Mouse Vascular Adhesion Protein 1 Is a Sialoglycoprotein with Enzymatic Activity and Is Induced in Diabetic Insulinitis

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The continuous recirculation of lymphocytes requires an adequate expression and function of the molecules mediating the cellular interactions between endothelium and lymphocytes. Human vascular adhesion protein 1 (hVAP-1) is an endothelial cell adhesion molecule that mediates the binding of lymphocytes to venules in peripheral lymph nodes as well as at sites of inflammation. Recently the mouse homologue of hVAP-1 has been cloned. It is a previously unknown molecule with a significant sequence identity to copper-containing amine oxidases. Besides the sequence, very little is known about the expression, structure, and function of mouse VAP-1 (mVAP-1). In this study we demonstrate that mVAP-1 is prominently expressed in endothelial and smooth muscle (but not in other types of muscle cells), as well as in adipocytes. mVAP-1 is a 220-kd homodimeric sialoglycoprotein that displays cell-type-specific differences in glycosylation. The expression of mVAP-1 is induced on inflammation in the vessels of the endocrine pancreas during the development of insulinitis, and the up-regulation correlates with the extent of the lymphocytic infiltrate. In general, different mouse strains displayed very similar VAP-1 expression, but the small differences seen in liver and gut suggest that immunostimulation may modulate VAP-1 synthesis in extrapancreatic organs as well. Finally, we show that mVAP-1 has a monoamine oxidase activity against naturally occurring substrates, implying a role in the development of vasculopathies. These data show that mVAP-1 and hVAP-1 are very similar molecules that nevertheless have certain marked differences in expression, biochemical structure, and substrate specificity. Thus mVAP-1 is a novel inflammation-inducible mouse molecule that has a dual adhesive and enzymatic function. (*Am J Pathol* 1999, 155:1613–1624)

Continuous recirculation is essential for lymphocytes to meet their antigens in the lymphoid organs. The extravasation of leukocytes is known to be an active multistep process, where the initial weak binding of leukocytes is

followed by activation, firm adhesion, and finally by transmigration into the tissue.^{1,2} Although several different adhesion molecules are known to mediate the sequential but overlapping interactions between leukocytes and the vessel wall, the currently known molecules do not explain all of the binding specificities observed in normal and inflammatory settings. For example, a peripheral lymph node addressin, PNA_d, that directs lymphocyte binding to peripheral lymph nodes (PLNs) is not sufficient to mediate all of the observed migration of lymphocytes to PLN, and therefore other molecules yet to be defined must exist.³

Human vascular adhesion protein 1 (hVAP-1) is a homodimeric endothelial cell molecule composed of two 90-kd subunits. It mediates subtype-specific, selectin-independent lymphocyte binding to endothelial cells.^{4–6} hVAP-1 is mainly expressed on high endothelial venules (HEVs) in PLN-type lymphatic tissues, but immunoreactive hVAP-1 can also be found in endothelial cells of other tissues as well as in smooth muscle cells and follicular dendritic cells.⁴ The expression of hVAP-1 is up-regulated during inflammation in the vessels of the skin, gut, and synovium.^{5,7,8} The sialic acids decorating VAP-1 are essential for its function, inasmuch as hVAP-1 has been shown to be nonfunctional in lymphocyte binding assays if these oligosaccharide modifications are removed.⁹ Under physiologically relevant shear stress VAP-1 has been shown by intravital microscopy to mediate the formation of the initial contacts of labeled human lymphocytes with inflamed rabbit mesenteric venules,⁵ suggesting that VAP-1 would function at an early step of the multistep adhesion cascade.

To obtain more information on the importance of VAP-1 in lymphocyte homing *in vivo* and to be able to manipulate genetically the expression of VAP-1, we have recently isolated the cDNA and gene encoding mouse VAP-1 (mVAP-1)^{10,11} and produced a mAb against it. Antibody stainings of frozen tissue sections from PLN and gut have shown that mVAP-1 is expressed on PLN HEVs, in lamina

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propria vessels, and in smooth muscle cells of the mouse gut. The analysis of the predicted mVAP-1 protein core revealed that it is a novel type II transmembrane molecule with an 83% identity to hVAP-1. Moreover, mVAP-1 displays significant identity to the semicarbazide-sensitive Cu-containing amine oxidase (SSAO) enzyme family. The members of this superfamily are enzymes that catalyze the oxidative deamination of different amines and have widely differing substrate specificities.^{12,13} Based on the expression and presence of a quinone cofactor, enzyme-bound copper, and enzyme activity only against primary amines or monoamines, the Cu-containing amine oxidases are clearly distinct from the flavinyl adenosine diamine (FAD)-containing intracellular (mitochondrial) monoamine oxidases.^{14,15} The true biological role of these enzymes has remained unclear, although they have been reported to be involved in the pathogenesis of different vasculopathies.^{16,17}

Although hVAP-1 has been shown to be inducible in clinical samples,⁸ it has not been possible to study the characteristics of VAP-1 in controlled animal models. Nonobese diabetic (NOD) mice are a good model system for a lymphocyte-dependent inflammatory reaction, because these mice spontaneously develop insulinitis and thereafter a syndrome with clinical findings resembling those of insulin dependent diabetes mellitus.¹⁸

In the present study we have for the first time in any species been able to examine the distribution of VAP-1 in formalin-fixed paraffin-embedded sections with good resolution and have analyzed many tissues for which no information on VAP-1 synthesis had been available earlier. We have also followed the expression of mVAP-1 during the development of insulinitis in the pancreata of NOD mice and shown that the expression of VAP-1 is induced in islet vessels during the formation of islet infiltrates and that this up-regulation of expression correlates with the amount of lymphocyte infiltration, suggesting a novel biological role for VAP-1 and SSAOs in the development of diabetes. Analyses of biochemical characteristics and enzyme activity of mVAP-1 in several tissues revealed tissue-specific differences in glycosylation of VAP-1 and the existence of naturally occurring substrates for this enzyme. The results show that there are important differences between human and mouse VAP-1. Finally, these data not only represent the first thorough cellular analyses of mVAP-1 but, in fact, any single molecular species of a whole large family of semicarbazide-sensitive monoamine oxidases.

Materials and Methods

Antibodies and Tissues

TK10-79 is a rat anti-mVAP-1 mAb, and its production has been described elsewhere.¹⁰ Briefly, a 117-bp C-terminal fragment of mVAP-1 was cloned into glutathione S-transferase (GST) gene fusion vector (Pharmacia Biotech, Uppsala, Sweden) to produce a soluble GST-mVAP-1 fusion protein, which was used to immunize rats. A new anti-hVAP-1 mAb TK8-18, which detects both the mono-

meric and dimeric forms of hVAP-1, was produced by immunizing BALB/c mice with immunoaffinity-purified hVAP-1 as described earlier.¹⁹ Hermes-1, a rat mAb against human CD44,²⁰ and 3G6, a mouse mAb against an antigen expressed on chicken peripheral T cells,⁴ were used as negative control antibodies in immunostainings and in immunoblottings. Anti-MAdCAM-1 mAb MECA-367²¹ was a kind gift from Dr. E. C. Butcher. Fluorescein isothiocyanate-conjugated goat anti-rat IgG and peroxidase-conjugated goat anti-rat IgG were from Sigma Chemical Co. (St. Louis, MO). Peroxidase-conjugated sheep anti-mouse IgG was from Dakopatts (Glostrup, Denmark).

Cell Culture and Expression of mVAP-1 cDNA

CHO (Chinese hamster ovary) cells were from the American Type Culture Collection and grown in α -MEM (Gibco BRL, Paisley, UK) plus CHO nucleosides supplemented with 20% fetal calf serum (FCS), 2 mmol/L glutamine (Biological Industries, Beit Haemek, Israel), 128 U/ml penicillin, and 128 μ g/ml streptomycin. Electroporation was used (Bio-Rad Gene Pulser apparatus; 0.3 kV, 960 μ F, 0.4 cm cuvette in RPMI plus 1 mM Na-pyruvate, 2 mM L-glutamine without serum) to transfect these cells (5×10^6 /transfection) with 20 μ g of expression vector pcDNA3 alone or with the vector containing full-length mVAP-1 cDNA. After transfection these stably transfected cells were selected in the presence of 0.5 mg/ml geneticin (Gibco BRL).

Fluorescence-Activated cell sorting (FACS) Analyses

Stably transfected CHO cells were detached from the culture flasks by trypsin-EDTA treatment. For the immunofluorescence stainings the cells were either left intact or permeabilized and/or fixed by incubation either in 100% methanol or acetone (2 minutes, -20°C) or in 2% glutaraldehyde in phosphate-buffered saline (PBS) (20 minutes, 20°C). After washing and blocking (20 minutes in RPMI1640 containing 10% FCS) the primary mAbs were added at 10 μ g/ml (diluted in PBS containing 1% FCS and 1 mM sodium azide). Fluorescein isothiocyanate-conjugated anti-rat IgG with 5% normal mouse serum was used as a second-stage reagent. The cells were finally fixed in PBS containing 1% formalin. Approximately 10,000 cells were analyzed with a FACScan cytometer (Becton Dickinson, Mountain View, CA).

Tissue Specimens and Immunohistochemistry

Tissue samples were freshly prepared from different organs of mice. BALB/c and NOD mice were obtained from the local colony in Turku, and they were fed with normal mouse chow and housed under specific pathogen-free conditions. Wild mice were caught with traps from local farms. The tissue samples were either snap-frozen or fixed in formalin. The formalin-fixed, paraffin-embedded

Figure 1. mAb TK10-79 recognizes recombinant mVAP-1. mVAP-1 cDNA or mock transfected CHO cells were stained with mAbs Hermes-1 (negative control) or with TK 10-79 (against mVAP-1) and analyzed by FACS. The *x* axis is the fluorescence intensity on a log-scale, and the *y* axis is the relative number of cells. A positive staining with mAb TK10-79 is obtained only after fixation of the transfectants with methanol.

BALB/c tissue sections were deparaffinized with xylene and rehydrated in a series of decreasing concentrations of ethanol before microwave treatments (3×2 minutes, 900 W (50% power) in 10 mM citric acid, pH 6.0). The endogenous peroxidase activity was removed by incubating the sections for 30 minutes in 1% H_2O_2 in PBS. The staining was done according to the standard protocol with the avidin-biotin complex technique (Vectastain ABC kit (rat IgG Elite), Vector Laboratories, Burlingame, CA). The primary mAbs TK10-79 (anti-mVAP-1) and Hermes-1 (control mAb) were used at a concentration of 2 μ g/ml at +7°C overnight. The sections were slightly counterstained with hematoxylin.

Because MECA-367 (against MAdCAM-1) does not work on paraffin-embedded tissue sections (data not shown), acetone-fixed serial cryostat sections of pancreata from 3- and 12-week NOD mice were stained by immunoperoxidase staining, using a protocol described earlier.¹⁰ Primary mAbs TK10-79, MECA-367, and Hermes-1 were used at a concentration of 100 μ g/ml. The stainings with TK10-79 and MECA-367 were read blindly by counting all islets in the sections and every vessel around and within the islets. To avoid counting the expression of the smooth muscle VAP-1, larger arteries and veins were omitted from the analysis of both mVAP-1 and MAdCAM-1 stainings. The staining intensity was scored semiquantitatively as follows: (–) negative staining, (+) weak staining, (++) moderate staining, (+++) strong staining. The number of infiltrating lymphocytes present in the islets of 12-week mice was scored as follows: (0) no lymphocytes present in the islet, (1) mild periislet insulinitis, (2) insulinitis occupying $\leq 50\%$ of the islet area, (3) islet filled with lymphocytes ($> 50\%$ of the area) as described previously.²² Because of the sample size, certain cate-

gories were finally combined as shown in the tables. The statistical analyses were performed with a χ^2 test.

Immunoblotting and Glycosidase Digestions

Samples from different BALB/c mouse tissues were minced and lysed in a lysis buffer (150 mM NaCl, 10 mM Tris-base, pH 7.2, 1.5 mM $MgCl_2$, 1% NP-40, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride), and after removal of the insoluble material by centrifugation, all of the supernatants, except the supernatant from adipose tissue, were depleted of endogenous immunoglobulins by incubation with protein G-Sepharose beads (Pharmacia, Sweden). Mouse smooth muscle cell lysate was prepared from aorta and the white adipose tissue lysate from abdominal fat. The precleared samples were then mixed with Laemmli sample buffer with or without reduction (5% 2-mercaptoethanol) before loading onto 5–12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gradient gels. The human smooth muscle lysate was prepared from tissue samples obtained from surgical operations and run in a parallel lane on the same gel as the mouse lysates. For glycosidase treatments the clarified lysate supernatants (mAb TK10-79 does not work in immunoprecipitation) were treated with different enzymes, using a protocol previously described for hVAP-1.⁹ In brief, for sialidase treatments 50- μ l aliquots of the lysate were incubated with *Vibrio cholerae* sialidase (Behring, 5 mU, 2 h, +37°C), which removes $\alpha 2,3$, $\alpha 2,6$, and $\alpha 2,8$ linked sialic acids.²³ Endo- α -N-acetylglucosaminidase (Genzyme, 16 mU, +37°C, overnight) was used to remove O-glycans from the sialidase-treated samples, and peptide: N-glycosidase F treatment (Gen-

Figure 2. Tissue distribution of mVAP-1. Staining of formalin-fixed paraffin-embedded mouse tissues with a negative control mAb Hermes-1 are shown in the last (narrower) micrograph of each series, and anti-mVAP-1 mAb TK10-79 stainings are shown in the first or first two micrographs of each series. **A:** Gut. Bw, bowel wall; Mm, muscularis mucosae. **Arrows** indicate lamina propria vessels. **B:** Peyer's patch. **Arrowheads** point to two mVAP-1-positive HEVs. **C:** Spleen. wp, white pulp. **Arrows** indicate two mVAP-1-positive splenic vessels. **D:** Bone. Sinusoidal vessels of bone marrow in femur are pointed out by **arrows**. Tr, bone trabeculae. **E:** Thymus. **Arrows** point to mVAP-1-positive vessels at the corticomedullary junction. **F:** Lung. Bronchus epithelium (**curved arrow**) lacks mVAP-1, whereas the smooth muscle layer of terminal bronchii (**arrowheads**) and endothelium of large veins (**arrows**) are mVAP-1 positive. **G:** Heart. Capillary endothelium (**arrows**) and larger vessels are mVAP-1 positive. **H:** Kidney. mVAP-1 is absent from glomeruli (gl), tubuli (one outlined with a **dashed line**), and intertubular vessels (**arrows**) but present on afferent arterioles (**arrowheads**). **I:** Liver. Sinusoidal endothelial cells are very faintly mVAP-1 positive (**arrowheads**), whereas central veins (**arrow**) are strongly mVAP-1 positive. **J:** White fat. The original magnifications were $\times 100$ (A1, C2, E2, H1), $\times 200$ (B1 and 2, C1, F1 and 3, G1), and $\times 400$ in the others.

zyme, 1.2 U, $+37^{\circ}\text{C}$, overnight) was used to digest N-linked oligosaccharides.

Amine Oxidase Assays

Stably transfected CHO cells ($5\text{--}10 \times 10^6$) were detached with trypsin-EDTA, washed with PBS, resuspended in 1 ml PBS, and finally lysed by using a Braun

sonicator (Melsungen, Germany). The enzyme activities were measured according to a radioisotopic method,²⁴ using 25 μl of the lysate in all reactions. Unlabeled substrates (benzylamine, β -phenylethylamine, tyramine, tryptamine, methylamine, and histamine; obtained from Sigma Chemical Co.) were added to the reaction at 1 mM as cold competitors before the addition of ^{14}C -labeled benzylamine. The reactions were incubated at 37°C for

Figure 2 continued.

60 minutes before extraction of the labeled aldehyde reaction products in toluene. Finally, the extracts were measured with a liquid scintillation counter.

Results

TK10-79 is a mVAP-1-Specific mAb

mAb TK10-79 has been raised against a bacterial expressed 39 amino acid peptide of mVAP-1.¹⁰ To confirm the specificity of the anti-mVAP-1 mAb TK10-79, mVAP-1 and mock transfected CHO cells were stained with this mAb and a control mAb (Figure 1). Neither of the untreated transfectants stained positively with mAb TK10-79 (Figure 1, untreated samples). No VAP-1 reactivity was observed with glutaraldehyde fixation or acetone permeabilization of the transfectants (Figure 1 and data not shown). However, when the cells were fixed with methanol, a positive signal was detected from mVAP-1 CHO transfectants, but not from mock cells, with TK10-79, while the control mAb stainings remained negative, showing that the mAb TK10-79 is immunoreactive against mVAP-1. These data indicate that mAb TK10-79 does not detect the native mVAP-1 in these transfectants, but

rather a certain epitope of the mVAP-1 protein core after fixation of the cells and denaturation of the protein.

Distribution of mVAP-1 in Mouse Tissues

Previously mVAP-1 has been shown to be expressed in gut and PLN by staining of frozen tissue sections, but the staining intensity was relatively weak.¹⁰ Expression of mVAP-1 in all other tissues and cells is unknown. However, during the course of this work we found out that mAb TK10-79 stained mVAP-1 well in formalin-fixed, paraffin-embedded tissue sections. In fact, mAb TK10-79 is the first anti-VAP-1 mAb (of 15) in any species that recognizes VAP-1 in these histological specimens of superior resolution. Therefore paraffin sections were prepared from different mouse organs to determine the distribution of mVAP-1. In all organs examined, mVAP-1 is expressed at least on a few endothelial cells. In addition, smooth muscle cells and fat cells are positive in all tissues studied. mVAP-1 is always absent from all types of epithelial cells, leukocytes, and fibroblasts.

The staining of paraffin sections of mouse gut revealed exactly the same staining pattern as previously detected from frozen sections. The smooth muscle cells and lam-

ina propria endothelial cells were mVAP-1 positive, as shown in Figure 2A. Some, but not all, HEVs in Peyer's patches were also VAP-1 positive (Figure 2B). In PLN HEVs mVAP-1 was expressed on both the endothelial cells and pericytes, whereas the lymphocytes were mVAP-1 negative. In germinal centers of the lymph nodes mVAP-1-positive dendritic cells were detected (data not shown). In spleen mVAP-1 is expressed in many vessels but not on splenocytes or marginal zone macrophages (Figure 2C).

No information on the expression of mVAP-1 or hVAP-1 in primary lymphoid organs has been available. As shown in Figure 2D, strong mVAP-1 expression was seen on the sinusoidal vessels in bone marrow, but mVAP-1 was absent from osteocytes and hematopoietic cells. We found mVAP-1 on the endothelial cells of thymic vessels in both the cortex and medulla in a conspicuously luminal pattern (Figure 2E).

mVAP-1 is present in multiple nonlymphoid tissues. In lung the capillary endothelial cells and alveolar epithelial cells were mVAP-1 negative, whereas the smooth muscle cells of the bronchii and endothelium of larger veins were mVAP-1 positive (Figure 2F). In heart, the cardiac muscle cells were mVAP-1 negative, but the endocardium and myocardial capillary endothelium expressed this antigen (Figure 2G). The endothelium of large arteries (aorta) was faintly mVAP-1 positive. In kidney, the glomerular endothelium, Bowman's capsule, peritubular capillaries, and renal tubuli were mVAP-1 negative, and the only reactivity was found in the muscle cell layer of the incoming glomerular artery (Figure 2H). In liver the sinusoidal endothelial cells lacked or had only a marginal mVAP-1 reactivity, whereas the central veins displayed endothelial VAP-1 positivity (Figure 2I). mVAP-1 was absent from hepatocytes and bile duct cells. In pancreas, exocrine and endocrine cells lacked mVAP-1, which was only faintly detectable in blood vessels. In brain, neurons and glial cells were mVAP-1 negative, and only occasional positive vessels were found. In skin, mVAP-1 was found only at low levels on a few dermal vessels. In both white (Figure 2J) and brown fat tissue the adipocytes were strongly mVAP-1 positive. mVAP-1 was absent from striated skeletal muscle cells (data not shown).

Our conclusion is that mVAP-1, like all other known adhesion molecules (reviewed in ref. 1), is present not only on endothelial cells but also on certain other cell types in different organs. The endothelial staining pattern is compatible with the possibility that VAP-1 functions at the sites of lymphopoiesis (thymus and bone marrow), in the physiological recirculation of lymphocytes (secondary lymphoid organs) and in leukocyte influx into sites of inflammation (nonlymphoid tissues).

mVAP-1 Is Expressed in Islet Vessels of Nonobese Diabetic Mice

To investigate the expression of mVAP-1 in inflammation we analyzed the well-established model of insulinitis in diabetic mice. In our colony, none of the animals has

insulinitis at 3 weeks, whereas by 12 weeks 80% of the islets are inflamed in females. In this disease MAdCAM-1 serves as a model of an inducible, functionally important adhesion molecule.^{22,25} To compare the distribution of mVAP-1- and MAdCAM-1-positive vessels in the endocrine pancreas of NOD mice, frozen tissue sections from 12-week-old animals with emerging insulitis were stained with mAbs TK10-79 and MECA-367 (an anti-MAdCAM-1 mAb). The expression of both molecules could be found in the vessels of endocrine and exocrine pancreas, and a similar staining pattern of mVAP-1 was seen on paraffin sections (data not shown). Because mAb MECA-367 does not work on paraffin sections, usage of frozen tissue sections was mandatory to compare the localization of these vascular addressins on islet vessels. The staining of serial sections revealed that mVAP-1 and MAdCAM-1 colocalize in the same vessels in many islets (two representative islets are shown in Figure 3), although vessels that were positive only for VAP-1 or MAdCAM-1 were also found.

mVAP-1 Is Induced in Islet Endothelium during Insulitis

The observation that mVAP-1- and MAdCAM-1-positive vessels colocalize led us to investigate whether the expression of mVAP-1, like that of MAdCAM-1,²⁵ is up-regulated during the pathogenesis of diabetes. To that end we compared the expression of mVAP-1 in the pancreata of NOD mice at the age when most of the islets are devoid of lymphocyte infiltration (3–4 weeks) and at the age when advanced insulitis occurs (12 weeks). As a positive control for the scoring, we also stained and analyzed the expression of MAdCAM-1 in the same pancreata. When the number of MAdCAM-1-positive vessels

Figure 3. The expression of mVAP-1 and MAdCAM-1 in pancreas. **A, B:** MAdCAM-1 is expressed both on vessels within the inflamed islets (IS) and on periislet vessels of pancreas from a 12-week NOD mouse. **C, D:** mVAP-1 staining of the same islets as in **A** and **B** in serial sections. Note that the vessels marked by arrows coexpress MAdCAM-1 and VAP-1. Original magnifications: (**A, C**) $\times 200$; (**B, D**) $\times 400$.

Table 1. VAP-1 and MAdCAM-1 Are Induced on Islet Vessels of NOD during the Development of Insulitis

Age	Staining intensity [†]			
	mVAP-1		MAdCAM-1	
	-/+	++/+++	-/+	++/+++
4 weeks*	81%	19% [#]	69%	31%
12 weeks**	50%	50% [#]	36%	64%

[†]Intensity was scored as: -/+, negative or weak; ++/+++, moderate/strong (see Materials and Methods).

*Six mice. The number of counted islets was 32 in MECA-367 stainings and 37 in TK10-79 stainings.

**Six mice. The number of counted islets was 72 in both MECA-79 in TK10-79 stainings. Each peri- and intraislet vessel was counted.

[#]The percentage of the vessels with moderate/strong (++/+++) VAP-1 expression in pancreata from 12-week-old mice was significantly increased ($P < 0.001$) when compared to that at 4 weeks.

was compared at the ages of 3–4 and 12 weeks, more vessels having moderate or strong MAdCAM-1 expression were found at 12 weeks (Table 1), confirming the earlier observations and hence our scoring system with the frozen sections. When the stainings for mVAP-1 were analyzed, there were statistically significantly ($P < 0.001$) more vessels modestly or strongly VAP-1 positive in 12-week-old than in 3–4-week-old animals (Table 1). These results indicate that during the development of insulitis the expression of both mVAP-1 and MAdCAM-1 is induced (Table 1).

To examine whether the up-regulation of mVAP-1 correlates with the degree of lymphocyte infiltration we next investigated the relationship between mVAP-1-positive vessels and the amount of lymphocytes present in the islets. A comparison of the islets of 12-week pancreata showed that strong mVAP-1 expression is found mostly in islets with high lymphocyte infiltration rather than in islets with no/mild infiltration (Figure 4 and Table 2). Thus the up-regulation of mVAP-1 in islet vessels of 12-week NOD mice correlates statistically significantly with a high degree of lymphocyte influx.

mVAP-1 Is a ~110/220-kd Molecule in Different Mouse Organs

To study the biochemical properties of mVAP-1 in different tissues, lysates from several mouse tissues were run in parallel on SDS-PAGE gel, transferred to a filter, and stained with the anti-mVAP-1 mAb. Under nonreducing conditions a specific 220-kd antigen was detected from most examined tissues (Figure 5A, lanes 1–13). In prolonged exposures (data not shown) the 110-kd antigen was also detectable in all tissues except PLN/MLN lymphocytes, which remained constantly negative. Under reducing conditions a prominent ~110-kd antigen was detected from the smooth muscle lysate (Figure 5B), but the ~220-kd antigen disappeared, and a large smear at higher molecular weights became reproducibly detectable. The probing with the control mAb remained negative. A similar result under reducing conditions was also seen from CHO-mVAP-1 transfectants (data not shown). Moreover, in all examined tissues mVAP-1 was roughly of

Figure 4. Lymphocyte infiltration correlates with the expression of mVAP-1 in islet vessels. **A:** In islet (IS) with no/very low lymphocyte infiltration the expression of mVAP-1 is undetectable, whereas islets (**B**) with a high number of lymphocytes have strongly mVAP-1-positive vessels (arrow). Original magnification, $\times 400$.

the same size, and the intensity of the signal corresponded well with the staining pattern. The comparison of the apparent molecular weight of mVAP-1 in nonreduced and reduced SDS-PAGE analyses to the predicted mass of the amino acid sequence and to the structure of hVAP-1 shows that most, if not all, mVAP-1 is in a dimeric form composed of two 110-kd subunits in the examined tissues.

To compare the molecular weight of mouse and human VAP-1, lysate from human smooth muscle cells was run in parallel (Figure 5A, lane 14) and detected with an anti-hVAP-1 mAb TK8-18. A predominant 170-kd molecule was seen together with larger forms typical of smooth muscle VAP-1 (unpublished results). Thus mVAP-1 has a clearly slower electrophoretic mobility than hVAP-1.

mVAP-1 Is a Sialoglycoprotein

There is a marked size discrepancy between the size of mVAP-1 monomer seen in immunoblotting (~110 kd) and the predicted molecular mass from the cDNA sequence (84.5 kd). Because mVAP-1 contains six potential *N*-glycosylation sites and six putative *O*-glycosylation sites,¹⁰ we next analyzed whether the difference in the two molecular mass estimates is due to oligosaccharide modifications. Cell lysates from peripheral lymph nodes, heart, adipose tissue, and gut smooth muscle were digested with different glycosidases. Sialidase treatment decreased, although quite modestly, the molecular weight of mVAP-1 in all examined tissues (Figure 6),

Table 2. mVAP-1 Expression Is Induced Mainly on Vessels of Islets with Severe Insulitis

Expression of mVAP-1 [†]	The degree of lymphocyte infiltration*	
	0/1	2/3
-/+	76%	49%
++/+++	24% [#]	51% [#]

[†]Intensity was scored as: -/+, negative or weak; ++/+++, moderate/strong.

*Number of infiltrating lymphocytes was scored as: 0/1 none or few peri-islet ones, 2/3 marked or massive (see Materials and Methods). The number of counted vessels was 46 in the 0/1 islets and 146 in the 2/3 islets.

[#]The intensity of mVAP-1 expression in vessels correlates in a statistically significant manner ($P < 0.001$) to the degree of lymphocyte influx within the islet.

Figure 5. TK10-79 recognizes a 110/220 kD molecule in different mouse tissues. Lysates from different mouse organs were separated by SDS-PAGE and thereafter subjected to immunoblotting with the indicated mAbs. **A:** Under nonreducing conditions a 220-kD band (arrow) can be detected, whereas the monomeric 110-kD band is detectable only from adipose tissue and smooth muscle lysate (in overexposures the 110-kD band can be seen also from lanes 1 and 3–13). hVAP-1 in smooth muscle lysate (arrowhead) is smaller than the corresponding mouse antigen (lane 9). **B:** Under reducing conditions, the monomeric ~110-kD form of mVAP-1 (arrowhead) and a high-molecular-weight smear from mouse smooth muscle lysate are detected. Molecular weight standards are listed on the left.

indicating that mVAP-1 protein core is decorated with sialic acids. Cleavage of the lysates with *N*-glycanase also increased the electrophoretic mobility of mVAP-1, showing that mVAP-1 contains N-linked oligosaccharide decorations. However, mVAP-1 may not have major O-linked side chains, because further digestions of sialidase-treated mVAP-1 with O-glycanase did not have any detectable effect on the mobility of mVAP-1 when compared to the samples treated with sialidase only. The change in the electrophoretic mobility of desialylated or de-*N*-glycosylated mVAP-1 varied to some extent between different tissues and was greatest in the adipose tissue. Thus mVAP-1 is a sialoglycoprotein that is differently glycosylated in distinct tissues.

mVAP-1 Has Enzyme Activity Against Naturally Occurring Amines

Because mVAP-1 has previously been shown to possess activity only against benzylamine, a synthetic amine not found *in vivo*,¹⁵ we tested whether any endogenously found amines could be oxidized by mVAP-1. Lysates from CHO-mVAP-1 transfectants were used in a radioisotopic assay as the source of the enzyme, and [¹⁴C]benzylamine was used as the substrate. The effect of different amines was tested by adding them unlabeled in the reaction and by analyzing the effect of possible competition on the oxidation of the labeled benzylamine. As

Figure 6. mVAP-1 is a glycoprotein. Lysates from the indicated mouse tissues were subjected to different glycosidase digestions before SDS-PAGE and immunoblotting with mAb TK10-79 under reducing conditions. All of the negative control stainings with Hermes-1 were negative. —, nontreated; sial, *V. cholerae* sialidase; O-Glyc, endo- α -N-acetylgalactosaminidase; N-Glyc, peptide:N-glycanase F is the name of the enzyme. The molecular weight standards are listed on the left.

expected, an excess of cold benzylamine almost completely inhibited the oxidation of the labeled benzylamine. Interestingly, mVAP-1 also binds β -phenylethylamine, tyramine, tryptamine, and methylamine, because these amines also inhibited the oxidation of labeled benzylamine (Figure 7). In contrast, histamine is not a substrate for mVAP-1. These data indicate that mVAP-1 can oxidatively deaminate naturally occurring primary amines.

Discussion

The purpose of this study was to examine the lymphoid and nonlymphoid expression of mVAP-1, to study the role

Figure 7. Substrate specificity of mVAP-1. Formation of labeled aldehyde product from radioactive benzylamine (Bz) in a mVAP-1-catalyzed reaction in the absence (—) or in the presence of competing amines was measured using scintillation. Quantification of the results (mean activity in cpm $\pm$ SEM) was obtained from two independent enzyme assays, each performed in duplicate with two different mVAP-1 CHO lines. PEA, phenylethylamine.

of mVAP-1 in the development of lymphocytic inflammation, to define the molecular characteristics of mVAP-1, and to compare mVAP-1 and hVAP-1. Our results show that mVAP-1 is a 110/220-kD glycosylated molecule in different mouse tissues and that it possesses monoamine oxidase activity against multiple naturally occurring amines. mVAP-1 is expressed in HEVs of the secondary lymphoid organs as well as in adipocytes and smooth muscle cells at various anatomical locations, and it is inducible during the development of insulinitis leading to the onset of diabetes.

The appearance of mononuclear cells (mainly lymphocytes) in the islets of Langerhans in the pancreas is a key event in the pathogenesis of insulin-dependent diabetes mellitus.^{26,27} This inflammatory response, called diabetic insulinitis, leads to the destruction of the insulin-secreting β cells and later to the onset of the disease. Previously it has been shown with NOD mice that MAdCAM-1, a mucosa-specific vascular addressin,^{21,28} is induced on islet vessels of these mice during the development of insulinitis and that it mediates the binding of lymphocytes to the islet endothelium.²⁵ The expression of MAdCAM-1 correlates with the degree of lymphocyte infiltration,²⁹ and furthermore, the incidence of diabetes can be reduced by blocking the function of MAdCAM-1 by an anti-MAdCAM-1 mAb,²² indicating that MAdCAM-1 is required for the development of diabetes. Here we have shown that mVAP-1 is expressed on islet vessels in NOD mice, its expression is induced on islet endothelium during the development of the insulinitis, and mVAP-1 is induced in islet vessels with a high degree of lymphocyte infiltration. Our findings show that VAP-1 may have a role similar to that of MAdCAM-1 in the pathogenesis of diabetes. Intriguingly, in humans inhibitory mAbs against inflammation-induced VAP-1 have been shown to block the function of VAP-1 *in vitro*.⁸ However, the functional role of mVAP-1 remains to be studied, because the anti-mVAP-1 MAb TK10-79 is noninhibitory. After development of a function blocking anti-mVAP-1 mAb, it will be of interest to use this model to investigate whether mVAP-1 is causally involved in the development of the disease.

In this study the expression of VAP-1 was investigated for the first time in formalin-fixed paraffin-embedded sections in any species. It allowed us to define the expression of mVAP-1 precisely and in multiple tissues not earlier amenable for analyses. In most tissues and cell types, the expressions of mVAP-1 and hVAP-1 were the same. There are, nevertheless, some clear distinctions. Immunoperoxidase stainings of human liver tissue sections have previously revealed strong VAP-1 positivity in the vascular endothelium of portal vessels and sinusoidal endothelium of the liver.^{8,30} In contrast, the sinusoids are mostly mVAP-1 negative in BALB/c mice. This is notable, because in humans, liver VAP-1 has been shown to mediate T-cell binding to hepatic endothelium and to support the binding of tumor-infiltrating lymphocytes to tumor endothelium in hepatocellular carcinomas.^{30,31} Because previous Northern blot analyses have revealed that the expression of mVAP-1 and hVAP-1 in liver does not differ at the mRNA level,^{6,10} one explanation for the lack of mVAP-1 staining is that in mouse liver, VAP-1 may have

Table 3. Differences between Mouse and Human VAP-1

	mVAP-1	hVAP-1
Expression		
Liver sinusoidal endothelium	-/+	++
Kidney peritubular capillaries	-	++
Structure		
Molecular weight (kd)	220/110	170–180/90
Sialic acids	+	++
N-linked oligosaccharides	+	+/-*
Substrates		
Methylamine	+	+
Tyramine	+	-
Tryptamine	+	-
β -Phenylethylamine	+	-

*Present only in smooth muscle hVAP-1.

different posttranslational modifications and therefore be poorly detectable by the antibody. Another possible explanation for the almost complete lack of mVAP-1 expression in sinusoidal endothelium is that the mice have been housed under specific pathogen-free conditions. Because the liver is a major lymphatic organ (reviewed in ref. 32), the lack of immunostimulation may explain the low level of mVAP-1 expression. This latter possibility is also supported by the observation that mVAP-1 seems to be more faintly expressed on PLN HEVs of these animals than hVAP-1 is in human PLN (data not shown). Another tissue where the expression of mVAP-1 differs from that of hVAP-1 is kidney. In humans the peritubular small vessels (through which lymphocytes infiltrate into a rejecting kidney) display strong VAP-1 positivity, but in mouse these capillaries are negative. Although it is possible that the modifications of renal VAP-1 may also be different, it should be kept in mind that genuine species-specific differences can also lie behind the detected differences in the expression of mouse and human VAP-1 (Table 3).

The role of immunostimulation for mVAP-1 expression was analyzed by simultaneously studying BALB/c mice, NOD animals, and wild mice caught on local farms (data not shown). The stainings showed that mVAP-1 expression was the same in most organs and tissues studied (PLN, MLN, gut, fat, heart, and kidney). However, in the liver sinusoids of wild mice the intensity of mVAP-1 staining was brighter than in BALB/c and NOD mice, but even in the wild mice the expression was minimal when compared to humans. Interestingly, the vessels in the lamina propria displayed more mVAP-1 reactivity in the NOD animals, in which the gut-associated immune system dominates, than in the other mice. These results suggest that immunostimulation may cause up-regulation of mVAP-1 at other anatomical sites in addition to the pancreas.

The SDS-PAGE separation and immunoblotting analysis of mVAP-1 in different tissues revealed that the apparent molecular weight of mVAP-1 is higher than that of hVAP-1. This is in accordance with previous reports stating that the molecular weight of different SSAOs varies between species.³³ The glycosidase digestions of lysates from different mouse tissues revealed that mVAP-1 contains at least some sialic acids and N-linked oligosac-

charides. Digestions with the same glycosidases lead to different changes in mobility in mVAP-1 and hVAP-1 on an SDS-PAGE gel analysis. Treatment of hVAP-1 with sialidase paradoxically increases the apparent molecular weight of hVAP-1, indicating that hVAP-1 is decorated with abundant sialic acid residues affecting the net charge of the molecule or that the removal of them causes a differential conformational change leading to an altered mobility.⁹ *N*-Glycanase treatment leads to a detectable change in the mobility of only smooth muscle-derived hVAP-1 but not that of HEV-originating hVAP-1. Moreover, smooth muscle and endothelial hVAP-1 consistently display different mobilities in SDS-PAGE. Interestingly, the glycosylation of mVAP-1 seems to differ not only between hVAP-1 and mVAP-1, but also between various mouse tissues. This indicates, together with our previous findings of tissue-specific mVAP-1 mRNA transcription start sites,¹¹ that there may be heterogeneity in the structure and presumably in the function of mVAP-1 in different cell types.

The substrate specificity and affinities of different SSAOs have been reported to vary unpredictably between different species.^{34,35} For example, certain amines like tyramine and tryptamine are good substrates for rat but poor substrates for human SSAO. Previously both human and mouse VAP-1 have been shown to have activity against benzylamine, but the activity of mVAP-1 toward amines present *in vivo* has remained unknown. This study reveals that mVAP-1 is active against methylamine, tyramine, tryptamine, and β -phenylethylamine. Thus the substrate specificity of mVAP-1 is different from that of hVAP-1 because only methylamine of the amines tested here has been shown to be oxidized by hVAP-1⁶ (Table 3). Furthermore, hVAP-1 has higher affinity toward methylamine than does mVAP-1 (unpublished observations). This study reveals for the first time physiological substrates for a mouse SSAO because, eg, tyramine is ingested in food and methylamine is formed endogenously from metabolic pathways involved in the degradation of sarcosine, creatinine, and adrenaline.¹³ Based on our observations the substrate specificity of mVAP-1 resembles the specificity of rat SSAO more than it does the specificity of human SSAO (hVAP-1). However, regardless of the substrate specificity, the aldehydes produced in the reaction have been shown to be important in vascular injury seen in pancreas, kidney, and arteries during inflammatory disorders.^{16,17} Moreover, the oxidation reaction of these amines results in the production of H₂O₂, which is a potent modulator of the local microenvironment. Therefore, it will be of interest to study the effects of mVAP-1-dependent H₂O₂ production because it has been shown that this oxygen radical affects the expression of other adhesion molecules^{36,37} and leukocyte rolling *in vivo*.³⁸

A prominent VAP-1 staining can be found in both mouse and human white adipose tissue, indicating that adipocytes widely express VAP-1 (Figure 2 and data not shown). This is in accordance with a recent (partial) cloning report of a rat membrane-bound adipocyte amine oxidase, which probably represents the rat homologue of mouse and human VAP-1, and the identity of which to

mVAP-1 is 95%.^{10,39} The very prominent mVAP-1 staining in the adipose tissue is of interest because some leukocyte adhesion molecules have been suggested to participate in the regulation of adipose tissue mass. For instance, intercellular adhesion molecule 1-deficient mice spontaneously become obese, and Mac-1 (CD11b/CD18)-deficient mice are susceptible to diet-induced obesity, indicating that leukocyte adhesion and fat metabolism may be linked phenomena.⁴⁰ Based on a recent finding that benzylamine has effects on glucose transport of cultured adipocytes,⁴¹ VAP-1 may also affect glucose metabolism, because we have now shown that mVAP-1 can deaminate not only benzylamine but also naturally found amines. Moreover, while the manuscript was being revised, a mouse SSAO cDNA sequence from adipocytes was reported,⁴² which was 100% identical to the mVAP-1 sequence we had published earlier. SSAO activity was shown to be involved in body mass regulation in rats. Therefore, it will be interesting to examine whether the VAP-1-deficient mice gain weight normally or if they are susceptible to obesity.

mVAP-1 is expressed in endothelial, smooth muscle, and fat cells. In addition to the restricted expression pattern, another level of functional control exists. In experiments with dogs and pigs, a cross-reacting anti-hVAP-1 mAb has been given *iv* to animals with skin inflammations, and the localization of the *in vivo* bound mAb has been visualized from tissue sections (Jaakkola et al, manuscript in preparation). The experiments showed that luminal VAP-1 is only found at sites of inflammation but not in resting vessels, which nevertheless express abundant cytoplasmic VAP-1.

Mouse and human VAP-1's are 83% identical proteins that share many common features in expression, biochemical structure, and enzyme activity. Nevertheless, there are several distinct parameters (summarized in Table 3) that should be taken into account when comparing studies between animals and humans. The expression of mVAP-1 in primary and secondary lymphatic tissues and its inducibility at sites of inflammation (eg, in insulinitis) make it a potential adhesion molecule that mediates both physiological lymphocyte recirculation as well as extravasation into sites of inflammation. On the other hand, the analyses described here represent the first detailed cellular and molecular study of any member of the large, but so far not well understood, class of semicarbazide sensitive monoamine oxidase family. Moreover, the activity of mVAP-1 against naturally occurring amines provides new clues to the biological role of these enzymes.

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