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A 90-Kilodalton Endothelial Cell Molecule Mediating Lymphocyte Binding in Humans

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Interactions between leukocyte surface receptors and their ligands on vascular endothelial cells control lymphocyte traffic between the blood and various lymphoid organs, as well as extravasation of leukocytes into sites of inflammation. A heretofore undescribed 90-kilodalton human endothelial cell adhesion molecule (VAP-1) defined by a monoclonal antibody 1B2 is described. The expression pattern, molecular mass, functional properties, and an amino-terminal amino acid sequence define VAP-1 as an endothelial ligand for lymphocytes. VAP-1 helps to elucidate the complex heterotypic cell interactions that direct tissue-selective lymphocyte migration in man.

Most mature lymphocytes continuously recirculate between the blood and lymphatic organs (*1*). Lymphocyte trafficking allows the full repertoire of lymphocyte specificities to be available for immune reactions throughout the body, and it also facilitates the cell-cell interactions required for the generation and control of immune responses. Lymphocytes leave the blood by recognizing and binding to the vascular endothelial cells. Thereafter, they migrate between the endothelial cells into the surrounding tissues. Lymphocyte adherence to endothelial cells is dependent on interactions between complementary adhesion molecules expressed on both cell types (*2–7*). Under normal conditions, lymphocytes mainly bind to specialized postcapillary venules called high endothelial venules (HEV). Functionally separate lymphocyte-HEV recognition systems mediate lymphocyte migration to peripheral lymph nodes, mucosal lymphoid organs, synovium, and skin in an organ-specific manner (*8–10*). In inflammation, activation of the endothelial cell results in changes of its adhesion molecule status, which largely determines the magnitude and type of leukocyte influx into the affected tissue. Thus, endothelial cell molecules are a key element in controlling the characteristics of local immune response, and a detailed understanding of the mechanisms regulating lymphocyte traffic and leukocyte extravasation can provide new means to clinically manipulate the inflammatory response. Because in man the endothelial cell ligands mediating tissue-selective lymphocyte homing are largely unknown, we made an attempt to identify such molecules by producing monoclonal antibodies (MAbs) to human synovial vessels (*11*).

Immunohistological stainings revealed that one of the MAbs produced, 1B2, strongly stained HEV-like venules in inflamed synovial membranes (Fig. 1, A and B). No staining was observed in infiltrating leukocytes or in connective tissue components of the synovial stroma. The antigen recognized by MAb 1B2 was named VAP-1 (for vascular adhesion protein–1). In peripheral lymph node and tonsil, MAb 1B2 reacted with majority of HEV (Fig. 1C). VAP-1 was intensely expressed at the luminal side of the endothelial cells (Fig. 1D). A granular staining was seen in the endothelial cell cytoplasm, and also the abluminal surface was MAb 1B2 positive. Especially in tonsil, the staining intensity notably varied between different HEV, and few individual HEV with a typical plump morphology were 1B2-negative. In appendix (Fig. 1E) and in lamina propria of the gut, only faintly staining venules were detected. Weak expression of VAP-1 was also seen on dendritic-like cells in germinal centers and on smooth muscle cells of arteries, veins, and bowel wall. In contrast, VAP-1 was practically absent from the luminal surface of large vessels. Like leukocytes in tissue sections, peripheral blood lymphocytes, monocytes, natural killer (NK) cells, granulocytes, and isolated tonsillar leukocytes were all completely 1B2-negative in fluorescence-activated cell sorter (FACS) analyses. T lymphoblastoid (CCRF-CEM), B lymphoblastoid (KCA and IBW-4), monocytic (U937), and leukemic (KG-1, KG-1a, and K 562) cell lines all lacked VAP-1. VAP-1 was absent from human umbilical vein endothelial cells (HUVEC), and 4-hour or 20-hour treatments with interleukin-1 (IL-1) (20 or 100 U/ml), tumor necrosis factor (TNF) (200 U/ml), or lipopolysaccharide (LPS) (0.1 or 1.0 μg/ml) could not induce its expression. Primary cultures of smooth muscle cells, fibroblasts, and keratinocytes, and an epithelioid (HeLa) cell line did not express VAP-1.

To determine the molecular weight of VAP-1, affinity-isolated molecule from tonsillar stroma was subjected to SDS–polyacrylamide gel electrophoresis (PAGE). Silver staining of the gel revealed a major band of apparent molecular size 90 kD under reducing conditions (Fig. 2). Analyses of immunoprecipitates from iodinated stromal cells of tonsil confirmed the reactivity of MAb 1B2 with a 90-kD molecule (and a slightly smaller species; presumably a degradation product) (Fig. 2). VAP-1 migrated slightly slower under nonreducing conditions (M_r ~ 100 kD).

The tissue distribution of VAP-1 on endothelial cells in vivo suggested that it might function as a specific recognition element for leukocytes. Therefore, the functional role of VAP-1 in HEV-binding was studied with the modified Stamper-Woodruff in vitro assay (*12*). By first treating frozen sections with MAb 1B2, lymphocyte binding to HEV was inhibited (Fig. 3). The inhibitory effect was most pronounced in tonsil and peripheral lymph node, but binding to synovial HEV was also significantly reduced. Lymphocyte binding to appendix HEV and granulocyte binding to tonsil HEV were less affected (Fig. 3). Thus, VAP-1 either mediates or associates closely with endothelial cell elements mediating lymphocyte recognition of peripheral lymph node, tonsil, and synovial HEV. To directly evaluate the involvement of VAP-1 in lymphocyte-endothelial cell interaction, we analyzed binding of lymphocytes to affinity-isolated VAP-1 (Fig. 4). Lymphocytes adhered efficiently to plate-bound VAP-1. Lymphocyte binding to VAP-1 was specifically inhibited with MAb 1B2 but not with a control MAb 3G6. MAb 1B2 did not prevent lymphocyte binding to another unrelated endothelial cell molecule (Fig. 4).

The expression pattern, molecular weight, and function of VAP-1 indicate that it is not identical with any of the previously defined endothelial molecules

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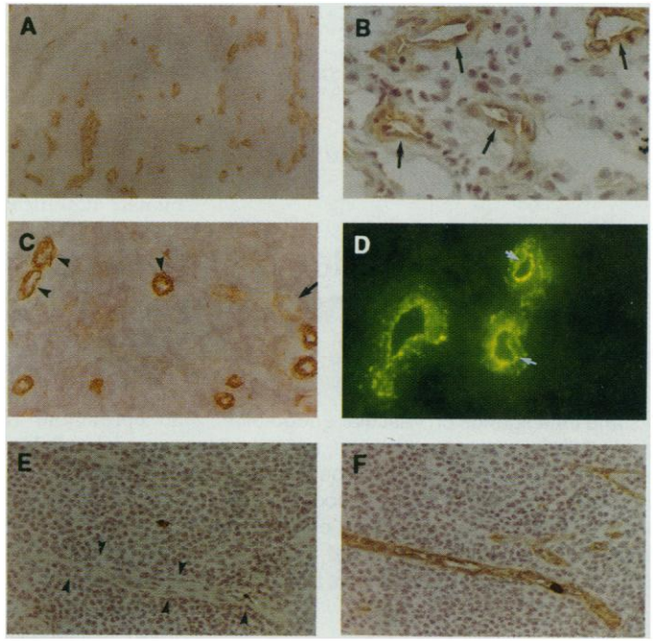

Fig. 1. Distribution of VAP-1 in human tissues. (**A**) In inflamed synovial membrane, MAb 1B2 abundantly stains HEV-like vessels and also venules in transition to HEV. (**B**) Higher magnification of the positive synovial venules (arrows). (**C**) In tonsil, expression of VAP-1 in HEV varies from intense (arrowheads) to weak (arrows) or negative. (**D**) Immunofluorescence staining of a tonsil shows the prominent expression of VAP-1 on the luminal surface of the vessels (arrows). (**E**) In appendix, only a few weakly staining HEV are seen. The largest HEV is pointed by arrowheads. (**F**) In a serial section (of E), we confirmed the identity of vessels in appendix by staining with anti-factor VIII antibody. Magnifications: (A) ×100; (C, E, and F) ×250; and (B and D) ×400. We determined the tissue distribution of VAP-1 by immunoperoxidase staining of cryostat sections. The sections were incubated with primary antibodies (1B2 and 3G6, a control mouse IgG_1 MAb against chicken T cells) for 30 min. After two washings in phosphate-buffered saline (PBS), peroxidase-conjugated rabbit antimouse IgG (Dakopatt, Denmark) in PBS containing 5% AB-serum was added for 30 min. Next, 3′,3′diaminobenzidine hydrochloride in PBS containing 0.03% hydrogen peroxide was used as a chromogen. After the staining, the sections were counterstained with hematoxylin. For immunofluorescence staining, 3-μm cryostat sections were overlaid with primary antibodies and FITC-conjugated sheep anti-mouse IgG (Sigma, St. Louis, Missouri) was used as a second-stage reagent. In every tissue type, 1B2 staining was analyzed from ten samples from separate individuals.

involved in lymphocyte binding. We confirmed the uniqueness of this molecule by subjecting immunoaffinity-purified VAP-1 to protein sequencing (*13*). An NH_2-terminal sequence of the 90-kD species (TEDGDMXLVNGASANEGXVE) was obtained, and it showed no significant homology to any known molecule in SwissProt and GenEMBL data banks.

Tissue distribution and HEV-binding results suggest that VAP-1 is mainly involved in lymphocyte trafficking to peripheral lymph node, tonsil, and synovium. In tonsil, lack of VAP-1 expression defines a minor subset of postcapillary venules, which are morphologically indistinguishable from venules that express 1B2. Because tonsils are intimately associated with the gastrointestinal tract, they may contain HEV-specificities of both mucosal (little or no VAP-1) and peripheral lymph node (VAP-1–positive) types. It remains to be determined how the phenotypic difference in VAP-1 expression correlates to lymphocyte binding capacity of each individual HEV. The scarcity of VAP-1 in mucosal lymphoid organs implies that this endothelial antigen may be differentially regulated in distinct lymphocyte recognition systems. Moreover, preliminary analyses indicate that the degree of inflammation correlates with VAP-1 expression in skin and bowel specimens (*14*).

Comparison of VAP-1 with the known endothelial cell molecules mediating leukocyte binding reveals several differences. Intercellular adhesion molecules–1 and –2 (ICAM-1 and ICAM-2), vascular cell adhesion molecule–1 (VCAM-1), E-selectin (ELAM-1), and P-selectin (CD62, PADGEM, and GMP 140) are all expressed on HUVEC either basally or after induction by inflammatory mediators (*2–6, 15–21*). In contrast, VAP-1 is neither constitutively expressed nor inducible by IL-1, TNF-α, or LPS treatments on HUVEC. Tissue distributions of these molecules are clearly distinct—also when analyzed on parallel sections of tonsil. ICAMs stain the luminal surface of most large and small vessels and certain leukocytes (*15, 22*). VCAM-1 and E-selectin, in contrast, are present only on a subset of venules in inflamed tissues (*23–25*). Instead, VAP-1 is strongly expressed on the vast majority of HEV at nonmucosal sites, and it is absent from all white cells and cell lines tested. The molecular size of the known adhesion molecules are also different from that of VAP-1, with the exception of ICAM-1 [ICAM-2 is a 60-kD molecule; VCAM-1, 110-kD molecule; E-selectin, 115-kD molecule; and P-selectin, 140-kD molecule, (*3, 4, 15*)]. Furthermore, VAP-1 is mainly involved in lymphocyte

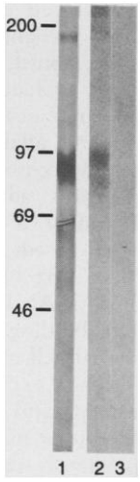

Fig. 2. VAP-1 is a 90-kD protein. Lane 1, silver staining of immunopurified VAP-1. Lanes 2 to 3. ^{125}I-labeled stromal cells of tonsil were immunoprecipitated (*27*) with either MAb 1B2 (lane 2) or control MAb 3G6 (lane 3). The bands in the area 180 to 200 kD are inconstant findings. Molecular size standards are indicated on the left in kilodaltons.

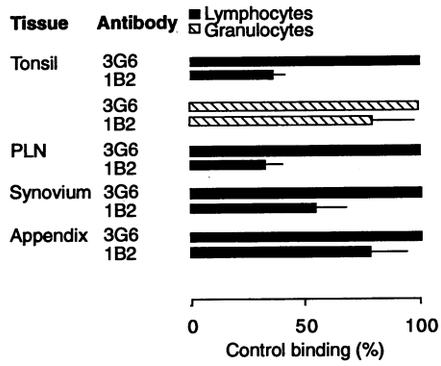

Fig. 3. VAP-1 is involved in lymphocyte binding to HEV. Binding of lymphocytes to tonsil, peripheral lymph node (PLN), synovial, and appendix HEV and binding of granulocytes to tonsil HEV were assessed in the presence and absence of MAb 1B2 with the in vitro frozen section assay. Results of three independent experiments are presented as percentages of control binding with standard errors (100% = number of bound cells on 3G6-treated sections). The details of this technique have been described (*12*). Cells bound to HEV on four to six sections per tissue per sample were counted (minimum of 100 HEV) single-blind. When determining granulocyte binding, the assay was done similarly, with the exception that purified granulocytes (isolated using Histopaque 1077/1119 gradient, Sigma) were kept in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (HBSS) until just before application onto the sections.

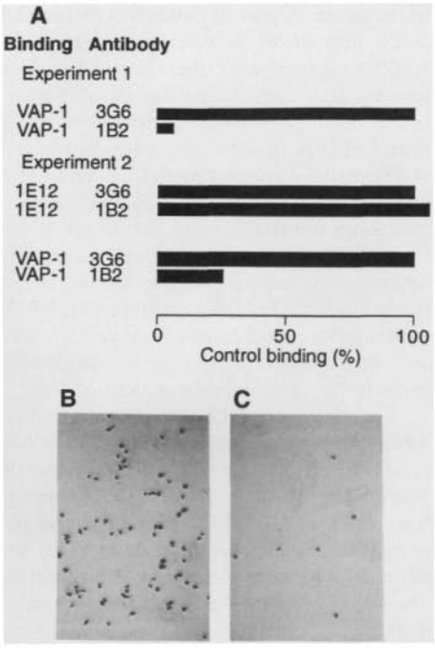

Fig. 4. Isolated VAP-1 supports lymphocyte binding. Immunopurified VAP-1 and control proteins [1E12, an unrelated endothelial cell molecule that supports lymphocyte binding, and bovine serum albumin (BSA)] were absorbed on glass, and lymphocyte binding was determined (*28*). (**A**) Results from two independent experiments are presented as percentages from control binding (100% = number of cells bound to plate-bound VAP-1 or 1E12 after MAb 3G6 treatment). Nonspecific background (binding to BSA) is subtracted from all analyses. (**B**) Lymphocyte binding to VAP-1–coated well in the presence of MAb 3G6. (**C**) Lymphocyte binding to VAP-1–coated well in the presence of MAb 1B2. Magnifications, ×250.

binding, whereas ICAMs, E- and P-selectins also efficiently (or solely) mediate adhesion of other leukocytes (*2–6*). The only endothelial adhesion molecule described so far that is involved in lymphocyte binding in man and is not expressed on HUVEC is the MECA-79–defined antigen (*26*). However, VAP-1 is not coexpressed in all MECA-79–positive venules and vice versa, and MAb 1B2 does not recognize purified MECA-79 antigen.

Finally, the NH_2-terminus of the 90-kD species of VAP-1 shows no significant similarity with the deduced amino acid sequences of any known protein. Thus, VAP-1 appears to be a new endothelial molecule involved in lymphocyte binding.

The description of VAP-1 underlines the necessity of developing other endothelial cell models in addition to HUVEC when determining the contribution of different adhesion molecules in leukocyte trafficking and extravasation. VAP-1 will be relevant to understanding of the physiological lymphocyte recirculation in man, and it will be especially valuable for dissecting the molecular mechanisms of tissue-selective lymphocyte homing.

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11. We produced MAb 1B2 by immunizing mice with stromal elements of inflamed synovium from rheumatoid arthritis patients. The immunogen in incomplete Freund's adjuvant was applied three times at 1-week intervals into the footpads of specific pathogen-free BALB/c mice. Lymphocytes from popliteal lymph nodes were isolated and were fused with the nonsecreting NS-1 mouse myeloma cells with standard protocols. Hybridomas were screened with immunoperoxidase staining of frozen sections. Twice we cloned one hybridoma [1B2, subclass immunoglobin G_1 (IgG_1)] producing an antibody reactive with vascular endothelium of synovium by limiting dilution and then selected it for further analyses.
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13. For protein sequencing, VAP-1 was affinity-isolated from human tonsils. Lymphocyte-depleted tonsillar extracts were solubilized in lysis buffer [150 mM NaCl, 10 mM tris-base, 0.15 mM $MgCl_2$, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% aprotinin] overnight at 4°C. The lysate was centrifuged at 10,000*g* for 30 min at 4°C. The supernatant was subjected to precipitation with $(NH_4)_2SO_4$ (20% w/v). We recovered the precipitate by centrifugation (30 min at 10,000*g*) and the precipitate was resuspended in lysis buffer. We precleared the protein concentrate by passing it over a Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column. Then it was sequentially applied to three CnBr-activated Sepharose 4B (Pharmacia) columns derivatized with normal mouse serum, with irrelevant IgG_1 MAb and with 1B2 MAb (5 mg/ml, 5 ml column volume). The column was washed extensively with the lysis buffer. Thereafter, the material bound to the 1B2 column was eluted with 50 mM triethanolamine and was lyophilized. Isolated VAP-1 molecule was resolved in SDS-PAGE (10%, reduced, at 4°C) and was transferred to polyvinylidine difluoride (PVDF) membrane (ProBlott, Applied Biosystems, Foster City, CA). The membrane was stained with Coomassie blue, and the 90-kD band was excised and was subjected for analysis in protein sequencer (Model 477A equipped with an on-line PTH amino acid analyzer 120A, Applied Biosystems). Starting from ten tonsils, the yield of purified antigen was approximately 10 μg. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
14. M. Salmi and S. Jalkanen, unpublished observations. Preliminary analyses included five normal and five inflamed (inflammatory bowel diseases) specimens, five punch biopsies from dermatitis lesions with control specimens (*5*) from the healthy areas of skin of the same individuals. We defined the degree of inflammation by evaluating the density of lymphocyte and/or polymorphonuclear leukocyte infiltration.
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27. For silver staining, VAP-1 was isolated and was separated by electrophoresis as indicated in (*13*). For iodine labeling, lymphocyte-depleted tonsillar extracts were digested in RPMI 1640 containing collagenase (100 U/ml) (type II from *Clostridium histolyticum*, Sigma, St. Louis, MO) 10% fetal calf serum (FCS) antibiotics, and 10 mM Hepes for 1 hour at 37°C with gentle stirring. After the collagenase digestion, cells were washed in HBSS, and surface-labeled with ^{125}I with the lactoperoxidase method. Iodinated cells were lysed with the lysis buffer, and the lysate was clarified by centrifugation at 10,000*g* for 15 min. The lysate was precleared for 16 hours at 4°C with CnBr-activated Sepharose 4B coupled to normal mouse serum. Immunoprecipitations were done with CnBr-activated Sepharose 4B beads conjugated with MAbs 1B2 or 3G6 (a negative control MAb against chicken T cells). The samples were analyzed with 7.5% SDS-PAGE under reducing (2-mercaptoethanol) conditions.
28. VAP-1 and 1E12 (an unrelated endothelial cell molecule) were affinity-purified from tonsillar extracts as indicated in (*13*). Purified VAP-1, 1E12, and heat-inactivated BSA were diluted in 20 mM tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ with 0.01% β-octyl glucopyranoside as detergent. Proteins (approximately 10 ng/mm^2, estimated from silver-stained gels) were added onto glass wells (Lab-Tek chamber slides, Nunc, Inc., Naperville, IL) for 16 hours at +4°C. After blocking in PBS containing BSA (1 mg/ml) for 30 min at room temperature, 1B2 or 3G6 supernatants were added into wells and incubation was continued for 30 min at room temperature. Meanwhile, freshly isolated peripheral blood mononuclear cells were incubated in RPMI 1640 containing 10% FCS and 10 mM Hepes for 1 hour at 37°C in tissue culture bottles to deplete the plastic adherent monocytes. Nonadherent lymphocytes (1.8×10^6 cells per well) in 100 μl RPMI 1640 were applied into each well. After 30 min of incubation at 37°C, the nonadherent cells were removed by flicking. The tops of the wells were removed, the slides were washed with a gentle stream of PBS, and were fixed in cold PBS-containing 1% glutaraldehyde. Thereafter, the cells were stained with the Diff-Quick stains. We quantitated the bound cells by visually scoring the number of cells in each well (total area of 50 mm^2 was counted per sample). Analyses were performed in duplicates, and standard errors were ≤8.8%.

29. We thank E. Butcher and E. Berg for discussions, for testing the reactivity of MAb 1B2 with isolated MECA-79 antigen, and for providing MAb MECA-79; T. Springer and D. Haskard for providing MAbs; J. Uksila, P. Vuorela-Miilunpalo, and L. Airas for help with the initial screening of the MAb 1B2; J. Hellman for analyzing the protein sequence; M. Niskala and M. Matikainen for culturing 1B2; and M. Skurnik for advice. Supported by the Finnish Academy, the Finnish Cancer Foundation, the Sigrid Juselius Foundation, the Rheumatism Research Foundation, and the Finnish Life and Pension Insurance Companies.

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