Circulating Form of Human Vascular Adhesion Protein-1 (VAP-1): Increased Serum Levels in Inflammatory Liver Diseases

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*J Immunol* 1998; 161:1549-1557;
http://www.jimmunol.org/content/161/3/1549
Circulating Form of Human Vascular Adhesion Protein-1 (VAP-1): Increased Serum Levels in Inflammatory Liver Diseases

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Vascular adhesion protein-1 (VAP-1) is a dimeric 170-kDa endothelial transmembrane molecule that under normal conditions is most strongly expressed on the high endothelial venules of peripheral lymph nodes and on hepatic endothelia. It is a glycoprotein that mediates tissue-selective lymphocyte adhesion in a sialic acid-dependent manner. In this study, we report the detection of a soluble form of VAP-1 in circulation. We developed a quantitative sandwich ELISA using novel anti-VAP-1 mAbs and used it to determine the levels of soluble VAP-1 (sVAP-1) in the serum of healthy individuals and in patients with inflammatory diseases. In healthy persons, circulating sVAP-1 concentrations were 49 to 138 ng/ml. Immunoblotting studies revealed that the apparent molecular mass of dimeric sVAP-1 is slightly (~10 kDa) higher than that of transmembrane VAP-1 under nonreducing conditions. In contrast, the electrophoretic mobilities of monomeric sVAP-1 and transmembrane VAP-1 were similar after reduction and boiling. Adhesion assays showed that the circulating sVAP-1 modulates lymphocyte binding to endothelial cells. Inflammation can cause an elevation of serum sVAP-1 levels, because sVAP-1 concentrations in patients with certain liver diseases were two- to fourfold higher than those in normal individuals. In contrast, rheumatoid arthritis and inflammatory bowel diseases were not associated with elevated levels of sVAP-1. These findings indicate that there is a functionally active, soluble form of VAP-1 in circulation and suggest that the serum level of sVAP-1 might be a useful marker of disease activity in inflammatory liver diseases. The Journal of Immunology, 1998, 161: 1549–1557.

The ability of leukocytes to extravasate from the vasculature to the tissue space is a fundamental feature of the normal immune system. Several cell adhesion molecules play a key role in this complex process of initial and stable adhesion and diapedesis of leukocytes across the endothelial barrier (1–4). Vascular adhesion protein-1 (VAP-1) is one of the endothelial cell adhesion molecules that mediates lymphocyte binding to the endothelium under shear conditions (5, 6). It is constitutively expressed in high endothelial venules in peripheral lymph nodes. Moreover, the expression of VAP-1 is induced/up-regulated by chronic inflammation in the vessels of the tonsil, gut, skin, and synovium (7). VAP-1 is also present on sinusoidal and vascular endothelia in the liver under both normal and inflammatory conditions (8); however, it is not found on any of the blood leukocytes (5). The mature VAP-1 molecule is a 170-kDa homodimeric glycoprotein that consists of two 90 kDa subunits held together by disulfide bonds (9). VAP-1 has a large extracellular domain, a single-pass transmembrane domain, and a short cytoplasmic tail (40). The molecule has abundant sialic acid decorations that are essential to its adhesive function, because VAP-1 is unable to mediate lymphocyte adhesion to desialylated vessels (9). The leukocyte ligand for VAP-1 is currently unknown.

Over recent years, many of the leukocyte-endothelial cell adhesion molecules, such as ICAM-1, VCAM-1, platelet endothelial cell adhesion molecule-1, selectins, and CD44, have been shown to circulate in soluble forms (10–18). Extracellular parts of CD44, L- and E-selectin, VCAM-1, and ICAM-1 are cleaved enzymatically from the cell surface and released into the bloodstream (19). This process of shedding is one mechanism by which soluble forms of adhesion molecules can be produced. In addition, circulating molecules can arise at the level of RNA processing, as has been demonstrated for P-selectin and platelet endothelial cell adhesion molecule-1, which have alternatively spliced mRNAs lacking the transmembrane domains (14, 20, 21). Soluble adhesion molecules may have distinct and contrasting physiologic effects. They may function as inhibitors of cell to cell adhesion by competing with their membrane-bound forms. For example, soluble L-selectin and soluble ICAM-1 reportedly inhibit lymphocyte adhesion in vitro (16, 22). Alternatively, soluble molecules can trigger responses in cells that bear their ligands (23). Furthermore, because the serum levels of these circulating adhesion molecules alter in different disease states (10, 12, 13, 15, 24, 25), the determination of their concentrations can be useful for the diagnosis and monitoring of certain diseases.

We hypothesized that VAP-1 might exist in a soluble form. In this study, we have identified and quantitated such a circulating...
form of VAP-1 in normal human serum (NHS) and have characterized the biochemical structure of soluble VAP-1 (sVAP-1). We also show that the level of sVAP-1 is elevated in patients with certain liver diseases but not in patients with rheumatoid arthritis (RA) or inflammatory bowel diseases (IBDs), raising the possibility that the determination of sVAP-1 may have clinical significance in the follow-up of inflammatory liver diseases. Moreover, our studies indicate that the presence of sVAP-1 enhances the binding of lymphocytes to VAP-1 transfectants, which suggests an adhesion-modulating function for sVAP-1.

Materials and Methods

Antibodies

New anti-VAP-1 mAbs were produced by immunizing mice with affinity-purified VAP-1. In brief, VAP-1 was immunoprecipitated from precleared 1% Nonidet P-40 lysates of tonsil stroma using the original anti-VAP-1 mAb IB2; this original mAb had been immobilized on cyanogen bromide (CNBr)-activated Sepharose 4B beads as an affinity matrix, as described below for other mAbs. After extensive washings, the beads were mixed with IFA and used to immunize specific pathogen-free BALB/c mice. After three s.c. injections into foot pads at 1-wk intervals, mice were sacrificed, and the popliteal lymph nodes were collected. The isolated lymphocytes were then fused with nonsecreting SP-2 myeloma cells (American Type Culture Collection, Manassas, VA) using standard procedures. Hybridomas were screened using immunofluorescence staining of stable VAP-1 cDNA transfectants, and positive hybridomas were subcloned at least twice by limiting dilution. The isotype of the mAbs was determined using ImmunoNoType strips (Sigma, St. Louis, MO). All new anti-VAP-1 mAbs, which were designated TK8–14, TK8–18, and 2D10, showed an identical tissue-staining pattern with the mAb IB2, and these mAbs reacted with a 170-kDa molecule during the immunoblotting of tonsil stroma under nonreducing conditions. The mAbs 3G6 and Hermes-3 were used as negative controls in these experiments (see Table 1).

Biotinylated TK8–14, Hermes-3, and 3G6 Abs were prepared according to standard procedures. Each Ab was initially diluted in PBS (1 mg/ml), and pH was adjusted to 8.2 to 8.6 with 7% NaHCO₃. A total of 180 µl of NHS-biotin (Calbiochem-Novabiochem, La Jolla, CA) at 1 mg/ml in DMSO was added to 1 ml of the Ab concentrate. After stirring for 4 h at room temperature, the reaction mixture was dialyzed against PBS at 4°C to remove free biotin.

Serum samples

Serum samples from 74 normal healthy volunteers between the ages of 21 and 68 (mean ± SD, 35 ± 13) were obtained and stored at −70°C. Of the donors, 34 were males and 40 were females. Blood was drawn into Terumo Venovenous serum tubes (Leuven, Belgium) with a 20-gauge needle and allowed to clot for at least 30 min. After centrifugation at 3000 rpm for 10 min, serum was collected, aliquoted, and kept frozen at −70°C until use in the sVAP-1 ELISA.

Serum samples from patients with different inflammatory diseases were also measured by the sVAP-1 ELISA. There were 11 patients with RA, 9 with IBDs (6 with ulcerative colitis and 3 with Crohn’s disease), and 41 with different kinds of liver diseases. The liver disease group contained patients with alcoholic liver disease (ALD) (n = 11), primary biliary cirrhosis (PBC) (n = 11), primary sclerosing cholangitis (PSC) (n = 8), hepatocellular carcinoma (HCC) arising in the noncirrhotic liver (n = 8) and colorectal hepatic metastases (CHM) (n = 3).

sVAP-1 ELISA

Wells of microtiter plates (96-well, flat-bottom, white Clinitest EP Lab-Sys, Helsinki, Finland) were coated with 100 µl of the anti-VAP-1 mAb TK8–14 at 10 µg/ml in 0.1 M NaHCO₃ buffer (pH 9.6) at 4°C overnight and then at 37°C for 1 h. The wells were washed six times with 0.1% Tween 20 in PBS (Tween/PBS) and then blocked to prevent nonspecific adsorption by the addition of 200 µl of PBS containing 1% gelatin and 1% nonfat milk powder (blocking solution) for 45 min at room temperature. After washing the wells six times with Tween/PBS, 175 µl of each serum sample (at 1:25 dilution in the blocking solution) was added into the wells, and the plates were left at room temperature for 1 h. The wells were then washed six times with Tween/PBS and incubated with 100 µl of the biotinylated anti-VAP-1 mAb TK8–14 or biotinylated control mAb Hermes-3 (10 µg/ml in the blocking solution) at room temperature for 1 h. After six washes with Tween/PBS, 100 µl of streptavidin-horse-radish peroxidase (Amersham Int., Buckinghamshire, U.K.; diluted 1:1000 in the blocking solution) was added into the wells, and the plates were allowed to incubate at room temperature again for 1 h. Thereafter, plates were washed six times with Tween/PBS and finally developed with chemiluminescence ELISA reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s instructions. The intensities of the chemiluminescence reactions in the wells were always measured after a 3-min incubation time with a Luminoskan 390 luminometer (Labsystems).

All serum samples were initially measured at a 1:25 dilution. However, some of the samples gave values that were out of the linear range of the assay when diluted to 1:25; therefore, those samples were remeasured after dilution so that the obtained values fit into the linear range. Each sample was measured in triplicate with both the anti-VAP-1 mAb and the negative control mAb. The specific VAP-1 value was calculated by subtracting the mean background value of the negative control from the mean value of VAP-1.

Each assay included a titration of the previously quantified tonsil lysate; this titration was used to generate a standard curve. To obtain a protein milieu similar to the serum samples, tonsil lysate standards were diluted in blocking solution that contained as much sVAP-1-depleted human serum as the test samples contained patient serum. Calculations of the amount of sVAP-1 in the serum samples were made by comparing the specific VAP-1 values with a standard curve of titrated tonsil lysate using linear regression analysis.

The amount of VAP-1 present in the standard tonsil lysate was determined as follows: Immunoaffinity-purified VAP-1 was obtained from tonsil lysate by column chromatography using CNBr-activated Sepharose 4B beads coupled with anti-VAP-1 mAb as described below. Aliquots of the affinity-purified sample were electrophoresed on 5 to 12.5% SDS-PAGE gels. The resolved proteins were then stained with two different protein dyes: AgNO₃ and Cypro Orange (Bio-Rad, Hercules, CA). The silver-stained gel was scanned using the Microcomputer Imaging device (Imaging Research, Ontario, Canada) and the Cypro orange-stained gel was scanned using a FluorImage imaging system (Molecular Dynamics, Grefeld, Germany). The densities of the VAP-1 bands were quantitated against the protein standard curves that had been generated with BSA, and an average of the two results obtained by staining with different protein dyes was calculated. The concentration of VAP-1 in the standard tonsil lysate was finally determined by comparing the ELISA signal from the same batch of immunoaffinity-purified VAP-1 with the signal from the titration of standard tonsil lysate.

Immunoaffinity purification and immunoblotting of VAP-1

sVAP-1 was purified by immunoaffinity column chromatography using AB serum obtained from the Finnish Red Cross Blood Bank. For purification, 200 ml of AB serum was precipitated with 25% (NH₄)₂SO₄ overnight at 4°C. After centrifugation at 10,000 rpm for 30 min at 4°C, the precipitate was resuspended in lysis buffer (10 mM Tris-Cl, 150 mM NaCl, 1 mM MgCl₂, and 1.5 mM MOPS, Molecular Dynamics, Grefeld, Germany). The densities of the VAP-1 bands were quantitated against the protein standard curves that had been generated with BSA, and an average of the two results obtained by staining with different protein dyes was calculated. The concentration of VAP-1 in the standard tonsil lysate was finally determined by comparing the ELISA signal from the same batch of immunoaffinity-purified VAP-1 with the signal from the titration of standard tonsil lysate.

Table 1. mAbs used in this study

<table>
<thead>
<tr>
<th>mAb</th>
<th>Ag</th>
<th>Monomer</th>
<th>Dimer</th>
<th>Isotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB2</td>
<td>VAP-1</td>
<td>+</td>
<td>Mouse IgM</td>
<td>5.26</td>
<td></td>
</tr>
<tr>
<td>TK8–18</td>
<td>VAP-1</td>
<td>+</td>
<td>Mouse IgG2a</td>
<td>˜˜</td>
<td></td>
</tr>
<tr>
<td>TK8–14</td>
<td>VAP-1</td>
<td>+</td>
<td>Mouse IgG2a</td>
<td>˜˜</td>
<td></td>
</tr>
<tr>
<td>2D10</td>
<td>VAP-1</td>
<td>−</td>
<td>Mouse IgG1</td>
<td>˜˜</td>
<td></td>
</tr>
<tr>
<td>Hermes-3</td>
<td>CD44</td>
<td>+</td>
<td>Mouse IgG2a</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>3G6</td>
<td>Chicken T cell</td>
<td>−</td>
<td>Mouse IgG1</td>
<td>5</td>
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</tr>
</tbody>
</table>

* mAbs described here.
the samples to sialidase treatment that was performed with 5 nM of *Vibrio cholerae* neuraminidase (Behringwerke AG, Marburg, Germany) at 37°C for 2 h. For gel electrophoresis, the samples were mixed with Laemmli sample buffer with or without reduction (5% 2-ME), incubated at either 37°C for 20 min (nonreduced) or 95°C for 5 min (reduced), and loaded on 5 to 12.5% SDS-PAGE gels. The resolved proteins were transferred onto nitrocellulose membranes (Hybond/Enhanced chemiluminescence (ECL), Amersham) with a Hoefer electroblotter (San Francisco, CA). The nitrocellulose sheets were then developed using the ECL detection system (ECL kit for Western blotting, Amersham) according to the manufacturer’s recommendations. In brief, the membranes were blocked with PBS containing 10% nonfat milk powder and 0.3% Tween 20, and the primary Abs were used at 2 μg/ml. VAP-1 Ag was also immunofinity-isolated from tonsil. For purification, tonsil stroma was lysed in lysis buffer supplemented with 1 mM EDTA, 1% aprotinin, and 1 mM PMSF as protease inhibitors, and insoluble material was removed by centrifugation. The supernatants were then precleared, passed over affinity columns, depleted of Igs, digested with sialidase, electrophoresed, and immunoblotted as described above. The tonsil lysate as such was also used as the source of membrane-bound VAP-1 Ag for immunoblotting. Lysate supernatants were depleted of Igs with protein G beads before electrophoresing on a SDS-PAGE gel, as described above for serum samples.

For an alternative analysis of sVAP-1, serum was depleted of most albumin by three successive incubations with Blue Sepharose beads (Pharmacia, Uppsala, Sweden) at 4°C overnight. Thereafter, the serum was incubated with protein G beads to remove Igs and mixed with Laemmli sample buffer. After heating at 37°C for 20 min, the samples were run on a 5 to 12.5% SDS-PAGE gel, and the resolved proteins were transferred onto a nitrocellulose membrane. Nitrocellulose strips were then developed using bio-tinylated primary mAbs and the ECL kit for Western blotting. Blocking was performed with 0.3% Tween 20 in PBS. The biotinylated mAbs were used at 1 μg/ml, and streptavidin-horseradish peroxidase was used as the secondary detection reagent.

**Adhesion assays**

Stable VAP-1 transfectants were generated by transfecting a eukaryotic expression vector, pcDNA3, containing the VAP-1 cDNA into a VAP-1− endothelial cell line, Ax, as described elsewhere (40). Mock transfectants were transfected similarly using pcDNA3 containing VAP-1 cDNA in an inverse orientation. The expression of VAP-1 on these cells was determined by immunofluorescence stainings with anti-VAP-1 and control mAbs and by FACS analyses using standard techniques. Adhesion assays were performed on microscopic slides. A wax pen circle (2 cm in diameter) was drawn on the slide, and the area delineated by the circle was precoated with 1% gelatin in PBS for 3 h at 37°C. A total of 20,000 AX VAP-1 or mock transfectants were seeded within the circles in RPMI 1640 medium containing 10% FCS, penicillin (100 IU/ml) and streptomycin (100 μg/ml), and 10 mM HEPES and grown into confluence.

A serum sample containing a high concentration of sVAP-1 was divided into two 0.5 ml aliquots. sVAP-1 was specifically depleted from one aliquot by two sequential incubations with CNBr-beads that had been coupled into two 0.5 ml aliquots. sVAP-1 was specifically depleted from one aliquot as described above for serum samples.

**Results**

**Development of a sandwich ELISA for sVAP-1**

A quantitative sandwich ELISA was developed, using newly generated anti-VAP-1 mAbs, to determine the amount of sVAP-1 in serum samples. The highest level of sensitivity was obtained using the anti-VAP-1 mAb TK8−18 as a capture Ab and biotinylated anti-VAP-1 mAb TK8−14 as a detecting Ab. Tonsil detergent lysate and pooled human AB serum were used as positive controls in the assay. The chemiluminescence signals obtained with 1:10 dilutions of tonsil lysate and AB serum were ~3000 and ~1500 U, respectively.

The specificity of the ELISA was confirmed by three different procedures: coating the wells with an irrelevant mAb 3G6, using serum samples that had been depleted of sVAP-1 by immunofinity chromatography, and detecting with an irrelevant biotinylated mAb, Hermes-3. No significant reactivity was observed in any of these controls (chemiluminescence values of 0.3−4 U). Since as many as 1 to 3% of normal individuals may have anti-mouse IgG Abs in their serum that can cross-link the capture and biotinylated detecting murine Abs in the absence of Ag, we chose to use the murine Abs Hermes-3 as a negative control for every serum sample in all subsequent experiments to eliminate all possible false positive results.

The obtained chemiluminescence signals were converted into nanogram/milliliter values by including a titration of a previously quantitated standard of tonsil lysate in each performed assay and using linear regression analysis (see Materials and Methods). Using one batch of tonsil lysate as a standard was found to be the best alternative in the absence of sVAP-1. This ELISA proved to be linear up to 1.1 ng of VAP-1/well, and as little as 0.005 ng of VAP-1/well was reliably detected with this assay. In addition, the inter- and intraassay variations were usually ~10%. The amount of immunoreactive sVAP-1 in serum was found to be unaffected by freezing-thawing cycles.

**Detection of sVAP-1 in NHS**

Sera from 74 healthy individuals between the ages of 21 and 68 (mean 35) were tested for the amount of sVAP-1 by this ELISA. The sVAP-1 concentrations in this population of 34 males and 40 females varied from 49 to 138 ng/ml (Fig. 1A), and the mean ± SD level for this group of normal controls was 88 ± 20 ng/ml. The sVAP-1 values did not correlate with the age (r = −0.076; p = 0.52) or sex (p = 0.30) of the donors. In addition, 12 of the blood donors were retested several months after the first measurement to evaluate the intra-individual variability of the sVAP-1 level. The observed changes in their values were between 0.1% and 20% (mean ~7%) (see Table II). In 8 of the 12 cases, the changes were within the variation limits of the assay; in the remaining 4 cases, the changes were <20%. Thus, the level of sVAP-1 in the serum of healthy individuals seems to be quite stable over time. Moreover, to study diurnal variations of the sVAP-1 level, four of the normal blood donors were tested for their sVAP-1 levels at four different time points: at 6 p.m., at 12 a.m., at 6 a.m., and at 12 p.m. As seen in Figure 1B, sVAP-1 concentrations tended to be higher in the evening than in the morning, but the differences were quite minor (<25%).
Biochemical structure of sVAP-1

SDS-PAGE and the immunoblotting analysis of affinity-purified sVAP-1 were performed to determine the molecular mass of the circulating VAP-1 molecule and to compare it with the membrane-bound form. Initially, nonreducing conditions and mild heating of samples were chosen because the mature dimeric VAP-1, which consists of two identical subunits held together by disulfide bonds, dissociates into its monomeric subunits by reduction and boiling (9). Two different anti-VAP-1 mAbs, 2D10 and TK8–18, were used as primary Abs in immunoblottings. 2D10 is an Ab that recognizes an epitope only in the intact dimeric form of VAP-1, whereas the epitope recognized by TK8–18 is present both in dimeric and monomeric forms (see below). When detected with the mAb 2D10, a specific, ~180-kDa band was seen in purified sVAP-1 samples (Fig. 2A, lane 3). The apparent molecular mass of circulating sVAP-1 (lane 3) was slightly higher (~10 kDa) than that of the membrane-bound tonsil form of VAP-1 (lanes 1 and 2). When the mAb TK8–18 was used to detect sVAP-1 in immunoblotting, a ~180-kDa band (Fig. 2A, lane 6) was observed as well. Hence, the electrophoretic mobility of dimeric sVAP-1 is slightly slower than that of transmembrane VAP-1 under nonreducing, nonboiling conditions.

Table II. sVAP-1 levels are stable over time

<table>
<thead>
<tr>
<th>Blood Donor</th>
<th>Value 1 (ng/ml)</th>
<th>Value 2 (ng/ml)</th>
<th>Change (%)</th>
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<td>12</td>
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a sVAP-1 concentrations were measured with sandwich ELISA in two samples that were drawn ~6 mo apart.

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**FIGURE 1.** Concentrations of sVAP-1 in sera of healthy individuals. A. Serum samples from 74 volunteers were tested for the amount of sVAP-1 by the sandwich ELISA as detailed in Materials and Methods. The mean and mean ± SD levels are indicated by the continuous and dotted lines, respectively. B. Time of day variations of sVAP-1 level. Four of the normal blood donors were tested for their sVAP-1 concentrations using blood samples drawn at the indicated time points.

**FIGURE 2.** Circulating VAP-1 is a dimeric, ~180-kDa molecule. sVAP-1 was immunoaffinity-isolated (I) from serum (S) with the anti-VAP-1 mAb TK8–18 as described in Materials and Methods, and the purified material was analyzed using SDS-PAGE and immunoblotting with the anti-VAP-1 mAbs TK8–18 and 2D10. 3G6 is a negative control mAb. Lysate (L) and immunoaffinity-purified (I) VAP-1 from tonsils (T) were used as controls. Samples were either mildly heated and analyzed under nonreducing conditions (A) or boiled and analyzed under reducing conditions (B). Molecular mass standards in kDa are indicated on the right.
FIGURE 3. The dimeric, ~180-kDa form is the only naturally occurring species of sVAP-1. Both serum that had been partially depleted of albumin and IgGs (S) and IgG-depleted tonsil detergent lysate (T) as a control were electrophoresed without reduction after mild heating. The biotinylated anti-VAP-1 mAb TK8–14 and the biotinylated negative control mAb 3G6 were used for the detection of immunoblotting. In the serum, only one specific band (arrow) was detected by the mAb TK8–14. Molecular mass standards in kDa are indicated on the right.

To analyze further the biochemical structure of the soluble and membrane-bound forms of VAP-1, gel electrophoresis and immunoblotting of reduced and boiled affinity-purified sVAP-1 were conducted. As mentioned above, reduction and boiling break up the dimeric VAP-1 molecule into its 90-kDa monomeric subunits (9). When the mAb TK8–18 was used as a primary Ab in the immunoblotting of tonsil stromal lysate, affinity-purified tonsil VAP-1, and affinity-purified sVAP-1, a specific band in the range of 90 kDa was seen in all cases (Fig. 2B, lanes 4–6). Thus, the monomeric subunits of membrane VAP-1 and sVAP-1 have similar molecular masses (see Discussion). No detection was seen with the mAb 2D10 in these reduced and boiled samples (Fig. 2B, lanes 1–3), indicating that the epitope recognized by this Ab was destroyed in monomeric VAP-1.

Additional immunoblotting experiments under nonreducing conditions were performed to determine whether the natural purification process affects the structure of the sVAP-1 molecule. Normal serum that had been depleted of most albumin and IgGs was used as the source of sVAP-1 Ag in SDS-PAGE and immunoblotting. The primary Ab used in immunoblotting was the biotinylated anti-VAP-1 mAb TK8–14. This Ab, like the mAb TK8–18, recognizes an epitope that is present in both the dimeric and monomeric forms of VAP-1 (our unpublished observations). The reason for using mAb TK8–14 instead of mAb TK8–18 was that biotinylated TK8–18 did not work properly in these immunoblotting experiments. One specific band was detected by mAb TK8–14 in the serum sample. The band was in the range of 180 kDa (Fig. 3), as was the case in affinity-purified sVAP-1 samples. Thus, these data indicate that the slower electrophoretic mobility of sVAP-1 compared with membrane VAP-1 under nonreducing conditions is not caused by effects of the affinity purification process (e.g. possible rearrangements of disulfide bonds) on the structure of the sVAP-1 molecule. Moreover, this experiment implies that there is only one naturally occurring form of VAP-1 in the circulation, which is the dimeric, 180-kDa species. To rule out the possibility that a small portion of sVAP-1 might be in a monomeric form, we confirmed the immunoblotting result with a more sensitive method. To that end, AB serum was depleted of dimeric VAP-1 by CNBr beads that had been coupled with the anti-VAP-1 mAb 2D10, and this serum sample was tested by the sVAP-1 ELISA in which both the capturing (TK8–18) and detecting (TK8–14) mAbs recognize both the dimeric and monomeric form of VAP-1. No reactivity was observed in this sample. Thus, monomeric-circulating VAP-1 was not detected by this method either. Consequently, most, if not all, sVAP-1 is dimeric.

Endothelial VAP-1 is a sialoglycoprotein whose electrophoretic mobility is changed after treatment with sialidase (9). Because the molecular masses of the intact dimeric sVAP-1 and the membrane VAP-1 from which sialic acids have been removed proved to be similar (~180 kDa), we suspected that the dimeric sVAP-1 may not contain any sialic acid residues. To test this hypothesis experimentally, immunoaffinity-isolated sVAP-1 was subjected to sialidase treatment before SDS-PAGE and immunoblotting. The sialidase digestion caused a remarkable change in the electrophoretic mobility of sVAP-1 samples (data not shown), indicating that sVAP-1 also contains abundant sialic acid decorations.

Increased levels of sVAP-1 in patients with inflammatory liver diseases

We hypothesized that patients with inflammatory disorders may show elevated levels of sVAP-1 because endothelial VAP-1 is up-regulated upon inflammation and is translocated to the luminal surface of vessels (7). Therefore, we tested samples from patients with different systemic inflammatory conditions, including RA and IBDs, by the sVAP-1 ELISA. However, the sVAP-1 values obtained from these patients did not differ significantly from the values of healthy controls (Fig. 4A). Because of the strong constitutive expression of VAP-1 on hepatic endothelia (8) we also tested sera from 41 patients with different liver diseases by the sVAP-1 ELISA. In contrast to other patients tested, many of the liver disease patients had sVAP-1 values that were clearly elevated when compared with healthy controls (Fig. 4A). The mean level ± SD for this liver disease group was 140 ± 61 ng/ml, and the range was 50 to 315 ng/ml. The difference of sVAP-1 concentrations between the liver patient group and normal controls was statistically highly significant (p < 0.0001). Moreover, when comparisons were made according to the nature of the liver disease, differences within the liver disease group became apparent. The highest values were found in patients with active cirrhosis due to ALD (Fig. 4B). Patients with PBC showed a less pronounced but statistically significant increase in the levels of sVAP-1 (p < 0.001), whereas the levels in patients with PSC did not differ significantly from those of normal controls. The increase in sVAP-1 levels in patients with HCC did not approach statistical significance, although some of these patients had elevated levels. In contrast, all of the patients with CHM had values within the normal range. Taken together, serum sVAP-1 concentrations can be modulated by certain inflammatory liver diseases.

sVAP-1 is functionally active

To investigate the physiologic role of circulating VAP-1, adhesion assays were performed. Since we were unable to isolate enough functionally intact VAP-1 from serum samples, and because we do not have rsVAP-1, the effect of native serum VAP-1 on the binding of PBLs to VAP-1 transfectant monolayers was analyzed. We have shown that PBLs bind >25 times better to VAP-1-transfected Ax
cells than to mock-transfected controls (40). PBLs were preincubated with a serum sample containing high levels of sVAP-1 (120 ng/ml) and with an identical serum sample from which the sVAP-1 was specifically depleted (0 ng/ml) by sequential immunoprecipitations. The rationale was that under these conditions sVAP-1 has the possibility to bind to its counter-receptor on lymphocytes and either inhibit adhesion to VAP-1 transfectants (if sVAP-1 is has an inhibitory role) or enhance binding to VAP-1 transfectants (if sVAP-1 has a triggering function).

In three independent assays using PBLs from different blood donors, PBL binding to VAP-1 transfectants was increased when sVAP-1 was present during the preincubation step (Fig. 5). Binding to mock transfectants was always negligible (<5 cells/mm²). Thus, these data indicate that circulating VAP-1 does not serve an adhesion-blocking function. Rather, sVAP-1 most likely enhances lymphocyte binding to endothelial cells by triggering the functional up-regulation of other adhesion molecules on lymphocytes.

**Discussion**

In the present study we have shown that VAP-1 circulates in a soluble form in serum. The concentrations of sVAP-1 in healthy individuals were found to be between 49 and 138 ng/ml when measured by an ELISA technique, suggesting that the variability of sVAP-1 levels within a healthy population is relatively small. Also, no age or sex correlations were observed. Furthermore, levels were stable over time, because the sVAP-1 detected when blood donors were retested several months later did not differ significantly from the original levels. Hence, the lack of variation in sVAP-1 levels in serum from healthy volunteers indicates that sVAP-1 is normally released into the circulation at a relatively constant rate.

Immunoblotting studies revealed that the apparent molecular mass of affinity-purified sVAP-1 is slightly higher than that of transmembrane VAP-1 under gentle, nonreducing conditions. The affinity purification process may affect the structure of VAP-1, because large aggregates were often seen in affinity-isolated tonsil VAP-1 samples (see Fig. 2A, lane 5). These aggregates are thought to be multimers of the VAP-1 molecule that have arisen during the purification process. However, the structure of affinity-isolated sVAP-1 was not modified by the purification process, because the same 180-kDa band that was seen in affinity-isolated sVAP-1 samples was observed in the serum sample that had only been depleted of most albumin and Igs (Fig. 3). The relatively weak intensity of the specific sVAP-1 band in Figure 3 is due to very small amount of Ag in the sample. After multiple preclearings, there is <4 ng of sVAP-1 in the 50-μl serum sample that can be loaded per well, and the blotting procedure is never very efficient with large molecules such as dimeric sVAP-1. In contrast to the size difference between sVAP-1 and membrane-bound VAP-1 that was seen under nonboiling, nonreducing conditions, the electrophoretic mobilities of VAP-1 from these sources were identical after reduction and boiling. Taken together, these data suggest that the monomeric subunits of these two forms of VAP-1 are very similar, if not identical, but that different rearrangements in the structure of dimeric membrane bound VAP-1 and sVAP-1 occur under nonreducing conditions, resulting in minor shifts in apparent SDS-PAGE mobilities. We also showed that there is no monomeric sVAP-1 in the circulation, because monomeric sVAP-1 was undetectable either by immunoblotting of sVAP-1 in serum or by ELISA in serum samples that had been depleted specifically of dimeric VAP-1. Based on these data, it seems probable that the dimeric 180-kDa species is the only naturally occurring form of sVAP-1 in the circulation.

**FIGURE 4.** Serum levels of sVAP-1 are increased in certain inflammatory liver diseases. Patient sera were tested for the amount of sVAP-1 by the sandwich ELISA. Each individual patient is shown as a circle; bold lines are the means, and thin lines are the means ± SDs of the indicated patient group. A shows results for the normal (NOR), RA, IBD, and liver disease (LIVER) groups. B shows results for the NOR, ALD, PBC, PSC, HCC, and CHM groups. The p values of each patient group as compared with normal controls are indicated.
It is likely that sVAP-1 is derived from the transmembrane form of VAP-1 by proteolytic cleaving. The location of the cleavage site just proximal to the membrane-spanning region would explain the similar molecular masses of sVAP-1 and membrane VAP-1 under reducing conditions. Our sequence data (40) indicate that deletion of the transmembrane and cytoplasmic domains would cause only an ~2 kDa decrease in the molecular mass of VAP-1, which would not be clearly demonstrated by SDS-PAGE. However, we cannot formally exclude the possibility that sVAP-1 is encoded by an alternatively spliced mRNA lacking the transmembrane sequence, or even that it is a secreted protein encoded by an independent gene.

**FIGURE 5.** sVAP-1 augments lymphocyte binding to VAP-1 transfectants. A, Expression of VAP-1 on Ax transfectants. Stable VAP-1 and mock transfectants were stained with anti-VAP-1 and nonbinding control mAbs, and the cells were analyzed using flow cytometry. The x-axis is the intensity of the fluorescence signal on a log scale, and y-axis is the relative number of cells. B and C, Lymphocytes were preincubated with serum containing high levels of sVAP-1 or with serum that had been specifically depleted of sVAP-1 and allowed to adhere to VAP-1 and mock transfectants as described in Materials and Methods. B, Representative photomicrographs illustrate the adhesion of PBLs (small round cells, some pointed out by arrows) to transfectant monolayers. C, Results from three independent adhesion assays in which the number of lymphocytes that bound to Ax-VAP-1 transfectant monolayers was analyzed. Binding to mock transfectants was negligible. PBLs from different donors were used in each experiment. VAP-1+ = lymphocytes preincubated with serum containing sVAP-1; VAP-1− = lymphocytes preincubated with serum depleted of all sVAP-1.
Like the membrane-bound endothelial VAP-1 (9), circulating VAP-1 contains abundant sialic acid decorations. The other post-translational modifications are likely to be very similar if not identical in these two forms of VAP-1 as well, since monomeric membrane-bound VAP-1 and sVAP-1 had apparently identical molecular masses. The finding of abundant sialic acid residues in sVAP-1 is particularly important, because the adhesive function of VAP-1 is dependent upon its sialic acids (9). Thus, our observation that sVAP-1 is heavily sialylated suggests that it might be functionally active.

The functional role for sVAP-1 was actually shown in adhesion assays. When sVAP-1 had been depleted from the serum in which PBLs were preincubated, lymphocyte binding to VAP-1-transfected endothelial cells decreased. These results suggest that sVAP-1 binds to its (uncharacterized) lymphocyte counter-receptor during the preincubation step and causes a change in the adhesive status of the lymphocyte. The mechanism of enhanced binding after sVAP-1 preincubation remains to be established. However, ligation of VAP-1R on lymphocytes might trigger a signal that results in the up-regulation of the expression and/or avidity of some other lymphocyte adhesion molecule(s). This could then lead to increased PBL adherence to endothelial monolayers by binding of lymphocyte adhesion receptors to their cognate counter-receptors, which are expressed on these endothelial transfectants. Since the very low level of binding of PBLs to mock transfectants apparently did not increase after preincubation in VAP-1′ serum, PBLs most likely recognize endothelial adhesion molecules whose expression is directly or indirectly dependent upon VAP-1 expression in these cells. Our results suggest that increased levels of sVAP-1 may lead to an increased adhesion of PBLs to vascular endothelium locally at sites of inflammation. This mechanism could serve to augment the immune response at the scene of inflammation, where the other components of the multistep adhesion cascade are also active. It should be noted that sVAP-1 may have a normal physiologic role in maintaining a proadhesive status of PBLs toward endothelia, since its concentration in normal individuals is within the range used in these adhesion experiments; the complete depletion of sVAP-1 resulting in decreased adhesion is an artificial condition used in this experimental setup.

Significantly elevated levels of sVAP-1 were found in patients with liver diseases. When these sVAP-1 values were correlated with the diagnosis of the patients, differences between the disease groups were observed. Patients with ALD had the most marked elevations in systemic circulation. Indeed, levels in these patients were high enough to suggest that local concentrations of sVAP-1 could cause biologic effects. Patients with PBC also had elevated levels, although levels in patients with PSC were not elevated when compared with controls. This finding is relevant, because these patients have marked cholestasis, which suggests that impaired bile flow per se does not cause the elevation of sVAP-1 levels. It was also interesting to note that the patients with HCC had higher sVAP-1 values than the patients with CHM, which fits well with the finding of high VAP-1 expression on primary liver tumors but not in liver metastases (28). The other nonhepatic inflammatory diseases we studied (i.e., RA and IBDs) were not associated with elevated levels of sVAP-1. These data suggest that the elevation of sVAP-1 concentrations is specific for certain inflammatory diseases, such as ALD. In contrast, levels of other soluble adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, have been demonstrated to be elevated not only in ALD but in practically all liver diseases (29–34). Moreover, elevated levels of these other circulating adhesion molecules have been reported in many other inflammatory disorders (24), including RA (35–37) and IBDs (38, 39). Thus, the specificity of elevated sVAP-1 levels for certain liver diseases warrants further study to determine the potential role of sVAP-1 in the follow-up of the inflammatory activity of these diseases.

In conclusion, we have shown that dimeric sVAP-1 circulates in the serum of healthy individuals at a level that is comparable with those of most other soluble endothelial adhesion molecules (24). Moreover, the level of sVAP-1 is increased in certain inflammatory liver diseases in a more disease-specific manner than the levels of other known circulating endothelial adhesion molecules (24). sVAP-1 may exert its biologic function by regulating the leukocyte-endothelial cell adhesion cascade.

Acknowledgments

We thank V. Arstila for irreplaceable help with computers, Drs. M. Viljanen and K. Granfors for helpful discussions about ELISAs, and R. Lehvenon for technical assistance.

References


