Vascular Adhesion Protein-1 Enhances Tumor Growth by Supporting Recruitment of Gr-1+CD11b+ Myeloid Cells into Tumors

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Abstract

Cancer growth is regulated by several nonmalignant cell types, such as leukocytes and endothelial cells, which reside in the stroma of the tumor. Vascular adhesion protein-1 (VAP-1) is an amine oxidase enzyme that is expressed on the surface of endothelial cells. It supports leukocyte traffic into inflamed tissues, but nothing is known about its possible role in cancer biology in vivo. Here, we report that B16 melanoma and EL-4 lymphoma remain smaller in VAP-1–deficient mice than in wild-type controls. We found an unexpected defect in tumor angiogenesis in the absence of VAP-1. VAP-1 also selectively enhanced the recruitment of Gr-1+CD11b+ myeloid cells into the tumors. Generation of mice expressing enzymatically inactive VAP-1 showed that the oxidase activity of VAP-1 was necessary to support neoangiogenesis, myeloid cell recruitment, and tumor growth in vivo. These data describe VAP-1 as the first adhesion molecule known to be involved in the recruitment of Gr-1+CD11b+ myeloid cells into tumors. They also suggest that VAP-1 is a potential new tool for immunotherapy of tumors that could be exploited to reduce tumor burden by controlling the traffic of Gr-1+CD11b+ myeloid cells. [Cancer Res 2009;69(19):7875–83]

Introduction

Leukocyte trafficking from blood to tissues is not only a prerequisite for mounting normal immune responses against microbes but also needed for immunosurveillance against malignantly transformed cells. Normally, leukocytes leave the blood using a multistep extravasation cascade involving many activation and adhesion molecules both on the leukocyte surface and on the endothelial lining (1). Endothelial adhesion molecules are needed for leukocyte extravasation into normal tissues as well as into tumors. Likewise, many of the chemokines presented on the endothelial cells guide the activation and chemotaxis of leukocytes both under physiologic conditions and at sites of tumor formation (2). Moreover, tumor cells of both leukocytic and nonleukocytic origin use these adhesion molecules and chemokines to facilitate their own dissemination (3).

Vascular adhesion protein-1 (VAP-1) is one of the endothelial molecules that support rolling, firm adhesion, and transmigration of various subsets of leukocytes into sites of inflammation (4, 5). Unlike the classic adhesion molecules, VAP-1 is a cell surface enzyme that has a catalytic site outside the plasma membrane (6). It belongs to a specific subgroup of amine oxidases known as semicarbazide-sensitive amine oxidases (SSAO; ref. 7). These enzymes catalyze oxidative deamination of amines into corresponding aldehydes in a reaction that also produces hydrogen peroxide and ammonium. The adhesive role of VAP-1 in leukocyte trafficking has been verified using VAP-1–deficient mice (8). In the absence of VAP-1, leukocyte rolling, firm adhesion, and transmigration are impaired in inflamed vessels. The defective leukocyte extravasation cascade in the VAP-1–deficient mice leads to blunted inflammatory responses in several models, including peritonitis, arthritis, and mucosal immunization (8–10).

VAP-1 gene is amplified in gastric cancer (11), and histologic analyses with head and neck, liver, and melanoma tumors have shown that vessels within the cancer express VAP-1 in humans (12–14). However, no information is available on the role of VAP-1 in tumor growth in vivo. VAP-1 might affect tumor progression by regulating the entrance of leukocytes into emerging tumors. Moreover, the biologically potent end products (e.g., hydrogen peroxide) produced through the catalytic activity of VAP-1 might have local effects in the tumor microenvironment. Here, we found that VAP-1 activity enhances tumor angiogenesis. It also enhances tumor growth by increasing the recruitment of myeloid leukocytes into the tumors. These data reveal VAP-1 as the first adhesion molecule involved in recruitment of these cells into tumors and suggest that blocking of VAP-1 may be a future tool for immunotherapy of cancer.

Materials and Methods

Mice. VAP-1–/– mice on C57BL/6 background (>10 generations of backcrossing from 129S6 to C57BL/6 background) have been previously described (8). Wild-type (wt) C57BL/6 mice were used as controls. We also generated a new transgenic mouse expressing enzymatically inactive human VAP-1 transgene (carrying the point mutation Y471F) in the endothelial cells of VAP-1–deficient mice (Supplementary Materials and Methods; Supplementary Fig. S1).

Age- and sex-matched animals were used in all studies. Mice were maintained in specific pathogen-free environment and had access to food and water ad libitum. All animal procedures were approved by the local ethical committees on the use of laboratory animals.

Tumor models. The B16-F10-luc-G5 melanoma cells (Xenogen), which are stably transfected with luciferase, were cultured in MEM/Earle’s balanced salt solution (HyClone) supplemented with 10% fetal bovine serum, nonessential amino acids, 200 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and MEM vitamin solution. EL-4 T-lymphoma cells (American Type Culture Collection) were grown in RPMI 1640 containing 10% FCS, L-glutamine, and penicillin-streptomycin. B16-F10-luc-G5 melanoma cells (4 × 105 per mouse) or EL-4 cells (10 × 106 per mouse) in 200 μL of RPMI 1640 were injected s.c. into the shaved abdominal area of anesthetized (ketamine and xylazine) mice. Tumor sizes were kinetically measured by an electronic caliper (Mitutoyo). The volume of the tumor spheroid was calculated according to the formula V = π/6 × (shortest diameter)2 × (longest diameter), as described (15).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Tumor Microenvironment
In certain experiments, the tumor sizes in wt and VAP-1–deficient mice were followed daily. Each tumor was collected when its size reached 1,000 mm³, regardless of the time needed to reach this predetermined, fixed tumor size.

Matrigel plugs and bioluminescence imaging. B16-Luc cells (4 × 10⁵ in 200 μL) were mixed with Matrigel (300 μL; a basement membrane extract; Becton Dickinson) and injected to the flanks of recipient mice according to the manufacturer's instructions. Real-time in vivo luciferase bioluminescence imaging was performed at days 0, 1, 4, 7, and 10 as described previously (16). In brief, mice were anesthetized with 2.5% isoflurane. α-Luciferin sodium salt (a luciferase substrate; Synchem) was injected i.p. (150 mg/kg) to mice 10 min before imaging. A black and white photographic image was acquired using the IVIS 50 workstation (Xenogen). Signal intensity was quantified as the photon counts from the region of interest and converted into a pseudocolor image using the Living Image software (Xenogen).

Antibodies and immunohistochemistry. Rat monoclonal antibodies (mAb) against mouse CD31 (MEC13,3), CD11b, Gr-1 (all from BD Pharmingen), mouse macrophage mannose receptor SD1i (gift from Dr. L. Martinez-Pomares, University of Nottingham, Nottingham, UK), MECA-32 (gift from Dr. E. Butcher, Stanford University, Stanford, CA), and VAP-1 (7-106) were used in immunostainings. Polyclonal rabbit antibodies reacting with mouse VAP-1, Lyve-1 (ReliaTech), and NG-2 (Chemicon) antigen were also used. A rabbit anti-human β-actin, cross-reactive with mouse β-actin, was from Sigma. As negative controls, 9B5 (rat IgG) and normal rabbit Ig were used. Primary antibodies were detected using Alexa Fluor 488–conjugated anti-rat IgG and Alexa Fluor 546–conjugated anti-rabbit IgG, as appropriate. Vectashield was used as the mounting medium, and the slides were examined using Olympus BX60 microscope equipped with epifluorescence or with Zeiss LSM510 Meta confocal microscope.

Quantifications of the stainings were performed by counting the numbers of positive cells in the whole tumor area, that is, ~120 high-power fields (original magnification, ×400) in each section (Figs. 1C, 2B, 3A, 4C, 5D, and 6D). Five (Fig. 1D) and nine (Figs. 4B and 5C) randomly chosen high-power fields, respectively, were examined from every animal. Imagej analysis program was used to define the area of positivity and the perimeter of the vessels.

Fluorescence-activated cell sorting analyses. Bone marrow cells, blood leukocytes, and splenocytes were isolated from melanoma-bearing wt and VAP-1–deficient mice using standard techniques. Tumor-infiltrating leukocytes were isolated from minced tumor pieces after collagenase D digestion (1 mg/mL, 37°C, 40 min; with DNase I). The cells were stained with LSRII flow cytometer (Becton Dickinson) anti-bodies: treated with 7-aminocinomycin D (7-AAD); and analyzed using LSRII flow cytometer (Becton Dickinson) with two different excitation wavelengths (488 and 635 nm). Viable leukocytes were defined based on CD45-APC-Cy7, CD11b-PE, and Gr-1-FITC (Becton Dickinson) anti-bodies, and the result was confirmed using a vascular endothelium–specific marker, MECA-32 (Fig. 1C). In contrast, NG-2 and Lyve-1 immunostainings revealed that the numbers of mature pericyte-containing blood vessels and the numbers of lymphatic vessels in the tumors were not altered in the absence of VAP-1 (Fig. 1B).

Enzyme essays. The SSOA activity from the cell and tissue lysates was measured using a previously described protocol (8, 17).

In vivo Gr-1 depletion experiments. Gr-1 antibody (LEAF-purified anti-mouse Ly-6G/Ly-6C, clone RB6-8C5) for in vivo use was purchased from Biolegend. HB151 served as an isotype-matched control antibody. The depletion protocol was modified from refs. 18–20. Briefly, the mice were treated with 250 μg of the mAbs i.p. at days −1, 1, 3, 5, and 7, and 9. Melanoma tumor cells were injected s.c. at day 0 to all mice, and the growth of the tumor volume was followed kinetically as described above. At day 10, the mice were killed, and blood was drawn via an intracardiac puncture and the tumors were collected. Leukocytes were released from the tumors as described above, and the intratumoral leukocytes and blood cells were subjected to four-color fluorescence-activated cell sorting (FACS) stainings as described above. Pieces of the tumor were snap frozen and used for histologic analyses as described above.

Ex vivo leukocyte-endothelial binding assay. Melanomas were induced in wt mice, and at day 10, the intratumoral leukocytes were isolated as described above. Myeloid cells were then separated using magnetic cell sorting (EasySep, Stem Cell Technologies) with the Gr-1 antibody according to the instructions of the manufacturer. The Gr-1– cells were resuspended in RPMI 1640 containing 10% FCS and overlaid on freshly cut frozen sections of melanoma tumors grown in wt and VAP-1–deficient mice. The ex vivo adhesion assay was then performed as described earlier (21, 22). Briefly, the myeloid cells were allowed to stick to vessels in the sections for 30 min at +4°C under rotatory conditions. Thereafter, the nonbound cells were gently decanted off from the section, and the bound cells were fixed in 2% glutaraldehyde. The leukocyte-interacting intratumoral blood vessels were then counted using dark-field microscopy. At least 100 vessels from three independent tumors were scored in both genotypes, and the average number of leukocytes bound per vessel in wt mice defines the relative adherence ratio 1.0.

cDNA microarray, quantitative PCR, vascular endothelial growth factor ELISA, and immunoblotting. cDNA microarray, quantitative PCR, vascular endothelial growth factor (VEGF) ELISA, and immunoblotting have been described in Supplementary Materials and Methods.

Statistics. All statistical analyses were performed using Mann-Whitney U tests. The statistical significance was set at P < 0.05.

Results

Tumor growth is regulated by VAP-1. We examined the role of VAP-1 in tumor growth by injecting B16 melanoma cells s.c. into wt and VAP-1–deficient (VAP-1−/−) mice. The preexisting peritumoral vessels and many of the newly formed intratumoral vessels were VAP-1+ in the wt mice (Fig. 1A). Kinetic analyses revealed that after an initial period of similar growth, tumors started to grow slover in VAP-1–deficient mice after day 4 (Fig. 1B). At day 10, the tumor volumes were 41 ± 5% smaller in VAP-1–deficient mice when compared with wt controls (P = 0.01; n = 23–24 mice per genotype). Thus, lack of VAP-1 impairs the growth of melanoma in vivo.

VAP-1 supports neoangiogenesis in tumors. Because VAP-1 was prominently present in tumor vessels in wt mice, we asked if the absence of VAP-1 would affect the vasculature in tumors. Strikingly, there was a 42 ± 5% reduction in the number of CD31+ vessels in the VAP-1–deficient tumors when compared with wt controls (P = 0.02; n = 6 mice per group; Fig. 1C). A significant reduction in the surface area of CD31+ cells and vessel perimeter within tumors was also found in the VAP-1–deficient mice (Fig. 1C). The diminished neoangiogenesis in the absence of VAP-1 was confirmed using a vascular endothelium–specific marker, MECA-32 (Fig. 1C). In contrast, NG-2 and Lyve-1 immunostainings revealed that the numbers of mature pericyte-containing blood vessels and the numbers of lymphatic vessels in the tumors were not altered in the absence of VAP-1 (Fig. 1D).

As another model of angiogenesis, we used luciferase-tagged B16 melanoma cells implanted in Matrigel plugs. The newly formed vessels were VAP-1+ in wt mice but lacked, as expected, VAP-1 in the VAP−/− mice (Fig. 2A). Quantification of CD31 immunostainings showed that the growth of new vessels was significantly impaired (by 53 ± 6%; P = 0.02; n = 8 mice per group) in the absence of VAP-1 (Fig. 2B). Kinetic bioluminescence imaging revealed a 68 ± 7% attenuation in tumor growth in the absence of VAP-1 activity at day 10 (P < 0.05; n = 8 mice per group; Fig. 2C and D). Together, these data reveal a novel role for a leukocyte trafficking molecule VAP-1 in tumor angiogenesis and growth in vivo.

VAP-1 is not indispensable for physiologic vessel formation. Vasculogenesis, angiogenesis, and lymphangiogenesis are vital for normal development (23). VAP-1 does not seem to be vital for the development of vessels because VAP-1–deficient mice reproduce normally. Moreover, we found that the numbers of CD31+ vessels in the normal skin (wt: 427 ± 15 vessels/mm²; VAP-1−/−: 423 ± 8 vessels/mm²) and heart (wt: 3,563 ± 178 vessels/mm²; VAP-1−/−: 3,683 ± 251 vessels/mm²; mean ± SE; n = 5–6 mice per group)
were similar in both genotypes. The number of Lyve-1+ lymphatic vessels in normal tissues was not altered in the absence of VAP-1 either (in lung, wt mice had 20 ± 1 vessels/mm² and VAP-1−/− mice had 20 ± 1 vessels/mm²; mean ± SE; n = 3 mice per group). Thus, physiologic formation of blood vessels during development and lymphangiogenesis is apparently VAP-1 independent, although many of the blood vessels express VAP-1.

**VAP-1 regulates recruitment of myeloid cells into the tumor.** In search of VAP-1–dependent alterations in tumors, we performed preliminary microarray and quantitative PCR experiments from melanomas growing in wt and VAP-1–deficient mice. We found decreased expression of transcripts highly expressed in myeloid cells [matrix metalloproteinase-9 (MMP-9), S100a8, and S100a9] in the absence of VAP-1 (Supplementary Fig. S2). This led us to test whether VAP-1 would be involved in the recruitment of proangiogenic and immune-suppressing Gr-1+CD11b+ cells into the tumors. Our quantitative histologic analyses in situ showed that the number of CD11b+ myeloid cells was reduced by 66 ± 9% (mean ± SE; n = 5 mice per group; P = 0.02) and the numbers of intratumoral Gr-1+ granulocyte-like cells were also reduced by >50% in the melanomas of VAP-1–deficient mice when compared with wt controls (Fig. 3A). Importantly, most CD11b+ cells coexpressed Gr-1 in the tumors (Fig. 3A), which defines them as myeloid-derived suppressor cells (24, 25). In contrast, the numbers

of type 2 macrophages, another immune-suppressing leukocyte population (26), were not altered in the tumors in the absence of VAP-1 (wt: 63.6 ± 8.3 and VAP-1−/−: 67.2 ± 13.1 macrophage mannose receptor–positive cells/mm2; mean ± SE; n = 6 mice per group).

To get further insight into VAP-1–dependent Gr-1+CD11b+ myeloid cell migration, we used four-color FACs analyses of bone marrow, blood, spleen, and tumor cells in melanoma-bearing wt and VAP-1–deficient mice. The analyses of cell numbers and percentages of Gr-1+CD11b+ cells showed that VAP-1 was not needed for the normal production of these cells in bone marrow, for their emigration into blood, or for their homing to the spleen (Fig. 3B). However, when the cells were isolated from the melanomas, the leukocyte number was reduced by 67 ± 14% in the absence of VAP-1, which is consistent with our histologic analyses. FACs analyses showed that >80% of the tumor-infiltrating CD11b+ leukocytes coexpressed Gr-1 (i.e., are myeloid-derived suppressor cells) in wt mice. In wt mice, 11.9 ± 2.9% of the tumor-infiltrating leukocytes were CD11b+Gr-1high (i.e., granulocyte-like cells), whereas only 1.4 ± 1.5% of cells had this phenotype in the absence of VAP-1 (Fig. 3C and D). The identity of these cells as granulocyte-like subpopulations of myeloid-derived suppressor cells (as opposed to CD11b+Gr-1intermediate monocyte-like myeloid-derived suppressor cells; ref. 27) was also verified using separate antibodies against Ly6G and Ly6C (data not shown). Together, the histologic and FACs data thus show that the production, emigration, or splenic homing of Gr-1+CD11b+ myeloid cells is not dependent on VAP-1. In contrast, in the absence of VAP-1, a significant 60% to 70% reduction of Gr-1+CD11b+ myeloid cells is seen in tumors, and the recruitment of granulocyte-like cells is particularly strongly affected.

Because the numbers and density of Gr-1+CD11b+ cells are typically smaller in small tumors than in large ones (24), the reduction of these myeloid cells in tumors of VAP-1–deficient mice might be either a secondary consequence of the reduced tumor size or a more causally linked to reduced VAP-1–dependent recruitment of these cells. To distinguish between these possibilities, we used three different approaches. First, we depleted the Gr-1+ cells from wt and VAP-1–deficient mice to see if VAP-1 affects the melanoma progression in a myeloid cell–dependent manner. The anti–Gr-1 antibody efficiently reduced the numbers of myeloid cells both in the blood and in the tumor in both genotypes in comparison with a negative control mAb treatment (Fig. 4A). The depletion of CD11b+Gr-1high cells was particularly good. Kinetic analyses of the tumor volumes showed that in the control mAb–treated VAP-1–deficient mice, the tumor growth was significantly slower when compared with control antibody–treated wt mice (61 ± 5% reduction at day 10; P = 0.009; n = 5 mice per group; Fig. 4B). This finding showed that repeated dosing of an irrelevant antibody into mice did not alter the original observation of reduced tumor progression seen in nonmanipulated VAP-1–deficient mice. In wt mice, depletion with the Gr-1 antibody significantly reduced the growth of tumors. In contrast, the anti–Gr-1 treatment had no significant effect on tumor growth in VAP-1–deficient mice. These data suggest that the myeloid cells do contribute to tumor burden in wt mice and that they use VAP-1 for their normal functions. This finding would be compatible with the possibility that VAP-1 is used in the recruitment of these cells into tumors.

As a second approach, we determined the numbers of Gr-1+CD11b+ cells in tumors of similar size in both genotypes. For this purpose, we collected the tumors from wt and VAP-1–deficient mice as soon as they reached the 1,000 mm3 size. Histologic analyses showed that there was a significant 51 ± 10% reduction (mean ± SE; n = 5 mice per group; P < 0.05) in the numbers of CD11b+ cells in these fixed-size tumors when the host lacked VAP-1 (Fig. 4C). These results suggest that the absence of VAP-1 impairs myeloid cell recruitment into tumors independently of the tumor size.

Finally, we used an ex vivo binding assay to study whether endothelial VAP-1 in the tumors would support myeloid cell
binding. We immunomagnetically isolated Gr-1+ myeloid cells from the melanomas grown in wt mice and analyzed their binding to vessels in frozen sections of melanoma, which were obtained from either wt mice (VAP-1+ endothelium) or VAP-1−deficient mice (VAP-1− endothelium). When the number of myeloid cells bound per intratumoral vessel was determined using microscopic countings, we found that statistically significantly fewer (40 ± 0.1% reduction; n = 3 tumors/genotype; P < 0.05) myeloid cells interacted with VAP-1−deficient vessels when compared with wt vessels (Fig. 4D). These data suggest that VAP-1 protein can be directly used as an adhesion molecule in the recruitment of myeloid cells into tumor or that VAP-1 activity alters the expression or function of some other endothelial molecules involved in tumor homing of myeloid cells.

CD11b+Gr-1+ cells are known to be proangiogenic and to produce VEGF (26) and could therefore provide a link between VAP-1 and angiogenesis. We found that the intratumoral VEGF concentrations were lower in VAP-1−deficient mice than in wt mice, whereas the circulating VEGF protein levels were similar in both genotypes (Supplementary Fig. S3). We also found that purified Gr-1+ cells from the wt and VAP-1−deficient mice produced similar levels of VEGF-A mRNA per cell basis (Supplementary Fig. S3). However, the perimeter and surface area of CD31+ vessels was not altered in wt mice after Gr-1 depletion in this model (Fig. 4B). Because many Gr-1+ intermediate cells resisted the depletion, these data do not provide definite evidence that the Gr-1+CD11b+ population could not contribute to the angiogenesis. Therefore, we conclude that decreased recruitment of myeloid cells in VAP-1−deficient animals might contribute to decreased local VEGF levels and reduced neoangiogenesis, but some other VAP-1−dependent mechanisms seem to dominate in the vessel growth in this model.

VAP-1 enhances local growth of lymphoma. We then asked whether VAP-1 is needed for normal expansion of different tumor cell types. We observed that after s.c. injections of EL-4 T-cell lymphoma cells, significantly smaller tumors developed in VAP-1−deficient mice when compared with wt hosts at days 14 and 17 (Fig. 5A). Neovessels within the lymphoma expressed VAP-1 in wt mice (Fig. 5B). Notably, the number of CD31+ vessels was significantly reduced within the lymphoma in the absence of VAP-1 (Fig. 5C). Significantly fewer Gr-1+CD11b+ myeloid cells (71 ± 3% reduction; n = 5 mice per group; P = 0.009) were found in the lymphomas in the absence of VAP-1 (Fig. 5D). These data thus suggest that VAP-1 supports angiogenesis, recruitment of Gr-1+CD11b+ myeloid cells, and tumor growth in several tumor types.

The oxidase activity of VAP-1 is necessary for its tumor-promoting effects. To pinpoint the mechanistic role of the catalytic activity of VAP-1 in tumor progression, we generated VAP-1 transgenic mice expressing enzymatically inactive VAP-1 protein (a single point mutation Tyr471→Phe471) in vessels of VAP-1−deficient mice (Fig. 6A and B; Supplementary Fig. S1). The Tie1 promoter directs VAP-1 expression specifically into endothelial cells, whereas the wt VAP-1 is present in the vessels both in the endothelial and pericyte/smooth muscle layer.
However, in immunohistochemical stainings, the VAP-1 expression was grossly similar in the vessels of melanomas and normal tissue (heart) in mice producing wt and catalytically inactive VAP-1, and based on immunoblot analyses, the VAP-1 protein levels were not statistically significantly different either, although clear interindividual variation was evident (Fig. 6A; Supplementary Fig. S4). We found that in these mutant mice, the expansion of melanoma tumors was significantly slower than in wt mice but indistinguishable from that seen in VAP-1+/− mice (n = 12 mice per group; Fig. 6C). The migration of Gr-1+CD11b+ myeloid cells into tumors in the mutant mice was also similar to that seen in VAP-1-deficient mice (Fig. 6D). Hence, the mice expressing enzymatically inactive VAP-1 point mutant provide genetic evidence that only the expression of VAP-1 protein with the catalytic activity is sufficient for supporting wt migration of Gr-1+CD11b+ myeloid cells into the tumors.

**Figure 4.** Gr-1+ myeloid cells mediate the VAP-1-dependent increase in tumor growth and adhere preferentially to VAP-1+ vessels. A, melanoma-bearing wt and VAP-1-deficient mice were treated with anti-Gr-1 or negative control mAb as detailed in Materials and Methods. At day 10, leukocytes were isolated from the blood and tumors and stained for CD11b, Gr-1, CD45, and 7-AAD. Representative FACS plots showing the expression of CD11b and Gr-1 on viable leukocytes. The percentages of CD11b+Gr-1high and CD11b+Gr-1intermediate populations were determined (n = 5 mice per group). B, the growth of melanoma and neoangiogenesis in the four different antibody-treated groups was analyzed using volume measurements and immunohistology (n = 5 mice per group). C, the numbers of CD11b+ cells in the fixed-sized tumors (≤1,000 mm3) growing in wt and VAP-1-deficient mice (no antibody treatments) were determined using immunohistochemistry (n = 3 mice per group). D, the binding of tumor-derived wt Gr-1+ cells to the vessels of melanoma grown in wt or VAP-1-deficient mice was determined using an ex vivo leukocyte-endothelial cell interaction assay. The number of myeloid cells bound per one intratumoral blood vessel is assigned a value of 1.0 in wt tumors by definition (n = 3 tumors/group). *, P < 0.05; **, P < 0.01.
Discussion

Here, we show that an endothelial adhesion molecule, VAP-1, (a) promotes tumor expansion by enhancing recruitment of Gr-1^CD11b^ myeloid cells into tumors and (b) increases tumor neoangiogenesis and that (c) the oxidase activity of VAP-1 (dependent on the modification of Tyr^471 to topaquinone) is needed for normal recruitment of Gr-1^CD11b^ myeloid cells, neoangiogenesis, and tumor progression in vivo. Our data reveal a novel role for amine oxidases in angiogenesis and Gr-1^CD11b^ myeloid cell biology and suggest that therapeutic inhibition of VAP-1 activity could be exploited to inhibit tumor growth.

We found that VAP-1 expression was retained in the neovessels within the tumors. VAP-1 protein supported recruitment of myeloid cells into tumors in a selective manner because VAP-1 played no role in the tumor homing of immune-suppressing type 2 (alternatively activated) macrophages or regulatory T cells (data not shown). In contrast, Gr-1^CD11b^ myeloid cell infiltrations were significantly diminished (by 60–70%) in VAP-1–deficient mice. Even when the tumor sizes were fixed, there were fewer Gr-1^CD11b^ cells in the tumors of VAP-1–deficient mice when compared with wt mice. Moreover, depletion of these myeloid cells in vivo significantly inhibited tumor growth in wt mice but had no effect in VAP-1–deficient animals. The tumor vasculature from wt mice also supported the adhesion of the myeloid cells better than that from VAP-1–deficient mice. Collectively, these data suggest that there may be a causal link between VAP-1–dependent recruitment of myeloid cells (Gr-1^high^ cells in particular) into tumor and tumor growth.

Gr-1^CD11b^ myeloid cells comprise immature macrophages, granulocytes, and dendritic cells of highly plastic nature (24, 26). Their production in bone marrow, their release into blood, or their homing to spleen in tumor-bearing mice was not affected by VAP-1. In contrast, the numbers of Gr-1^CD11b^ myeloid cells were significantly decreased in tumors in the absence of VAP-1, and the “granulocyte-like” cell types were preferentially affected. Similar to other leukocyte types (1), Gr-1^CD11b^ myeloid cells probably also use the multistep extravasation cascade to enter the tumors. Although the role of chemotactic factors and activation signals such as S100A8, CCR2, and Bv8 in Gr-1^CD11b^ myeloid cell production and migration is starting to emerge (28–31), to our knowledge, VAP-1 is the first adhesion molecule and the first endothelial molecule known to be involved in the recruitment of these cells.

Gr-1^CD11b^ myeloid cells have multiple tumor-promoting effects (24, 32–34). They show high proangiogenic activity as they secrete actively MMP-9, which facilitates endothelial cell migration and liberates matrix-bound growth factors such as VEGF-A (28). The ability of Gr-1^CD11b^ cells to induce vessel formation has also been confirmed in vitro (35). Gr-1^CD11b^ myeloid cells may also differentiate into endothelial cells (36). We also found decreased MMP-9 and VEGF levels and diminished neoangiogenesis in tumors growing in VAP-1–deficient mice, which have reduced numbers of these myeloid cells in the tumors. Nevertheless, the Gr-1 depletion experiments suggest that angiogenic stimuli originating from other cell types in the tumor, or from Gr-1^intermediate^ cells, may dominate in our melanoma model. Importantly, Gr-1^CD11b^ myeloid cells inhibit tumor-specific T-lymphocyte responses through various mechanisms.

Figure 5. VAP-1 supports the recruitment of myeloid cells, angiogenesis, and tumor growth in lymphoma.
A, EL-4 T-lymphoma cells were injected s.c. into wt and VAP-1–deficient mice and the growth of the lymphomas (volume) was measured kinetically. B and C, immunohistochemical stainings and quantification of (B) VAP-1 and (C) CD31 expression in the tumor vessels in EL-4 lymphomas at day 17 (quantification from nine high-power fields per mouse). Some positive (brown) vessels are pointed out by arrows. Note that the lymphoma cells also express low levels of CD31. Bar, 50 μm. D, the numbers of CD11b^ cells were determined using immunohistochemistry (>100 high-power fields/mouse). Columns, mean of five to six mice per groups; bars, SE. *, P < 0.05; **, P < 0.01.
mechanisms (29, 37), and hence, the overall reduction in the numbers of these cells in the tumors of VAP-1–deficient mice suggests that in the absence of VAP-1, the immune responses against tumor can be more efficient.

Gr-1+CD11b+ myeloid cells required the oxidase activity of VAP-1 in genetically modified mice for successful recruitment into the tumors because transgenic expression of the enzymatically inactive VAP-1 mutant did not revert the tumor growth to wt tumor growth pattern. This suggests that the end products of VAP-1–driven catalytic reaction, including aldehydes, ammonium, and hydrogen peroxide, are biologically relevant in this context. Notably, at least hydrogen peroxide is an emerging signaling molecule, which regulates leukocyte migration and angiogenesis (38–41).

Lack of VAP-1 did not lead to abnormal vasculogenesis, angiogenesis, or lymphangiogenesis during normal development. The function of VAP-1 is thus different from systems such as VEGF-VEGF receptor, which is required for both developmental and pathologic processes (42). However, other angiogenic molecules, such as placental growth factor and Rgs-5, only seem to contribute to the vessel formation under pathologic conditions (15, 43). Targeting of this latter group of molecules, including VAP-1, may thus more selectively affect neovascularization in tumors.

Our findings in mice may have clinical relevance because VAP-1 protein is found in vessels in clinical tumor specimens (12–14), and VAP-1 gene amplification has been found in the genome of cancer patients (11). Moreover, a soluble form of VAP-1 in serum may serve as a predictive marker in cancer because increased soluble VAP-1 levels were found in colorectal patients, and soluble VAP-1 proved to be an independent marker for predicting metastatic disease (44). Thus, the membrane-bound VAP-1 may serve as a recruitment molecule for myeloid cells on tumor endothelium, whereas the soluble VAP-1—the cellular origin, mechanism of formation, and functions of which remain to be elucidated—may be a surrogate marker of beneficial tumor-induced inflammation.

In conclusion, these data indicate a novel function of amine oxidases in controlling migration of Gr-1+CD11b+ myeloid cells, angiogenesis, and tumor growth. They also suggest that VAP-1 could be a target to inhibit trafficking of this unique leukocyte population and, consequently, to inhibit tumor progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 6. Oxidase activity of VAP-1 is needed for recruitment of myeloid cells into melanomas. A, immunohistologic analysis of VAP-1 (green) and CD31 (red) protein expression in the B16 melanomas in wt (VAP-1+/+), VAP-1−/−, and VAP-1 transgenic mice carrying enzymatically inactive human VAP-1 under the Tie1 promoter (VAP-1Y471F). Note the prominent VAP-1 protein expression in endothelial cells in the transgenic mice. Bar, 100 μm. B, SSAO activity in the gene-modified mice was analyzed using enzyme assays (n = 12 mice per group). Growth of melanoma (volumes, n = 12 mice per group; C) and numbers of intratumoral CD11b+ cells (n = 5 mice per group; >100 high-power fields per mouse; D) in the gene-modified mice. Columns and points, mean of the indicated number of mice; bars, SE. *, P < 0.05; **, P < 0.01.
References


VAP-1 Supports Tumor Growth
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