

Induction and Function of Vascular Adhesion Protein-1 at Sites of Inflammation

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Summary

Emigration of leukocytes from the blood into the tissues is critical in controlling lymphocyte patrolling in different lymphatic organs and in leukocyte accumulation at sites of inflammation. During the first stage of the extravasation process, leukocytes bind to the endothelial lining of vessels. At the molecular level, several adhesion molecules on leukocytes and endothelial cells function as receptor-ligand pairs in mediating this dynamic interaction. Recently, we have identified a novel human endothelial cell molecule, vascular adhesion protein 1 (VAP-1), that mediates lymphocyte binding (Salmi, M., and S. Jalkanen. 1992. *Science [Wash. DC]* 257:1407). VAP-1 was initially characterized by mAb 1B2 which inhibits lymphocyte adhesion to high endothelial venules (HEV) and to purified VAP-1 protein. Here we report the location and function of VAP-1 in normal and inflamed tissues in humans. VAP-1 is abundant in HEV of lymphatic organs belonging to the peripheral lymph node system, but considerably less is expressed in vessels of mucosa-associated lymphatic tissues. A subset of venules in most normal nonlymphatic tissues like skin, brain, kidney, liver, and heart is also VAP-1 positive. In addition to vessels, VAP-1 is distributed on a few other cell types, most notably in dendritic-like cells of germinal centers. At sites of inflammation, such as in inflammatory bowel diseases and chronic dermatoses, expression of VAP-1 is clearly increased. The induced VAP-1 is functional, since mAb 1B2 inhibits lymphocyte binding to inflamed lamina propria venules by ~60%. Thus VAP-1 is an endothelial adhesion molecule that under normal conditions is expressed mainly in HEV of lymphatic tissues. However, expression of functional VAP-1 in vivo is upregulated during an inflammatory reaction at other sites as well. Inducibility of VAP-1 suggests that it may play a significant role, not only in recirculation of lymphocytes, but also in controlling entry of leukocytes into sites of inflammation.

The efficiency of immune defense is largely dependent on the ability of leukocytes to continuously survey different anatomical locations in the body and to rapidly accumulate at sites of antigenic insult. To perform this function, leukocytes must first bind to vascular endothelium and then transmigrate between the endothelial cells into the underlying tissue. Leukocyte-endothelial cell interactions are mediated by regulated function of several adhesion molecules on both cell types (for reviews see references 1-8). Engagement of certain endothelial cell adhesion receptors by their leukocyte counterparts results in a complex series of events where initial weak and transient adherence is followed by stable binding and extravasation of leukocytes (6-8). On human endothelium, at least E-selectin (9), P-selectin (10, 11), intercellular adhesion molecule 1 (ICAM-1) (12), ICAM-2 (13), vascular cell adhesion molecule 1 VCAM-1 (14), and peripheral lymph node addressin (PNAd)/glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) (15, 16) have a well-established role in supporting leukocyte binding during different steps of the migration cascade.

We have recently characterized a novel endothelial cell adhe-

sion antigen called vascular adhesion protein 1 (VAP-1) that mediates lymphocyte binding to high endothelial venules (HEV) in peripheral lymph nodes, tonsils, and inflamed synovia in humans (17). In contrast, VAP-1 plays only a marginal role in adherence of lymphocytes to normal mucosa-associated lymphatic tissues and in the binding of neutrophils to tonsillar HEV. VAP-1 is distinct from all the other currently known endothelial cell ligands involved in leukocyte binding when judged by several criteria like tissue and cell distribution, molecular weight, function, and NH₂-terminal amino acid sequence (17). In the present study we have examined the distribution of VAP-1 in several normal and inflamed organs and tissues in humans and its function in inflammation.

Materials and Methods

Antibodies. 1B2 is an inhibitory mAb (mouse IgG1) against VAP-1 (17). Polyclonal rabbit Ab against Factor VIII (Dako, Glostrup, Denmark) and mAb against CD31 (Becton Dickinson & Co., Mountain View, CA) were used to assist the identification of endothelial cells. mAb 3G6 (mouse IgG1) against chicken T cells was used as a negative control. Peroxidase-conjugated rabbit anti-mouse

Ig and swine anti-rabbit Ig were from Dako, and FITC-conjugated sheep anti-mouse Ig from Sigma Chemical Co. (St. Louis, MO).

Tissue Specimens. Normal tissue samples from peripheral lymph node (from two patients), large and small intestine (noninvolved bowel segments from nine patients with carcinomas of gut), kidney (from three patients), and heart (from five patients) were freshly obtained from surgical operations or from organ donors. Tonsils were from 20 tonsilectomies, and other tissues (liver, brain, spinal cord, peripheral nerve, aorta, vena cava inferior, thyroid, and adrenal) were from autopsies. All these specimens, except tonsils, were histopathologically determined to be noninflamed.

Skin samples from noninvolved and diseased area were obtained from punch biopsies of nine patients suffering from chronic dermatoses (psoriasis, atopic exzema, and lichen ruber planus). Inflamed gut specimens were from patients with inflammatory bowel diseases (Crohn's disease and ulcerative colitis) who underwent surgery for therapeutic purposes.

Immunohistochemistry. Frozen sections were stained by the immunoperoxidase method as previously described (17). VAP-1 expression in bowel and skin samples (normal and inflamed) was analyzed by two independent readers from coded samples without knowledge on the diagnosis. The number of VAP-1 positive venules was semiquantitatively scored as follows: (-) no positive vessels in the sample; (+) occasional positive vessels in the sample—less than five vessels/ $\times 100$ field; (++) 5–10 positive vessels/ $\times 100$ field; (+++) 11–19 positive vessels/ $\times 100$ field; and (++++) 20 or more positive vessels/ $\times 100$ field.

For confocal microscopy, frozen sections from tonsil were stained with 1B2 and FITC-conjugated sheep anti-mouse Ig. Samples were mounted in 50% glycerol, 2 \times PBS, 0.1% sodium azide, and 100 mg/ml DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma Chemical Co.). Analyses were performed using a beam scanning confocal fluorescent microscope (EMBL compact confocal microscope with Zeiss Axiovert/10 microscope and $\times 100/1.3$ oil Plan-Neofluor objective) with the laser emission at 488 nm.

Endothelial Cell Binding Assays. The in vitro frozen section assay was done as previously described (18). In brief, frozen sections from tonsil and inflamed gut were treated with mAbs 1B2 and 3G6 (control) under constant rotation for 30 min at 6°C before adding freshly isolated PBL (3×10^6 /section) onto tissues. After 30 min, nonadherent cells were gently tipped off, and adherent cells were fixed with 2% glutaraldehyde in cold PBS. Sections were then stained with FITC-conjugated anti-Factor VIII Ab, and only lymphocytes adherent to Factor VIII-positive venules were counted using dark-field microscopy and epi-illumination. At least 150 vessels were counted per sample.

In Vitro Induction Experiments. Human hybrid endothelial cell line (HEC; a gift from H. Holthöfer, University of Helsinki, Helsinki, Finland) was grown in RPMI 1640 supplemented with 10% FCS, 4 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, and 100 μ g/ml streptomycin in tissue culture bottles at 37°C. HEC were induced with IL-1 β (10 and 100 U/ml, Genzyme Corp., Cambridge, MA), IL-2 (10 and 100 U/ml; gift from P. Karnani, Lääke-Farmos, Turku, Finland), IL-4 (10 and 100 U/ml), IL-6 (10 and 100 U/ml), TNF- α (10 and 200 U/ml), IFN- γ (10 and 200 U/ml, all from Genzyme Corp.) and LPS (10 and 100 ng/ml, from *Escherichia coli* serotype O:55, Difco Laboratories, Detroit, MI) for 4 and 20 h, and with dibutyryl cAMP (0.5 and 5 μ g/ml), PMA (1 and 10 ng/ml), thrombin (0.1 and 1 U/ml; from human plasma), histamine (1 and 10 μ g/ml, free base, all from Sigma Chemical Co.), C5a (1:80 and 1:800, gift from K. Hartiala, University of Turku, Turku, Finland), and FMLP (10^{-9} and 10^{-7} M, Sigma Chemical Co.) for 5 min and 2 h. All cytokines were human recom-

Table 1. Tissue Distribution of VAP-1

Organ/tissue	VAP-1 expression
Tonsil, peripheral lymph node*	
HEV	+++ [†]
Interfollicular T cells	-
Interdigitating cells	-
Germinal center lymphocytes	-
Follicular dendritic cells	++
Follicle mantle cells	-
Crypt epithelia (tonsil)	-
Appendix, large intestine	
HEV (Peyer's patches)	+
Lamina propria vessels	+
Enterocytes, Goblet cells	-
Smooth muscle	++
Skin	
Keratinocytes, melanocytes	-
Sweat gland cells	-
Liver	
Hepatocytes	-
Sinus lining endothelium	+
Bile duct cells	-
Kidney	
Glomerular cells	-
Bowman's capsule	-
Tubular cells	-
Intertubular vessels	++
Brain, spinal cord, nerves	
Neurons	-
Glial cells	-
Skeletal muscle	
Muscle cells	-
Large arteries and veins	
Endothelial cells of intima	-
Media	++
Adventitia	++
Heart	
Endocardium (endothelial cells)	+
Muscle cells	+/-
Epocardium	-
Thyroid, adrenal	
Secretory epithelium	-

* In all organs, few VAP-1 positive vessels were observed.

[†] Intensity of staining: + + +, strong; + +, moderate; +, weak; -, no staining.

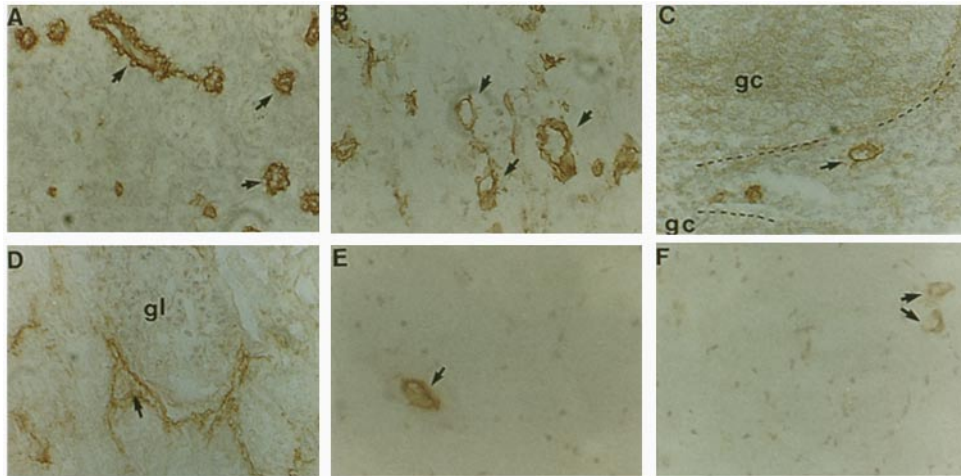


Figure 1. Expression of VAP-1 in normal human tissues. VAP-1 is prominently expressed in HEV (arrows) in tonsil (A) and peripheral lymph node (B), whereas lymphocytes are negative. Dendritic-like cells in germinal centers are also VAP-1 positive (C; two germinal centers [gc] in tonsil are outlined [dashed lines] and one HEV in the interfollicular area is pointed out [arrow]). In kidney, glomerular (gl) endothelium lacks VAP-1, but intertubular vessels (arrow) display positive reactivity (D). VAP-1 is absent from neurons (E, brain) and skeletal muscle cells (F), but occasional venules (arrows) at these sites express VAP-1. Original magnification $\times 250$.

binant forms. Inductions were terminated by quickly rinsing the monolayer twice with ice-cold PBS. Cells were detached with 5 mM EDTA, stained for immunofluorescence, and analyzed using a FACScan® (Becton Dickinson & Co.).

Results and Discussion

Distribution of VAP-1 in Normal Human Tissues. Localization of VAP-1 in normal human organs is summarized in Table 1. In tonsil (Fig. 1 A) and peripheral lymph node (Fig. 1 B), VAP-1 was expressed mainly in vessels. The most prominent staining was seen in endothelial cells of HEV through which most lymphocyte trafficking in these organs takes place. In large-sized vessels (e.g., aorta, vena cava), VAP-1 was confined to smooth muscle cells of the vessel wall rather than to the endothelial cell layer. VAP-1 was absent from capillaries. In germinal centers, VAP-1 was present in follicular dendritic cells (Fig. 1 C). In gut, only a few faintly staining HEV in organized lymphatic tissues (Peyer's patches and appendix) and flat-walled venules in lamina propria were observed. Leukocytes, fibroblasts, and epithelial cells were all VAP-1 negative.

In nonlymphoid organs, VAP-1 was detected mainly in endothelial cells of a few small-sized venules and in the smooth muscle layer of larger vessels. In kidney, arterial endothelium in glomeruli was VAP-1 negative, whereas intertubular vessels expressed this antigen (Fig. 1 D). In liver, sinusoidal endothelium was faintly VAP-1 positive, whereas parenchymal and ductal cells exhibited no staining. Occasional venules expressed VAP-1 both in the central nervous system (cerebrum, cerebellum, and spinal cord) and in peripheral nerves (sciatic nerve), whereas all neuronal and stromal cells at these locations were negative (Fig. 1 E). Smooth muscle cells from several anatomical locations expressed VAP-1 rather prominently in a granular-like pattern, whereas striated muscle cells (Fig. 1 F) were negative. In heart, VAP-1 was expressed in the endothelial cells of endocardium, and in some small vessels within the myocardium, but VAP-1 was absent from the epicardium. Cardiac muscle cells displayed marginal VAP-1

reactivity. All epithelial cell types studied (including both squamous and glandular epithelium) lacked VAP-1. Thus, VAP-1 is mainly expressed on HEV in tissues belonging to the peripheral lymph node endothelial recognition system. However, as is the case with all other currently known endothelial adhesion molecules (11–13, 15, 19–20), VAP-1 is not specific for endothelial cells: it is present in dendritic-like cells in germinal centers and in smooth muscle cells. Interestingly, a functional role for VCAM-1 in dendritic and muscle cells has been reported (21–23), emphasizing the diversity of functions that can be mediated by a single adhesion molecule.

VAP-1 Is Localized in Cytoplasmic Granules and on the Cell Surface of HEV. Using confocal microscopy, it was readily discernible that VAP-1 was present on the luminal surface of HEV as well as in discrete granules within the cytoplasm (Fig. 2). Granular staining suggests that VAP-1 may be stored intracellularly. The identity of VAP-1 positive vesicles remains unknown at present, but it will be interesting to determine

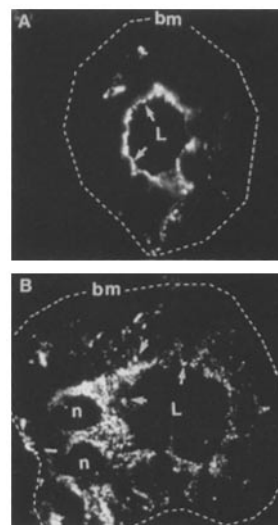


Figure 2. Location of VAP-1 in HEV. In tonsil HEV, VAP-1 is present both (A) on luminal cell surface and (B) in cytoplasmic granules of endothelial cells (arrows). XY-scans from two different HEV of one tonsil were obtained using confocal microscopy. In the images cross-sections of the vessels are shown. (L) Lumen of the vessel; (n) endothelial cell nuclei; (bm) basement membrane (dashed line). Original magnification $\times 2500$ (optical plus electronic).

whether they are Weibel-Palade bodies, for example, where P-selectin is known to reside (10, 11).

VAP-1 Is Inducible In Vivo. Since many of the known endothelial adhesion molecules (ICAM-1, VCAM-1, E- and P-selectin; 3, 4, 24, 25) are inducible in inflammation, we next investigated the possible association between VAP-1 synthesis and inflammatory status of tissues. As described above, VAP-1 was only expressed at a low level in some venules of normal, noninflamed gut (Fig. 3 A). In contrast, gut specimens from patients with inflammatory bowel diseases displayed a markedly increased expression of VAP-1 (Table 2). In the inflamed mucosa, VAP-1 was induced both in the flat-walled venules of the lamina propria (Fig. 3 B) and in the HEV-like venules in organized lymphatic follicles (Peyer's patches, Fig. 3 C).

In skin, chronic inflammation was also accompanied with increased synthesis of VAP-1. In samples from the dermatosis lesion and from macroscopically noninvolved skin area of the same patient that were simultaneously biopsied and stained, the number of VAP-1 positive venules in the upper dermis was consistently higher in the inflamed samples than in the controls. Using the semiquantitative scoring system, VAP-1 expression increased from + to ++ in one, from + to +++ in two, from ++ to +++ in two, from ++ to ++++ in two, and from +++ to ++++ in two patients. Moreover, prominent perivascular leukocyte infiltrates were constantly associated with VAP-1 positive vessels (data not shown). The expression of VAP-1 in normal gut and skin specimens may be due to the systemic nature of the diseases from which the tissue donors were suffering.

VAP-1 Mediates Binding to the Inflamed Mucosa. To study the function of the in vivo induced VAP-1 in the inflamed

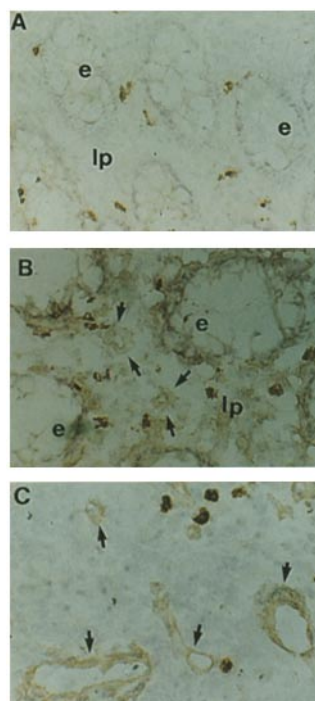


Figure 3. VAP-1 is upregulated in the inflamed gut. In normal gut, only a few faintly positive vessels in the lamina propria are observed (A); in this area, venules are practically negative for VAP-1). In the inflamed gut (ulcerative colitis), numerous VAP-1 positive venules (arrows) are seen both in (B) lamina propria and in (C) organized lymphoid follicles. Endogenous peroxidase-containing cells (mast cells) show nonspecific reactivity. (e) Epithelial cells of the gut; (lp) lamina propria. Original magnification $\times 250$.

Table 2. Induction of VAP-1 in Inflammatory Bowel Diseases

Specimen	n	VAP-1				
		-	+	++	+++	++++
Normal	9	0	5	3	1	0
Crohn	7	0	0	2	3	2
Ulcerative colitis	7	0	0	0	5	2

Bowel specimens were from patients operated on for Crohn's disease, ulcerative colitis, and tumors (uninvolved area of tumor samples represents normal samples). Number of VAP-1 positive venules in each sample was scored (- to +++) as described in Materials and Methods.

mucosa, endothelial cell binding assays were performed. mAb 1B2 inhibited lymphocyte binding to small Factor VIII-positive venules in the lamina propria by $\sim 60\%$ in two different gut samples that abundantly expressed VAP-1 (Fig. 4). Thus, expression of VAP-1 is markedly increased in chronic inflammation, and VAP-1, together with other endothelial adhesion molecules, appears to be capable of targeting appropriate leukocyte influx into sites of immunological challenge at different anatomical locations.

VAP-1 Expression In Vitro Is Not Induced by Known Inflammatory Mediators. 13 inflammatory cytokines, mitogens, and

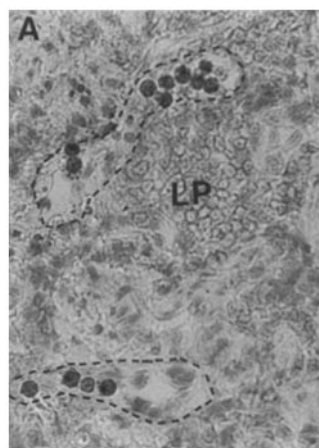
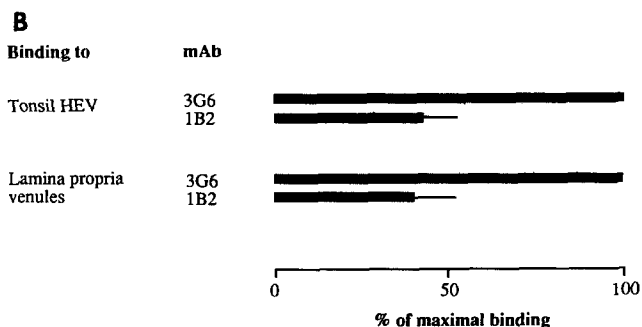


Figure 4. The inflammation-induced VAP-1 mediates lymphocyte binding. A frozen section binding assay was performed, in which binding of PBL to Factor VIII positive venules in inflamed lamina propria was analyzed. (A) Specificity of the assay. PBL (dark spots) adhere to venules (broken line), but not to the leukocyte-rich stroma between the vessels. The focus of the micrograph is at the level of lymphocytes, and therefore the underlying tissue seems blurred. (LP) Lamina propria. (B) Inhibition assays were done by preincubating tissue sections with mAbs 1B2 and 3G6. Results are presented as percent control binding with standard errors (i.e., binding of PBL in the presence of mAb 3G6 defines 100% binding).



secretagogues were tested with HEC in an attempt to define the mediators that are responsible for the induction of VAP-1 in vivo. HEC (Factor VIII-positive, P-selectin-positive cells, on which ICAM-1 is inducible with TNF- α) seem to resemble human umbilical vein endothelial cells that are commonly used as endothelial cell models (data not shown). HEC cells were induced with two different concentrations of each mediator for two time periods (see Materials and Methods). However, none of the mediators tested, many of which are known to be involved in the regulation of expression of other endothelial cell antigens, had any significant effect on VAP-1 expression on the HEC surface (net mean fluorescence intensity [MFI] after induction ranged between -2.3 and +3.0, mean -0.1; for comparison, ICAM-1 had a net MFI 23.2

after 20 h TNF- α [200 U/ml] induction; data not shown). These results suggest that VAP-1 is responsive to some other type of mediator or, alternatively, a combination of factors may be necessary for VAP-1 induction.

In conclusion, VAP-1 is present in a subset of endothelial cells in several normal organs and is clearly upregulated in venules in the setting of inflammation. The inflammation-induced VAP-1 is functionally intact since a mAb against it can effectively inhibit lymphocyte binding at these sites. A detailed understanding of the regulation of VAP-1 expression may prove helpful in the future for designing tools, e.g., mAbs or ligand analogs, to selectively control leukocyte influx at sites of inflammation.

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