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Vascular Biology, Atherosclerosis, and Endothelium Biology

Highly Invasive Melanoma Cells Activate the Vascular Endothelium via an MMP-2/Integrin αvβ5–Induced Secretion of VEGF-A

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Tumor cell extravasation is a critical step in the metastatic cascade and requires interaction between the tumor cell and the endothelium. Although cancer progression depends on a complex network of mechanisms, including inflammation and coagulation, the involvement of tumor-induced endothelium activation and the subsequent release of procoagulatory factors in this process are not well understood. Using tissue sections from patients with malignant melanoma, immunofluorescence studies for the presence of von Willebrand factor (VWF) clearly demonstrated endothelium activation and the formation of ultralarge VWF fibers in these patients. *In vitro* analyses revealed that supernatants from highly invasive melanoma cells induced an acute endothelium activation measured by VWF, P-selectin, and angiopoietin-2 release. Proteome profiling identified vascular endothelial growth factor A (VEGF-A) as the main mediator of endothelium activation. Inhibition and knock-down of VEGF-A in melanoma cells led to a rigorous decrease in VWF exocytosis. Selective small-interfering RNA to matrix metalloproteinase-2 (MMP-2) inhibited endothelium activation, and this effect correlated with reduced VEGF-A content in the supernatants of melanoma cells. Further experiments showed that active MMP-2 regulates VEGF-A in melanoma cells on a transcriptional level via an integrin αvβ5/phosphoinositide-3-kinase–dependent pathway. In conclusion, these results indicate an important role of VEGF-A in acute endothelium activation and provide clear evidence that MMP-2 plays a pivotal role in the autocrine regulation of VEGF-A expression in melanoma cells. ***(Am J Pathol 2012, 181: 693–705; http://dx.doi.org/10.1016/j.ajpath.2012.04.012)***

Human malignant melanoma is a highly metastatic tumor with poor prognosis and high resistance to treatment.[1,2] Tumor progression and, therefore, metastasis formation involves a complex network of several independent mechanisms, such as angiogenesis, immune responses, inflammation, and coagulation.[3] It is well-known that patients with cancer hold a high risk of thrombosis and that factors of the coagulation system (such as platelets, tissue factor, and plasmin) have a high impact on tumor spreading.[4,5] These observations are underlined by clinical studies showing that low-molecular-weight heparins have a significant benefit in patients with tumor.[6–8] One of the crucial steps in this process is the capacity of tumor cells to interact with the endothelial cell (EC) layer and to migrate to the surrounding tissue (transendothelial migration). Thus, although a variety of factors have been identified in the pathogenesis of cancer, little is known about the molecular mechanisms of such tumor cell–EC interactions and their consequences on cancer cell extravasation.

We postulate that tumor-derived factors induce a prothrombotic, proinflammatory, and adhesive milieu by activation of ECs. On EC activation, the regular repressive function on inflammation and coagulation subsides and the endothelium converts to a proinflammatory and procoagulatory surface. Accordingly, prothrombotic and

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proinflammatory conditions were shown to increase tumor progression.[9,10] In this context, we showed that acute EC activation is followed by secretion of the proinflammatory and prothrombotic content of Weibel-Palade bodies (WPBs), including IL-8, angiopoietin-2 (Ang-2), P-selectin, and the glycoprotein von Willebrand factor (VWF).[11] On the one hand, it is known that P-selectin, which is present on the surfaces of activated platelets and ECs after α-granula and WPB exocytosis, respectively, is involved in the initial adhesion of tumor cells to platelets or ECs.[12] Thereby, P-selectin plays a pivotal role in helping tumor cells evade the cytotoxic activity of natural killer cells and supports tumor cell extravasation from blood to the surrounding tissue.[13,14] On the other hand, we demonstrated that melanoma cell–derived factors induce massive release and immobilization of ultra-large VWF fibers at the luminal EC membrane, which efficiently immobilizes platelets even under high shear flow conditions.[15,16] This binding likely contributes to microvascular arrest and activation of platelets, which is known to be involved in the establishment of metastasis.[12] Moreover, we and other groups have shown that matrix metalloproteinase-1 (MMP-1), secreted by highly metastatic tumor cells or tumor-activated fibroblasts, activates proteinase-activated receptor-1 and, thereby, mediates EC activation associated with the release of WPB content.[11,17,18] To evaluate the molecular mechanisms of tumor-induced EC activation, we recently demonstrated that all the melanoma cells analyzed expressed tissue factor that enabled melanoma cells to generate thrombin.[19] Thrombin is the main agonist of platelet- and EC-expressed proteinase-activated receptor-1 and was shown to activate platelets and ECs, followed by P-selectin expression and VWF release.[19] This is in accordance with different animal studies showing that thrombin, tissue factor, and proteinase-activated receptor-1 enhance hematogenous metastasis.[14,20,21] Besides this thrombin-dependent pathway of melanoma-EC interaction, we previously demonstrated that some melanoma cells can activate ECs by a second, thrombin-independent pathway.[19]

Therefore, in the present study, we analyzed the molecular mechanism of the thrombin-independent pathway of melanoma-EC interaction. Herein, we identified that vascular endothelial growth factor (VEGF) is the only melanoma-derived molecule that can directly (-independent of thrombin) activate ECs. These data show that melanoma-derived VEGF targets endothelial VEGF receptor 2 (VEGFR-2), leading to prompt EC activation reflected by WPB exocytosis. These data were confirmed by using tissue sections from patients with malignant melanoma that were stained for VWF and ECs clearly showing EC activation in those patients. Finally, these data indicate that VEGF expression is mainly regulated by melanoma-derived active MMP-2 that activates $\alpha v\beta 5$ integrin in an autocrine manner.

Materials and Methods

Cell Culture

The human metastatic melanoma cell lines WM9 (established by Meenhard Herlyn, D.V.M., D.Sc., The Wistar Institute, Philadelphia, PA, from a metastatic melanoma lymph node), MV3 (developed from lymph node metastases),[22] BLM (subline of BRO, derived from a primary human melanoma, isolated from lung metastasis after subcutaneous inoculation of nude mice with BRO cells),[23,24] WM451LU (human melanoma cells isolated from lung metastasis of the mouse-bearing human melanoma xenograf),[25] SB-CL-2 (established in culture from primary cutaneous melanoma and are a poorly tumorigenic and nonmetastatic line in nude mice),[26] and IGR37 [established from the lymph node metastasis (groin) of a 26-year-old man with malignant melanoma][27] were gifts from Dagmar Kulms, Institute of Molecular Immunology, University of Stuttgart, Stuttgart, Germany. The cells were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (WM9) or RPMI 1640 medium (for other cell lines) containing enriched Earle's salts, nonessential amino acids, 1% glutamic acid, 1% antibiotics (penicillin and streptomycin), and 10% heat-inactivated fetal calf serum (Boehringer Mannheim, Mannheim, Germany). Human umbilical vein ECs (HUVECs) were isolated and cultivated as described previously.[19] Human dermal microvascular ECs were purchased from PromoCell (Heidelberg, Germany) and were maintained in EC growth medium MV (PromoCell) at 37°C, 5% CO_2 and cultivated maximally up to the third passage.

Generation of Melanoma-Derived Supernatants

For standardized generation of supernatants, 0.5×10^6 melanoma cells were grown in a T-75 flask to confluence, the culture medium was removed, and the cells were intensely rinsed twice with HEPES-buffered Ringer's solution (HBRS) consisting of 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 5 mmol/L glucose, and 10 mmol/L HEPES. After incubation in 5 mL of HBRS for 8 hours, the conditioned melanoma-derived supernatant was harvested and centrifuged to remove cell debris. Finally, the supernatants were frozen at −80°C until the experiments were performed; melanoma cells were counted to get comparable results.

Stimulation of ECs

For all the experiments, ECs were grown to confluence in commercially available tissue culture 24-well plates. For the experiments, culture medium was removed, and cells were washed twice in HBRS. For stimulation, stimuli [melanoma supernatants, recombinant human $VEGF_{165}$ (R&D Systems, Wiesbaden, Germany)] alone or combined with inhibitors [0.65 mg/mL of bevacizumab (Roche Diagnostics GmbH, Mannheim, Germany), 6 μg/μL of the MMP-2/MMP-9 inhibitor BiPS ([2*R*]-[(4-biphenylylsulfonyl) amino]-N-hydroxy-3-phenylpropionamide), 10 μmol/L MMP inhibitor GM6001 (Calbiochem, Darmstadt, Germany), and 0.1 mmol/L VEGFR-2 inhibitor Ki8751 (Tocris Bioscience, Bristol, UK)] were added to the cells at indicated concentrations. The supernatants of ECs of experimental and control groups were harvested after 15 minutes, cleared of cell debris by centrifugation, and immediately stored at −20°C for later VWF, Ang-2, and P-selectin analysis.

ELISA

Release of VWF was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) technique as described elsewhere.[19] VEGF-A ELISA was performed according to the manufacturer's instruction (human VEGF-A; Bender MedSystems, Vienna, Austria). The relative amounts of VEGF-A were calculated according to standard procedures. Ang-2 and P-selectin levels were measured by the human Ang-2/P-selectin DuoSet ELISA (R&D Systems), according to the manufacturer's instruction.

Proteome Profiler Array

The Proteome Profiler array human angiogenesis array kit (R&D Systems) was used to measure the relative levels of melanoma-released proteins in the supernatant. The assay was performed according to the manufacturer's instructions. The melanoma supernatants of BLM and SB-CL-2 cells were prepared as described previously herein. An equal volume (1 mL) of each supernatant was used for the assay. The density of the dot blots was analyzed using ImageJ software. The experiments were performed in duplicate.

Transfection Techniques

The melanoma cell knock-down experiments were performed according to the recommended HiPerFect transfection reagent traditional protocol (Qiagen GmbH, Hilden, Germany). Briefly, for each experiment, melanoma cells (1.5×10^5) were seeded in 6-well plates 1 day before the transfection procedure. After 24 hours of incubation at 37°C, the cells were transfected with specific MMP-2 small-interfering RNA (siRNA) (Hs_MMP2_5, Hs_MMP2_10), membrane-type 1 (MT1)–MMP siRNA (Hs_MMP14_1, Hs_MMP14_6), VEGF-A siRNA (Hs_VEGF_5), αv integrin siRNA (Hs_ITGAV_2, Hs_ITGAV_3), β5 integrin siRNA (Hs_ITGB5_3, Hs_ITGB5_5), or a nonspecific siRNA (Qiagen GmbH) at a final concentration of 5 nmol/L in a mix with 12 μL of HiPerFect transfection reagent (Qiagen GmbH) and 100 μL of serum-free RPMI 1640 medium. After 48 hours of incubation at 37°C, the cells were used for further experiments. Knock-down efficiency was checked by quantitative RT-PCR (RT-qPCR), VEGF-A ELISA, or fluorescence-activated cell sorting (FACS) analysis.

HUVECs were transfected using the Nucleofector I device (Lonza, Cologne, Germany). For this purpose, HUVECs (5.0×10^5) and 170-nm nonspecific siRNA (Qiagen GmbH) or VEGFR-1 or VEGFR-2 siRNA (Ambion, Darmstadt, Germany) were mixed with 100 μL of Nucleofector solution (HUVEC Nucleofector kit; Lonza). After transfection, cells were transferred into endothelial growth medium-2 (without VEGF-A) (Lonza) and were cultured on 12-well plates under standard conditions for 40 hours. The transfection efficiency was proved by FACS analysis.

FACS Analysis

The surface expression of MT1-MMP and αvβ5, β1, β3, β5, β6, and β8 integrins on melanoma cells was analyzed by FACS. Cells were harvested with EDTA (pH 7.3), washed with HBRS, and incubated with 1 μg of a primary rabbit anti-human MT1-MMP, mouse anti-human integrin αvβ5 (Millipore, Darmstadt, Germany), mouse anti-human integrin β1 (ImmunoTools, Friesoythe, Germany), mouse anti-human integrin β3 (Novus Biologicals, Cambridge, UK), mouse anti-human integrin β5 (eBioscience, Frankfurt, Germany), mouse anti-human integrin β6-PE (R&D Systems), or mouse anti-human integrin β8 (R&D Systems) antibody or the isotype-specific antibodies in HBRS + 1% bovine serum albumin for 1 hour on ice. The cells were washed and incubated with a phosphatidylethanolamine-conjugated secondary antibody for 30 minutes on ice in the dark. After the staining procedure 10,000 cells were analyzed using FACSCanto and FACSDiva software (Becton Dickinson, Heidelberg, Germany).

RNA Preparation and RT-qPCR

Total RNA was extracted 48 hours after transfection or 24 hours after incubation using the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (Cayman Chemical, Tallinn, Estonia), the recombinant human MMP-2 (rhMMP-2) (Calbiochem), or 0.1% dimethyl sulfoxide using the RNeasy mini kit (Qiagen GmbH), following the manufacturer's directions. The cDNA was synthesized from 1 μg of total RNA per sample using the QuantiTect reverse transcription kit (Qiagen GmbH). To determine the mRNA transcript level from cDNA, RT-qPCR was performed using specific primers for MMP-2 (Hs_MMP2_1_SG QuantiTect primer assay), MT1-MMP (Hs_MMP14_1_SG QuantiTect primer assay), VEGF-A (Hs_VEGFA_5_SG QuantiTect primer assay), αv integrin (Hs_ITGAV_1_SG QuantiTect primer assay), β5 integrin (Hs_ITGB5_1_SG QuantiTect primer assay), β1 integrin (Hs_ITGB1_1_SG QuantiTect primer assay), or the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs_GAPDH_2_SG QuantiTect primer assay) (Qiagen GmbH). The expression of each product was normalized to GAPDH mRNA expression and is shown as the ratio of the target gene to *GAPDH* gene expression, calculated by ΔC_T.

Gel Zymography

For gel zymography, 0.5×10^6 melanoma cells were grown to confluence, the culture medium was removed, and the cells were intensely rinsed twice with HBRS. The melanoma supernatants were mixed with an equal amount of nonreducing sample buffer, and for detection of gelatinase activity, equal amounts of samples were electrophoretically separated on 7.5% SDS-PAGE gels co-polymerized with 2 mg/mL of gelatin (Sigma-Aldrich, Taufkirchen, Germany) under nonreducing conditions. Gels were incubated in washing buffer containing 2.5% Triton X-100 (Roche Diagnostics GmbH) and then were incubated for 40 hours in developing buffer (50 mmol/L Tris-HCl, 10 mmol/L $CaCl_2$, 0.02% NaN_3) at 37°C before staining with Coomassie Blue R-250 (250 mL of methanol, 35 mL of acetic acid, 1.25 g of Brilliant Blue) for 1 hour. Gels were destained in 5% acetic acid and 25% methanol until clear bands manifesting gelatinolysis appeared on the blue background. Mixed human

MMP-2 and MMP-9 standards (CC073; Chemicon, Darmstadt, Germany) were used as positive control. Relative gelatinolytic activity was quantified via measurement of optical density using ImageJ software.

Cell-Based Zymography

Cellular gelatinolytic activity was assessed using a commercially available kit (Molecular Probes, Darmstadt, Germany). For the gelatinase assay, cells were seeded onto coverslips in a 12-well plate for 24 hours. The culture medium was removed, and the cells were intensely rinsed twice with HBRS. Cells were incubated for 6 to 8 hours at 37°C in HBRS containing a reaction buffer and the gelatin fluorescein conjugate (50 μg/mL) without washing and then were fixed in ice-cold acetone for 30 seconds. Nuclei were stained with DAPI diluted 1:5000 in PBS for 10 minutes. Coverslips were embedded with Mowiol mounting medium (Polysciences Europe GmbH, Eppelheim, Germany) containing 50 mg/mL of 1.4-diazabicyclo-[2.2.2]octane (DABCO; Sigma-Aldrich). Fluorescence imaging was performed using a Zeiss Axiovert 200 microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) connected to a charge-coupled device camera system (CoolSNAP HQ; Photometrics, Munich, Germany), and the data were analyzed using Image-J.

Immunofluorescence

HUVECs were grown on gelatin-coated coverslips in a 12-well plate, followed by 24 hours of incubation. After incubation, cells were stimulated for 15 minutes with the supernatant of BLM or with HBRS as negative control. Coverslips were fixed in ice-cold methanol for 30 minutes, washed with HEPES, and blocked in 2% bovine serum albumin in an incubation buffer (0.1% bovine serum albumin in HBRS consisting of 140 mmol/L NaCl, 5 mmol/L KCl, and 1 mmol/L $MgCl_2$; 1 mmol/L $CaCl_2$, 5 mmol/L glucose, and 10 mmol/L HEPES; and 0.3% Triton X-100) for 1 hour at room temperature. Incubation with the primary antibody rabbit anti-human VWF (Dako, Hamburg, Germany) was performed with a dilution of 1:150 in incubation buffer for 1 hour at room temperature. After washing, cells were incubated with secondary fluorescein isothiocyanate–conjugated goat anti-mouse IgG antibodies (BD Pharmingen, Heidelberg, Germany) diluted 1:400 in incubation buffer at room temperature for 1 hour.

Tumor cryosections (10 μm) from patients with melanoma were fixed in ice-cold methanol for 30 minutes, washed in 0.1 mol/L PBS, and blocked for 1 hour in 10% (v/v) goat serum dissolved in 0.1 mol/L PBS containing 0.1% Tween 20. Incubation with the primary antibody rabbit anti-human VWF (Dako) was performed with a dilution of 1:150 in PBS containing 0.1% Tween 20 for 1.5 hours at room temperature. After washing, sections were incubated with secondary fluorescein isothiocyanate–conjugated goat anti-rabbit IgG antibodies (BD Pharmingen) diluted 1:250 in blocking solution at room temperature for 1 hour. Co-staining was performed overnight at 4°C with mouse anti-human CD31 (Dako) diluted 1:40 in PBS containing 0.1% Tween 20 and detected by incubation with Alexa555–conjugated goat anti-mouse for 1 hour in blocking solution (Invitrogen, Darmstadt, Germany). Nuclei were stained with DAPI diluted 1:5000 in PBS for 10 minutes. Finally, sections were embedded with Mowiol–glycol solution with freshly added 50 mg/mL of DABCO (Sigma-Aldrich). Microscopy was performed using a Zeiss Axiovert 200 microscope connected to a charge-coupled device camera system and the data were analyzed using ImageJ.

Statistical Analysis

Data are expressed as mean ± SEM. Group data were compared by analysis of variance, followed by the Bonferroni *t*-test to assess differences between groups. $P < 0.05$ was considered statistically significant.

Results

Human Malignant Melanoma Tissue Exerts Acute EC Activation in Tumoral Vasculature

In an attempt to characterize the *in vivo* relevance of acute EC activation in tumor progression, we analyzed VWF release in vascular structures of human malignant melanoma. In this context, stock frozen human tissue from malignant melanoma tumors were stained using anti-CD31 and anti-VWF antibodies to identify ECs and VWF. As shown in Figure 1, we identified EC-derived VWF fibers in the lumen of tumor blood vessels, indicating EC activation. In contrast, skin of healthy volunteers as control tissue showed typical localization of VWF in ECs without VWF fiber formation in the vessel lumen (data not shown). Thus, we hypothesize that *in vivo* melanoma growth and/or metastasis formation is associated with activation of ECs in the microenvironment of human melanoma tumors.

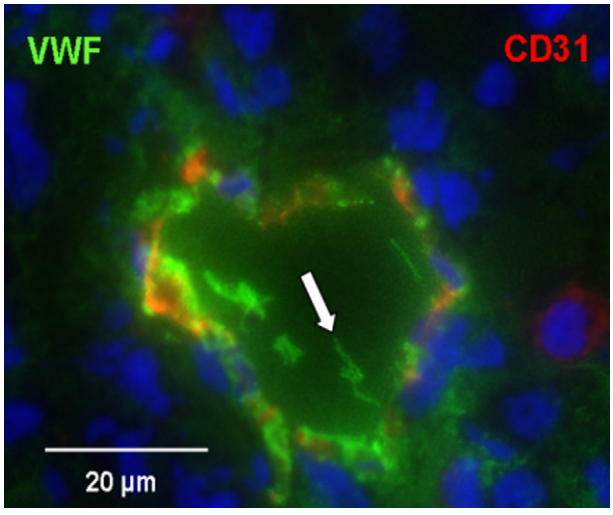

Figure 1. Patient-derived malignant melanoma tissue reveals intraluminal VWF and VWF fiber formation. At least six cryosections of three different human melanoma tissues were stained for VWF, and blood vessels were visualized by using anti-CD31 antibodies. Nuclei were stained with DAPI. The representative picture demonstrates that VWF (green) was localized within single ECs (red). Moreover, in tumoral blood vessels, VWF and VWF fibers are detectable in the vessel lumen, indicating EC activation (**arrow**).

Distinct Melanoma Cells Mediate VWF Release by EC Activation

To study the underlying molecular mechanisms of tumor-induced EC activation, we analyzed several human melanoma cell lines from different progression stages[28] for their ability to induce WPB exocytosis in ECs *in vitro*. Therefore, the EC supernatants were harvested 15 minutes after stimulation with the melanoma-derived supernatant and were used for quantitative analysis of VWF, Ang-2, and P-selectin release by ELISA, as described in *Materials and Methods*. The results demonstrated that the supernatants of MV3, BLM, and WM451LU melanoma cells lead to an acute induction of VWF, Ang-2, and P-selectin release by HUVECs, comparable with HUVECs stimulated with the positive control thrombin (0.5 U/mL) (Figure 2A and B). In contrast, the supernatants of other melanoma cell lines, such as WM9, SB-CL-2, and IGR37, could not activate ECs within 15 minutes. To determine whether BLM-mediated VWF release correlates with the formation of ultra-large VWF, an indicator of WPB exocytosis, we performed immunofluorescence staining against VWF. Untreated HUVECs showed VWF stored in WPBs, whereas stimulation of ECs with the supernatant of BLM for 15 minutes induced WPB exocytosis associated with the formation of ultra-large VWF fibers on the endothelial surface (Figure 2C). Taken together, these results demonstrate that soluble factors secreted by the highly invasive melanoma cell lines MV3, BLM, and WM451LU activate ECs in a direct, thrombin-independent manner, which led to an acute release of VWF, Ang-2, and P-selectin.

Melanoma Cell–Derived VEGF-A Correlates with Acute EC Activation

To investigate which soluble factors released by melanoma cells are responsible for this acute EC activation, the supernatants of two distinct melanoma cell lines were analyzed using the human angiogenesis array kit from R&D Systems, which allows detection of the relative levels of 55 proteins involved in angiogenesis, vascular morphogenesis, and tissue remodeling. For the assay, we used the supernatants of the melanoma cell lines BLM and SB-CL-2, which differ in their ability to directly activate the endothelium (Figure 2, A and B).

Both cell lines secreted most of the analyzed proteins in a comparable amount, including angiogenin, glial cell line–derived neurotrophic factor, granulocyte-macrophage colony-stimulating factor, IL-8, Serpin E1, tissue inhibitor of matrix metalloproteinases-1, and urokinase plasminogen activator. In contrast, the cytokines heparin-binding epidermal growth factor and insulin-like growth factor binding protein-2 and the monocyte chemotactic protein-1 were detectable only in SB-CL-2 cells. Surprisingly, BLM cells differ from SB-CL-2 cells only by the presence of two additional proteins (VEGF-A and pentraxin 3) (Figure 3A). Pentraxin 3 plays a nonredundant role in inflamma-

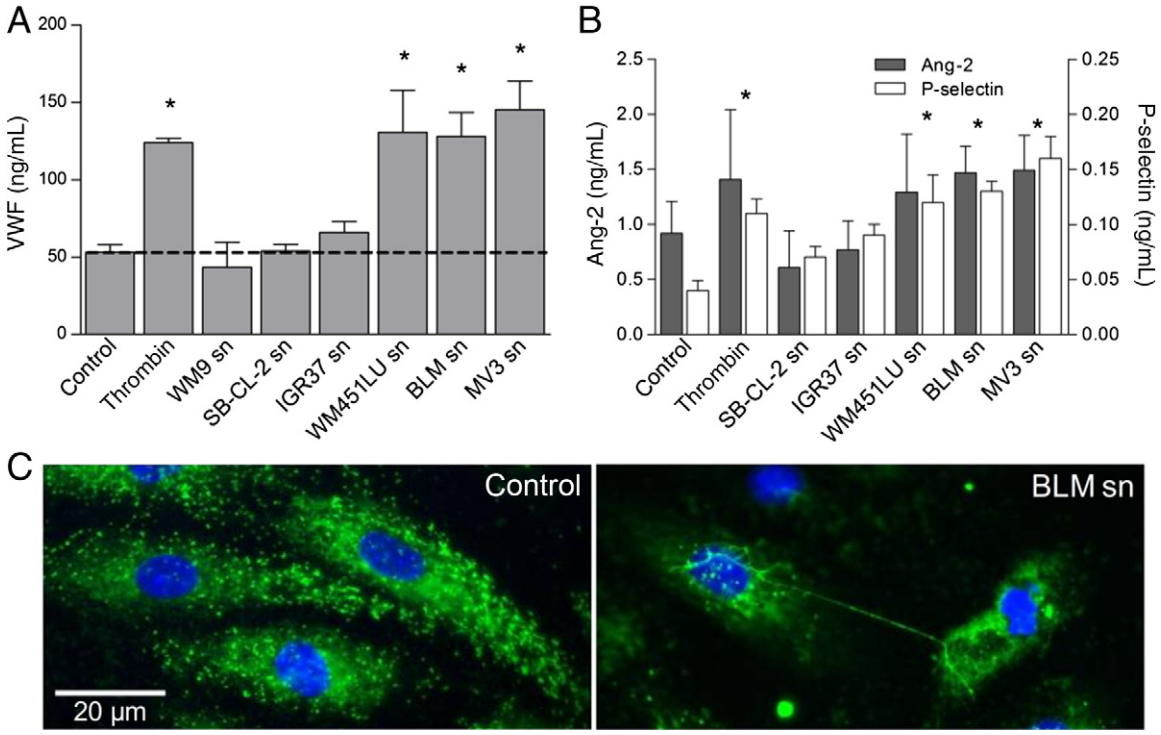

Figure 2. Distinct melanoma cell lines induce acute EC stimulation. **A** and **B:** HUVECs were stimulated for 15 minutes with the supernatants (sn) of different melanoma cell lines. The efficiency of the melanoma-induced EC stimulation was quantified by measurement of VWF, Ang-2, and P-selectin release. The MV3, BLM, and WM451LU melanoma cell sn can induce EC activation. In contrast, the sn of WM9, SB-CL-2, and IGR37 cannot induce acute EC activation. Thrombin (0.5 U/mL) was used as positive control. Data are presented as the mean ± SEM of at least five independent experiments. The **dashed line** in **A** represents the basal level of constitutive VWF, Ang-2, or P-selectin release without stimulation (control). $^*P < 0.05$. **C:** HUVECs were stimulated for 15 minutes with the BLM sn or with HBRS as negative control and were stained for VWF (green) and DAPI. Control cells showed intracellular localization of VWF in WPBs. Exposure to the melanoma sn correlated with the formation of ultra-large VWF fibers. Representative pictures of at least three independent experiments are shown.

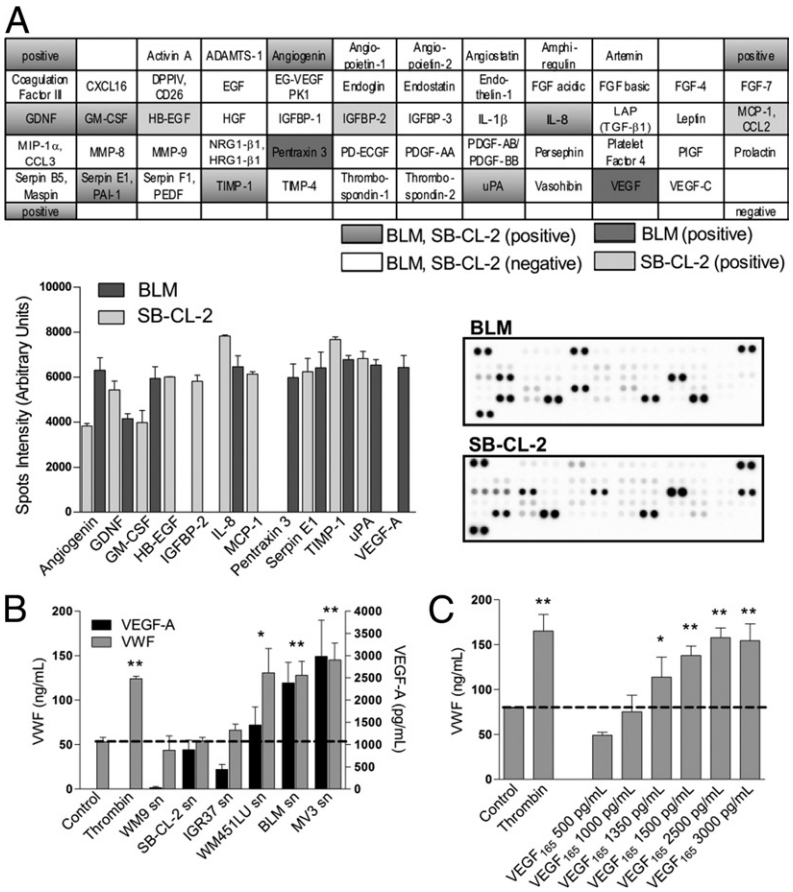

Figure 3. VWF release correlates with the amount of VEGF-A in the melanoma supernatant (sn). **A:** The R&D Systems human angiogenesis proteome profiler array was used to screen the melanoma cells lines BLM and SB-CL-2 for proteins released into the sn within 8 hours. Proteome Profiler array was performed with the collected melanoma sn according to the manufacturer's instructions. Dot plot signals were analyzed using ImageJ software. Array images of 10-minute exposure and profiles created by quantifying the mean spot pixel density are shown. Data are given as mean ± SEM ($n = 2$). GDNF, glial cell line–derived neurotrophic factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HB-EGF, heparin-binding epidermal growth factor; IGFBP-2, insulin-like growth factor binding protein-2; MCP-1, monocyte chemotactic protein-1; TIMP-1, tissue inhibitor of matrix metalloproteinases-1; uPA, urokinase plasminogen activator. **B:** The melanoma cell sn were analyzed for VEGF-A release by ELISA. The MV3, BLM, and WM451LU sn show considerably higher concentrations of VEGF-A compared with the other melanoma cells, indicating a correlation to VWF release. To enable efficient comparison, the data on VWF release from Figure 2A are included in this figure. Data are given as mean ± SEM ($n = 5$). The **dashed line** represents the basal VWF release from nonstimulated HUVECs (control). $^{*}P < 0.05$, $^{**}P < 0.005$ versus control. **C:** ECs were stimulated with different amounts of rhVEGF-A_{165} (500 to 3000 pg/mL). Data are given as mean ± SEM ($n = 3$). The dashed line represents the basal VWF release from nonstimulated HUVECs (control). $^{*}P < 0.05$, $^{**}P < 0.005$ versus control.

tion and acts as a natural angiogenesis inhibitor by binding the angiogenic fibroblast growth factor-2.[29]

Therefore, we hypothesized that VEGF-A is mainly responsible for melanoma-induced acute EC activation. Consequently, we screened the melanoma cell lines for VEGF-A secretion and compared these data with their ability to directly activate ECs. The supernatants of the melanoma cell lines MV3, BLM, and WM451LU contained much higher concentrations (1485 to 3000 pg/mL) of VEGF-A compared with the supernatants of the WM9, SB-CL-2, and IGR37 cell lines (30 to 900 pg/mL) (Figure 3B). As shown in Figure 2, A and B, melanoma cells MV3, BLM, and WM451LU could activate ECs. Stimulation of ECs with recombinant human $VEGF_{165}$ in varying concentrations confirmed that VEGF-A activates EC and indicates that a minimal concentration of 1350 pg/mL of VEGF-A is required for this process (Figure 3C).

To further demonstrate the relevance of melanoma-released VEGF-A, several experiments to inhibit VEGF-A signaling were performed. In the first set of analyses, we knocked-down VEGF-A in high-metastatic BLM cells using specific siRNA. VWF ELISA was performed on the supernatants harvested from HUVECs or human dermal microvascular ECs after a treatment of 15 minutes with the melanoma supernatants from transfected BLM cells. The data show that BLM cells lacking VEGF-A secretion could not activate ECs. However, stimulation of ECs with the supernatants from untreated BLM cells and cells transfected with a nonspecific siRNA (siCTL) leads to a high amount of VWF release comparable with ECs incubated with thrombin (0.5 U/mL) (Figure 4A). In agreement with these findings, completion of the BLM supernatant with the inhibitory VEGF-A antibody bevacizumab completely inhibited BLM supernatant–mediated EC stimulation (Figure 4A). The same results were obtained for the melanoma cell lines MV3 and WM451LU (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). To investigate the signal transduction pathways in ECs, activation experiments were performed in the presence of the selective VEGFR-2 inhibitor Ki 8751 (0.1 mmol/L) (Figure 4A) or after knock-down of VEGFR-2 in ECs (Figure 4B). This inhibition abolished BLM supernatant–mediated VWF release by ECs. In contrast, knock-down of VEGFR-1 in ECs did not alter BLM-induced EC activation (Figure 4B). These data indicate that a defined concentration of melanoma-derived VEGF-A is required for acute EC activation by its receptor VEGFR-2.

Melanoma Cell–Expressed MMP-2 Alters VEGF-A Expression

Prompted by previous studies indicating that MMP-1, derived from metastatic cancer cells or tumor-activated fibroblasts, is a factor involved in EC stimulation via proteinase-activated receptor-1,[11,17,18] we studied the role of MMPs in an experimental setting. Therefore, gelatin-substrate gel zymography was performed to assess the

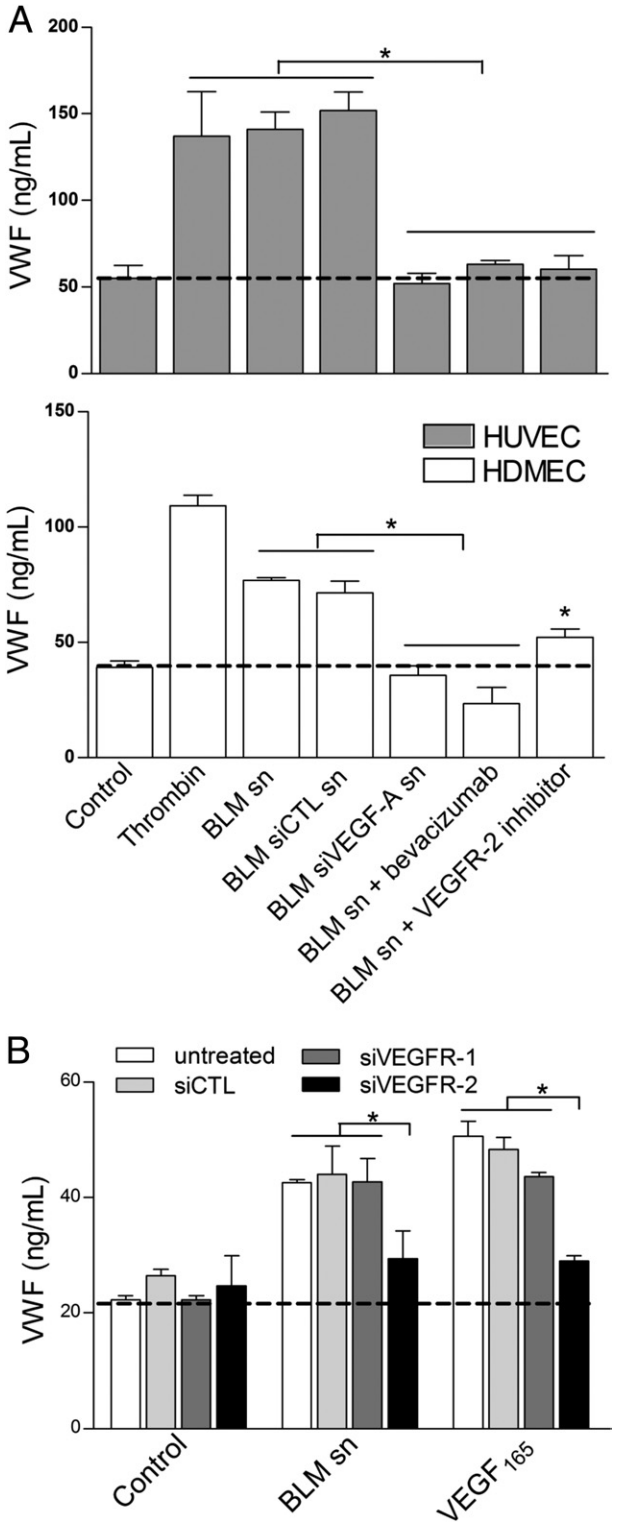

Figure 4. Melanoma-derived VEGF-A mediates acute EC stimulation via VEGFR-2. **A:** ECs were stimulated for 15 minutes with the BLM cell supernatant (sn), with the sn of VEGF-A siRNA (siVEGF-A) or siCTL-treated BLM cells or the BLM sn supplemented with 0.65 mg/mL of bevacizumab. Additionally, ECs were preincubated with 0.1 mmol/L of the VEGFR-2 inhibitor Ki 8751 for 30 minutes and, after washing with HBRS, were stimulated with the BLM sn. Thrombin was used as a positive control, and HBRS served as a negative control. HDMEC, human dermal microvascular EC. **B:** HUVECs were transfected with siRNA against VEGFR-1 (siVEGFR-1) or VEGFR-2 (siVEGFR-2) or with siCTL using the Nucleofector I device. After 40 hours, the transfected HUVECs were stimulated with the BLM sn or with rhVEGF-A_{165} (3000 pg/mL). Data are given as mean ± SEM ($n = 3$). The **dashed line** represents the basal VWF release under nonstimulated conditions (control). *$P < 0.05$.

presence of MMPs in the melanoma-derived supernatants. MMP-2 was detected in all the tested cell lines, independent of their invasiveness and their capability to activate ECs, and no other MMPs were detected in the different melanoma supernatants by zymography (Figure 5A). Next, we knocked-down MMP-2 in BLM cells and used the harvested supernatants for EC stimulation. Analysis of VWF release after EC stimulation by these supernatants of transfected cells showed an almost complete inhibition of EC activation (Figure 5B). The same results were also obtained for the high-metastatic melanoma cell lines MV3 and WM451LU (see Supplemental Figure S2 at *http://ajp.amjpathol.org*).

The addition of MMP inhibitors (the MMP-2/MMP-9 inhibitor BIPS or the MMP broad spectrum inhibitor GM6001) to the BLM cell supernatant (note: there is no incubation of the inhibitors with the melanoma cells) did not attenuate EC activation (Figure 5B). To further investigate the role of MMP-2 in VEGF-A–mediated EC stimulation, we measured VEGF-A concentrations in the melanoma cell supernatants on MMP-2 knock-down. After transfection of BLM cells with MMP-2 siRNA (siMMP-2), the melanoma cell supernatants were collected and analyzed. As shown in Figure 5C, the VEGF-A concentration in the supernatant of MMP-2 knock-down BLM cells was reduced in contrast to untreated cells and cells transfected with siCTL. The concentrations of VEGF-A in the supernatants of MMP-2 and VEGF-A knock-down cells ranged from 120 to 1100 pg/mL. In contrast, the supernatants of untreated BLM cells and cells transfected with siCTL exhibited mean ± SEM VEGF-A concentrations of 3500 ± 330 pg/mL (Figure 5C). As already mentioned, these data indicate that a VEGF concentration ≥1350 pg/mL is needed to activate ECs. Quantification of VWF exocytosis as an indicator of VEGF-A–induced EC activation directly correlated with the measured VEGF-A concentrations. Thus, the supernatants of VEGF-A siRNA– or siMMP-2–transfected BLM cells could not induce VWF release in ECs (Figure 5C). Similar results were obtained for the MV3 and WM451LU melanoma cell lines (see Supplemental Figure S2 at *http://ajp.amjpathol.org*).

To analyze whether MMP-2 knock-down alters VEGF-A mRNA content and, thus, likely VEGF-A expression in melanoma cells, we performed RT-qPCR using total RNA of control BLM cells and BLM cells after MMP-2 knock-down. As shown in Figure 5D, mean ± SEM VEGF-A mRNA expression was reduced to 22% ± 1.6% in siMMP-2–treated cells compared with in siCTL-treated cells. These results point to an important role of MMP-2 in regulating VEGF-A expression in melanoma cells and, therefore, on melanoma-induced EC activation.

MMP-2 Alters VEGF-A mRNA Content via an Integrin αvβ5/Phosphoinositide-3-Kinase–Dependent Pathway in Melanoma Cells

Recently, it was shown that MMP-2 regulates VEGF expression via an αvβ3 integrin–induced, PI3K/AKT-mediated signaling cascade in lung cancer cells.[30] Therefore, we analyzed the involvement of integrins in MMP-2–regu-

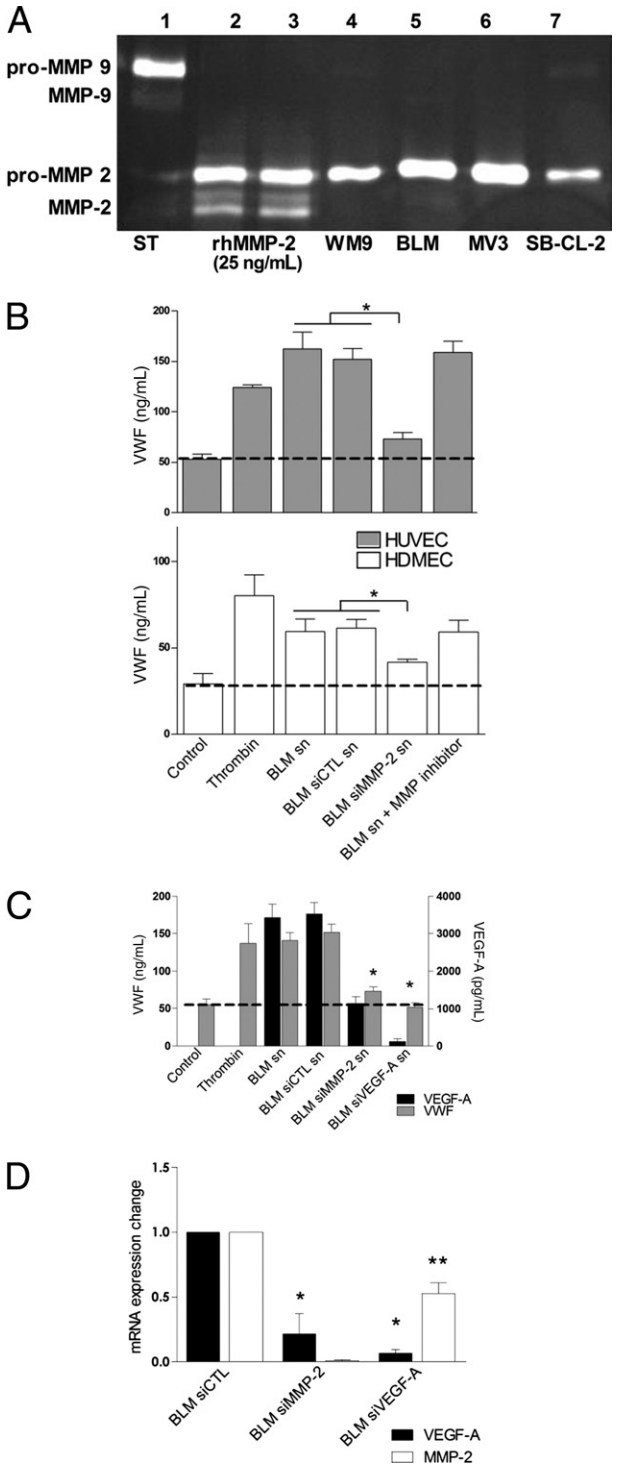

Figure 5. MMP-2 knock-down in melanoma cells affects the amount of melanoma-derived VEGF-A. **A:** Representative gelatin zymography demonstrating released MMPs from different melanoma cell lines. Pro-MMP-2 was presented in all the tested melanoma cell supernatants (sn), and no activated MMP-2 or other MMPs were detected. Bands corresponding to MMP-9 were observed in the standard as positive control only (ST). **B:** ECs were stimulated with the sn of MMP-2–depleted BLM (siMMP-2) cells or the BLM sn supplemented with 6 μg/μL of the MMP-2/MMP-9 inhibitor BIPS, or 10 μmol/L of the broad spectrum MMP inhibitor GM6001. Cells transfected with siCTL served as control. The **dashed line** represents the basal VWF release under nonstimulated conditions (control). **C:** VEGF-A ELISA was performed with the sn of siMMP-2– or siVEGF-A–treated BLM cells. The **dashed line** represents the basal VWF release under nonstimulated conditions (control). **D:** RT-qPCR analysis of VEGF-A and MMP-2 mRNA expression was performed 48 hours after transfection of the cells with siRNA. Data were normalized to siCTL-treated cells (set to one) and are represented as the mean ± SEM of at least three independent experiments. $^{*}P < 0.005$, $^{**}P < 0.05$.

lated VEGF-A expression in melanoma cells. By FACS analysis, we screened BLM cells on the surface expression of different integrins. BLM cells were positive for β1, β5, and $\alpha v\beta$5 and negative for β3, β6, and β8 integrin subunits (Figure 6A). Therefore, we proved the involvement of β1, β5, and αv integrin subunits on VEGF-A expression.

The knock-down of the integrin subunits αv and β5 in BLM cells by siRNA decreased mean ± SEM VEGF-A mRNA expression, which was, 48 hours after transfection, reduced to 25% ± 1.0% (siαv) and 18% ± 1.2% (siβ5) compared with the siCTL-treated cells. In contrast, β1 knock-down did not alter VEGF-A expression (Figure 6B). The transfection efficiency of αv and β5 knock-down was also proved by FACS analysis. As shown in Figure 6A, $\alpha v\beta$5 integrin was presented on the surface of siCTL-treated BLM cells with a mean ± SEM fluorescence intensity shift of 39.5 ± 6.07. The mean ± SEM fluorescence intensity shift of the $\alpha v\beta$5 integrin surface expression was 4.9 ± 0.71 after αv knock-down and 5.5 ± 1.34 after β5 knock-down. The amount of released VEGF-A in the supernatant of siαv-, siβ5-, or siβ1-treated BLM cells was also reduced only in the case of αv and β5 knock-down and not after β1 knock-down (Figure 6C).

To study whether the decrease in VEGF-A mRNA expression depends on MMP-2 activity, we added rhMMP-2, 25 ng/mL, to the transfected cells and incubated them for 24 hours. The VEGF-A mRNA expression reduced by $\alpha\nu$ and β5 knock-down, however, could not be recovered by the addition of rhMMP-2, indicating a pivotal role of $\alpha\nu\beta$5 in MMP-2–mediated VEGF-A expression in melanoma cells (Figure 6B).

Furthermore, we analyzed the involvement of PI3K in the VEGF-A expression regulated by MMP-2 in BLM cells. BLM cells were incubated for 24 hours with 50 μmol/L PI3K inhibitor (LY294002). The subsequent RT-qPCR showed a reduction in VEGF-A mRNA expression down to 48% ± 0.6% in cells treated with the PI3K inhibitor than in the untreated control (Figure 6D). The reduced VEGF-A expression after PI3K inhibitor treatment was also shown on the protein level by VEGF-A ELISA (Figure 6D).

To link the regulating effect of MMP-2 and PI3K to VEGF-A expression, BLM cells were transfected with siMMP-2 for 48 hours and were incubated with rhMMP-2 or rhMMP-2 plus LY294002 (50 μmol/L) for another 24 hours. The addition of rhMMP-2 to siMMP-2–transfected BLM cells partially restored mean ± SEM VEGF-A mRNA expression to 68% ± 0.8%, whereas rhMMP-2 did not increase VEGF-A mRNA content in the presence of LY294002 (Figure 6E). These findings show that MMP-2 regulates VEGF-A expression in an autocrine manner by an integrin $\alpha v\beta$5–induced, PI3K-dependent signaling pathway in high-metastatic BLM melanoma cells.

MT1-MMP–Mediated Activation of MMP-2 at the Cell Surface Correlates with EC Activation

To analyze whether melanoma-derived MMP-2 is mainly responsible for autocrine VEGF expression, we performed gelatin-substrate gel zymography on different melanoma cells. As shown in Figure 5A, all the melanoma

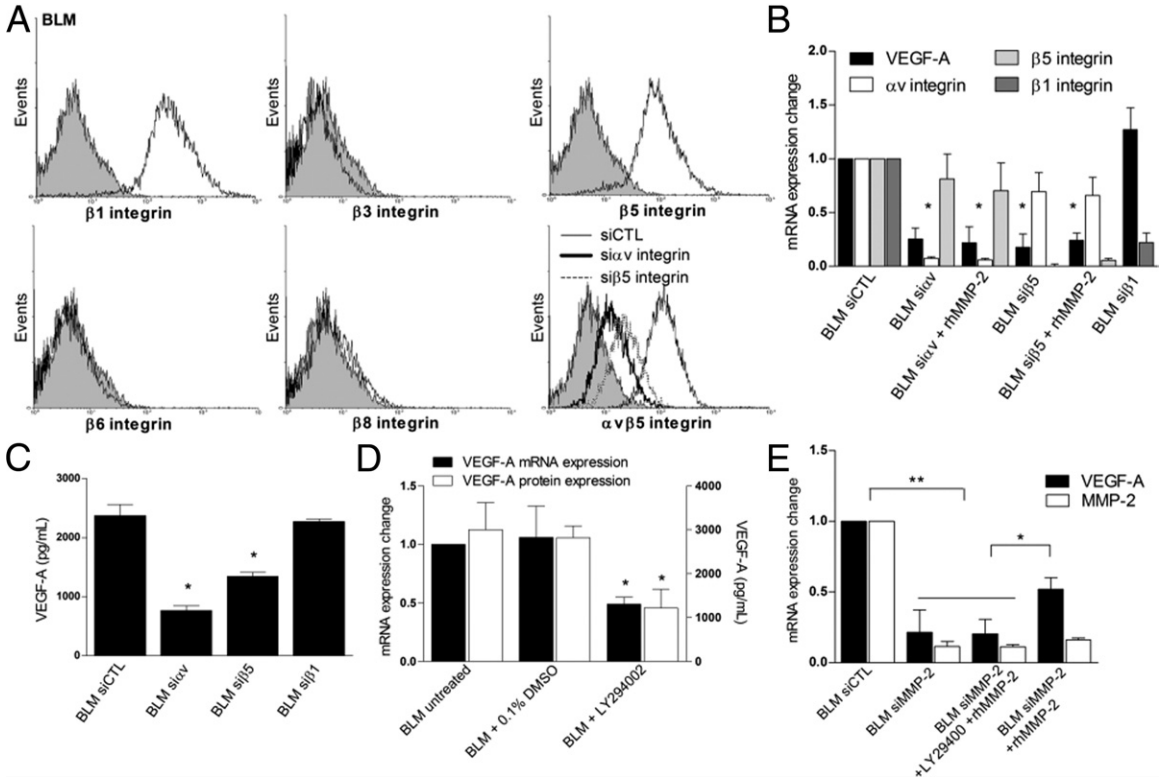

Figure 6. MMP-2 regulates VEGF-A mRNA content in melanoma cells via $\alpha v\beta 5$ and PI3K. **A:** FACS analysis of BLM cells on $\beta 1$, $\beta 3$, $\beta 5$, $\beta 6$, and $\beta 8$ integrins and of the surface expression of $\alpha v\beta 5$ integrin after knock-down of αv (siαv) or $\beta 5$ (si$\beta 5$) and on BLM cells treated with siCTL. The histograms show the fluorescence intensity (x axis) projected against the cell number (y axis) of the stained cells (open black line) compared with an isotype control (filled gray histograms). The data show one representative of three independently performed experiments. **B:** RT-qPCR analysis of VEGF-A, αv, $\beta 5$, or $\beta 1$ mRNA expression in siαv-, si$\beta 5$-, or si$\beta 1$-transfected BLM cells (±25 ng/mL of rhMMP-2) or in cells treated with siCTL. **C:** VEGF-A amount released in the supernatant of siαv-, si$\beta 5$-, si$\beta 1$-, or siCTL-treated BLM cells, measured by VEGF-A ELISA. **D:** RT-qPCR and VEGF-A ELISA of VEGF-A mRNA and protein expression were performed after treatment of BLM cells with the PI3K inhibitor LY294002 (50 μmol/L) or 0.1% dimethyl sulfoxide (DMSO). **E:** RT-qPCR analyses of VEGF-A and MMP-2 mRNA expression of BLM cells transfected with siMMP-2 (±25 ng/mL of rhMMP-2 or rhMMP-2 + LY294002 treatment for 24 hours) or siCTL-treated cells were performed. Data were normalized to siCTL-treated cells (set to one) and are represented as the mean ± SEM of at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.005$.

cells secrete latent pro-MMP-2 only. Therefore, we hypothesized that the active form of MMP-2 may be decisive in autocrine-induced VEGF expression. To prove the presence of active MMP-2 on the cell surface, we performed a cell-based zymography assay and assessed subcellular gelatinolytic activity of the melanoma cell lines BLM and SB-CL-2. Gelatinase activity was barely detectable in the low metastatic melanoma cells SB-CL-2. However, high levels of gelatin-degrading enzymes were present in the malignant BLM cell line, and they had a punctate distribution (Figure 7A).

As MMP-2 activation occurs at the cell surface of melanoma cells, where MMP-2 is associated with MT1-MMP (MMP-14), we performed FACS analysis for MT1-MMP in BLM and SB-CL-2 cells. In highly metastatic BLM cells (mean ± SEM fluorescence intensity shift, 3.4 ± 1.0), surface expression of MT1-MMP was more pronounced than in SB-CL-2 cells (mean ± SEM fluorescence intensity shift, 1.3 ± 0.1) (Figure 7B), directly correlating to the amounts of active MMP-2 on the cell surface of both cell lines (Figure 7A).

To investigate the possibility that surface-bound MMP-2 mediates VEGF-A release in an MT1-MMP–dependent manner, we next analyzed the effect MMP-2 and MT1-MMP depletion on gelatin degradation. Knock-down of either enzyme resulted in a significant decrease in gelatinolytic activity compared with the untreated control (data not shown). Similar to depletion of MMP-2, knock-down of MT1-MMP led to mean ± SEM 40% ± 11% reduced VEGF-A mRNA expression. The mean ± SEM level of VEGF-A mRNA expression could be significantly restored to 78% ± 9% of the control by the addition of rhMMP-2 (Figure 7C), which includes active MMP-2 as shown by gel zymography (Figure 5A).

Additionally, we showed that treatment of BLM cells with the broad spectrum MMP inhibitor GM6001 (10 μmol/L) or with recombinant tissue inhibitor of matrix metalloproteinases-2 (2 μg/mL) for 72 hours led to decreased VEGF-A release down to a mean ± SEM of 50% ± 5.5% and 10% ± 4.2%, respectively, from these melanoma cells (Figure 7D). Taken together, these data indicate that VEGF-A expression in BLM cells depends on autocrine MMP-2, which is activated in an MT1-MMP–dependent process.

Discussion

Malignant melanoma is a particularly aggressive tumor with a high probability of metastasis. Most patients with melanoma die due to metastases that are resistant to conventional therapies.[31,32] Critical steps in the meta-

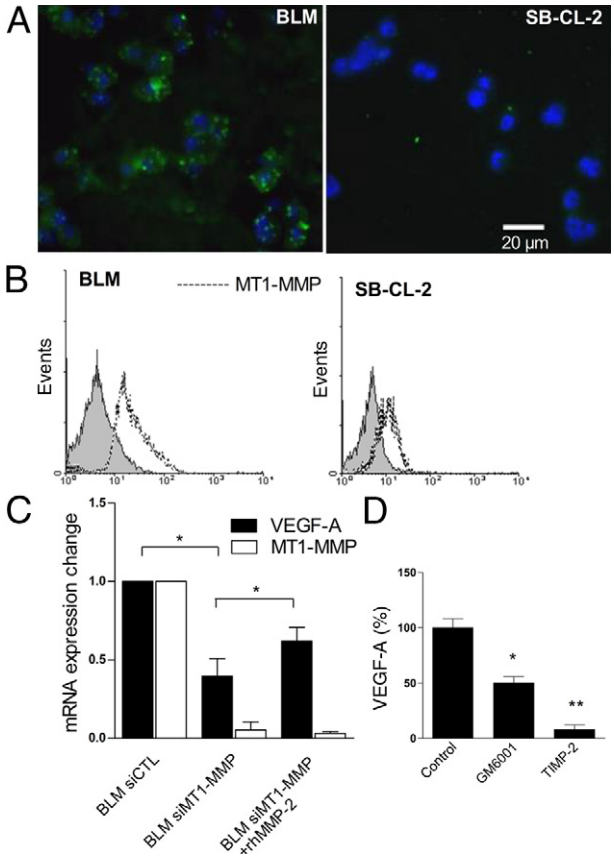

Figure 7. MT1-MMP–mediated activation of MMP-2 at the surface of BLM cells. **A:** *In situ* zymography shows increased gelatinolytic activity on highly malignant BLM cells. Cells were incubated with gelatin–fluorescein isothiocyanate substrate for 3 hours, and nuclei were co-stained with DAPI. BLM cells show a punctate distribution of gelatin degradation. In contrast, the SB-CL-2 melanoma cell line had no gelatin-degrading enzymes. The pictures show one of two independently performed experiments. **B:** FACS analysis of BLM and SB-CL-2 cells on MT1-MMP surface expression. $n = 3$. **C:** RT-qPCR analyses of VEGF-A and MT1-MMP mRNA expression in siMT1-MMP–transfected BLM cells (±25 ng/mL of rhMMP-2) or cells treated with siCTL were performed. Data were normalized to siCTL-treated cells (set to one) and are represented as the mean ± SEM of at least three independent experiments. **D:** BLM cells were treated for 72 hours with the broad spectrum MMP inhibitor GM6001 (10 μmol/L) or with recombinant tissue inhibitor of matrix metalloproteinases-2 (TIMP-2) (2 μg/mL). The supernatants of these cells were collected and analyzed by VEGF-A ELISA. Data are given as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.005$.

static cascade, once the tumor cell enters the circulation, are tumor cell adhesion to blood vessel endothelium and subsequent transendothelial migration. Recent studies have shown the importance of activated endothelium for melanoma cell adhesion to the EC layer.[13,33,34] In this context, activated ECs express P-selectin on their surface after WPB exocytosis. P-selectin is suggested to be mainly responsible for tumor cell adhesion and extravasation. Heparins were shown to interact with P-selectin and, therefore, to inhibit tumor cell arrest at the vessel wall. Using different animal models, it was demonstrated that heparin injection attenuates tumor spreading, reflected by a decrease in pulmonary filiae of up to 90%.[35,36] Moreover, clinical studies using low-molecular-weight heparins in patients with cancer suggest a benefit in tumor-associated morbidity and mortality.[8,37] For this reason, we were interested in the interaction between tumor cells and the EC layer and its effect on acute EC activation and WPB exocytosis.

In the present work, analysis of tissue sections from patients with malignant melanoma that were stained for VWF and ECs revealed EC activation in the tumor vasculature of those patients. In strong correlation, these *in vitro* data demonstrate that the tumor cell–derived supernatants of human high-metastatic melanoma cell lines induce a rapid release of vasoactive VWF from ECs, an indicator of WPB exocytosis and an activated endothelium. As already shown, elevated plasma levels of VWF correlate with enhanced tumor progression in patients.[38] In line with these findings, the application of VWF-blocking antibodies attenuated melanoma cell progression in a mouse model,[39] whereas in a recent publication, lung colonization by cancer cells was enhanced in VWF knockout mice,[40] pointing toward a crucial role of this procoagulatory protein in tumor progression. Although many tumor-derived cytokines or enzymes (such as tumor necrosis factor-α, IL-1, VEGF, transforming growth factor-β, etc) have been hypothesized to activate ECs,

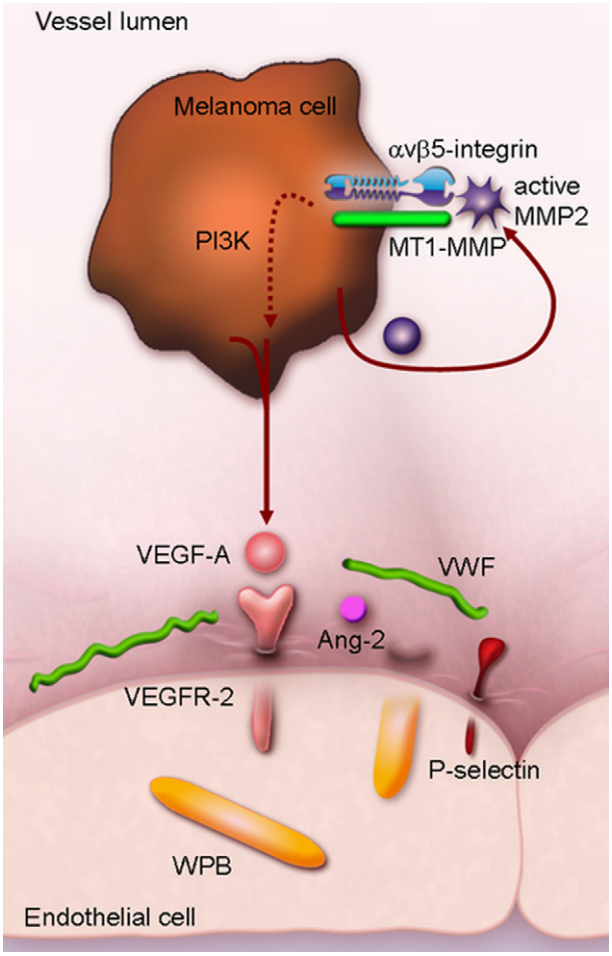

Figure 8. Schematic diagram of melanoma-derived VEGF-A targets endothelial VEGFR-2 promoting WPB exocytosis. VEGF-A expression in melanoma cells is regulated by $\alpha v\beta 5$ integrin. On pro-MMP-2 activation by melanoma surface-bound MT1-MMP, activated MMP-2 induces an $\alpha v\beta 5$ integrin– and PI3K-dependent increase in VEGF-A expression, thereby releasing highly metastatic melanoma cells. VEGF-A targets endothelial VEGFR-2, leading to WPB exocytosis followed by Ang-2, P-selectin, and VWF secretion.

the present data represent the first direct experimental evidence of such a mechanism. To be clear, we excluded all the molecules for this communication pathway and are left with VEGF as the only possible candidate to consistently explain the data. These results demonstrate that the amount of VEGF-A released from the different melanoma cell lines strongly correlates with their capability for acute EC activation. Only the high-metastatic melanoma cell lines MV3, BLM, and WM451LU could release sufficient amounts of VEGF-A to activate ECs. In this context, application of specific siRNA to VEGF-A or the humanized monoclonal anti-VEGF-A antibody bevacizumab enabled us to identify VEGF-A as the major activator of the endothelium in the experimental setup. The threshold VEGF-A concentration required for VEGF-A–mediated EC activation is remarkable and is evident in the capability of some tumor cell lines to induce acute EC stimulation, whereas others, with only minor amounts of VEGF-A, cannot.

In fact, patients with metastatic melanoma with VEGF-A–positive tumors have a worse prognosis than do those with VEGF-A–negative tumors.[41,42] It has recently been demonstrated that VEGF may also inhibit tumor immunity, whereas active immunotherapy is documented to envision a survival benefit in patients with melanoma.[43] It has been shown that antibodies against VEGF, Ang-1, and Ang-2 inhibit angiogenesis and monocyte chemotaxis, followed by prolonged survival in patients. Moreover, tumor infiltrating $CD4^+$ T cells block tumor angiogenesis by an interferon-γ– and tumor necrosis factor receptor–dependent pathway, attenuating tumor growth and progression, as shown in murine models and in patients with melanoma.[44,45] Recent mechanisms are independent of VEGF and MMP-9 and indicate that inhibition of EC activation needs to be followed by targeting of immune cell– and tumor cell–mediated EC activation.

In this study, we identified an integrin and MT1-MMP/MMP-2–dependent process regulating VEGF-A production and secretion in melanoma cells, as the knock-down of MT1-MMP, MMP-2, or $\alpha v\beta 5$ significantly attenuated VEGF-A mRNA expression in BLM cells. Obviously, MT1-MMP on the melanoma cell surface cleaves and, thus, activates secreted pro-MMP-2.[46] As shown, the VEGF-A amount in the melanoma-derived supernatants depends on MMP-2 activity; MMP-2 apparently regulates VEGF-A expression and secretion at the transcription level (Figure 8). In accordance with this interpretation, the knock-down of MMP-2 largely inhibits EC activation by the BLM cell supernatant. In addition, the application of MMP inhibitors to the melanoma supernatant does not alter EC activation, indicating that secreted MMP-2 is unable to directly activate ECs.

In this context, we showed that the VEGF-A amount in the melanoma-derived supernatants depends on MMP-2 activity, whereby down-regulation of VEGF-A apparently occurs at the transcription level and depends on PI3K. Therefore, the reduced EC activation mediated by the MMP-2–depleted BLM supernatants is an indirect effect derived from a decrease in VEGF-A levels in these melanoma supernatants.

As shown in previous studies, MMP-2 expression in melanoma cells highly correlates with the hematogenous metastatic spread and low survival rates of patients with malignant melanoma.[47–51] In accordance with these findings, it was shown that overexpression of MT1-MMP in tumor cells up-regulates VEGF-A production.[52] In tumor cells that overexpress MT1-MMP, integrin $\alpha v\beta 3$ binds pro-MMP-2 and promotes its conversion to the active form.[46,53,54] Furthermore, it was shown that MMP-2 alters VEGF-A expression via an $\alpha v\beta 3$ integrin–mediated, PI3K/AKT signaling–dependent pathway in cancer cells.[30,55] The present data point to a similar signaling cascade in melanoma cells by which MMP-2 regulates VEGF-A expression in an $\alpha v\beta 5$ integrin– and PI3K-dependent manner (Figure 8).

Taken together, we demonstrate that melanoma cells can induce acute endothelium activation, VEGF-A being the key mediator. A distinct threshold of VEGF-A is needed to activate ECs. In highly invasive melanoma cells, VEGF-A expression is regulated by an autocrine loop apparently activating secreted pro-MMP-2 by MT1-MMP, resulting in an increase in VEGF-A mRNA content via an $\alpha v\beta 5$ integrin–induced and PI3K-mediated signaling cascade. As shown herein, interference with any step in this autocrine loop can suppress EC activation by melanoma cells and, therefore, envisions new avenues in melanoma treatment.

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