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Small-Molecule Inhibitors of Vascular Adhesion Protein-1 Reduce the Accumulation of Myeloid Cells into Tumors and Attenuate Tumor Growth in Mice

Fumiko Marttila-Ichihara,*,† Karolien Castermans,‡ Kaisa Auvinen,*,† Mirjam G. A. oude Egbrink,§,† Sirpa Jalkanen,*,† Arjan W. Griffioen,‡ and Marko Salmi*,†,‖

Vascular adhesion protein-1 (VAP-1) is an endothelial, cell surface–expressed oxidase involved in leukocyte traffic. The adhesive function of VAP-1 can be blocked by anti–VAP-1 Abs and small-molecule inhibitors. However, the effects of VAP-1 blockade on antitumor immunity and tumor progression are unknown. In this paper, we used anti–VAP-1 mAbs and small-molecule inhibitors of VAP-1 in B16 melanoma and EL-4 lymphoma tumor models in C57BL/6 mice. Leukocyte accumulation into tumors and neoangiogenesis were evaluated by immunohistochemistry, flow cytometry, and intravital videomicroscopy. We found that both anti–VAP-1 Abs and VAP-1 inhibitors reduced the number of leukocytes in the tumors, but they targeted partially different leukocyte subpopulations. Anti–VAP-1 Abs selectively inhibited infiltration of CD8-positive lymphocytes into tumors and had no effect on accumulation of myeloid cells into tumors. In contrast, the VAP-1 inhibitors significantly reduced only the number of proangiogenic Gr-1+CD11b+ myeloid cells in melanomas and lymphomas. Blocking of VAP-1 by either means left tumor homing of regulatory T cells and type 2 immune-suppressing monocytes/macrophages intact. Notably, VAP-1 inhibitors, but not anti–VAP-1 Abs, retarded the growth of melanomas and lymphomas and reduced tumor neoangiogenesis. The VAP-1 inhibitors also reduced the binding of Gr-1+ myeloid cells to the tumor vasculature. We conclude that tumors use the catalytic activity of VAP-1 to recruit myeloid cells into tumors and to support tumor progression. Small-molecule VAP-1 inhibitors therefore might be a potential new tool for immunotherapy of tumors.


Leukocyte migration into tumors can have two opposite outcomes in tumor progression. Extravasation of cytotoxic leukocytes, such as CD8-positive T cells and NK cells, can lead to enhanced antitumor immune responses, which may limit, and in certain cases even prevent, tumor growth. However, infiltration of the immune-suppressing leukocyte types, such as regulatory T cells or type 2 macrophages, into the malignancy leads to defective immune responses. In fact, tumor cells themselves actively secrete molecules that lead to the recruitment of these immune-suppressing leukocyte types into the tumors. Thus, the tumors are able to use misguided leukocyte traffic as one way of tumor evasion from the immune system (1).

Leukocytes normally use a multistep adhesion cascade to enter tissues (2, 3). Several endothelial adhesion molecules engaging their cognate leukocyte ligands play an important role in this process. However, the molecules mediating interactions between tumor endothelial cells and various leukocyte types involved in antitumor immunity remain incompletely understood.

Among the endothelial adhesion molecules, a cell surface–expressed amine oxidase vascular adhesion protein-1 (VAP-1) supports leukocyte emigration into sites of inflammation (4). VAP-1 is expressed in normal tissues in the endothelial cells of veins, in the pericytes and smooth muscle cells, and also in adipocytes (5, 6). In endothelial cells, it is most abundant in high endothelial venules but also present in flat endothelial cells. In these cells, it is mainly present in intracellular vesicles, which then translocate the molecule onto the luminal surface upon certain inflammatory stimuli (7). VAP-1 belongs to the family of semicarbazide-sensitive amine oxidases (SSAOs), which catalyze oxidative deamination of primary amines in the reaction R-CH2-NH2 + O2 + H2O → R-CHO + NH3 + H2O2. The adhesive function of VAP-1 can be inhibited using function-blocking mAbs or enzyme inhibitors. The anti–VAP-1 Abs inhibit leukocyte–endothelial interactions in several in vitro and in vivo models (8–14). The small-molecule SSAO inhibitors also effectively attenuate inflammation by diminishing leukocyte rolling, firm adhesion, and transmigration through multiple types of endothelial cells (9, 13, 15–20). Notably, the anti–VAP-1 Abs do not inhibit the enzymatic activity of VAP-1, and the enzyme inhibitors do not alter the mAb-defined surface epitopes of VAP-1 (8, 15). It thus is envisaged that VAP-1 supports leukocyte extravasation by serving as both a traditional adhesion molecule (mAb-defined epitopes) and an enzyme (reacting with amine groups displayed on the surfaces of leukocytes) (21).

We have found that tumor progression is retarded in VAP-1–deficient mice (22). In this study, we therefore evaluated the effects of anti–VAP-1 Abs and SSAO inhibitors on leukocyte recruitment into tumors and tumor progression. We show that two different enzyme inhibitors, but not the anti–VAP-1 Abs, attenuated myeloid
cell accumulation in the tumors and impaired tumor development and neoangiogenesis. Because blocking of VAP-1 function effectively attenuates inflammatory reactions in vivo, therapeutics aimed at inhibition of VAP-1 are being actively developed to treat inflammatory disorders (17, 23, 24). These data suggest that the SSAO inhibitors may be a future adjuvant for immunotherapy in cancer.

Materials and Methods

Mice

Six- to eight-week-old female wild-type C57BL/6 mice were used in all of the inhibitor and Ab treatment experiments. Mice were kept in a specific pathogen-free environment, and they had free access to food and water throughout the experiments. All of the experimental procedures were approved by the local ethical committees.

Tumor models

B16F10 melanoma cells (hereafter called B16 melanoma cells) were obtained from Caliper Life Sciences (Hopkinton, MA), and maintained in MEM with Earle’s balanced salts (HyClone, Logan, UT) supplemented with 10% FCS (Integro, Zaandam, The Netherlands), nonessential amino acids, 200 mM l-glutamine, 1 mM sodium pyruvate, and MEM vitamin solution (all from Gibco Invitrogen, Carlsbad, CA). EL-4 T lymphoma cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 containing 10% FCS, l-glutamine, and penicillin/streptomycin (Gibco Invitrogen).

Mice were anesthetized with ketamine (Pfizer, Sollentuna, Sweden) and xylazine (Bayer, Leverkusen, Germany), and an injection area on the abdominal skin was shaved. Tumor cells (4 × 10^5 B16 melanoma cells per mouse and 10 × 10^5 EL-4 lymphoma cells per mouse) were injected in RPMI 1640 (200 μl) s.c. The growth of the tumor was followed by measuring the tumor dimensions using an electronic caliper (Mitutoyo, Kawasaki, Japan). The size of the tumor sphere was estimated using the formula V = π/6 × (shortest diameter)^3 × (longest diameter) as described previously (25).

Anti–VAP-1 Ab and SSAO inhibitor treatments

Wild-type mice were injected with 200 μg blocking-anti-mouse VAP-1 mAb (100 μg clone 7-106 + 100 μg clone 7-8/36) or with 200 μg control mAb (HB151 against human HLA-DR5 (American Type Culture Collection) per mouse (26) i.p. every other day. All of the Abs contained <0.1 IU endotoxin per milliliter as determined by the Limulus assay.

SSAO inhibitors ZSE5302 ([I(S),S,S]-2-(1-methylhydrizinyl)-l-indanol, also known as BTT-2052; a gift from Dr. F. Fülo¨p from University of Szeged, Szeged, Hungary) (15) and LPJ1586 ([S,3-fluoro-2-(4-methoxyphenyl)allyl]aminobenzyl)trimethylenemethane; a gift from M. Linnik, La Jolla Pharmaceuticals, Solana Beach, CA) were maintained in Vectashield (Vector Laboratories, Burlingame, CA) and examined using a BX 60 microscope (Olympus, Melville, NY) equipped with epifluorescence or an LSM510 Meta confocal microscope (Zeiss, Oberko- chen, Germany).

Intravital videomicroscopy

B16 melanoma cells (kindly provided by Dr. Isaiah J. Fidler, University of Texas, Houston, TX) were cultured and used to grow tumors in the flanks of C57BL/6 mice by s.c. injection of 10^5 cells in 100 μl in 0.9% NaCl solution (Sigma-Aldrich, St. Louis, MO). Intravital microscopic measurements were performed as described previously (27). Briefly, murine recombinant TNF-α (500 ng; R&D Systems, Minneapolis, MN) was injected i.p. 4 h before measurements, because leukocyte adhesion is hardly present in the absence of cytokines. Rhodamine 6G solution (Molecular Probes, Eugene, OR; 10–20 μl of 1 mg/ml solution in sterile 0.9% NaCl per mouse) was injected into the tail vein to label leukocytes. Tumor vessels were visualized using an intravital microscope (Leitz, Wetzlar, Germany), adapted for telescopic imaging and equipped with an SW25 objective lens (Leitz; numerical aperture 0.60). Microscopic images were recorded using a charge-coupled device camera (C3077; Hamamatsu, Ichinoco, Japan), coupled to an intensifier unit (C2400-80; Hamamatsu), and stored on a digital versatile disc (DVR-7000; Panasonic, Secaucus, NJ) for offline analysis.

The number of interacting leukocytes and experimental fluid dynamic parameters were determined as described previously (28). In brief, vessel diameters were measured using an image-sheeting device built in-house. Centerline blood flow velocity was determined by frame-to-frame analysis, using the fastest passing fluorescent leukocyte as a marker. The level of leukocyte rolling was determined by counting the number of rolling cells passing a vessel segment per minute. Leukocytes were considered as rolling along the vessel wall when their velocity was at least an order of magnitude lower than that of the free flowing blood cells. The level of leukocyte adhesion was assessed in a 100-μm vessel segment and ex-pressed as the number of cells per endothelial surface area (assuming the cross section of the venules to be circular). Leukocytes were considered adherent when they remained stationary for at least 30 s. The total number of leukocytes interacting with the vessel wall at a particular moment was determined as well. This parameter includes not only the numbers of ad-hering and rolling leukocytes but also the velocity with which the latter roll along the endothelium within a vessel segment of 100 μm in length. When leukocytes roll slowly, the number of interacting leukocytes observed in such a vessel segment will be higher than that when they roll faster, due to similar numbers of rolling and adhering leukocytes. To determine this parameter, we counted in a frozen video frame the total number of rolling and adhering leukocytes in a 100-μm vessel segment. At each time point, this count was performed in four randomly chosen video frames, and the data were averaged.

Immunohistochemistry

Five-micrometer frozen sections were cut, fixed in acetone, and used for immunofluorescence staining. Primary Abs were rat mAbs against mouse CD31 (MEC13.3), CD45, CD3, CD4, CD8, CD11b, Gr-1 (all from BD Pharmingen, San Diego, CA), F4/80 (AbD Serotec, Oxford, U.K.), FoxP3 (eBiosciences, San Diego, CA), mouse macrophage mannose receptor SD3 (gift from L. Martinez-Pomares, University of Nottingham, Nottingham, U.K.), and VAP-1. As a negative control, 9B5 (rat IgG) was used. Alexa Fluor 594-anti-rat IgG was used as the secondary reagent. The slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined using a BX 60 microscope (Olympus, Melville, NY) equipped with epifluorescence or an LSM510 Meta confocal microscope (Zeiss, Oberko- chen, Germany).

Zinc buffer (for CD31 and CD45) and formalin-fixed (for VAP-1), paraffin-embedded sections were used for immunoperoxidase staining using Vectastain kits (Vector Laboratories). For determination of the microvessel density, the sections were incubated with biotin-labeled Ulex europaeus agglutinin lectin (Sigma-Aldrich) followed by incubation of the sections with freshly prepared avidin–biotin complex (DakoCytomation, Carpin- teria, CA). The peroxidase activity was detected using diaminobenzidine (Sigma-Aldrich).

Quantifications of the staining were performed by counting the positive cells in the whole tumor area (i.e., ~120 high-power fields in each section; Figs. 2A, 3A, 4B, 4E, 4F, and 5B) or in five high-power fields (Fig. 5G) from every animal.

FACS analyses

Leukocytes were released from the melanomas using a collagenase D digestion for 30 min. The cells in suspension were stained using CD45-allophycocyanin-Cy7, CD11b-PE, Gr-1-FITC, and 7-aminoactinomycin D (as a viability marker). The expression of CD11b and Gr-1 among the viable cells and tumor cells (i.e., ~120 high-power fields in each section) was quantified using an LSRII flow cytometer (BD Biosciences, San Jose, CA).

To analyze the effect of SSAO inhibition on the expression of homing molecules on the myeloid cells, splenocytes were isolated from tumor-bearing vehicle- and SSAO532-treated mice and stained as above in the presence of mAbs against CD62L (MEM-14, FITC), CD16/32 (2PH1, PE), and VCAM-1 (clone 7DATK32-PE), and CD11a (2D7, FITC) (all obtained from BD Pharmingen). The cells were analyzed using an LSRII flow cytometer.

To study whether blockade of SSAO activity alters the expression of other endothelial adhesion molecules, we transduced HUVEC with adeno-niral vectors encoding for VAP-1, as described previously (15). The VAP-1-positive cells then were treated with the vehicle or SSAO532 and cultured for 2 d. For the first 15 h, the cultures were additionally stimulated or not with 30 U/ml recombinant human TNF-α. The expression of ICAM-1 and VCAM-1 on the cells after different treatments was analyzed using FACS staining with mAbs 5C3 (29) and P8B1 (from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), respectively.

To determine the possible effects of SSAO inhibition on the tumor cells, B16 melanoma cells and Chinese hamster ovary cells stably transfected with mouse VAP-1 were used for FACS analyses. The cells were stained without (surface) or with permeabilization (surface + cytoplasmic). A treatment for 20 s with ice-cold acetone followed by two washes in RPMI 1640 containing 10% FCS was used for the permeabilization. Primary Abs were used at concentrations of 10 μg/ml, and FITC-conjugated anti-rat IgG was used as a second stage. At least 10,000 cells per sample were analyzed using a FACS Calibur and CellQuest software (BD Biosciences).

Enzyme essays

The Amplex red assay (Molecular Probes) was used to measure SSAO activity from the cell and tissue lysates. Briefly, cells were lysed in 0.2%
NP-40 (Sigma-Aldrich) in PBS, and 100 μg clarified supernatant was used in the assays. The samples were incubated with or without semicarbazide (an SSAO inhibitor), and then benzylamine (an SSAO substrate) was added. Formation of hydrogen peroxide was followed kinetically after addition of the Amplex red detection mixture by measuring the fluorescence using a Tecan Ultra fluoropolarimeter (Tecan, Männedorf, Switzerland). Specific SSAO activity was counted by subtracting the emission values in semicarbazide-treated wells from those in nonpretreated wells and converting the signals to hydrogen peroxide production (picomoles per milligram of lysate per hour) using standard curves (9, 30).

Cell counts and proliferation assays

After treatment for 10 d with SZE5302 or vehicle, blood was drawn, and lymphatic organs were collected from the euthanized mice. The blood cell counts (leukocytes, erythrocytes, platelets, neutrophils, eosinophils, and basophils) and biochemistry (hemoglobin and hematocrit) were analyzed using an automated hemocytometer (Celltac α; Nihon Kohden, Tokyo, Japan) and differential counts. Leukocytes were teased out from minced lymphoid organs through a 44-μm steel mesh, and the number of cells was counted microscopically using trypan blue (Sigma-Aldrich).

The proliferation of B16 melanoma cells in the presence of SZE5302 in vitro was studied by analyzing the numbers of cultured cells using repeated alamarBlue (BioSource International, Camarillo, CA) measurements according to the manufacturer’s instructions. To study the role of SSAO activity in the growth of Gr-1+ myeloid cells, melanomas were inoculated into wild-type mice, and the tumors were digested as above at day 10. The cell suspension then was incubated with the anti–Gr-1-PE Ab (BD Biosciences), and the positive cells were isolated magnetically using the Easysep PE selection kit as suggested by the manufacturer (StemCell Technologies, Vancouver, British Columbia, Canada). The isolated cells were counted, divided into two, and cultured in RPMI 1640 containing 10% FCS, penicillin/streptomycin, and 0.9% NaCl (vehicle) or 40 μM SZE5302. The number of cells was counted microscopically at the beginning of the culture and after 1 d.

ELISA and immunoblots for cytokines and growth factors

The effect of SSAO inhibition on the production of different chemokines, cytokines, and other growth factors was evaluated using Proteome Profiler Mouse Cytokine Array Panel A kit (R&D Systems) according to the manufacturer’s instructions. In brief, spleens from wild-type mice were collected, and cell suspensions were made using mechanical teasing. The cells cultured in RPMI 1640 containing 10% FCS, penicillin/streptomycin, and 100 μM 2-ME in 96-well plates. At the beginning of the culture, the cells were stimulated with 100 ng/ml LPS (to induce cytokine synthesis) and treated with 10% NaCl (vehicle), 0.9% NaCl, 40 μM SZE5302, or 40 μM LPIP586. After being cultured for 20 h, the supernatants were collected. The samples were incubated on the membranes and developed using ECL. The intensities of the dots were measured using Gel Doc 2000 (Bio-Rad, Hercules, CA), and the background intensity was subtracted from all of the values. Vascular endothelial growth factor (VEGF) was measured from the sera of vehicle- and SZE5302-treated mice by ELISA and immunoblots (R&D Systems) according to the manufacturer’s instructions.

Frozen section adhesion assay

Binding of intratumoral myeloid cells into the tumor vasculature was analyzed ex vivo using a modification of the Stamper–Woodruff assay (31). In brief, melanoma tumors from vehicle- and SZE5302-treated mice were collected and stored as frozen blocks. Then, fresh melanomas were inoculated into wild-type mice (no treatment, 15 animals), and the Gr-1+ myeloid cells were isolated from the tumors using Easysep purifications at the end of the experiment as detailed above. The isolated cells were resuspended in the binding medium (RPMI 1640 supplemented with 10% FCS), and 0.5 × 10⁶ cells were overlaid on a 7-micron-thick frozen section encircled with a wax pen. After rotation for 30 min on an orbital shaker, the nonadherent cells were tilted off, and the bound cells were fixed to the sections by overnight incubation in a glutaraldehyde fixative. The number of leukocytes bound to vessels in vehicle- and SZE5302-treated melanomas was counted using dark-field microscopy. The number of leukocytes per vessel in the vehicle-treated tumor was assigned a value of 1.0 (relative adherence) by definition. At least 100 vessels in both tumor types were scored.

Statistical analysis

The effect of the test substance in comparison with an appropriate negative control treatment (control mAb versus anti–VAP-1 mAb treatment; vehicle versus inhibitor treatment) was analyzed using the Student t test (two-tailed, unpaired). For intravital videomicroscopy, Mann-Whitney U tests were used. The statistical significance was set at p < 0.05.

Results

VAP-1 is expressed in tumor vasculature but not in melanoma cells

Several endothelial adhesion molecules, including CD54/ICAM-1 and CD106/VCAM-1, are downregulated in tumors. In previous publications, it has been suggested that the diminished synthesis can be caused by VEGF, which is produced in large quantities in tumors (27, 32). Therefore, we first studied the expression and VEGF responsiveness of VAP-1 in tumors and normal tissues. When VEGF pellets were implanted into normal mouse tissues, many of the newly formed vessels synthesized VAP-1 protein in vivo (Fig. 1A). In B16 melanomas, ~20% of tumor vessels were found to be VAP-1-positive in vivo (Fig. 1B). Notably, the B16 melanoma cells themselves were VAP-1-negative. Thus, VAP-1, in contrast to many other endothelial molecules, is not downregulated in tumors, and it therefore might be important on the tumor vasculature for recruiting leukocytes into the tumors.

Anti–VAP-1 Abs attenuate leukocyte extravasation into tumors

To study the effect of function-blocking anti–VAP-1 mAbs on leukocyte extravasation into tumors, B16 melanomas were injected into recipient mice, and anti–VAP-1 mAb treatment was started at the same time. A treatment for 10 d with function-blocking anti–VAP-1 mAbs diminished leukocyte accumulation in the melanomas in a leukocyte-subclass–selective manner. The overall numbers of CD45+ intratumoral leukocytes were reduced significantly by >50% (Fig. 2A). Among the lymphocyte subpopulations, the accumulation of CD3- and CD8-positive T cells was reduced significantly, whereas the numbers of CD4-positive lymphocytes and T regulatory cells (FoxP3-positive cells) were unaltered. The anti–VAP-1 Ab treatment also did not affect the numbers of myeloid cells (F4/80-positive macrophages, macrophage mannose receptor–positive type 2 macrophages, Gr-1–positive, or CD11b-positive myeloid cells; Fig. 2A). Thus, when the adhesive epitope of VAP-1 is blocked by the Ab in vivo, the accumulation (recruitment, cell turnover, or both) of cytotoxic or suppressor lymphocytes into the tumors is selectively inhibited.

![FIGURE 1. VAP-1 is expressed in neangiogenic vessels in vivo. A, Immunohistochemical determination of microvessel density, number of VAP-1–positive vessels, and percentage of VAP-1–positive vessels in TNF-α–stimulated cremaster muscles treated with control (PBS, n = 7) and VEGF (n = 4) pellets for 3 d. B, Expression of VAP-1 in muscle and melanoma was determined by immunohistochemistry. The percentage of VAP-1–positive vessels was quantified (seven mice for muscle and four for tumor). All data are mean ± SEM. Original magnification ×100; insets, original magnification ×400. *p < 0.05 compared with control.](Image 332x127 to 511x280)
Table I. Fluid dynamic parameters in B16 melanoma vessels

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Anti–VAP-1 mAb</th>
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<tbody>
<tr>
<td>Number of mice</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Number of vessels</td>
<td>37</td>
<td>43</td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>30 (25–43)</td>
<td>30 (25–45)</td>
</tr>
<tr>
<td>Centerline velocity (mm/s)</td>
<td>1.7 (1.3–1.8)</td>
<td>1.6 (1.3–2.2)</td>
</tr>
<tr>
<td>Reduced velocity (U, s⁻¹)</td>
<td>27 (19–40)</td>
<td>35 (22–44)</td>
</tr>
<tr>
<td>Flow (Q, mm³/s)</td>
<td>9 (5–16) × 10⁻⁴</td>
<td>7 (5–11) × 10⁻⁴</td>
</tr>
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Data are presented as median values with interquartile ranges.
reduction in the growth of B16 melanoma was observed on days 7 and 10 (Fig. 5A). The inhibitor treatment was very efficient, because it led to an almost complete blockade of the SSAO activity in the mice (Fig. 5A). We found that the inhibitor treatment significantly reduced the number of CD31+ blood vessels in the tumors (Fig. 5B). Similarly, the treatment with LJP1586 blocked SSAO activity in vivo and attenuated significantly the growth of the melanomas in mice (Fig. 5C). Notably, although there was a significant correlation between the tumor size and the number of CD11b+ cells, tumors of a similar size had clearly more CD11b+ cells in the vehicle-treated groups when compared with those in the SSAO inhibitor-treated groups (Fig. 5D). These data suggest that the decreased numbers of myeloid cells in the SSAO inhibitor-treated mice is not secondary to the decreased tumor volumes.

On the basis of our earlier observations (12, 30), VAP-1 could play a direct role in supporting myeloid cell binding to the tumor vessels. To test this experimentally, we analyzed in an ex vivo frozen section adhesion assay the binding of purified tumor-infiltrating Gr-1+ myeloid cells to the tumor vessels obtained from tumors grown in vehicle- and SZE5302-treated mice. The results showed that significantly fewer Gr-1+ myeloid cells adhered to SSAO inhibitor-treated vessels than to control-treated vessels (Fig. 5E). These data suggest that VAP-1 in tumor vessels may directly support myeloid cell recruitment or that the SSAO activity modulates the expression, function, or both of other adhesion molecules in the microvascular environment into a more proadhesive phenotype.

The SZE5302 inhibitor also slowed down the expansion of EL-4 lymphoma cells in mice (Fig. 5F). Furthermore, it also significantly reduced the number of CD31-positive vessels in this model (Fig. 5G). Thus, inhibition of the neoangiogenesis and myeloid cell accumulation in the SSAO inhibitor-treated mice correlated positively with the reduced tumor growth in two different cancer models in vivo.

SSAO inhibitors selectively target host VAP-1 in vivo

The SSAO inhibitors could potentially directly target tumor cells, if they expressed VAP-1. Immunohistological analyses suggested that B16 tumors (and EL-4 lymphoma cells) in vivo are negative for VAP-1 (Fig. 1B and data not shown), but we wanted to address this question using more sensitive techniques. FACS staining showed that VAP-1 was absent from the surface and cytoplasm of B16 cells (Fig. 6A). Enzymatic assays also showed no SSAO activity in these cells (Fig. 6B). Moreover, in vitro proliferation of B16 cells was not altered in the presence of an SSAO inhibitor (Fig. 6C).

The inhibitors did not affect the production of any of the tested 40 chemokines and recruitment factors in the mice (Fig. 7A). The VEGF concentrations in the sera were comparable in the control- and SSAO inhibitor-treated mice (Fig. 7B). The numbers of Gr-1+ myeloid cells (VAP-1-negative by themselves) also were unaltered when cultured in the presence or absence of SSAO inhibition (Fig. 7C), suggesting that it does not affect the survival or turnover of these cells. The SZE5302 inhibitor, which was used in most experiments due to availability reasons, did not have nonspecific effects on the leucocyte counts in vivo because total leucocyte counts, the percentages of different subclasses in the blood, and the numbers of leucocytes in lymphoid organs (thymus, spleen, mesenteric lymph nodes (MLNs), peripheral lymph nodes, and Peyer’s patches) were similar to those in vehicle-treated mice (Fig. 7D). The numbers of erythrocytes and platelets in the blood or hemoglobin and hematocrit values were not affected (data not shown).

The expression of all of the leucocyte homing molecules studied (CD62L, CD11a, CD44, PSGL-1, CD49d, and α4β7) on the myeloid cells was similar in vivo in the absence and presence of SSAO inhibition (Table II). Moreover, the expression of endothelial adhesion molecules CD54/ICAM-1 and CD106/VCAM-1 also was unaffected by SSAO inhibitor treatment of mice in vivo. ICAM-1 was readily detectable on the vessels and leucocytes in MLNs of both vehicle- and SZE5302-treated mice, and VCAM-1 was abundant in dendritic cells in MLNs in both treatment groups. Notably, the intratumoral vessels lacked any ICAM-1 or VCAM-1 expression (Table II). The expression of all of the leukocyte homing molecules studied (CD62L, CD11a, CD44, PSGL-1, CD49d, and α4β7) on the myeloid cells was similar in vivo in the absence and presence of SSAO inhibition (Table II). Moreover, the expression of endothelial adhesion molecules CD54/ICAM-1 and CD106/VCAM-1 also was unaffected by SSAO inhibitor treatment of mice in vivo. ICAM-1 was readily detectable on the vessels and leucocytes in MLNs of both vehicle- and SZE5302-treated mice, and VCAM-1 was abundant in dendritic cells in MLNs in both treatment groups. Notably, the intratumoral vessels lacked any ICAM-1 or VCAM-1 expression (Table II).
VCAM-1 expression in these same assays (data not shown). Therefore, we confirmed the effect of SSAO inhibition on endothelial adhesion molecule expression in VAP-1–transfected HUVECs. Although SZE5302 treatment inhibited SSAO activity by ∼90% in these cells, neither the constitutive nor the TNF-α–induced synthesis of ICAM-1 or VCAM-1 was affected by the inhibitor (Table III).

We thus conclude that the catalytic activity of host VAP-1 is needed for normal growth of melanoma and lymphoma in vivo. Moreover, these data suggest that the most likely function of SSAO inhibitors is the direct blockade of VAP-1 in endothelial cells and the subsequent impairment of myeloid cell infiltration into tumors.

FIGURE 5. SSAO inhibitors attenuate tumor growth and neoangiogenesis. A, Melanoma-bearing mice were treated with the VAP-1/SSAO inhibitor SZE5302 or vehicle (n = 12–13 mice per group), and the growth of the tumors (volumes) was measured kinetically (left panel). The efficacy of SSAO inhibition was measured by determining the production of SSAO-derived hydrogen peroxide in the control- and SZE5302-treated mice at the end of the experiment (right panel). B, CD31-positive vessels (green) in melanomas were visualized in vehicle- and SZE5302-treated mice using immunohistochemistry. Blue is DAPI. The number of intratumoral CD31-positive vessels also was quantified (n = 5 mice per group, the entire tumor area from each). Scale bars, 50 μm. Original magnification 3200.

C, Proliferation of B16-Luc cells (mean ± SEM) in vitro in the presence or absence of SZE5302 was studied using alamar blue assay. *p < 0.01.

D, The numbers of intratumoral CD11b-positive myeloid cells were plotted against the tumor volumes from the vehicle- and SSAO inhibitor-treated mice bearing melanoma. The green lines highlight the mice that had tumors of a similar size range in the two treatment groups. Note that among these tumors the control mice had more CD11b+ cells than the SSAO inhibitor-treated mice. E, Intratumoral Gr-1+ cells were separated magnetically from wild-type mice bearing melanomas, and the adherence of these cells to vessels in melanomas harvested from vehicle- and SZE5302-treated mice was studied in vitro (n = 3 mice per group). F, Mice were treated with SZE5302 inhibitor, and the growth of EL-4 lymphoma was studied (n = 10 mice per group) (left panel). The efficacy of SSAO inhibition was measured by determining the production of SSAO-derived hydrogen peroxide in the control- and SZE5302-treated mice at the end of the experiment (right panel). G, The number of CD31-positive vessels within the EL-4 tumors was quantified from immunohistochemical staining. All of the data are mean ± SEM. *p < 0.05; **p < 0.01.

FIGURE 6. SSAO inhibitors selectively block VAP-1 in the host. A, FACS analysis of VAP-1 expression on the surface and in the permeabilized (total) B16-Luc melanoma cells. Cells were stained with an anti-mouse VAP-1 mAb (7-106) and a negative control mAb (9B5). Chinese hamster ovary cell transfectants expressing mouse VAP-1 served as a positive control. A representative experiment out of three with similar results is shown. B, B16-Luc cells were grown in vitro, and SSAO activity was measured from lysates (n = 5) using enzyme assays. VAP-1 expressing lysates from Chinese hamster ovary cell transfectants were used as a positive control. C, Proliferation of B16-Luc cells (mean ± SEM) in vitro in the presence or absence of SZE5302 was studied using alamar blue assay. *p < 0.01.
Discussion

Our results demonstrated that blocking the catalytic oxidase activity of VAP-1 impairs infiltration of selective leukocyte subtypes in tumors. The oxidase activity also supported tumor neoangiogenesis and growth in vivo. The anti-VAP-1 Abs, however, inhibited the migration of certain leukocyte types into the tumor but did not modify angiogenesis or tumor growth.

Several endothelial cell adhesion molecules, such as CD54/ICAM-1, CD106/VCAM-1, and CD62E/E-selectin, are down-regulated in tumors (27). VAP-1, in contrast, was still expressed in the newly formed vessels in both normal tissues upon VEGF induction and tumors. This suggests that VAP-1 may partly take over the function of other endothelial adhesion molecules in tumor vessels in supporting leukocyte migration into tumors. Our real-
Table II. Homing molecule expression in leukocytes is preserved after SSAO inhibition in vivo

<table>
<thead>
<tr>
<th>Ag</th>
<th>Vehicle</th>
<th>SZE5302</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%(a)</td>
<td>MFI(b)</td>
</tr>
<tr>
<td>CD62L</td>
<td>84 ± 4</td>
<td>2279 ± 779</td>
</tr>
<tr>
<td>CD11a</td>
<td>100 ± 0</td>
<td>4367 ± 142</td>
</tr>
<tr>
<td>CD44</td>
<td>100 ± 0</td>
<td>34,161 ± 5289</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>97 ± 2</td>
<td>31,886 ± 5714</td>
</tr>
<tr>
<td>CD49d</td>
<td>95 ± 3</td>
<td>8878 ± 1570</td>
</tr>
<tr>
<td>α4β7</td>
<td>66 ± 8</td>
<td>10,590 ± 1959</td>
</tr>
</tbody>
</table>

1Melanoma-bearing mice were treated with vehicle or SZE5302 (50 mg/kg) for 10 d.
2Percentage of positive cells from splenic Gr-1\(^+\)CD11b\(^+\)CD45\(^+\) cells in FACS analyses.
3Mean fluorescence intensity (MFI) for the given Ag of splenic CD45\(^+\)CD11b\(^+\) cells.

Table III. SSAO inhibition does not alter the expression of endothelial adhesion molecules

<table>
<thead>
<tr>
<th>Ag</th>
<th>Vehicle without TNF-α</th>
<th>Vehicle with TNF-α</th>
<th>SZE5302 without TNF-α</th>
<th>SZE5302 with TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%(a)</td>
<td>MFI(b)</td>
<td>%(a)</td>
<td>MFI(b)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>4 ± 2</td>
<td>4 ± 1</td>
<td>50 ± 7</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>1 ± 0</td>
<td>3 ± 0</td>
<td>45 ± 5</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

\(^a\)Percentage of positive cells in FACS.

\(^b\)MFI of cells in FACS.
We showed that binding of intratumoral myeloid cells to the tumor vessels is diminished when the target tumor vasculature originates from an SSAA inhibitor-treated animal. These data support the idea that VAP-1/SSAO activity directly supports leukocyte adhesion to endothelial cells or that it modulates the microenvironment or other molecules involved in cell immigration in a manner that renders it more proadhesive. In contrast, SSAA inhibition did not alter the growth of purified Gr^1^+ myeloid cells. In conjunction with previous publications demonstrating a role for VAP-1 in leukocyte recruitment, we favor the idea that also in tumors VAP-1 may regulate cell recruitment rather than cell proliferation or death in the tumor.

Our data suggest that use of small-molecule inhibitors to block SSAO activity might offer a new way to inhibit trafficking of Gr^1^+ CD11b^+ myeloid cells and inhibit tumor neoangiogenesis and tumor growth. This may be clinically relevant, because human tumor vasculature also expresses VAP-1 at least in the two cancer types studied (i.e., hepatocellular carcinoma and head and neck carcinomas) (41, 42). Moreover, VAP-1 gene amplification has been found in gastric cancer patients (43). Targeting ectoenzymes, such as VAP-1, is relatively easy, because the catalytically active site is outside the plasma membrane and the crystal structure of the molecule has been solved (44). In fact, multiple small-molecule inhibitors have been developed recently, and they are being tested for anti-inflammatory purposes (17, 23, 24). However, anti–VAP-1 inhibitors have been developed recently, and they are being studied (i.e., hepatocellular carcinoma and head and neck carcinomas) (41, 42). Moreover, VAP-1 gene amplification has been found in gastric cancer patients (43). Targeting ectoenzymes, such as VAP-1, is relatively easy, because the catalytically active site is outside the plasma membrane and the crystal structure of the molecule has been solved (44). In fact, multiple small-molecule inhibitors have been developed recently, and they are being tested for anti-inflammatory purposes (17, 23, 24). However, anti–VAP-1 Abs that are also being tested as anti-inflammatory drugs should not have potential tumor immunity-related side effects, inasmuch as they do not affect angiogenesis or tumor growth. Because SSAO inhibitors inhibit angiogenesis through a unique mechanism targeting immune cell trafficking, they alone, or in combination with therapies targeting vascular growth factors, might be useful for boosting antiangiogenic therapies.

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Disclosures

S.J. is a shareholder of Bioite, a Finnish biotech company developing anti–VAP-1–based anti-inflammatory agents.

References


