Vascular Adhesion Protein 1 Mediates Binding of Immunotherapeutic Effector Cells to Tumor Endothelium[1]

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Tumor-infiltrating lymphocytes (TIL) can be used as an immunotherapeutic tool to treat cancer. Success of this therapy depends on the homing and killing capacity of in vitro-activated and -expanded TIL. Vascular adhesion protein 1 (VAP-1) is an endothelial molecule that mediates binding of lymphocytes to vessels of inflamed tissue. Here, we studied whether VAP-1 is involved in binding of TIL, lymphokine-activated killer (LAK) cells, and NK cells to vasculature of the cancer tissue. We demonstrated that VAP-1 is expressed on the endothelium of cancer vasculature. The intensity and number of positive vessels varied greatly between the individual specimens, but it did not correlate with the histological grade of the cancer. Using an in vitro adhesion assay we showed that VAP-1 mediates adhesion of TIL, LAK, and NK cells to cancer vasculature. Treatment of the tumor sections with anti-VAP-1 Abs diminished the number of adhesive cells by 60%. When binding of different effector cell types was compared, it was evident that different cancer tissues supported the adhesion of TIL to a variable extent and LAK cells were more adhesive than TIL and NK cells to tumor vasculature. These data suggest that VAP-1 is an important interplayer in the antitumor response. Thus, by up-regulating the expression of VAP-1 in tumor vasculature, it can be possible to improve the effectiveness of TIL therapy. ***The Journal of Immunology,*** **2001, 166: 6937–6943.**

Despite the improvements in both surgery and radiotherapy and development of several new anticancer drugs, many cancers are still incurable. One example is locally advanced or metastasized squamous cell carcinoma of the head and neck (SCCHN),[3] the prognosis of which is still poor. Recurrent or metastatic disease is usually incurable and use of chemotherapy is limited to palliation of symptoms (1). Therefore, new treatment methods, including immunotherapy, are actively under investigation.

One form of immunotherapy is to use tumor-infiltrating lymphocytes (TIL), which are the lymphocytes that have migrated into the tumor of a patient presumably to kill the malignant cells. Even if the cancer can overcome this antitumor response, it is nevertheless possible to isolate these TIL, to increase their killing potential and expand them by culturing them in vitro with specific growth factors (IL-2), and finally to give them back to the patient. The idea is that these TIL would find their way back to the tumor tissue, where they now as progeny of the activated effector cells would be more effective in killing the malignant cells. The therapeutic efficacy of TIL naturally depends on their ability to succeed in both homing and killing. TIL treatment has already been in experimental use and even >30% response rates among patients with end-stage melanoma have been reported (2).

To find their way back to the cancer tissue, TIL must leave the blood circulation within the tumor. Lymphocyte extravasation is a multistep process which includes four main phases: 1) tethering and rolling, 2) activation, 3) firm adhesion, and finally 4) transendothelial migration. This carefully controlled lymphocyte-endothelial interaction cascade is mediated by several lymphocyte surface molecules and their ligands on endothelial cells (3–5). Although the molecular usage of different leukocyte adhesion molecules in interactions with vascular endothelial cells in normal lymphoid tissues and at sites of inflammation is well understood, very little is known about which lymphocyte surface molecules or their ligands on tumor vasculature are needed for efficient endothelial binding of immunotherapeutically important effector cells in humans.

One potential candidate for mediating adhesion of effector lymphocytes to tumor vasculature is vascular adhesion protein 1 (VAP-1). It is an endothelial adhesion molecule that is up-regulated at sites of inflammation and mediates lymphocyte binding to inflamed endothelium (6). VAP-1 is preferentially involved in adherence of $CD8^+$ (T killer) and $CD16^+$ (NK) lymphocytes to high endothelial venules (HEV) in peripheral lymph nodes and tonsils (7). Since TIL activated according to our protocol are predominantly $CD8^+$ (2, 8), it would be possible that VAP-1 participates in homing of TIL. In fact, sinusoidal endothelial cells in liver express VAP-1, which mediates binding of TIL to hepatocellular carcinomas. Hepatic metastases of colorectal malignancies in contrast are devoid of VAP-1 and are also less infiltrated with T cells (9).

In this work, we analyzed the expression of VAP-1 in vessels of SCCHN samples and its importance in adhesion of TIL therein. Moreover, the role of VAP-1 in controlling the adherence of lymphokine-activated killer (LAK) and NK cells to tumor vessels was evaluated for the first time. The results revealed an essential role for VAP-1 in binding of immunotherapeutic effector cells to tumor endothelium.

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[3] Abbreviations used in this paper: SCCHN, squamous cell carcinoma of the head and neck; TIL, tumor-infiltrating lymphocyte; VAP-1, vascular adhesion protein 1; HEV, high endothelial venule; LAK, lymphokine-activated killer; SCC, squamous cell carcinoma; MAdCAM-1, mucosal addressin cell adhesion molecule 1; PNAd, peripheral node addressin; RAR, relative adherent ratio.

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Materials and Methods

Tissue samples

Tumors of 33 patients suffering from malignancies of the head and neck were removed for diagnostic and therapeutic purposes. Twenty-nine of the tissues were squamous cell carcinomas (SCC): 16 primary tumors, 4 neck metastases, and 9 recurrent tumors. These recurrent tumors had all received radiation therapy as part of the treatment for the primary tumor (2–23 years before the recurrence of the tumor). The remaining four tumors were plasmacytoma, Merkel cell carcinoma, mucoepidermoid carcinoma, and acinar cell carcinoma. Three SCC samples were taken from gynecologic tumors (vulval SCC).

The specimens were received directly from the operating room and were divided into three pieces. One was Formalin fixed for diagnostic purposes, one was snap-frozen in liquid nitrogen for immunohistochemistry and in vitro adhesion assays, and one was used to isolate TIL. All cell and tissue sampling protocols used in this study were approved by institutional review boards and informed consent of the patients was obtained.

Propagation of TIL from tumors and LAK and NK cells from PBL

Approximately 1-mm^3 pieces were cut from the cancer samples. Pieces were placed in 96-well tissue culture plates containing RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10 mM HEPES, 12.5% AB serum (Finnish Red Cross Blood Service, Helsinki, Finland), 4 mM L-glutamine, 17 μg/ml PHA, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1000 IU human rIL-2 (a kind gift from P. Karnani, Orion-Farmos Pharmaceuticals, Turku, Finland). During the next few days ,T cells present in the tumor started to proliferate. Cancer pieces were removed after 5–7 days, and after ~3 wk the TIL population was large enough for in vitro adhesion assays.

PBL were isolated using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation, and LAK cells were propagated from PBL for 3 days under similar conditions described above for TIL. NK cells ($CD16^+$ PBL) were separated with superparamagnetic beads directly coupled with mAb against CD16 (MACS; Miltenyi Biotec, Auburn, CA) according to the protocol suggested by the manufacturer. The specificity of the selection was confirmed using flow cytometer (FACScan; Becton Dickinson, Mountain View, CA) with a PE-conjugated anti-CD16 mAb. L3T4-PE (α-mouse CD4; PharMingen, San Diego, CA) served as a negative control. More than 90% of the positively selected cells were CD16 positive.

Immunohistochemistry

For immunohistochemistry, 6-μm-thick frozen sections were cut from tumors, air dried, and acetone fixed. Sections were overlaid with first-stage mAbs (20–100 μg/ml) and incubated for 20 min at room temperature in a humidified chamber. After two washings in PBS, peroxidase-conjugated rabbit anti-mouse Ig in PBS containing 5% AB serum was added. 3,3-Diaminobenzidine (Polysciences,Warrington, PA) in PBS containing 0.03% hydrogen peroxide was used for 3 min to develop the reaction. Finally, the sections were counterstained in hematoxylin (Sigma, St. Louis, MO), dehydrated, cleared in xylene, and permanently mounted in DePex (BDH Limited, Pool, Dorset, U.K.). As primary Abs we used mAbs 2D10 (anti-VAP-1) (10), 2C8 (anti-CD31) (11), MECA-79 (anti-peripheral node addressin (PNAd)) (12), WAPS12.2 (α-P-selectin/CD62P) (13) (MECA-79 and WAPS12.2 are kind gifts from Prof. E. Butcher, Stanford, CA) and 10A6 (antimucosal addressin cell adhesion molecule 1 (MAdCAM-1), a kind gift from M. Briskin, Millenium Pharmaceuticals, Boston, MA) (14). ICAM-1/CD54 was detected by 5C3 (15) and 6.5B5 (16), VCAM-1/CD106 by P8B1 (DSHB) and 1G11 (17), and E-selectin/CD62E by P2H3 (DSHB) and 1.2B6 (16) (6.5B5, 1.2B6, and 1G11 were kind gifts from Prof. D. Haskard, Hammersmith Hospital, London, U.K.). 7C7 (against bursal epithelial cells) or 3G6 (against chicken T cells) were used as negative control Abs. Sections were analyzed single blind with an Olympus microscope (Olympus, New Hyde Park, NY) using a magnification of ×200. Staining intensity was semiquantitatively scored from 0 to +++ (0 corresponds to negative, + to weak, ++ to moderate, and +++ to strong staining). The number of positive vessels in the sample was counted from nine microscopy fields in the most positive area of the sample. The area analyzed was 2.4 mm^2 in each sample. The extent of inflammatory infiltrates in the cancer samples was analyzed from van Gieson-Weigert-stained sections and scored semiquantitatively from 0 to +++ (0 corresponds to negative, + to weak, ++ to moderate, and +++ to abundant infiltrate).

Detection of VAP-1 mRNA

Cancer samples were snap-frozen in liquid nitrogen, homogenized, diluted to 1 ml Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX) and the RNA was extracted according to the manufacturer's instructions. The RNAs were further purified by ethanol precipitation.

One-step RT-PCR was done using a GeneAmp Gold RNA PCR Reagent kit (PE Biosystems, Foster City, CA) according to the protocol suggested by the manufacturer with VAP-1-specific primer pairs: forward primer, ACT CAG ATC TCT ACT CGC ACT and reverse primers, TCT CCC AGC TGA AGC CTC TCG and ATA TGC AGA AAA CCA GCT GTC. Samples were run on a 1% agarose gel and Southern blotting was performed using a Zeta-Probe GT Genomic Tested Blotting Membrane (Bio-Rad, Hercules, CA). A VAP-1-specific probe was labeled with *redi*prime II kit (Amersham Pharmacia Biotech, Little Chalfont, U.K.) using [^{32}P]dCTP according to the protocol suggested by the manufacturer and hybridized to the membrane. Autoradiography was used for detection of the signal.

In vitro adhesion assay

The in vitro Stamper-Woodruff type of adhesion assay was performed as described elsewhere (18). In brief, 8-μm frozen sections were cut from VAP-1-positive samples. Sections were incubated with saturating levels of 1B2 (anti-VAP-1) and TK8-14 (anti-VAP-1) (6, 10) Abs simultaneously (these Abs see different epitopes of VAP-1 and both are function blocking) under constant rotation for 30 min at 7°C. To compare the contribution of VAP-1 to that of other adhesion molecules, we also tested the effect of an Ab pool containing the following function-blocking Abs: 5C3 (anti-ICAM1/CD54), P8B1 (anti-VCAM-1/CD106), P2H3 (anti-E-selectin/CD62E), WAPS12.2 (anti-P-selectin/CD62P), and MECA-79 (anti-PNAd). HB95 against HLA-ABC (American Type Culture Collection, Manassas, VA) and 7C7 were used as class-matched negative controls. TIL, LAK cells, NK cells, and PBL from patients and PBL from healthy subjects in suspension were overlaid, and the sections were incubated under constant rotation for another 30 min at 7°C. The adherent cells were fixed in 1% glutaraldehyde. In these assays, cancer tissues from five patients, TIL lines from six patients, LAK cells from three of the same patients as TIL, fresh PBL from three patients, and NK cells of a healthy control were used. Each assay contained PBL from the same individual to standardize the assays performed on different days. Although there is no marked difference in binding of PBL of healthy persons to HEV (18), use of the same standard minimized all possible day-to-day variations. In the assays where binding (and inhibition) to VAP-1-positive and -negative vessels was analyzed separately, Ab suspensions (containing anti-VAP-1 or negative control Ab) were removed after 30 min of preincubation. Thereafter, a predetermined number of sheep anti-mouse IgG Dynabeads (Dynal, Oslo, Norway) were added with cell suspensions. Approximately three to five magnetic beads (<10% of the surface area of a vessel) were observed to bind to VAP-1-positive vessels, still leaving enough space for cells to bind.

The number of lymphocytes bound to vessels was counted single blind under dark-field illumination. With each lymphocyte population, the adhesion was counted to a minimum of 100 vessels in each cancer in every experiment. For example, to analyze binding of TIL altogether 2512 vessels were counted. To facilitate direct comparison among different cell types and among different experiments, the relative adherent ratios (RAR) were determined. The RAR is the calculated number of sample cells bound to a vessel normalized to the number of reference lymphocytes (control PBL) bound under the same conditions. RAR value 1.0 was arbitrarily designated for PBL adherence. The results of the inhibition assays are presented as a percentage of control binding (the number of cells adherent to vessels in the presence of control mAb defines 100% adherence).

The in vitro binding assay accurately reflects the in vivo homing capacity and specificity of different leukocyte subtypes as demonstrated by several animal studies. The results of mAb inhibition studies using the Stamper-Woodruff assay have also been readily reproduced in in vivo homing assays (reviewed in Ref. 19). The binding assay mimics the shear conditions under which the initial recognition between blood-borne leukocytes and vascular lining takes place in vivo, and the members from all adhesion molecule families have been shown to function properly under these conditions (reviewed in Ref. 20).

Statistical analyses

Paired Student's *t* test was used for analysis of adhesion assays and Spearmen's rank correlation for comparison of staining intensity, number of positive vessels, and inflammatory infiltrations.

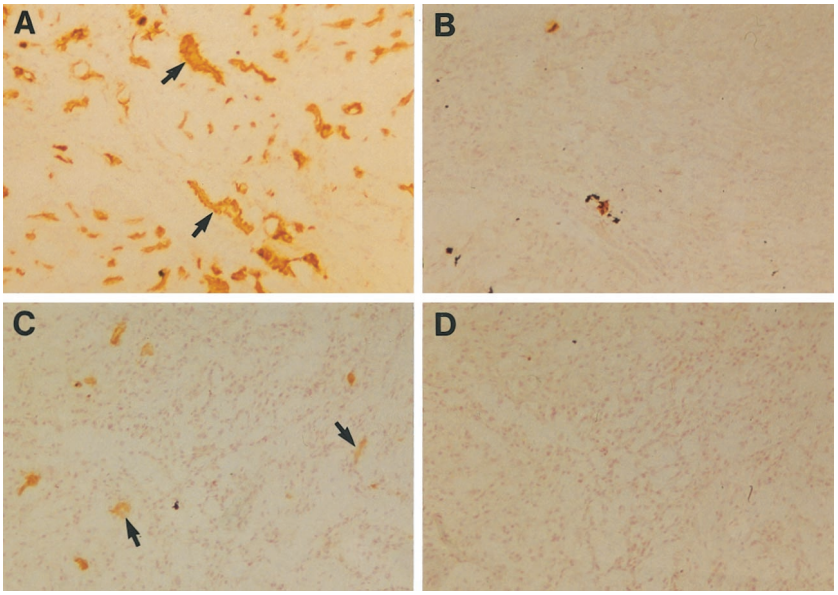

FIGURE 1. The number and intensity of VAP-1-positive vessels vary greatly between different cancer tissues. *A*, Cancer tissue with a high number of brightly VAP-1-positive vessels (two of which are pointed out by arrows) while *C* is an example of a cancer with a low number of moderately VAP-1-positive vessels (arrows). *B* and *D* are the same cancer tissues as in *A* and *C* stained with a negative control Ab. Immunohistochemical staining; original magnification, ×100).

Results

VAP-1 is synthesized in all SCCHN samples

Since the aim of the expression analysis was to see whether there are differences in expression of VAP-1 at different stages of cancer, primary tumors as well as metastases and recurrences were studied. We tested immunohistochemically 33 different malignant tissues from the head and neck area using anti-VAP-1 Ab. Anti-CD31 Ab was used to evaluate the total number of vessels in the sections. Four of the recurrent tumors had a high level of nonspecific staining that made the evaluation unreliable, and they were excluded from further analyses. All of the malignant tissues tested had VAP-1-positive vessels, whereas other cell types, including the malignant cells, remained negative. To analyze whether VAP-1 was also expressed in vasculature of SCC located outside the head and neck area, we tested three vulval SCC. They all contained high numbers of VAP-1-positive vessels. The number and intensity of VAP-1-positive vessels varied greatly between different SCC samples of the head and neck area. The staining of the vasculature was strong (+++) in 10, moderate (++) in 12, and weak (+) in 7 of the tumor samples. There was a significant ($p < 0.0001$) correlation between the number of VAP-1-positive vessels and their staining intensity (Fig. 1). Samples with high numbers of VAP-1-positive vessels tended to have extensive lymphocyte infiltrations. Neither the intensity nor the number of positive vessels correlated to the grade of the cancer (Table I). Patients with recurrent cancer had been given radiation therapy as a part of the therapy for the primary tumor, but the historical radiation therapy did not seem to have any effect on the number of VAP-1-positive vessels.

To test whether VAP-1 positivity correlates with the expression of other known endothelial cell adhesion molecules, certain samples were stained with Abs against P-selectin, E-selectin, VCAM-1, ICAM-1, MAdCAM-1, and PNAd. Individual tumors expressed variable levels of these molecules except MAdCAM-1, which was absent from all samples. There was no clear correlation between expression of VAP-1 and other adhesion molecules (data not shown).

To analyze whether VAP-1 found on the cancer vasculature is also produced locally, we performed RT-PCR with two different primer pairs and detected the product with a VAP-1-specific probe. Two products of expected sizes (500 and 700 bp) were detected (data not shown), indicating that tumor vasculature is able to synthesize VAP-1.

Different effector cells can bind to endothelia of tumor vasculature

The adherence of different effector cell types to tumor vasculature was originally tested on three cancer tissues. Strongly VAP-1-positive tissues were selected to find out the role of VAP-1 in this adhesion. The cell types included were TIL, LAK cells, and NK cells, while PBL served as a control population in every assay. Paramagnetic beads were used to purify the NK cells. The presence of these minute (50 nm diameter, 8×10^{-6} volume of a lymphocyte) beads on the cells did not functionally interfere with their endothelial adherence, since each purified subpopulation bound well to cancer vessels in the sections pretreated with the negative control mAb. A total of 49.7 ± 2.8 of vessels supported

Table I. *VAP-1-positive vessels in different cancer tissues*

Carcinoma Grade	No. of Cases	No. of Positive Vessels				
		≤10	10 < × ≤40	40 < × ≤70	70 < × ≤110	× >110
SCC grade I	5	1	2	1		1
SCC grade II	11	1	3	3	4	
SCC grade III	5		2	1		2
SCC neck metastasis	4		2	2		
Other type[a]	4		1	1	2	
Total	29	2	10	8	6	3

[a] Plasmacytoma, Merkel cell carcinoma, mucoepidermoid carcinoma, and acinar cell carcinoma.

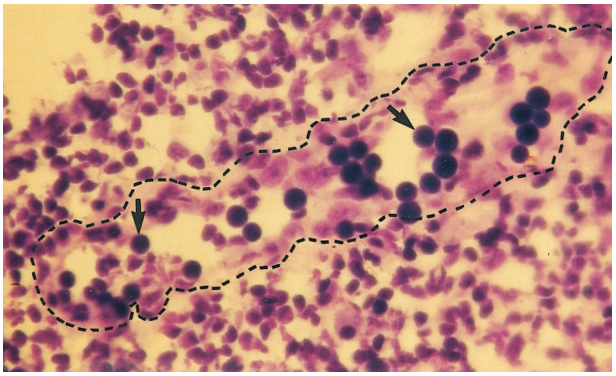

FIGURE 2. TIL specifically adhere to tumor endothelium. Twenty-five TIL (dark round large cells, two pointed out by arrows) binding to a vessel in a cancer are shown in the micrograph. Dashed line outlines the lilac-appearing basement membrane of the vessel. The focus of the photograph is a compromise between the plane of the tissue section and the bound cells on the top of it. Diff-Quik (Baxter, Düdingen, Germany) staining; original magnification, ×400.

binding of TIL and the number of TIL bound to a vessel varied from 1 to 25 depending on the size of the vessel (average, 2.35 ± 0.11 cells/vessel). Binding of TIL to tumor endothelium is illustrated in Fig. 2.

Of the cell types studied, LAK cells showed the best binding to all tested tumor tissues. The tumor tissues had a different capacity to support lymphocyte adherence. This was most evident with TIL, since 1.7 and 2.3 times higher binding of TIL was observed to vasculature of tumor 3 than to other tumors (Fig. 3). Notably, in every case, binding of immune effector cells to tumor vasculature was equal or superior to that of unselected PBL.

Next, we analyzed whether there is any difference in binding of distinct TIL to tumor tissues. Data from these experiments did not reveal any significant differences in the capacity of individual TIL to bind to tumor vasculature. In contrast, LAK cells from patient C bound significantly less than LAK cells from two other patients (Fig. 4). Thus, the ability of vessels in different cancers to support the binding of effector cells as well as the capacity of different effector cell types to adhere to tumor endothelium varies.

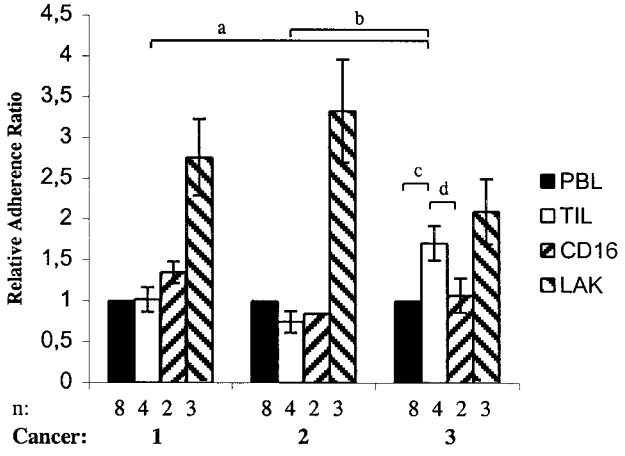

FIGURE 3. PBL, NK cells, LAK cells, and TIL all bind to tumor endothelium. Binding of all other cell types was compared with that of PBL, which were used as a control in every assay and were given the RAR value of 1.0. Cancer 3 supports binding of TIL more efficiently than other cancers (*a*, $p = 0.014$ and *b*, $p = 0.008$). Binding of TIL to cancer 3 is also significantly better than that of PBL (*c*, $p = 0.04$) and $CD16^+$ cells (*d*, $p = 0.01$). The results are presented as RAR ± SEM.

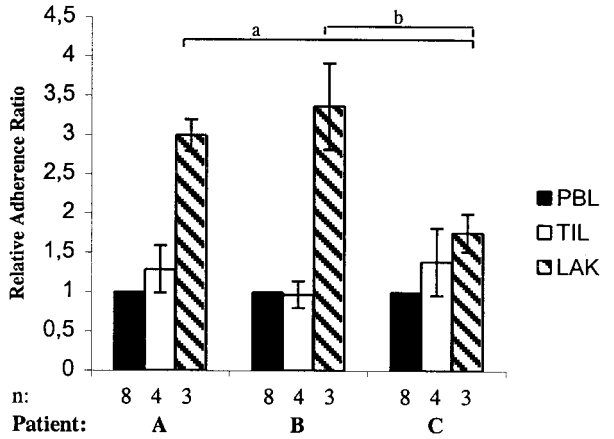

FIGURE 4. Adherence of TIL and LAK cells vary between different patients. Binding of TIL and LAK cells from three patients was compared with that of PBL, which was the control in each assay (RAR = 1.0). Binding of all cell types was tested with three different cancer tissues. LAK cells of patient C bound more poorly than those of two other patients (*a*, $p = 0.0006$ and *b*, $p = 0.035$). The results are presented as RAR ± SEM.

TIL and LAK cells were also generated from one of the cancers used for the binding assays. This allowed us to test whether the binding efficiency is dependent on histocompatibility Ags. This was not the case, because the binding of TIL and LAK cells to autologous cancer vasculature did not significantly differ from the binding to allogenic cancer vessels. Neither were we able to find any difference between the binding capacity of PBL from healthy individuals (RAR = 1.0) and autologous PBL from patients (RAR = 1.076 ± 0.089). Therefore, all data with allogenic and autologous cells were pooled.

Binding of immunotherapeutic effector cells to endothelium of cancer vasculature is VAP-1 dependent

Brightly VAP-1-positive samples served as a useful tool to measure the importance of VAP-1 in mediating the binding of different lymphocytes to cancer vasculature. Treatment of the sections with anti-VAP-1 Abs significantly reduced the adherence of all of these cell types to all cancer tissues. The averages of inhibition of PBL, TIL, NK cell, and LAK cell adherence were 59.1, 58.0, 65.2, and

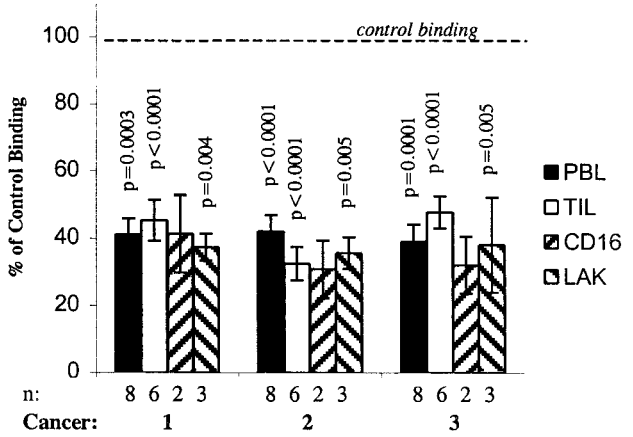

FIGURE 5. VAP-1 dependence of immune effector cell adhesion to tumor endothelium. The control binding level (100%, dashed line) represents the binding of the cell types to endothelium in the presence of negative control Abs (7C7 and HB95). The bars below show the binding in the presence of anti-VAP-1 Abs (1B2 and TK8-14). The results are presented as percentages of control binding ± SEM.

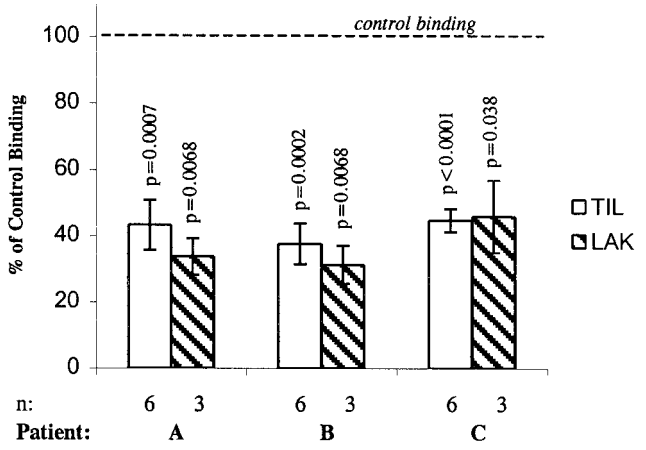

FIGURE 6. VAP-1-dependent binding of TIL and LAK cells from different patients. Binding of TIL and LAK cells from each patient was tested with three different cancer tissues. The results are presented as percentages of control binding ± SEM.

62.9%, respectively, when compared with treatment with negative control Abs (Fig. 5). These results show that VAP-1 on cancer vasculature is functionally active.

We also wanted to see whether individual TIL lines and LAK cells rely on VAP-1-mediated binding to different extents and, therefore, the binding of each TIL and LAK cells to three different cancer tissues was tested. There were no marked differences in the ability of TIL and LAK cells from different patients to bind to tumor endothelium in a VAP-1-dependent manner (Fig. 6). Notably the binding was significantly VAP-1 dependent in all patients.

Since the binding of lymphocytes to tumor vasculature endothelium is not apparently entirely VAP-1 dependent, we wanted to analyze the binding of cells separately to VAP-1-positive and -negative vessels. For this purpose, tumors expressing high and low numbers of VAP-1-positive vessels were selected. VAP-1-positive vessels were detected with anti-VAP-1 Ab followed by small numbers of Dynabeads coupled to anti-mouse IgG, because glutaraldehyde used for fixing prevents the usage of fluorescently labeled anti-VAP-1 Abs. As serial sections were treated with anti-VAP-1 and control Abs, the numbers of TIL bound to VAP-1-negative and -positive vessels could be counted. The adhesion to VAP-1-positive vessels was almost exclusively VAP-1 dependent, since the number of adherent cells decreased by 85.2% with anti-VAP-1 Abs. In contrast, binding to VAP-1-negative vessels increased 12.4% when the anti-VAP-1 Ab was used (Fig. 7). This suggests that blocking of the binding to VAP-1-positive vessels slightly facilitates lymphocyte binding to VAP-1-negative vessels.

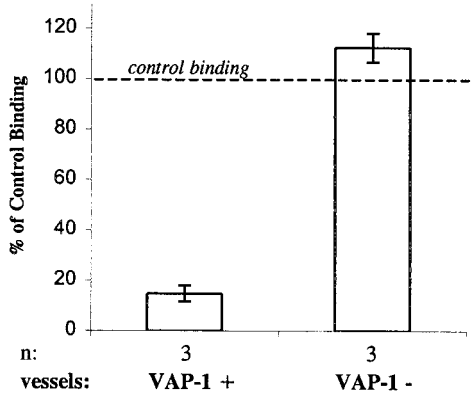

FIGURE 7. VAP-1-dependent binding of lymphocytes to VAP-1-positive and -negative vessels. Binding of cells to VAP-1-positive and -negative vessels was tested with three different cancer tissues. The results are presented as percentages of control binding ± SEM.

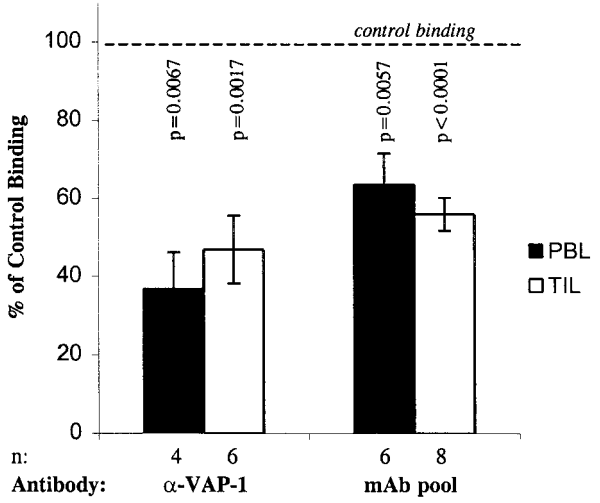

FIGURE 8. Contribution of VAP-1 and other known endothelial adhesion molecules to binding of TIL and PBL to endothelium of the tumor vasculature. The pool of mAbs contained anti-ICAM-1, anti-VCAM-1, anti-PNAd, anti-E-selectin, and anti-P-selectin Abs. The results are presented as percentages of control binding ± SEM.

To compare the contribution of VAP-1 to that of other adhesion molecules in the adherence of TIL to tumor vasculature, we performed in vitro adhesion assays parallel with anti-VAP-1 Abs and an Ab pool, which contained anti-ICAM-1, anti-VCAM-1, anti-PNAd, anti-E-selectin, and anti-P-selectin Abs using one of the original tumors (see above) and two additional tumor tissues and TIL from three additional patients. Inhibition of adhesion was of the same magnitude with anti-VAP-1 Abs (53.1%) and with the Ab pool (44.0%; Fig. 8) .

Discussion

The major findings of this work were that VAP-1 known to be up-regulated at sites of inflammation was abundantly present in a group of tumors, and all tumors expressed VAP-1 to certain extent. Most importantly, up-regulated VAP-1 was functional in tumors, because it efficiently mediated binding of immunotherapeutic effector cells to the vascular endothelium within the tumor tissues. Our results further show that although the capacity of TIL, LAK cells, and NK cells to adhere to tumor endothelium varies, they all use VAP-1 to bind to vessel walls in the tumors.

In general, tumor endothelium is thought to be at an activated state. A few vessels in the tumor even show resemblance to HEV normally present only in lymphoid organs and at sites of chronic inflammation (8). Activation status can also be demonstrated by high expression of certain endothelial cell adhesion molecules such as ICAM-1, E-selectin, P-selectin, VCAM-1, and peripheral lymph node addressins in tumor vasculature (8, 21–23). There are, however, marked differences in the expression patterns of these endothelial cell molecules in different tumors suggesting that individual tumor tissues have different arrays of inflammatory mediators, angiogenic factors, and cytokines capable of inducing only selected endothelial cell molecules (21, 24). Up-regulation of VAP-1 seems also to be controlled by a limited set of mediators, because VAP-1 is expressed at high levels only in a subgroup of tumors and its expression did not correlate with the expression of other adhesion molecules (Table I; data not shown). Although it is not known which mediators directly regulate the expression of

VAP-1, the presence of the NF-κB binding sequences in the promoter region of VAP-1 suggests that at least TNF-α is most likely able to control VAP-1 expression (our unpublished data). This hypothesis is also in line with the data obtained from tonsil organ cultures, in which VAP-1 can be induced in HEV by TNF-α (25).

It is feasible to think that altering the cytokine levels within a tumor tissue may facilitate expression of endothelial cell adhesion molecules required for optimal homing of TIL (26). There are already examples of experiments in which exogenously given mediators change the outcome of tumors. For example, administration of IL-12 augments the migration of T cells into the malignant tissues in certain tumor models. It is suggested that, at least partially, the effects of this treatment are at the level of vasculature, resulting in altered expression of endothelial adhesion molecules. On the other hand, there are other experimental tumors in which IL-12 treatment is ineffective (27). This suggests that so far unknown characteristics of tumors may determine whether the critical elements are responsive to the treatment given. Regarding homing, vascular endothelial cells, which may differ quite remarkably between different tumor beds, are at a key position. If up-regulation of VAP-1 on tumor endothelium is attempted, more studies are needed for identification of the most potent mediator or combination of them to be effective in a large variety of tumors. However, VAP-1 itself on tumor endothelium may be used to target endothelial cells with an anti-VAP-1 Ab or ligand-coupled liposomes or other vehicles containing gene constructs encoding the selected mediator. VAP-1 is especially appropriate for targeting, because according to animal studies VAP-1 is practically absent from vessel walls in normal conditions but is translocated from intracellular granules onto the vessel wall upon inflammation (our unpublished data). Thus, even a low level of expression of VAP-1 on the tumor endothelium at the beginning of the therapy directs the treatment rather specifically into the tumor tissue. Based on the selectivity of VAP-1 expression, it can also be envisioned as a target for cytotoxic drug delivery.

Although there are few studies reporting the expression status of different endothelial cell adhesion molecules in tumors, the functional significance of these molecules in mediating lymphocyte homing to tumor tissues has remained largely unknown. Animal studies indicate that leukocytes can interact with tumor endothelium in a multistep fashion using P- and E-selectin and β_2 integrin (28), and also LFA-1-ICAM-1 and very late activation Ag 4-VCAM-1 pathways participate in homing of T cells into the tumor tissues (27). Due to ethical reasons, in vivo studies elucidating the molecular requirements of proper homing of TIL into the tumor tissues are practically impossible to perform in humans. Therefore, the in vitro adhesion assay provided us with an opportunity to address the significance of VAP-1 in binding of TIL to the human tumor vessels. Despite inherent limitations of in vitro assays, the Stamper-Woodruff type assay performed in this study has been shown to reflect well in vivo homing of different cell populations (29–31). Since our results unambiguously showed that VAP-1 is a central molecule in mediating binding of different effector lymphocyte populations in this in vitro assay, we believe that VAP-1 is fundamentally contributing to TIL binding to tumor endothelium in vivo in those tumors that express significant levels of VAP-1. Significant VAP-1-dependent binding of PBL to tumor endothelium may reflect the fact that $CD8^+$ cells and NK cells from unselected PBL adhere especially willingly to vessels within the tumor.

Among the immunotherapeutic effector cells studied in this work, LAK cells showed the most efficient adhesion capacity to tumor endothelium. This is not surprising in the light that IL-2-activated effector cells rather slowly lose the expression of certain molecules important in mediating binding to vascular endothelium. For example, expression of L-selectin is still at a moderate level after 3 days of activation, whereas 2 wk in culture leads to the disappearance of L-selectin and diminished expression of CD44 (32). This may also have clinical consequences, because in in vitro experiments LAK cells bind efficiently to isolated endothelial cells in culture. However, LAK cells do not discriminate between different endothelial cells in their binding (33), nor do they preferentially home into the tumor tissue when given i.v., as is the case with TIL, at least in a subgroup of patients receiving TIL therapy (34–36). When injected intraarterially LAK cells, however, show preferential accumulation in the tumor microcirculation (37). Thus, due to the high expression levels of homing-associated molecules, LAK cells may efficiently get trapped in the lungs and liver when administered i.v. and never get sufficient chances to interact with the tumor endothelium.

One important reason for rather moderate results with TIL therapy is that the in vitro-expanded cells are not able to find their way back into the tumor. This can either be due to down-regulation of the original TIL surface molecule(s) they used to enter the tumor site for the first time or to the lack/disappearance of the proper endothelial cell molecule(s) from the growing tumor. VAP-1 may be such a molecule which is highly expressed on the tumor vasculature but down-regulated in some tumors during the growth of malignancy or not up-regulated at all in others. Since VAP-1 has an unambiguous role in binding of TIL to tumor vessels, one can envision that up-regulating VAP-1 in tumors with a low level of VAP-1 expression may improve the success rate of TIL therapy.

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