In Vivo Detection of Vascular Adhesion Protein-1 in Experimental Inflammation

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Vascular adhesion protein-1 (VAP-1) is an inflammation-inducible endothelial glycoprotein which mediates leukocyte-endothelial cell interactions. To study the pathogenetic significance of VAP-1 in inflammatory disorders, an *in vivo* immunodetection method was used to detect the regulation of lumenally expressed VAP-1 in experimental skin and joint inflammation in the pig and dog. Moreover, VAP-1 was studied as a potential target to localize inflammation by radioimmunosintigraphy. Up-regulation of VAP-1 in experimental dermatitis and arthritis could be visualized by specifically targeted immunosintigraphy. Moreover, the translocation of VAP-1 to the functional position on the endothelial surface was only seen in inflamed tissues. These results suggest that VAP-1 is both an optimal candidate for anti-adhesive therapy and a potential target molecule for imaging inflammation. (Am J Pathol 2000, 157:463–471)

Leukocyte migration into tissues is vital for efficient defense against insulting pathogens and foreign antigens. Nevertheless, the same phenomenon is also crucial to inappropriate inflammation and tissue destruction in several types of acute and chronic inflammatory and autoimmune diseases such as rheumatoid arthritis, inflammatory bowel diseases, organ transplant rejection, and ischemia-reperfusion injury. Leukocytes enter from the

blood circulation into the tissues by passing through the walls of blood vessels. An essential step in this process is binding of leukocytes to the innermost layer of the blood vessel wall, the endothelium, by adhesion molecules. Multiple adhesion molecules on the leukocytes interact concertedly with their counter-receptors on the endothelium during the adhesion and the subsequent transmigration process.^{1,2} A change in the functional expression of adhesion molecules on the endothelial surface is an early and specific indicator of inflammation. In fact, recent studies suggest that radioactively labeled monoclonal antibodies against specific endothelial adhesion molecules can be used in the diagnosis of inflammation by nuclear imaging methods.^{3,4}

Human vascular adhesion protein-1 (VAP-1), originally defined by 1B2 monoclonal antibody, is a 170-kd endothelial sialoglycoprotein.⁵ VAP-1 is inflammation inducible and mediates the early phases of interaction between lymphocytes and endothelium.⁶ The expression pattern of VAP-1 in normal and inflamed human tissues has been described^{7,8} and the role of VAP-1 in human leukocyte adhesion has been shown *in vitro*.^{5,9} However, practically nothing is known about the translocation of VAP-1 from the inside of the cells to the functional position on the cell surface as well as the significance of VAP-1 in leukocyte-endothelium interactions *in vivo*.

The anti-human-VAP-1 mAb 1B2 does not recognize VAP-1 of small laboratory animals such as mouse, rat, or rabbit. However, preliminary screening experiments revealed that 1B2 antibody does recognize porcine and canine blood vessels. That encouraged us to study whether the antigens recognized by 1B2 are the porcine and canine homologues of human VAP-1 and to develop inflammation models in these animals. The ultimate goal of this work was, for the first time, to gather novel data about the regulation of VAP-1 *in vivo* for further therapeutic

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tic use. Also as a direct clinical application of VAP-1 induction in diseased tissue we investigated whether VAP-1 can be used as a target for immunoscintigraphic imaging of inflammation.

Materials and Methods

Antibodies

Mouse anti-human VAP-1 antibodies (1B2⁵, IgM; 2D10,¹⁰ IgG1; TK8–14⁸, IgG2a) and a mouse-human chimeric antibody, all against human VAP-1 were used to detect the porcine and canine forms of the antigen. The V-region domains of the chimeric anti-VAP-1 antibody were taken from TK 8–14 (Laukkanen et al, submitted). The control antibodies included 7C7, a mouse IgM monoclonal antibody that recognizes bursal epithelium of chicken; 3G6, a mouse IgG1 specific to chickens T cells;⁵ and 7E8, a mouse IgG1 against human TIE growth factor receptor.¹¹ For the imaging experiments a nonbinding human chimeric antibody was engineered for use as a negative control. The antigen-binding site of the control antibody consists of domains from two different antibodies. The variable region of heavy chain is taken from an antibody against the hapten nitrophenylacetyl whereas the variable region of light chain is from an antilysozyme antibody. The constant region used in both the experimental VAP-1-specific and the control chimeric antibodies was a slightly modified form of human IgG2.¹² In this constant region of the IgG2 residues A330 and P331 have been replaced by the residues S330 and S331 as found in human IgG4, and this has been shown to reduce the binding of the antibody to human Fc receptors and also to prevent human complement activation.¹² For the canine experiments the antibodies were purified from serum-free culture supernatants by precipitation using ammonium sulfate. The mouse antibodies for pig experiments were produced in bioreactors and purified chromatographically as described.¹³ The chimeric antibodies were purified from the cell culture supernatants by using protein-A affinity chromatography. A peroxidase-conjugated goat anti-mouse Ig (DAKO, Glostrup, Denmark) and tetramethylrhodamine B isothiocyanate (TRITC)-conjugated goat anti-mouse IgM (Zymed, San Francisco, CA) antibody were used in the detection of mouse antibodies in immunohistochemistry. A mouse IgG1 antibody against porcine CD31 (Serotec Ltd., Oslo, Norway) and fluorescein isothiocyanate-conjugated F(ab')₂ of sheep antibody against mouse IgG (Sigma Chemical Co., St. Louis, MO) were used to identify endothelial cells.

Radiolabeling of Antibodies

The chimeric anti-VAP-1 and control antibodies were labeled with I-123 and I-131, respectively, using the standard chloramine-T method. Briefly, an adequate amount of 123-I or 131-I in 100 to 150 μ l of 0.18 mol/L phosphate buffer at pH 7.5 and 100 μ g of antibody were mixed with 0.15 μ g chloramine-T. After 5 minutes, the radiolabeled

antibody was purified using PD-10 Sephadex G-25 size exclusion column (Pharmacia Biotech, Uppsala, Sweden) with 2% albumin/0.9% sodium chloride mobile phase. The purity of the radiolabeled immunoconjugate was determined by instant thin layer chromatography with 20% trichloro acetic acid as a solvent.

The biological activity of the labeled anti-VAP-1 antibody was studied from each labeling lot by an *in vitro* binding assay¹⁴ using VAP-1 and control-transfected Ax cells. The labeling method was adjusted to always leave the anti-VAP-1 antibody with a biological activity of >60%.

Immunohistochemistry

The tissue samples were immediately immersed in RPMI 1640 solution (Gibco BRL, Life Technologies Ltd., Paisley, UK), refrigerated, embedded in OCT compound (Tissue-Tec; Miles Inc., Elkhart, IN), frozen in liquid nitrogen, and, finally, serial sections were cut. The acetone-fixed sections were stained using immunohistochemical methods as described earlier.⁵ Briefly, sections were sequentially incubated for 40 minutes with 100 μ l of primary mAb and appropriate peroxidase-conjugated secondary antibodies. The specimens from the animals that had received mouse antibodies intravenously were incubated with the peroxidase-conjugated anti-mouse Ig antibody. The immunoreaction was visualized using 3,3'-diaminobenzidine (Polysciences, Inc., Warrington, PA) as a chromogen. Finally, the sections were lightly counterstained with hematoxylin and mounted in DePeX (BDH, Poole, UK) for permanent records.

To allow better discrimination of the endothelium and other components of the vascular wall, a confocal microscope (Leica TSC 4D confocal system connected to Leica RXA microscope; Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) was used. The specimens from the pigs that had received 1B2 or 7C7 intravenously were prepared in the following manner: 20- μ m frozen sections were cut, air dried, fixed in -20°C acetone, and stored at -40°C . Sections were sequentially incubated with anti-CD31 and fluorescein isothiocyanate-labeled anti-mouse IgG or TRITC-labeled anti-mouse IgM secondary antibodies in phosphate-buffered saline containing 2% normal porcine serum. After the washes, the coverglasses were mounted with Fluoromount (Southern Biotechnology Associates, Inc., Birmingham, AL).

Immunoblotting

The samples from canine, porcine, and human peripheral lymph nodes, wall of gut (representing smooth muscle), and human tonsil tissues were lysed in 10 mmol/L Tris, pH 7.0, containing 0.15 mmol/L MgCl_2 , 5 mmol/L ethylenediaminetetraacetic acid, 2% Nonidet P-40, 1% Aprotinin (Sigma Chemical Co., St. Louis, MO), 1 mmol/L phenylmethylsulfonyl fluoride (Sigma), and 5 mmol/L NaN_3 as described elsewhere.¹⁰ The centrifuged lysate was diluted in Laemmli sample buffer, and a sodium dodecyl sulfate-polyacrylamide gel electrophoresis was

Figure 1. Experimental design. In the dog experiments, expression of VAP-1 in healthy canine tissues and in experimental inflammation were studied in samples from six beagles. Two of these dogs were healthy and served as donors of tissue samples for conventional immunohistochemical studies of the baseline expression of VAP-1. To study the kinetics of VAP-1 up- and down-regulation on the cell surface of vascular walls, skin lesions of different ages were studied. The inflammation was induced to four beagles according to the protocol I. Two of these animals received 2 mg/kg (total of 24 mg) of 1B2 whereas an equal amount of a negative control antibody 7E8 was administered to the remaining two dogs. There were 17 pigs in the pig experiment study. Three different experimental protocols (II, III, and IV) were used. In the first pig group (II) two animals received 2 mg/kg of 1B2, two pigs received 2 mg/kg control antibody 7C7 in 10 to 20 ml saline in a 10-minute bolus, and two pigs received saline only. To ensure complete distribution of the administered antibody to all tissues, the antibody was left to circulate 6 hours before the tissue sampling. The other six pigs (experimental protocol III) received 1B2 or 7C7 antibodies (3 + 3) 2 mg/kg (1.9 mg/ml in saline) in 10 ml of intravenous infusion twice during the experiment. Five pigs participated in the imaging study (experimental protocol IV). The skin inflammation was induced to all pigs 8 hours and 1 hour before the administration of the radiolabeled antibodies whereas the arthritis was induced to four pigs 8 hours before the antibody administration. To three pigs both the I-123-labeled chimeric anti-VAP-1 and the I-131-labeled chimeric control antibodies were given simultaneously to compare the accumulation of specific and unspecific antibodies in exactly equal settings. To two pigs only I-123-labeled chimeric anti-VAP-1 antibody was given. Abbreviations: I, induction of skin inflammation; J, induction of joint inflammation; M, administration of the mouse monoclonal antibodies; H, administration of the mouse-human chimeric antibodies; S, tissue sampling; IMG, imaging. The pigs simultaneously participated in studies in which lung inflammation (^A) or myocardial ischemia (^B) were induced.

run in a standard way. The proteins were transferred from the gel to a Hybond nitrocellulose membrane (Amersham, Buckinghamshire, UK) with Hoefer Transphor Electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). The membranes containing canine tissues were probed 1 hour with 1B2 or 7C7 antibodies (2 µg/ml). The bound primary antibodies were detected with the Vistra enhanced chemifluorescence Western blotting system (Amersham). FluorImager 595 (Molecular Dynamics, Sunnyvale, CA) was used to visualize the chemifluorescence. The membranes on which porcine VAP-1 was transferred were probed with TK8-14 and 3G6 antibodies, and the enhanced chemiluminescence (Amersham) system was used to visualize the human and porcine forms of VAP-1.

Inflammation Models

The skin inflammation was induced with 4 drops of 5% (w/v) dinitrochlorobenzene (1-chloro-2,4-dinitrobenzene, DNCB; Sigma) in acetone applied to 3 × 3-cm skin area. Control areas were painted with acetone only.

Figure 2. Molecular masses of canine and porcine VAP-1 are close to that of the human. Shown are immunoblots with 1B2 anti-VAP-1 and 7C7 (control) antibodies on lysates from human and canine tissues (lanes 1–8) and with TK8-14 anti-VAP-1 and 3G6 (control) antibodies on human and canine tissues (lanes 9–14). A difference in molecular weight of VAP-1 between human tonsil (lane 1) and gut (lane 2) is visible. A smaller but obvious difference can be seen also between gut and lymph node forms of canine VAP-1 (lanes 3 and 4). In an other gel, in which human and porcine VAP-1 were compared, VAP-1 from human tonsil tissue (lane 9) is slightly heavier than VAP-1 from porcine gut and lymph node (lanes 10 and 11, respectively). The control membranes shown (lanes 5–8 and lanes 12–14) are identical duplicates from the preceding lanes and were run in the same gels. (lanes 1, 5, 9, and 12, human tonsil; lanes 2 and 6, human gut; lanes 3 and 7, canine gut; lanes 4 and 8, canine lymph node; lanes 10 and 13, porcine gut; and lanes 11 and 14, porcine lymph node.)

Table 1. VAP-1 Expression in Tissues of Dog and Pig Detected with Immunohistochemistry on Frozen Sections

Organ/tissue	VAP-1 expression		
	Dog	Pig	Man*
Heart			
Muscle cells	–	–	–
Endocardium	+/–	+	+
Epicardium	–	–	–
Blood vessels	+	+	+
Lung			
Airway epithelium	–	–	–
Blood vessels	+	+	+
Skeletal muscle			
Muscle cells	–	–	–
Blood vessels	+	+	+
Liver			
Hepatocytes	–	–	–
Sinusoidal lining	+/–	+	+
Blood vessels	+	+	+
Intestine			
Epithelium	–	–	–
Smooth muscle	+	+	+
Blood vessels	+	+	+
Skin			
Epithelium	–	–	–
Blood vessels	+	+	+
Lymph node			
Lymphocytes	–	–	–
Blood vessels	+	+	+

–, Negative; +/–, weakly positive; +, clearly positive.
 *The human expression from Salmi et al 1993.⁷

Aseptic arthritis was induced by intra-articular injection of 400 μ g of phytohemagglutinin (Sigma) in 1 ml of RPMI 1640 8 hours before the administration of the radiolabeled antibody. Only RPMI 1640 was injected to the contralateral knee.

The design of the experimental inflammation studies and the respective antibody treatment protocols are explained in detail in Figure 1. All animal experiments were approved by the Committee of Animal Care in Research of the University of Turku.

Pharmacokinetic Analyses

From the pigs (receiving unlabeled antibodies) three blood samples were collected in the beginning, at 60 minutes, and at the end of the experiments to reveal the lowest antibody concentration during the experiments. To determine the pharmacokinetics of the monoclonal antibodies more precisely, three additional pigs received antibodies at 3 mg/kg (two 1B2 pigs, one 7C7 pig, experimental protocol III in Figure 1) and blood samples were collected just before injection of antibody at 2, 10, 20, 40, 60, 90, 120, 180, and 240 minutes after the injection, and at the end of the experiment. The serum was separated and the samples were stored at -40°C until analyzed. Concentrations of the mouse antibodies were measured by time-resolved immunofluorometric assay using microtitration wells coated with rabbit anti-mouse immunoglobulin. The purified 1B2 and 7C7 mAb produced earlier and the sera taken before antibody administrations were used as standards. Europium-labeled sheep anti-mouse Ig (Boehringer-Mannheim, Mannheim, Germany) and enhancement solution (Wallac, Turku, Finland) were used for detection. The intensity of fluorescence was measured by Fluorometer (Wallac). The results were converted to $\mu\text{g/ml}$ using the known standards. The pharmacokinetic modeling of the antibody concentration data were done using two-compartment disposition model.¹⁵

Imaging

During the induction of the joint inflammation and imaging the animals were kept under light anesthesia with ketamine hydrochloride (Ketalar, 50 mg/ml; Parke-Davis,

Figure 4. Pharmacokinetics of the anti-VAP-1 antibody. The antibody elimination curve from an 1B2-treated animal is shown as an example.

Solna, Sweden) and diazepam (Stesolid Novum, 5 mg/ml; Dumex/Kabi Pharmacia, Sweden).

The single- and dual-isotope scintigraphies were performed with Siemens Diacam (Siemens Gammasonics Inc., Des Plaines, IL) γ -camera. Immediately after the intravenous administration of the labeled chimeric antibodies dynamic imaging was started (3 hour imaging using 6×10 minutes and 6×20 minutes frames) and a 30-minute static image was acquired 24 hours later. In the dual-isotope imaging the energy windows were set as 159 keV (window width, 20%) for I-123 and 364 keV (15% window) for I-131. A third energy window for images used in correction of the crosstalk and scatter artifact in dual-isotope experiments was set as 210 keV (20% window). The locations of the skin inflammations were marked in each series of the images with a radiation source positioned outside the animals. At the end of the last scan, the animals were humanely sacrificed with i.v. administrations of pentobarbital (Mebunat, 60 mg/ml; Orion-Farmos, Turku, Finland) and potassium chloride. To determine the kinetics of the radiolabeled antibody accumulation, the regions of interest were drawn on the areas of skin and joint inflammation and on corresponding healthy control areas on the contralateral side. The 3-hour dynamic image sets and the second day static images were visually analyzed for maximal tracer uptake.

Figure 3. Blood vessels of inflamed skin, but not normal skin, bind the *in vivo* circulating anti-VAP-1 antibody. Because only the cells that have VAP-1 on their surface can bind circulating antibody, our result shows that surface expression of VAP-1 is limited to inflamed areas. The binding seems to be VAP-1-specific because the control antibody is not bound. Shown are inflamed (8-hours-old inflammation; **a** and **b**, **e** and **f**) and noninflamed (**c** and **d**) skin specimens from 1B2- (**a-d**) and 7C7- (**e** and **f**) treated pigs. Sections shown in **a**, **c**, and **e** have been stained with anti-mouse second stage antibody only while the first stage antibody against VAP-1 or the appropriate control antibody were administered intravenously. In the corresponding serial sections (**b**, **d**, and **f**), both primary anti-VAP-1 mAb and the second stage antibody were sequentially used. In these sections the intracellular VAP-1 was also able to bind the anti-VAP-1 primary antibody. **a**: An intense accumulation of circulating anti-VAP-1 (1B2) antibody to blood vessels of inflamed skin (**arrows**) of a 1B2-treated animal is detected. **b**: Shown is the serial section that has been immunostained with anti-VAP-1 antibody (several blood vessels indicated with **arrows**). **c**: From an uninfamed area of the same pig as in **a** and **b** no mouse antibody is seen with peroxidase labeled anti-mouse second stage antibody while in the serial section (**d**) the blood vessels carry intracellular VAP-1 as detected after staining the section with anti-VAP-1 antibody (**arrows**). **e**: An inflamed skin is shown from a 7C7-treated pig where no positive blood vessels are visible in the section treated with anti-mouse antibody. Again, the blood vessels of the serial section (**f**) contain intracellular VAP-1 (**arrows**). The same magnification in **a-f**; bar shown in (**a**) = 50 μm . In (**g**) and (**h**) two confocal microscopic images from blood vessels of inflamed skin of an 1B2-treated pig are shown. Red = TRITC-labeled anti-mouse IgM antibody visualizing bound anti-VAP-1 antibody; green = anti-CD31 + fluorescein isothiocyanate-labeled anti-mouse IgG. **g**: A blood vessel is shown in which anti-VAP-1 and anti-CD31 reactivities co-localize (visualized as yellow color; **arrowhead**). **h**: The partially abluminal localization of anti-VAP-1 antibody (two cells indicated with **arrowheads**) suggests that VAP-1 is also detected in smooth muscle/pericyte layer of the blood vessel wall. Original optical magnification, $\times 63$ (**g** and **h**).

Figure 5. Inflammation is visualized with I-123-labeled chimeric anti-VAP-1 antibody. Anti-VAP-1 images obtained at 2 and 24 hours after intravenous injection of labeled anti-VAP-1 antibody. At 2 hours (**a**) the inflamed skin area (**arrowhead**) is already detectable. In the 24-hour image (**b**), the inflamed joint shows clear accumulation of the radioactivity (**arrow**) whereas the contralateral noninflamed knee does not. The areas of skin inflammation are pointed out with **arrowheads**.

Figure 6. In the imaging experiments, more anti-VAP-1 antibody than control antibody accumulated to sites of inflammation. Three animals received both chimeric I-123-labeled anti-VAP-1 and I-131 control antibodies. From each animal two to four inflamed skin samples were paired with normal skin samples and one inflamed synovial sample was paired with normal synovial sample for analysis of I-123-labeled anti-VAP-1- and I-131-labeled control antibody accumulation. The data points connected with the **solid line** represent I-123 and I-131 results of the paired skin samples and the **dashed lines** indicate the paired synovial samples. **▲**, Ratio of I-123-labeled anti-VAP-1 antibody per gram inflamed tissue *versus* the same antibody per gram comparable to noninflamed tissue of the same animal; **▼**, ratio of I-131-labeled control antibody per gram inflamed tissue *versus* the same antibody per gram comparable to noninflamed tissue of the same animal.

Tissue Distribution of Radiolabeled Antibodies

The tissues of the animals that had received radioactively labeled antibodies were biopsied after the scintigraphies for detailed analysis of radioactivity. Biopsies were obtained from the normal and inflamed skin areas as well as from synovial membranes of inflamed and uninflamed knees. The skin samples were obtained as a whole skin thickness, whereas duplicate samples were taken from the most superficial part of the skin containing only epidermis and part of the dermis. The samples were weighed and the radioactivities of different iodine isotopes were determined with a multichannel analyzer and germanium crystal detector (Harshaw, Bricron, Washougal, WA) capable of recognizing isotopes by their characteristic γ radiation spectra. The results were converted to percentages of the injected dose per gram of tissue and compared using Wilcoxon signed-rank test.

Results

Expression of VAP-1 in Dog and Pig

To analyze the usefulness of dog and pig as animal models in which to study the characteristics of VAP-1 *in vivo* we first determined immunohistochemically the expression of VAP-1 in canine and porcine tissues using 1B2 monoclonal antibody. The main results are summarized in Table 1. The expression of VAP-1 in dog and pig closely resembles the published results for VAP-1 expression in humans.⁷ A baseline expression of VAP-1 was seen in a subpopulation of small blood vessels in all

of the tissues studied. Moreover, smooth muscle cells of larger vessels are VAP-1-positive although the endothelium of those blood vessels remains VAP-1-negative. Also other smooth muscle cells as in the wall of the gastrointestinal tract and in the myometrium are also VAP-1-positive. 1B2 also stains other cell types that may be considered related to smooth muscle cells like pericytes of blood vessels and myoid cells of testis. Heart and skeletal muscle cells, however, are negative for VAP-1.

Molecular Characteristics of Canine and Porcine VAP-1

To study the molecular nature of the canine and porcine VAP-1 more precisely, the molecular weight of VAP-1 of dog and pig were studied by immunoblotting and compared with that of humans. The molecular weights of dog and pig VAP-1 are 10 to 20 kd smaller (Figure 2) when compared with humans.

Induction of VAP-1 in Skin Inflammation

The DNCB dosage that caused acute dermatitis categorized by observation of erythema and edema but no bullae was used in all experiments. After intravenous antibody administration, only the blood vessels in the specimens from the inflamed skin of the 1B2-treated animals bound the fluorescently- or peroxidase-labeled secondary anti-mouse antibodies. The noninflamed skin and all other tissues of the same animals remained completely negative. Also, no staining was seen in the inflamed skin or in the other tissue specimens from the animals treated with the control antibodies. These results indicate that 1B2 antibody on the vessel wall was a result of specific binding of the antibody in the areas of VAP-1 up-regulation and in noninflamed areas VAP-1 remained completely intracellular and thus, nonaccessible for the circulating antibody (Figure 3, a–f). Confocal microscopic studies showed that in some vessels the anti-VAP-1 and anti-CD31 reactivities colocalized, whereas in some other vessels the CD31 expression was more luminal than that of VAP-1 suggesting that in addition to endothelial VAP-1, the VAP-1-positive pericytes/smooth muscle cells also contributed to the positive immunoreaction seen in the blood vessels (Figure 3, g and h). The skin inflammation model was also used to study the regulation of VAP-1 during inflammation (experimental protocol I, Figure 1). The translocation of VAP-1 to the cell surface was already seen in the skin samples 60 minutes after the topical application of DNCB, the maximum positivity was in the samples taken 8 hours after the induction, and decreasing intensity of immunoreaction was observed at 24 and 48 hours of inflammation.

Pharmacokinetics of Antibodies in Pigs

The pharmacokinetics of 1B2 and 7C7 antibodies were studied in pigs. Complete sets of data allowing pharmacokinetic modeling were obtained from three animals,

two of which had received 1B2 and one 7C7 antibodies. The elimination curve of both antibodies was biphasic. A pharmacokinetic curve after administration of 1B2 antibody is given in Figure 4. The half-lives of the initial rapid distribution phase were 11, 26, and 8 minutes in the animals which had received 1B2, 1B2, and 7C7, respectively. The corresponding half-lives of the elimination phase were 240, 431, and 278 minutes. The calculated apparent distribution volumes were 0.035 and 0.045 l/kg for 1B2 and 0.121 l/kg for the 7C7 antibody.

Imaging and Distribution of Radiolabeled Antibodies

Administration of both the chimeric anti-VAP-1 and control antibodies resulted in enhanced tracer uptakes at the sites of inflammation when compared with background activity. Based on the immunostaining experiments described above (Figure 3), the accumulation of the control antibody is considered to be an indicator of increased blood flow as well as the endothelial leakage. The scintigraphies with simultaneous administrations of chimeric anti-VAP-1 and control antibodies and dual-label imaging protocol showed that specific accumulation of the anti-VAP-1 antibody occurred at the areas of skin and joint inflammation as well as in the liver. When the dynamic images were analyzed, the accumulation of anti-VAP-1 antibody had a cumulative trend at the inflamed areas whereas at the control areas the radioactivity followed the radioactivity of blood (measured by drawing regions of interest on the heart) in decreasing manner (data not shown). As seen also in Figure 5, in the 24-hour images the contrast between inflamed and noninflamed tissues is better than in the first day images. Analysis of the radioactivity in the skin and synovial samples, obtained immediately after 24-hour imaging, showed that the accumulation of anti-VAP-1 antibody to sites of inflammation was 10.38 ± 1.32 (mean $\pm$ SEM) times greater than to comparable noninflamed control tissues. With the control antibody the same ratio was 6.55 ± 1.06 , respectively (Figure 6). There is a statistically significant difference between the accumulation of anti-VAP-1 and control antibodies at sites of inflammation (Wilcoxon signed-rank test, $P = 0.0019$). In two additional animals that received only chimeric anti-VAP-1 antibody, the accumulation to inflamed sites was 9.8 ± 3.1 times greater than to noninflamed control areas.

Discussion

An ideal target for anti-adhesive therapy or imaging inflammation would be an adhesion molecule that is absent from the endothelium of normal tissues but is induced at the onset of inflammation. Our data show that VAP-1 in the pig and dog well fulfills these requirements. Moreover, we were able for the very first time to draft a time sequence for the induction of luminal VAP-1 expression in inflammation. The early translocation of VAP-1 onto the cell surface within an hour after the stimulus suggests

that the function of VAP-1 may be connected to the early recruitment of polymorphonuclear leukocytes, later also lymphocytes to the area of inflammation. However, prolonged expression of VAP-1 on the cell surfaces suggests that successive anti-adhesive therapy could be targeted against VAP-1 still after the very first phase of the inflammatory disorder.

This study was undertaken as part of an effort to find a suitable animal model for anti-adhesive drug development. Our working hypothesis was that the molecular characteristics and regulation of VAP-1 in man, dog, and pig would be comparable and these animal species could be used in such studies. The molecular weight of human VAP-1 is distinct in different organs and even in one organ under pathological conditions.²⁵ The changes are believed to be because of alterations in carbohydrate structures of the molecules that are essential for the adhesive function of VAP-1.⁹ Indeed, in canine and porcine tissues the molecular weight close to that of human VAP-1 and the expression pattern of the antigen detected in immunohistochemistry in both species together suggest that the molecules recognized by mAb 1B2 in canine and porcine tissues really are the homologues of human VAP-1. VAP-1 has been found to be up-regulated in areas of inflammation when compared to the baseline expression.^{7,16,17} Importantly, in this present study, *in vivo* immunodetection experiments and immunohistochemical stainings showed that expression of VAP-1 on the luminal surface of endothelium is limited to areas of inflammation. These observations are consistent with the potential role of VAP-1 as a therapeutic target in inflammatory disorders and hold promise for relevance of our animal models for drug development purposes.

After translocation onto the cell surface, endothelial adhesion molecules may become shed or taken into various intracellular compartments.^{18–22} Our previous observations and current *in vivo* immunodetection experiments suggest that a significant proportion of up-regulated VAP-1 and anti-VAP-1 antibody complexes remain attached to the cell surface or become internalized.^{7,23} This observation forms the fundamental basis of using VAP-1 to target therapeutic compounds into inflamed tissues. In addition, we have shown here that VAP-1 can be used as a target in imaging inflammation. Radioactively labeled polyclonal (nonspecific) antibody can be used for that purpose because of the leakage of plasma proteins to extracellular space at sites of inflammation. Our results with the chimeric control antibody are consistent with that. However, when the ratios of the radioactivities in inflamed skin and synovium and healthy control specimens were studied, the amount of chimeric anti-VAP-1 antibody accumulation was approximately twofold higher when compared with the accumulation of the non-specific antibody. The methodological strengths of our imaging study are that we were able to use a chimeric frame-matched control antibody in a dual-isotope protocol in which specific and nonspecific accumulation of the antibodies could be detected simultaneously. The dual-isotope scintigraphy is also a sophisticated way to overcome the biological variation between individual experiments. With these methods we were able to identify

correctly the antigen-specific binding and nonspecific accumulation of the antibodies that otherwise inevitably complicates the interpretation of the results. Based on these results, anti-VAP-1 antibody is a more sensitive tool for imaging inflammation than a nonspecific antibody alone. Because the specific accumulation of anti-VAP-1 antibody may also happen in mild inflammation in which the mechanisms behind the nonspecific enhancement of the inflamed tissue may be less significant, we believe that using VAP-1 as a target in immunoscintigraphy deserves further studies.

Our present results show that circulating anti-VAP-1 antibody binds selectively to blood vessels in diseased tissue. However, besides its endothelial cell expression, VAP-1 is also present on the smooth muscle cells and pericytes of the vascular wall. The smooth muscle VAP-1 differs from endothelial VAP-1 in its carbohydrate modifications and it does not have adhesive function.¹⁰ Because the endothelium is permeable to the plasma proteins to a certain extent even in a noninflamed stage,²⁴ the smooth muscle cells/pericytes of the vascular wall should bind anti-VAP-1 antibody if VAP-1 was on the cell surface. The fact that despite 6 hours of *in vivo* treatment with the 1B2 antibody the smooth muscle cells remain unstained in noninflamed tissues, suggests that the smooth muscle cells of the wall of blood vessels do not express VAP-1 on their surface in noninflamed conditions but, as in the endothelium, seem to relocate VAP-1 onto the cell surface in inflammation. This is especially beneficial for tissue targeting, because the blood vessels are capable of binding more diagnostic or therapeutic compounds to the inflamed tissue than in the case when only endothelial cells express the antigen.

In conclusion, our data show that VAP-1 in the pig and dog is an intracellular molecule in the noninflamed state and it is translocated onto the cell surface only after a proinflammatory stimulus. Therefore, VAP-1 well fills the requirements for a potentially effective target for anti-adhesive and diagnostic purposes. The results further indicate that the dog and pig can be used as experimental animals when biology of VAP-1 is studied.

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