of our experiments. Furthermore, although the total number of adherent cells was consistently reduced when inhibition of VAP-1 was combined with inhibition of E-selectin, the number of rolling cells was the same as that seen with E-selectin blockade alone, suggesting that these receptors are acting at different points in the adhesion cascade.

In addition to inhibiting adhesion, blockade of VAP-1 had a marked inhibitory effect on lymphocyte transendothelial migration that was independent of its ability to support adhesion and was seen with TNF-α- and LPS-activated HSEC as well as TNF-β-treated cells. This effect was specific for VAP-1 or ICAM-1 Abs and did not occur with control Ab or Abs against VCAM-1 or E-selectin. Comparatively little is known about the molecules that regulate transendothelial migration of adherent lymphocytes. There is evidence that β2 integrins and their ligands are involved (42, 43), although other studies suggest that they act predominantly at the firm adhesion step rather than directly regulating transmigration (44). Several molecules detected preferentially at endothelial cell junctions, including CD31 and JAM-1, have been proposed to mediate transmigration of adherent leukocytes (45). The role of CD31 in lymphocyte transmigration is contentious, and because CD31 and its putative receptor αvβ3 are not expressed on all lymphocyte subsets, it cannot provide a general mechanism for transmigration (46, 47). Moreover, mice that are deficient in CD31 exhibit normal lymphocyte migration (48). In the present study we found that both anti-ICAM-1 and anti-VAP-1 inhibited lymphocyte transmigration under conditions of flow. We were careful to assess transendothelial migration as a proportion of adherent cells to allow an accurate assessment of transmigration independently of the contact and arrest steps, allowing us to state that both ICAM-1 and VAP-1 are directly involved in transendothelial migration in our model. These results support a role for VAP-1 and ICAM-1 in lymphocyte recruitment to the liver and also suggest that VAP-1 could be involved in transmigration at other sites where it is upregulated during inflammation (24).

VAP-1 is a monoamine oxidase belonging to the subfamily of semicarbazide-sensitive amine oxidases. We have recently shown that the previously reported increased monoamine oxidase activity in serum of patients with chronic liver disease (49–51) is, in fact, SSAO activity derived from soluble VAP-1 protein (40). Recent reports suggest that the adhesive and enzymic functions of VAP-1 are intimately linked, and we therefore investigated whether this was the case in our system. We used specific inhibitors to block enzymic activity of VAP-1 and found that the specific semicarbazide-sensitive amine oxidase inhibitor (semicarbazide) and a broad-acting monoamine oxidase inhibitor (hydroxylamine) both markedly reduced lymphocyte adhesion and transmigration to levels comparable to those observed with Ab blockade. These data differ from those from our previous studies (33), which found no
role for the enzymatic capacity of VAP-1 in the adhesion of lymphocytes to VAP-1-transfected cells. However, the transfected cells have relatively low monoamine oxidase activity (S. Jalkanen, unpublished observations), and there may clearly be other differences between transfectants and our primary endothelial cells. We conclude that native VAP-1 expressed by hepatic endothelial cells has both adhesive and enzymatic functions.

This effect was specific for hepatic endothelial cells that express VAP-1, since adhesion and transmigration of lymphocytes on HUVEC were not decreased by either treatment. Other molecules have been reported to share adhesive and enzymatic properties, including CD26, CD73, and the adamalysins, but VAP-1 is the only adhesion molecule with amine oxidase activity (30, 52). We have not yet elucidated a mechanism by which enzymatic catalysts can support adhesion, but two of us (M. Salmi and S. Jalkanen) have recently proposed that the active site of S1AS in VAP-1 may mediate adhesion via interactions with immobilized amine residues on the lymphocyte surface (30). The fact that the inhibitors had a similar effect on transendothelial migration is the first demonstration of enzymatic regulation of this process.

In summary, we have shown that VAP-1 is expressed on human hepatic endothelial cells in culture and that its expression is regulated by a limited number of factors. The ability of VAP-1 to support lymphocyte adhesion and transendothelial migration under conditions of shear stress comparable to those found in the hepatic sinusoids in vivo suggest that it is functionally important in regulating lymphocyte recruitment to the human liver.

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