Induction of Vascular Adhesion Protein-1 during Liver Allograft Rejection and Concomitant Cytomegalovirus Infection in Rats

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Vascular adhesion protein-1 (VAP-1) is an adhesion molecule controlling lymphocyte recirculation through high endothelial venules of the lymph nodes. It has also been shown to be induced and to mediate lymphocyte adhesion at sites of inflammation. We studied the expression of VAP-1 and two other inducible adhesion molecules ICAM-1 and VCAM-1 in our experimental model of rat liver allograft rejection and, in addition, the effect of concomitant rat cytomegalovirus (RCMV) infection on this expression. Expression of VAP-1, ICAM-1, and VCAM-1 was studied in rat liver allografts with or without RCMV infection, isografts, and normal rat liver. Immunoperoxidase technique and monoclonal antibodies including a novel anti-VAP-1 reagent were used. VAP-1 expression was induced by acute rejection in sinusoids, hepatocytes, and also in bile ducts, when compared to the isografts or normal liver, where only blood vessels were consistently positive. Sinusoidal and hepatocyte expression of VAP-1 was prolonged by the presence of RCMV. ICAM-1 and VCAM-1 expression was also induced by acute rejection. However, RCMV increased sinusoidal VCAM-1 expression compared to uninfected grafts. The present experimental study shows that VAP-1 is up-regulated in acute rejection of liver allografts, and that this up-regulation is prolonged by RCMV infection. (Am J Pathol 2000, 157:1229–1237)

The hallmark of rejection is the influx of inflammatory cells, mainly lymphocytes and monocytes/macrophages into the graft. This process involves sequential adhesive interactions between the leukocyte and the endothelium. Initial physiological rolling is followed by reversible and then firm adhesion when endothelial activation by inflammatory cytokines and leukocyte activation by the interaction with activated endothelium and/or chemokines take

place. The complex process of adhesion and diapedesis of leukocytes into the tissue sites of inflammation is coordinated by several adhesion molecules.¹ A distinct array of adhesion molecules expressed in endothelia in a particular tissue or during a certain type of inflammatory response enables great selectivity of the traffic of inflammatory cells.¹ Adhesion molecules also play a role in many other cell-cell interactions such as antigen presentation and T-cell activation. In rejection of the liver, endothelia are induced to express adhesion molecules such as ICAM-1, VCAM-1, and E-selectin, but in addition to endothelia, the hepatocytes and bile duct cells may express ICAM-1.^{2,3} Cytomegalovirus (CMV) infection has been shown to be associated with acute and chronic rejection of the liver and also to affect the expression of adhesion molecules.^{3–7}

Vascular adhesion protein-1 (VAP-1) is a dimeric endothelial transmembrane protein that has been demonstrated to mediate lymphocyte binding to peripheral lymph node high endothelial venules and also to be induced at sites of inflammation.^{8,9} VAP-1 may play a significant role in controlling entry of lymphocytes into sites of inflammation.⁹ VAP-1 has been demonstrated to be up-regulated by proinflammatory cytokines in the tonsillar organ culture system.¹⁰ Serum levels of the soluble form of VAP-1 have been shown to be elevated in certain inflammatory liver diseases.¹¹

We have previously characterized the rejection response in a rat liver transplantation model with a donor-recipient strain combination of PVG(RT1^c) into BN(RT1ⁿ).¹² We wanted to study whether VAP-1 expression would be induced in acute liver allograft rejection. We have also previously shown that rat CMV (RCMV) increases inflammation, bile duct destruction, and sinusoidal VCAM-1 expression in rat liver allografts with concomitant rejection.¹³ Therefore we were also interested whether CMV infection would have an effect on the expression of VAP-1 in concomitant acute rejection.

Supported by grants from The Finnish Academy, Helsinki University 350th Anniversary Foundation, The Sigrid Juselius Foundation, and the Helsinki University Hospital Research Funds (EVO).

Accepted for publication July 13, 2000.

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Materials and Methods

Rats

A donor-recipient combination of PVG(RT1^c) into BN (RT1ⁿ) with a previously observed mean survival time of 37 days was used in liver grafting.¹² BN to BN syngeneic transplantations were performed for syngeneic controls. The rats were fed with regular rat food and tap water *ad libitum*. The animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health). The study was approved by the committee for experimental research of the Helsinki University Central Hospital and the regional authorities.

Transplantation

Liver transplantation under ether anesthesia was performed using the technique introduced by Kamada and Calne,¹⁴ supplemented with reconstruction of the hepatic artery. Cold (4°C) heparinized 0.9% saline was used for perfusion and preservation of the graft. On day 1 after transplantation the animals in the infection group were infected with rat CMV (see below). No immunosuppressive drugs were given to any of the animals. Grafts were harvested at 1 week (12 in the CMV group and five in the uninfected group) and at 4 weeks (seven in the CMV group and seven in the uninfected group) to be able to observe both the peak of inflammation and the more prolonged phase of acute rejection. In addition, a liver from normal PVG and BN rats and three syngeneic grafts were harvested at 1 week and three at 4 weeks after transplantation. A piece of fresh liver tissue was embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen, and stored at -70°C.

RCMV Infection

The rats were infected by inoculation with 10⁵ pfu of RCMV (Maastricht strain) intraperitoneally 1 day after liver transplantation. The procedure for culturing and inoculating the RCMV and the characteristics of RCMV and the RCMV infection have been described in detail previously.¹⁵ Briefly, the virus was passaged in rats, and harvested 4 to 6 weeks after inoculation by homogenization of the salivary glands which are the organ containing high titers of the virus. Quantification of the rat virus was done by plaque assay, as described previously by Bruggeman et al.¹⁶ The infectious virus was stored at -70°C.

Demonstration of CMV Infection

Viral Culture

The presence of RCMV infection in the graft was demonstrated by culturing the virus from material obtained from the graft by fine-needle biopsies. The fine-needle sample was aspirated from the graft into RPMI 1640

culture medium containing albumin. A fentanyl-fluanisone anesthesia was used for the fine-needle sampling. The virus was cultured in rat embryo fibroblasts under standard virus culture conditions. After the appearance of cytopathic effect, the RCMV infection of the cells was confirmed by immunofluorescence technique as described below for frozen tissue sections.

Detection of RCMV Antigens in the Liver

The presence of RCMV-specific antigens in the liver grafts was demonstrated by immunofluorescence in frozen tissue sections. Monoclonal antibodies (mAbs) against RCMV early and late antigens and a goat anti-mouse antibody conjugated to fluorescein isothiocyanate (Eappel/ICN Pharmaceuticals, Aurora, OH) were used. The specificity of these RCMV mAbs has been described previously in detail.¹⁷

Antibodies against VAP-1

New VAP-1-specific mAbs were produced by immunizing mice with affinity-purified VAP-1. To that end stromal elements of tonsils (containing VAP-1-positive blood vessels) were lysed in a buffer containing 10 mmol/L Tris-base (pH 7.4), 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, and 1% Nonidet P-40. After centrifugation the lysate supernatant was precleared by passing it through Sepharose CL4B beads. Thereafter VAP-1 was immunoaffinity purified by passing the precleared lysate sequentially through three columns packed with 1 ml of CNBr-activated Sepharose 4B beads that had been coupled with bovine serum albumin, normal rat serum, and anti-VAP-1 mAb JG2.10.¹⁸ After extensive washing with the lysis buffer, the JG2.10 beads were washed with 10 ml of ice-cold water. Then the beads were mixed with incomplete Freund's adjuvant and used to immunize specific pathogen-free BALB/c mice. After three subcutaneous injections into footpads at 1-week intervals, the mice were sacrificed. Lymphocytes isolated from popliteal lymph nodes were then fused with nonsecreting SP2/0 myeloma cells using standard procedures. The resulting hybridomas were screened for VAP-1 positivity using VAP-1 transfectants and immunofluorescence stainings (see below), and positive hybridomas were subcloned twice by limiting dilution. The selected mAbs were grown in serum-free medium (HB101) and the mAbs were purified by ammonium sulfate precipitation. After dialysis against phosphate-buffered saline (PBS), the protein concentration was determined with a bicinchoninic acid assay (Pierce, Rockford, IL).

Immunofluorescence Stainings and Flow Cytometry

Chinese hamster ovary cells transfected with the full-length VAP-1 cDNA in an eukaryotic expression vector pcDNA3 or with the vector alone have been described.¹⁹ The stable transfectants were detached with trypsin-eth-

ylenediaminetetraacetic acid and washed in PBS containing 2% fetal calf serum and 0.01% sodium azide. Thereafter, the cells were reacted with a negative control mAb 3G6, a positive control anti-VAP-1 mAb 1B2,⁸ or with the new mAbs from the hybridomas (10 μ g/ml of purified mAbs or 100 μ l of conditioned culture medium). As the second stage, reagent fluorescein isothiocyanate-conjugated sheep anti-mouse Ig supplemented with 5% normal AB-serum was used. Finally the cells were fixed in PBS containing 1% formaldehyde.

The stained cells were analyzed with a FACScan flow cytometer (Becton-Dickinson, Palo Alto, CA). Routinely, data from 10,000 cells were collected and analyzed using CellQuest software.

Immunohistochemistry

Expression of VAP-1, ICAM-1, and VCAM-1 was studied in frozen sections from the liver grafts. Tissue sections of 5- μ m thickness were cut with a cryostat. A three-layer indirect immunoperoxidase technique was used.

To study the expression of VAP-1, the mAb TK 8-110 (see above) reacting against rat VAP-1 was used in a final dilution of 1 mg/ml. As a negative control, an isotype-specific irrelevant antibody (mouse anti-human CD 44, Hermes-3, Ig2a) was used in parallel.²⁰

For studying ICAM-1 expression, monoclonal mouse antibody (BSA 1; R&D Systems Europe, Abingdon, UK) was used. The antibody against rat VCAM-1 was a generous gift from Dr. R. Lobb, Biogen Inc., Cambridge, MA.

The frozen liver-tissue sections were first incubated with the monoclonal mouse antibody, then with peroxidase-conjugated rabbit anti-mouse antibody (DAKO, Copenhagen, Denmark), and thereafter treated with a peroxidase-conjugated goat anti-rabbit antibody (Zymed Laboratories, San Francisco, CA). The reaction was revealed by a 3-amino-9-ethyl carbazole solution containing hydrogen peroxide. Mayer's hemalum was used as a counterstain.

The intensity of expression of VAP-1, ICAM-1, and VCAM-1 in veins and arteries in the portal fields, bile ducts, hepatocytes, and sinusoidal endothelium were blindly scored from – to +++ (– corresponds to no staining, + to weak staining, ++ to moderate staining, and +++ to intense staining of the cell) (see Figure 2).

To identify arterioles and bile ductules in the portal areas, sequential sections were stained with a polyclonal rabbit antibody against von Willebrand factor/FVIII (DAKO) and a monoclonal mouse antibody against cytokeratin (DAKO), respectively. The secondary and tertiary peroxidase-conjugated antibodies were as above for the cytokeratin staining. For the FVIII immunostaining, the secondary and tertiary antibodies were peroxidase-conjugated goat anti-rabbit (Zymed Laboratories) and swine anti-goat (Caltag, San Francisco, CA).

Quantification of VAP-1 mRNA by Fluorogenic Polymerase Chain Reaction (PCR)

A piece of liver tissue was minced aseptically, embedded in guanidium isothiocyanate buffer, and stored at –70°C.

Pieces from the same livers were processed for immunohistochemistry. RNA was extracted from six normal PVG rat livers, from one normal BN liver, from four liver allografts without CMV, from eight liver allografts with CMV at 1 week, and from three liver allografts with CMV at 4 weeks. Total RNA extraction was done by the guanidium isothiocyanate-phenol-chloroform extraction.²¹ RNA was DNase (Life Technologies Inc., Rockville, MD) treated before converted to cDNA. cDNA synthesis was performed using the First Strand cDNA synthesis kit (Superscript Preamplification System; Life Technologies Inc.) following the manufacturer's instructions. The quantitative PCR was optimized and performed in 20- μ l volumes using 4 mmol/L MgCl₂, 1 $\times$ DNA master SYBR green I mix (Roche Molecular Biochemicals, Indianapolis, IN), 11 ng/ μ l anti-*Taq* antibody, 0.4 pm/ μ l of oligo Rssao-354 (5'-gac cct cgg aca act gtg tct t) and Rssao-673 (5'-gcg ttt gta gaa gca aca gtg a). The primer sequences were obtained from the published partial rat VAP-1 sequence.²² The DNA amplification, data collection and analysis were performed with LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). The program was optimized and performed finally as denaturation at 95°C for 30 seconds followed by 40 cycles of amplification (95°C for 0 seconds, 63°C for 4 seconds, 72°C for 30 seconds). The temperature ramp rate was 20°C/second. At the end of each extension step, the fluorescence of each sample was measured to allow the quantification of the PCR product. After the PCR was completed, the melting curve of the product was measured by temperature gradient from 60°C to 96°C at 0.2°C/second with continuous fluorescence monitoring to produce a melting profile of the primers.

For quantification of mRNA copy number, a 900-bp PCR product was amplified by oligo Rssao-2 (5'-gct gct ctg ctc ggc cta) and oligo Rssao-910 (5'-ctc aaa cat gtc ctc cag ctg a). The PCR product was then purified by a PCR purification column (Qiagen, Inc., Valencia, CA) and the OD 260-nm value was measured to estimate the DNA copy number. Serial dilutions of this standard were then included in the PCR along with the liver samples to produce a standard curve, from which the original mRNA copy numbers could be estimated.

RNA copy numbers were then correlated to the VAP-1 positivity of the frozen sections from the same grafts. The expression of VAP-1 was blindly scored semiquantitatively from 0 (–) to 3 (+++) reflecting the total positivity of the whole graft, including blood vessels, hepatocytes, sinusoids, and bile ducts. Normal liver with the positive vessels but no other VAP-1-positive structure was given a composite score 0.5 (+/–).

Results

The RCMV infection of liver allografts in the infected group proceeded as we have previously described.¹³ All RCMV-infected animals had a positive culture from the graft at 1 week. Immunofluorescence from the explanted grafts demonstrated that RCMV-positive hepatocytes, endothelial, and inflammatory cells could be found in the

Figure 1. mAb TK8-110 recognizes VAP-1. Chinese hamster ovary cells transfected with VAP-1 cDNA or with the vector only were stained with a negative control mAb 3G6, the mAb TK8-110, and with the positive control mAb IB2 and analyzed by FACS. The x axis is the fluorescence intensity at a logarithmic scale and y axis is the relative amount of cells.

liver grafts of the infected animals at 1 week. At 4 weeks only occasional RCMV-positive leukocytes could be found. The grafts of the uninfected animals were negative for RCMV antigens.

A Novel Anti-Human VAP-1 Antibody Cross-Reacting with Rat VAP-1

New anti-VAP-1 mAbs were produced by immunizing mice with affinity-purified VAP-1. One mAb, designated TK8-110, was found that stained Chinese hamster ovary transfectants expressing human VAP-1 but not mock-transfected cells (Figure 1). In human tissues it stained the same cell types as other known anti-VAP-1 mAbs (data not shown). When screening a reactivity with other species, mAb TK8-110 was also found to cross-react with rat VAP-1. Hence, this new anti-VAP-1 mAb allowed us to use an animal model to study the regulation of VAP-1 during organ rejection.

Expression of VAP-1, ICAM-1, and VCAM-1 in Normal Liver and Isografts

In normal rat liver, VAP-1 was quite strongly expressed in the endothelium of veins in the portal area and in the media of the arteries. Locally, there was also faint sinusoidal staining (Figure 2a).

In isografts, no histological signs of acute rejection such as portal inflammation, endothelitis, or cholangitis were seen. In the isografts the expression of VAP-1 was essentially similar to normal liver (data not shown), ie, strong staining of portal blood vessels, occasional sinusoidal staining, but negative hepatocytes and bile ducts (Table 1). ICAM-1 was expressed at a low level in sinusoidal endothelium of normal liver and isografts. VCAM-1 was not expressed in normal liver or isografts. Stainings with the isotype-specific control antibody were negative in all groups.

VAP-1 Is Induced in a Time-Dependent Manner during Acute Liver Rejection

VAP-1 Expression at 1 Week

In the acute rejection at 1 week after transplantation, at the peak of inflammation, there was a clear induction of VAP-1 expression on hepatocytes and sinusoids, while the expression in the vessels of the portal tract was essentially similar to the normal liver (Figure 2b). In addition, occasionally bile duct cells expressed VAP-1 moderately (Table 1). In arteries both endothelium and media stained strongly positive.

ICAM-1 and VCAM-1 Expression at 1 Week

The sinusoidal ICAM-1 staining was greatly intensified by rejection. ICAM-1 expression was strongly induced by acute rejection on portal veins and arteries. Weak to moderate ICAM-1 synthesis was found on hepatocytes and bile duct cells. At 1 week low levels of VCAM-1 expression was induced by acute rejection in veins, arteries, and sinusoidal endothelium (Table 1).

Expression of VAP-1, ICAM-1, and VCAM-1 at 4 Weeks

In the later time point, 4 weeks after transplantation, the induction of VAP-1 on hepatocytes had diminished somewhat in the noninfected group. Otherwise the expression was quite similar as at 1 week.

The vascular staining for ICAM-1 was weaker than at 1 week although sinusoidal expression remained at high level. VCAM-1 expression was still seen in the vascular endothelium, but sinusoidal expression was now almost totally absent (Table 2). At this phase the grafts demonstrated moderate to intense portal mononuclear inflammation, slightly milder than at 1 week.

Concomitant CMV Infection and Acute Rejection Induce Long-Lasting VAP-1 Expression

VAP-1 Expression in CMV-Infected Grafts at 1 Week

At 1 week there was no clear difference between infected and noninfected groups in the intensity or localization of expression of VAP-1. Also in CMV-infected animals VAP-1 expression was strongly induced on hepatocytes and sinusoids, and occasionally bile duct cells were positive (Table 1).

ICAM-1 and VCAM-1 Expression in CMV-Infected Grafts at 1 Week

Induction of ICAM-1 expression was similarly intensive as in the uninfected group. VCAM-1 expression was also essentially similar as in the noninfected group, but the vascular staining was slightly stronger (Table 1).

Figure 2. **a:** VAP-1 expression in a normal rat liver. There is intense staining of the portal vessels, but parenchyma and sinusoids are negative. **b:** VAP-1 expression in acute liver allograft rejection in the rat, at 1 week after transplantation. In addition to vascular VAP-1 expression there is moderate to intense staining of hepatocytes, increased sinusoidal staining, and some staining in bile ductules. Immunoperoxide staining. Original magnification, $\times 200$ (**a** and **b**). **c–e:** Sequential tissue sections from 4 weeks after transplantation (acute rejection plus CMV) demonstrating bile ducts expressing VAP-1. **c:** VAP-1 staining shows positive tubular structures in the portal field, some of which appear like bile ducts (**straight arrows**) and some like blood vessels (**curved arrow**). **d:** Cytokeratin staining identifying the bile ductules (**straight arrows**). **e:** Factor VIII staining identifying endothelial cells (**curved arrow** shows the same vessel as in previous sections). The bile ducts (**straight arrows**) are negative. Original magnification, $\times 400$ (**c–e**). **f–h:** Demonstrates the scoring of the intensity. VAP-1 expression of hepatocytes: +, weak (**f**); ++, moderate (**g**); and +++, intense (**h**). Original magnification, $\times 200$ (**f–h**).

Expression of VAP-1, ICAM-1, and VCAM-1 in CMV-Infected Grafts at 4 Weeks

At 4 weeks, the hepatocyte expression of VAP-1 in the CMV group was still almost as intense as at the peak of inflammation. Also the sinusoidal staining was moderate

to intense in the CMV group at this later time point and somewhat stronger than in the uninfected group. After CMV infection the bile duct positivity was clearer at this later time point (Figure 2c and Table 2).

To be sure that bile ducts indeed could be VAP-1-positive, cytokeratin and F VIII stainings on sequential

Table 1. VAP-1, ICAM-1, and VCAM-1 Expression in Liver Allografts 1 Week Posttransplantation

	AcRX*	AcRX + CMV	Isograft	Normal liver
VAP-1				
Portal veins	++ [†]	++/+++	++	++/+++
Arteries	++	++	++	++
Bile ductules	-/+	-/++	-	-
Hepatocytes	++/+++	+++	-	-
Sinusoids	++	++	-/+	-/+
ICAM-1				
Portal veins	+/++	++	-	-
Arteries	+/++	++	-	-
Bile ductules	-/+	+	-	-
Hepatocytes	+	+	-	-
Sinusoids	+++	+++	+	+
VCAM-1				
Portal veins	+	+/++	-	-
Arteries	+	+/++	-	-
Bile ductules	-	-/+	-	-
Hepatocytes	-	-	-	-
Sinusoids	+	+	-	-

*AcRX, acute rejection; AcRX + CMV, acute rejection and concomitant CMV infection.

[†]Staining was scored as -, no staining; +, weak; ++, moderate; and +++, intense staining.

sections were performed to identify bile ducts and blood vessels, respectively. These identified some of the VAP-1-positive structures in the portal fields as bile ducts (Figure 2, d and e).

There was no difference between uninfected and RCMV-infected grafts with respect to ICAM-1 expression at 4 weeks. However, the sinusoidal VCAM-1 expression was found to be much stronger than in the uninfected group at the later time point. Hepatocytes were VCAM-1-negative in all groups.

VAP-1 mRNA

To see whether increased VAP-1 staining in liver allografts represents up-regulation of VAP-1 synthesis or decreased internalization or shedding, we analyzed ex-

pression of VAP-1 mRNA in these samples. The quantitative reverse transcriptase-PCR analysis demonstrated a significant positive correlation between the composite immunohistological VAP-1 positivity and the VAP-1 mRNA expression in the livers (Figure 3).

Discussion

In the present study acute rejection was found to cause a strong induction of VAP-1 expression on hepatocytes and sinusoidal endothelium in rat liver allografts. The induction was strong at 7 days, which is the time point when the lymphocytic infiltration into the graft reaches its peak in our model.¹² At the later phase of rejection, at 4 weeks, there was still VAP-1 expression in hepatocytes and si-

Table 2. VAP-1, ICAM-1, and VCAM-1 Expression in Liver Allograft Rejection 4 Weeks Posttransplantation

	AcRX*	AcRX + CMV	Isograft
VAP-1			
Portal veins	++ [†]	++	++
Arteries	++	++	++
Bile ductules	+	++	-
Hepatocytes	+/++	++/+++	-
Sinusoids	+/++	++/+++	-/+
ICAM-1			
Portal veins	+	+	-
Arteries	+	+	-
Bile ductules	+	+	-
Hepatocytes	+	+	-
Sinusoids	+++	+++	+
VCAM-1			
Portal veins	+	++	-
Arteries	+	++	-
Bile ductules	+/-	+/-	-
Hepatocytes	-	-	-
Sinusoids	-/+	++	-

*AcRX, acute rejection; AcRX + CMV, acute rejection and concomitant CMV infection.

[†]Staining was scored as -, no staining; +, weak; ++, moderate; and +++, intense staining.

Figure 3. Blindly scored total VAP-1 positivity in frozen sections correlates with VAP-1 mRNA expression. mRNA was isolated from seven normal livers, from four liver allografts without CMV, from eight liver allografts with CMV at 1 week, and from three liver allografts with CMV at 4 weeks. Quantitative PCR was used to analyze VAP-1 mRNA in these samples, and semiquantitative immunohistochemistry was used to score the staining intensity in the same livers. RNA copy number plotted against immunohistological score. Regression line, correlation coefficient, and 95% confidence archs are shown.

nusoidal endothelium, but in the uninfected group it was clearly milder than at the peak of inflammation. However, in the grafts concomitantly infected with RCMV, the VAP-1 expression of sinusoids and hepatocytes was still moderate to intense at the later stage of rejection. Also some bile duct epithelial cells expressed VAP-1 in the allografts. In isografts as well as in the normal rat liver, only the vascular structures and a small proportion of the sinusoids were VAP-1-positive. ICAM-1 and VCAM-1 expression was induced in acute rejection alone and with CMV infection as has been previously described.^{2,3,7,12}

In man, VAP-1 expression is reported to be similar in both uninfamed livers and liver grafts with acute or chronic rejection, and in patients with primary biliary cirrhosis.²³ In the same study it was demonstrated by an *in vitro* adhesion assay that VAP-1 is important in mediating T-cell adhesion to liver endothelia.²³ Also in hepatocellular carcinoma in humans the tumor endothelium is strongly VAP-1-positive, and this has been found to correlate to T-cell infiltration in the tumor.²⁴ Thus, there is strong constitutive sinusoidal VAP-1 expression in man, contrasting the low-level expression in normal rat liver, as well as in mouse liver.²⁵ In addition, we saw a clear induction of sinusoidal VAP-1 expression in rat-liver allograft rejection, as well as VAP-1 induction in hepatocytes and bile duct cells. In our model, no immunosuppression is given, which may explain some of these differences compared to the human liver transplant patients that are always immunosuppressed. However it does not explain the difference in VAP-1 expression in the sinusoids of normal liver of humans and rodents. The strong constitutive VAP-1 expression of the sinusoids in humans might imply that the functional role of VAP-1 is greater in humans than in rodents.

In acute liver allograft rejection most of the lymphocyte extravasation takes place in the portal areas, while the sinusoidal area/liver parenchyma is relatively spared. This implies that the endothelia of the portal vessels, and not the sinusoids, express the array of adhesion molecules that mediates lymphocyte recruitment to the liver in acute rejection.^{2,26} In this study and also in man,²³ the expression of VAP-1 in portal vessels seems to be constitutive. Therefore the up-regulation of VAP-1 in the sinusoids and hepatocytes in our model probably does not play a direct role in the development of the portal lymphocytic infiltrates in acute rejection. The relative inactivity of the sinusoidal endothelium has been attributed to the lack or low level of expression of E- and P-selectin and VCAM-1 in the sinusoid,² while ICAM-1 is constitutively expressed. In advanced rejection the VCAM-1 expression can break through in the sinusoids.² VAP-1 is able to support the initial transient tethering of leukocytes to endothelium²⁷ hence acting very early in the adhesion cascade. ICAM-1 and VCAM-1 act later, in the phase of strong adhesion after integrin activation.¹ However, VCAM-1 has been shown to by-pass the selectin step and to be able to mediate all steps of the adhesion cascade for lymphocytes.²⁸ Because of the absence of selectins VAP-1 could possibly be the sole molecule to mediate the early steps of the adhesion cascade in the sinusoid, and ICAM-1 and VCAM-1 could mediate the

stable adherence. In our rejection model some parenchymal lymphocytic inflammation also is seen although the histological pattern is dominated by the portal inflammation.¹² Whether these lymphocytes are recruited by the sinusoidal adhesion molecules is not clear.

In our rat model we found VAP-1 expression also in the bile duct cells in acute rejection. Bile duct epithelial cells are prime targets for immune cells in both acute and chronic liver allograft rejection.²⁹ VAP-1 has been reported to be important in the adhesion of cytotoxic T lymphocytes²⁷ and could possibly enhance the immune attack against the bile ductules in rejection. Bile duct cells are known to be active cells in various inflammatory conditions of the liver as they have been reported to express adhesion molecules, cytokines, and MHC class II molecules in response to various inflammatory stimuli.^{2,30} Alternatively one could hypothesize that bile duct cells and hepatocytes could bear the receptor for VAP-1 and bind the soluble form of VAP-1 that is increased in various liver disorders.¹¹ VAP-1 has not previously been described to be expressed on any epithelial cell types.

Although we could demonstrate a clear induction of VAP-1 expression by acute rejection, these results do not yet prove a functional role for VAP-1 in liver allograft rejection. To formally establish a functional role for VAP-1 in this setting will require *in vivo* blocking experiments with an anti-VAP-1 reagent to see whether graft rejection could be inhibited or modulated by abolishing lymphocyte binding to VAP-1.

VAP-1 is stored in granules of endothelial cells,³¹ and therefore its surface expression can possibly be rapidly up-regulated without the delay of protein synthesis. In a pig model, VAP-1 is completely absent from luminal surface in uninfamed vessels. At inflammatory sites, in contrast, rapid translocation of intracellular VAP-1 onto the luminal surface of endothelial cells is evident. Therefore, in the liver allografts the subcellular localization of VAP-1 in portal veins may also be different in rejecting organ from that in normal liver.³² VAP-1 expression cannot be induced in cultured endothelial cells, but in tonsil organ culture, tumor necrosis factor- α , interleukin-1, interleukin-4, and interferon- γ induced VAP-1, indicating that a correct micromilieu is required, and that VAP-1 can be induced by cytokines at sites of inflammation.¹⁰ These proinflammatory cytokines, eg, tumor necrosis factor- α , interleukin-1, and interferon- γ are also abundantly expressed in rejecting liver allografts³³ and responsible for the up-regulation of ICAM-1. Further *in vivo* studies are needed to establish the regulatory factors for VAP-1 expression. Here we found that VAP-1 mRNA synthesis is increased in allograft rejection. In fact, this is the first time when induction of VAP-1 expression has been attributed to increased transcription rather than decreased disappearance of the synthesized protein (eg, by decreased shedding or internalization). The prolonged kinetics of VAP-1 and the induction of VCAM-1 in RCMV-infected allografts could possibly reflect the ongoing stimulation of cytokine expression, because CMV has been reported to up-regulate, for example, tumor necrosis factor- α .³⁴ Alternatively RCMV could directly stimulate adhesion molecule expression, as has been reported *in vitro* for ICAM-

1.³⁵ In our study, however, no difference could be seen in ICAM-1 expression, which was maximally induced in all allografts because of unmodulated acute rejection.

In conclusion, the present experimental study shows, that VAP-1 mRNA and protein synthesis can be up-regulated in acute rejection, and that this up-regulation is prolonged by RCMV infection. It remains to be elucidated whether the up-regulation of VAP-1 in rejection has functional significance or is merely a marker of activation and the increase in cytokine levels. Demonstration of the pathophysiological role for VAP-1 in liver allograft rejection requires *in vivo* experiments using blocking antibodies or other methods to inhibit expression.

Acknowledgments

We thank Mrs. Teija Kanasuo, Kaarina Inkinen, Raisa Loginov, and Miia Mäkijouppila for excellent technical assistance; and Kari Savelius for the animal care. We also thank Dr. Roy Lobb, Biogen Inc., Cambridge, MA, for kindly providing the antibody against rat VCAM-1; and Professor Eugene Butcher for providing the antibody JG2.10; and Gennady Yegutkin, University of Turku, for drawing the correlation plot.

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