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Differential Effects of Vascular Endothelial Growth Factor A Isoforms in a Mouse Brain Metastasis Model of Human Melanoma[1]

Benno Küsters, Robert M. W. de Waal, Pieter Wesseling, Kiek Verrijp, Cathy Maass, Arend Heerschap, Jelle O. Barentsz, Fred Sweep, Dirk J. Ruiter, and William P. J. Leenders[2]

Departments of Pathology [B. K., R. M. W. d. W., P. W., K. V., C. M., D. J. R., W. P. J. L.], Radiology [A. H., J. O. B.], and Chemical Endocrinology [F. S.], University Medical Centre St. Radboud, 6500 HB Nijmegen, the Netherlands

ABSTRACT

We reported previously that vascular endothelial growth factor isoform A (VEGF-A) expression by Mel57 human melanoma cells led to tumor progression in a murine brain metastasis model in an angiogenesis-independent fashion by dilation of co-opted, pre-existing vessels and concomitant enhanced blood supply (B. Kusters *et al.*, Cancer Res., *62:* 341–345, 2002). Here, we compare the activities of the 121, 165, and 189 VEGF-A isoforms in this model by transfecting Mel57 cells with the respective cDNAs and by injecting the resulting stably transfected cell lines in the internal carotid arteries of nude mice ($n = 10$ for each isoform). Although the three isoforms had similar potency to induce endothelial cell proliferation, $VEGF_{121}$ expression did not result in sprouting angiogenesis but rather led to extensive vasodilation and increased permeability of pre-existing, predominantly peritumoral vessels. Sometimes, proliferating endothelial cells accumulated in vessel lumina, giving these a microvascular, glomeruloid, proliferation-like appearance. Expression of $VEGF_{165}$ or $VEGF_{189}$ was associated with induction of an intratumoral neovascular bed. In $VEGF_{165}$-expressing tumors, daughter endothelial cells were distributed among newly formed vessels that were extensively dilated. This also occurred in $VEGF_{189}$ tumors, but there, vasodilation was less pronounced. Using contrast-enhanced magnetic resonance imaging, the different vascular phenotypes were visualized on characteristic radiological images. $VEGF_{165}$ expression was the most unfavorable of the three. Mice carrying $VEGF_{165}$ tumors became moribund earlier than those carrying $VEGF_{121}$-expressing tumors (16 ± 4 days *versus* 22 ± 3 days). Our data demonstrate that VEGF-A isoforms differ in angiogenic properties that can be visualized by contrast-enhanced magnetic resonance imaging.

INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a complex process that is regulated by a vast array of stimulators and inhibitors. Of these, VEGF-A[3] is regarded as the most potent proangiogenic factor. Via binding and activation of the tyrosine kinase receptors VEGFR1 and VEGFR2 (also named Flt-1 and KDR/Flk-1, respectively), endothelial cell proliferation, migration, and expression of tissue factor and proteases are induced (1, 2). These concerted actions lead to blood vessel permeability, followed by extravascular deposition of a fibrin network that serves as a provisional matrix for newly formed daughter endothelial cells (3).

VEGF-A is member of a family of growth factors also comprising VEGF-B,-C,-D,-E; placental growth factor; and the PDGFs (PDGF-A and B) (1). Alternative splicing of VEGF-A pre-mRNA leads to secretion of six variants of this homodimeric molecule consisting of 121, 145, 165, 183, 189, and 206 amino acid residues, respectively (4, 5). In mice, these isoforms have one amino acid residue fewer. With the exception of $VEGF_{121}$, these proteins have affinity for heparin and HSPGs that increases with length because of the presence of positively charged domains encoded by exon 7 in $VEGF_{165}$ and exons 6 and 7 in $VEGF_{189}$ and $VEGF_{206}$ (6–8). The exon 7-encoded sequence also confers affinity upon $VEGF_{165}$ for the coreceptors neuropilin 1 and neuropilin 2. In cell culture $VEGF_{189}$, and to a lesser extent $VEGF_{165}$, are sequestered by HSPGs on the cell surface. It is assumed that in this way the extracellular matrix functions as a reservoir of biologically inactive VEGF-A that, when needed, can be released quickly as NH_2-terminal bioactive fragments by the action of proteases (9–11).

Although $VEGF_{121}$ itself does not bind to heparin or HSPGs, the latter are required for efficient receptor activation (6). Both VEGFR1 and VEGFR2 have heparin-binding domains, suggesting that receptor-bound HSPGs stabilize the ligand-receptor complex (11–13). *In vitro*, $VEGF_{121}$ binds to VEGFR1 with 20-fold lower affinity than $VEGF_{165}$ (14), and it is also less potent in activating VEGFR2 (10). Remarkably, $VEGF_{121}$ and $VEGF_{165}$ have been reported to have similar angiogenic activities *in vivo* (15, 16). The presence of ligand-receptor complex-stabilizing HSPGs *in vivo* that are absent *in vitro* may account for the apparent difference in $VEGF_{121}$ potencies in both settings.

In vitro studies have unraveled in detail the molecular biology and cell biology of VEGF/VEGFR signaling. However, only recently has attention turned to the *in vivo* activities of individual VEGF-A isoforms. Data obtained by the group of Carmeliet (17, 18) using knock-in mice, which selectively express individual VEGF-A isoforms in cardiac muscle, showed that $VEGF_{164}$ is sufficient for normal embryonic vascular development. In hearts where only $VEGF_{188}$ was expressed, arterial development was significantly affected, whereas venous development appeared normal. Exclusive $VEGF_{120}$ expression led to lethal disturbances in vascular development (17, 18).

Contradictory reports exist on the functional importance of $VEGF_{121}$ in tumor biology. When overexpressed in a colon tumor xenograft model, $VEGF_{121}$ increased vascularity and enhanced tumor growth (19), whereas in recent reports expression of $VEGF_{165}$ and $VEGF_{189}$, but not $VEGF_{121}$, correlated with increased vascular density and poor prognosis (20–22). Interestingly, Guo *et al.* (23) described that the ability of $VEGF_{121}$ to induce angiogenesis depended on the site of tumor growth; $VEGF_{121}$ minimally enhanced angiogenesis in and growth of s.c. gliomas, whereas in brain the same $VEGF_{121}$-expressing tumor generated an angiogenic response. Some reports described that $VEGF_{121}$ and $VEGF_{165}$ expression in brain tumor models led to cerebral hemorrhage and $VEGF_{189}$ caused sprouting angiogenesis, whereas other reports stated that $VEGF_{189}$ lacked any angiogenic activity (24, 25).

We reported previously that the human melanoma cell line Mel57 had low background production of angiogenic factors and grew in

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[2] To whom requests for reprints should be addressed, at University Medical Centre St. Radboud, Department of Pathology, 6500 HB Nijmegen, the Netherlands. Phone: 31-243614322; Fax: 31-243540520; E-mail: W.Leenders@pathol.umcn.nl.

[3] The abbreviations used are: VEGF-A, vascular endothelial growth factor isoform A; VEGFR, VEGF receptor; PDGF, platelet-derived growth factor; HSPG, heparan sulfate proteoglycan; CE-MRI, contrast-enhanced magnetic resonance imaging; 3D, three-dimensional; MR, magnetic resonance; USPIO, ultrasmall superparamagnetic iron oxide-coated dextran; Gd-DTPA, gadolinium diethylenetriaminepenta-acetic acid; Glut-1, glucose transporter-1.

murine brain parenchyma by co-option of pre-existing brain vessels, *i.e.* without inducing an angiogenic response and without notably affecting pre-existing vessels (26). In these tumors, even after having reached considerable size, hardly any hypoxia developed. This feature makes this system particularly convenient to study angiogenic activities of different factors, because results are not biased by up-regulation of endogenous VEGF-A, an event known to occur in response to hypoxia. Using this model, we described previously that recombinant VEGF-A was not able to induce an angiogenic response but instead modulated pre-existing vessels by dilation, induction of hyperpermeability, loss of blood-brain barrier markers, and up-regulation of a number of molecules, characteristic of activated endothelial cells (26). In CE-MRI scans, VEGF-A-expressing tumors were easily recognized, whereas parental Mel57 tumors were undetectable (27).

Here we report on the vascular phenotypes and MRI behavior of Mel57 brain tumors expressing either of the three VEGF-A isoforms.

MATERIALS AND METHODS

Cell Lines and Transfections. Mel57 human melanoma cells were maintained in DMEM (Life Technologies, Inc., Breda, the Netherlands) supplemented with 10% FCS and penicillin/streptomycin. cDNAs encoding the 121-, 165-, and 189-amino acid VEGF isoforms (human origin) were cloned in vector pIRESneo (Clontech) to generate plasmids pIRESneoVEGF$_{121}$, pIRESneoVEGF$_{165}$, and pIRESneoVEGF$_{189}$. All sequences were verified by DNA sequencing (AbiPrism; Applied Biosystems). Plasmid pIRESneoVEGF$_{165}$ was different from the VEGF$_{165}$ construct published before (26), because the cDNA in that plasmid had a slightly modified 3′ end. Plasmids were stably transfected into Mel57 cells as described (26). The presence of recombinant proteins in conditioned media was confirmed by SDS-PAGE and Western blotting experiments using antibody VEGF-A20 (Santa Cruz Biotechnology, Santa Cruz, CA). In the case of VEGF$_{189}$, 5 IU/ml heparin (Leo Systems, Breda, the Netherlands) were included in the culture medium before analysis. VEGF concentrations were also determined using an ELISA developed in-house (28).

Animal Experiments. All experiments were approved by the Animal Experiment Committee of Nijmegen University. The hematogenous brain metastasis protocol has been described previously (29). In short, 10^5 Mel57 (transfectant) cells were microsurgically injected into the right internal carotid artery of BALB/c nude mice (n = at least 10 for each cell line). After 14–21 days, depending on which isoform was expressed, animals became moribund because of cachexia or development of neurological defects. At this stage, CE-MRI was performed (see below), after which animals were killed by i.v.

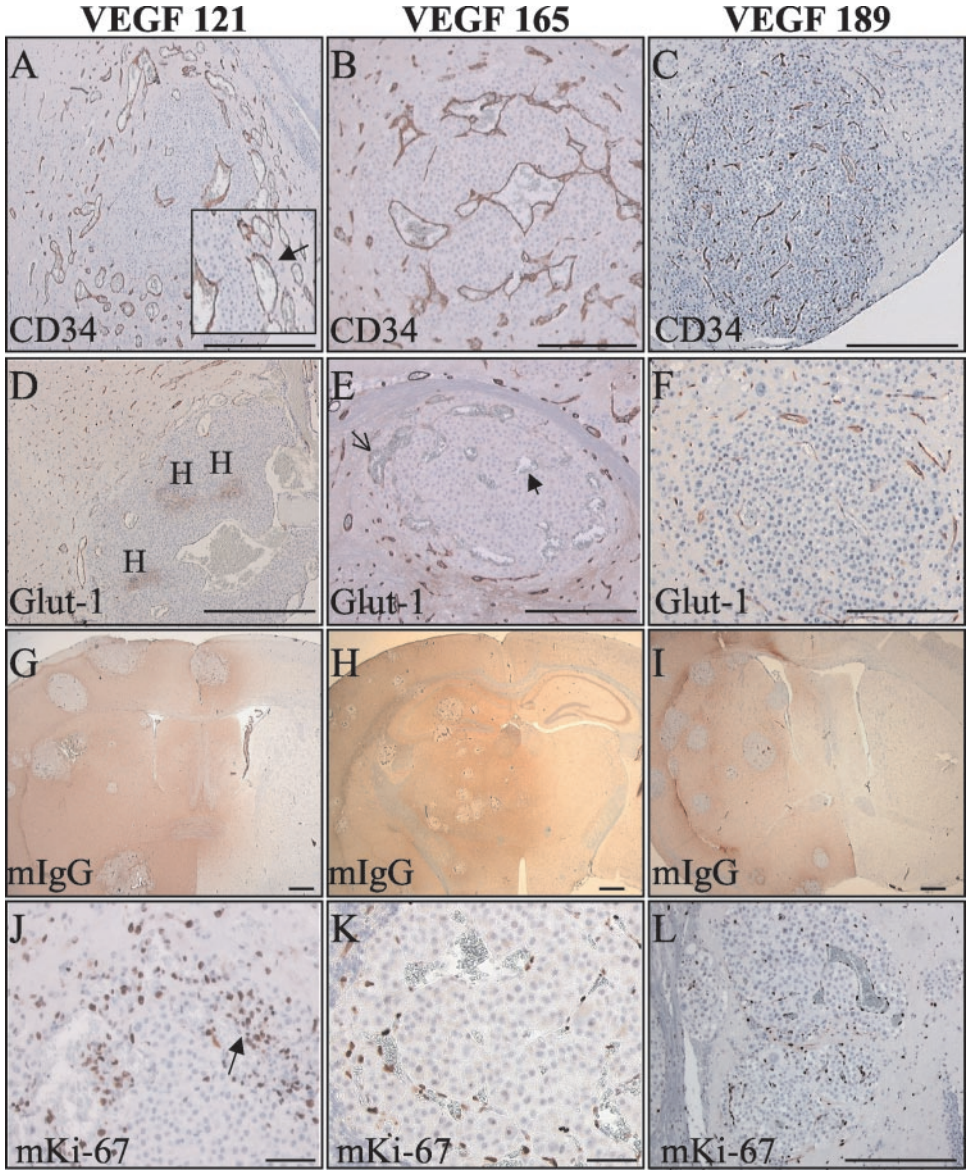

Fig. 1. Immunohistochemical analysis of mouse brain Mel57 lesions expressing VEGF isoforms as indicated. *A–C*, CD34 endothelial staining. *D–F*, staining for Glut-1, a blood-brain barrier-specific protein. *G–I*, staining for murine IgG (*mIgG*) showing cumulative leakage of immunoglobulins from permeable vessels. *J–L*, staining for the murine proliferation marker Ki67 (*mKi-67*). The *arrow* in *J* points at a glomeruloid-like vascular proliferation, whereas in *K* and *L*, daughter endothelial cells are redistributed over newly formed vessels. Note the predominantly peritumoral effects on the vasculature by VEGF$_{121}$ (*A*), whereas expression of the two other isoforms lead to predominantly intratumoral vascular effects (*B* and *C*). The *arrows* in *E* point at Glut-1 negative (*arrow*) and mosaic (*small arrowhead*) vessels. *H*, hypoxia, which manifests as Glut-1 positivity. *Bars*, 0.5 mm.

injection of an overdose of barbiturates. Brains were removed and either fixed in formalin or snap-frozen in liquid nitrogen.

3D Reconstruction of Tumor Vessels by Ink Perfusion. Moribund tumor-bearing mice (three from each group) were anesthetized and received injections microsurgically into the supra-aortic branches with 5 ml of India ink that contained 5000 units/ml heparin. After this procedure, brains were removed and fixed in buffered formalin for 24 h, and 0.5-mm sections were cut and examined under a transmission microscope with bright light. By focusing through different layers of the section, a good impression of the 3D vascular structure was obtained.

Immunohistochemistry. Immunostainings were performed using antibodies directed against murine Ki67 as a proliferation marker (Dianova, Hamburg, Germany), the endothelial markers CD31 and CD34 (Hycult, Uden, the Netherlands), VEGFR2 (Santa Cruz Biotechnology), and the blood-brain barrier marker Glut-1 (DAKO, Glostrup, Denmark). Antibodies against the pericyte marker α-smooth muscle actin were obtained from Sigma (Zwijndrecht, the Netherlands). Extravascular mouse immunoglobulins were detected with a biotin-labeled anti-mouse IgG. Secondary biotin-labeled antibodies were visualized by avidin-biotin complex staining according to standard protocols (Vector, Burlingame, CA).

Magnetic Resonance Imaging. Mice were anesthetized (1.3% isoflurane; 1:1 (v/v) N_2O:O_2 mixture), catheterized in the tail vein, and placed in an MR spectrometer (S.M.I.S. console equipped with a Magnex Scientific 7T/200 mm horizontal bore magnet and a 150-mT/m gradient set). Body temperature was maintained at 37°C with a circulating warm water bed. After initial monitoring of the mouse brain with fast gradient-echo scout images using a 12-mm surface coil, 20 contiguous, high-resolution coronal MR images were acquired with T_2-weighted multislice spin-echo imaging (T_E, 50 ms; T_R, 3000 ms; field of view, 40 × 40 mm; matrix size, 512 × 512; slice thickness, 1 mm) before and 2 min after i.v. injection of 0.2 ml of USPIO (Sinerem®; Guerbet, Roissy-Charles-de-Gaulle, France; 12.5 mg/kg). In the case of Gd-DTPA contrast enhancement, 16 contiguous images were acquired with a T_1-weighted multislice gradient-echo sequence (T_E, 8 ms; T_R, 100 ms; field of view, 25 × 25 mm; matrix size, 256 × 256; slice thickness, 1 mm) before and at several time points (0, 2, 10, and 20 min) after bolus injection (0.2 ml; 0.2 mmol/kg) of Magnevist® (Schering, Berlin, Germany).

RESULTS

Effects of VEGF-A Isoforms on Growth of Mel57 Brain Lesions. To examine the effects of the 121, 165, and 189 isoforms of VEGF-A on tumor growth in mouse brain, we generated stable transfectants of the human melanoma cell line Mel57, expressing each isoform *in vitro* at levels of 30–100 ng/ml per 10^6 cells in 48 h (as determined by ELISA, not shown). Recombinant proteins were of the correct molecular weights as determined by Western blotting (not shown). Before transfection, this cell line expressed very low endogenous levels of angiogenic factors, especially VEGF-A (30, 31). After intracarotid injection of parental Mel57 cells, metastatic lesions formed in the parenchyma grew by vascular co-option without notable vascular changes (26, 27). In tumors expressing any of the three VEGF-A isoforms, we found up-regulation of the endothelial markers CD31, CD34, VEGFR2, and the activated pericyte marker α-smooth muscle actin (Fig. 1, *A–C* and not shown). There was no significant effect of VEGF-A isoform expression on the number of lesions that developed (not shown). All isoforms induced proliferation of endothelial cells, as demonstrated by mouse-specific immunostaining for the proliferation marker Ki67 (Fig. 1, *J–L*). Furthermore, staining for murine IgGs, which normally do not pass the blood-brain barrier, revealed that all isoforms caused extravasation of macromolecules, indicating vessel hyperpermeability (Fig. 1, *G–I*). Besides these common VEGF-A effects, striking differences were found between vascular morphologies, as described below. *In situ* hybridization with an antisense VEGF-A RNA probe revealed that also *in vivo*, expression levels of the three isoforms were comparable (not shown), indicating that the differences were qualitative.

$VEGF_{121}$. After injection of Mel57-$VEGF_{121}$ cells, tumors developed with a combined infiltrative and expansive phenotype. Animals were moribund because of cachexia or neurological defects 3–4 weeks after injection of tumor cells; these effects were reproducible. Immunostaining for the endothelial marker CD34 revealed that in the peritumoral zone, irregularly dilated vessels were present (Fig. 1*A*). Tumor cells could often be observed along these vessels, indicating migration in the perivascular space (*arrow* in Fig. 1*A*, *inset*). Vessel densities within tumor nests were relatively low, as was demonstrated by CD34 immunohistochemistry (Fig. 1*A*) and by 3D visualization established by ink perfusion (Fig. 2*B*). The low intratumoral vessel density and the morphology of peritumoral vessels indicated a lack of sprouting angiogenesis. In some tumors, the low intratumoral vessel density resulted in central hypoxia and necrosis [Fig. 1*D*: note that Glut-1, besides being a marker for the blood-brain barrier (see below), also is up-regulated on hypoxic cells]. Although $VEGF_{121}$ clearly lacked the capacity to induce sprouting angiogenesis, it was yet a strong inducer of endothelial proliferation (see the murine Ki67 staining in Fig. 1*J*). Focally, glomeruloid-like microvascular proliferations consisting of endothelial cells and pericytes were present (Fig. 1*J*,

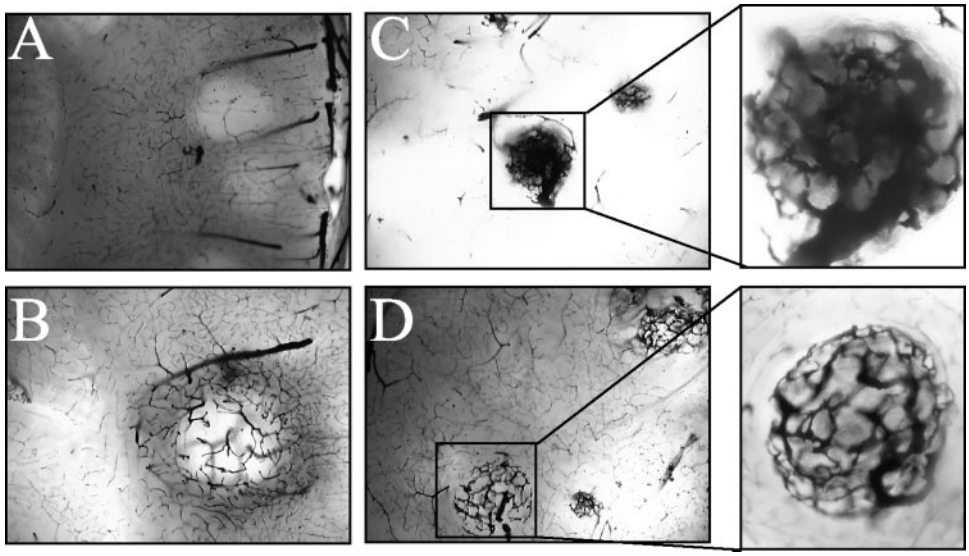

Fig. 2. 3D visualization of brain tumor vasculature. Mice carrying parental Mel57 (*A*), Mel57-VEGF121 (*B*), Mel57-VEGF165 (*C*), and Mel57-VEGF189 (*D*) tumors were perfused with India ink as described in "Materials and Methods." Brains were cut into 0.5-mm sections and examined under a transmission light microscope. Lesions were easily identified on the basis of different optical breaking indices compared with normal brain tissue. *White areas* in *A* and *B* represent the poorly vascularized lesions ×50. *Insets*, ×200. Because mice carrying $VEGF_{165}$ lesions became moribund 14 days after injection of the tumor cells, *i.e.*, 1 week earlier than the other mice (see text), $VEGF_{165}$ lesions were relatively small. Because of the amount of light needed to visualize these relatively small, very dark lesions, the surrounding brain tissue is hardly visible in *C*.

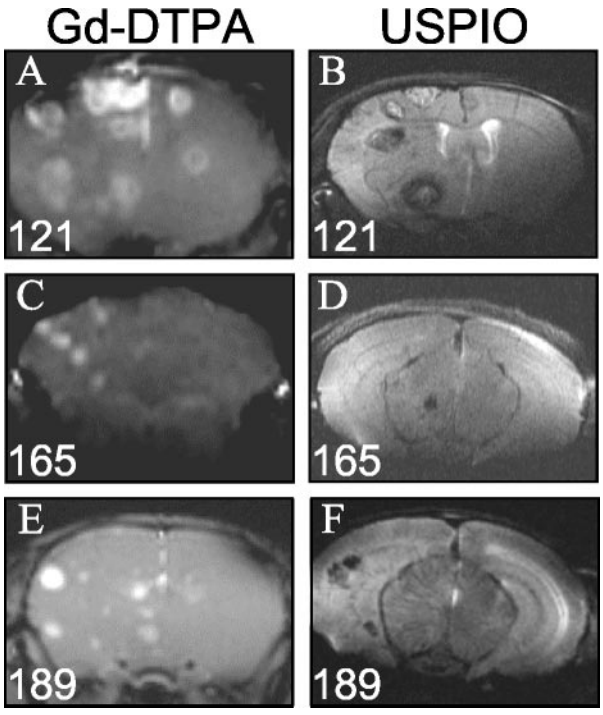

Fig. 3. CE-MRI of murine brains carrying Mel57 lesions expressing the different VEGF isoforms. T_1 gradient echo (*A*, *C*, and *E*) or T_2 spin echo sequences (*B*, *D*, and *F*) were used to detect Gd-DTPA and USPIOs, respectively. The images shown were acquired 2 min after i.v. bolus injection of both contrast agents.

arrow), resembling a phenotype that is often encountered in high-grade glial brain tumors such as glioblastoma multiforme (32). Glut-1 is a membrane protein that is specifically expressed on endothelial cells of brain vessels and is regarded as a blood-brain barrier marker (33). The dilated vessels in and around $VEGF_{121}$ lesions had a mosaic Glut-1 phenotype, some endothelial cells being positive and others negative (Fig. 1*D*), consistent with a loss of blood-brain barrier function.

$VEGF_{121}$-induced peritumoral vascular effects were reflected in CE-MRI. Intravenous injection of the paramagnetic, low molecular weight compound Gd-DTPA in mice carrying $VEGF_{121}$-expressing lesions led to a predominantly circumferential signal increase immediately after injection, whereas centrally in tumor nests, no notable enhancement was observed (Fig. 3*A*). Peritumoral enhancement was entirely attributable to expression of $VEGF_{121}$, because it was absent in mice carrying parental Mel57 lesions (27). A similar pattern of enhancement was obtained after i.v. injection of USPIOs that, because of their large size, are assumed to remain in the intravasculature (34). Again, predominantly circumferential enhancement occurred (Fig. 3*B*), although individual, dilated, intratumoral vessels were also highlighted (note that the nature of the MR sequence used causes a drop of signal intensity). This enhancement pattern lasted for up to 4 h after injection, whereas Gd-DTPA enhancement was much more transient, having disappeared within 20 min after injection (not shown).

$VEGF_{165}$ and $VEGF_{189}$. When $VEGF_{165}$ or $VEGF_{189}$ was expressed by Mel57 brain tumors, mice developed terminal cachexia and neurological defects approximately 2 and 3 weeks after injection, respectively. The tumor vasculature that had formed was very different from that induced by $VEGF_{121}$. Now, extensive neoangiogenesis had occurred (see CD34 staining in Fig. 1, *B* and *C*, and 3D visualization in Fig. 2, *C* and *D*), whereas peritumoral vascular effects were hardly observed, in contrast to the $VEGF_{121}$ situation (Fig. 2*B*). $VEGF_{165}$-expressing lesions displayed prominent vessel dilation and permeability with numerous proliferating endothelial cells (Fig. 1, *B*, *E*, and *K*). Consistent with the high vascular volume in these tumors, necrosis was never observed. In the border regions of these tumors, vessels were partly Glut-1 negative (Fig. 1*E*, *thin arrowhead*), whereas this protein was completely absent in vessels in the tumor center (*bold arrowhead*). In the $VEGF_{189}$-expressing metastases, a dense network of microcapillaries had formed that were leaky and had diminished Glut-1 expression, whereas vessel dilation was minimal. Consistent with the high intratumoral vascular volumes, $VEGF_{165}$- or $VEGF_{189}$-expressing tumor nests were entirely contrast enhanced in Gd-DTPA- and USPIO-enhanced MR images (Fig. 3, *C–F*). The wash-out rates of Gd-DTPA were not notably different from that of the $VEGF_{121}$-expressing tumors, because 10–20 min after injection, signals had returned to basal levels (not shown). Because of the earlier time point at which mice carrying $VEGF_{165}$ lesions became moribund, these lesions were generally smaller than $VEGF_{121}$ lesions.

DISCUSSION

We report here that, in striking contrast to $VEGF_{165}$ and $VEGF_{189}$, $VEGF_{121}$ is not able to induce angiogenic sprouting in metastatic melanoma in mouse brain. Rather, the small isoform caused extensive dilation and permeability of pre-existing blood vessels, a phenotype that is very similar to what we described previously for $VEGF_{165}$ (26). However, in that study we used a $VEGF_{165}$ molecule that carried a mutation of the COOH terminus. At that time, we considered this a wild-type protein because the COOH terminus had not been recognized in the literature as functionally important. Furthermore, this protein was indistinguishable from commercially obtained wild-type $VEGF_{165}$ in a number of *in vitro* functional and receptor binding assays. To our surprise, we have now found that when the COOH terminus was unmodified, a different vascular phenotype developed than we described previously, indicating that the COOH terminus is critical for full VEGF activity. These findings imply that different biological activities of $VEGF_{165}$ can be dissected using these mutants.[4]

Interestingly, in a recent report Bates *et al.* (35) described the identification of $VEGF_{165b}$, a splice variant that lacks the COOH-terminal exon 8 sequence. Despite intact binding domains for VEGFR1, VEGFR2, and neuropilins, this protein behaved as a VEGF receptor antagonist, thus confirming the importance of the COOH terminus for VEGF activity. How exactly exon 8-encoded sequences contribute to VEGF-A activity is still enigmatic.

In contrast to $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ induced extensive sprouting angiogenesis in our model. The phenotypes of tumors expressing these larger isoforms could also be distinguished because $VEGF_{165}$ induced profound vasodilation, whereas $VEGF_{189}$ failed to do so.

Interestingly, expression of $VEGF_{165}$ appeared to correlate with worse clinical outcome because we consistently found that mice carrying $VEGF_{165}$-expressing brain tumors became symptomatic after ~2 weeks, 1 week earlier than those expressing $VEGF_{121}$ or $VEGF_{189}$. The reason for this difference is not clear; it is not likely to be a result of enhanced growth of the $VEGF_{165}$-expressing tumors. In a time-matched experiment, we found no significant differences between the sizes of the various brain lesions, although within individual animals, tumor sizes showed a high degree of heterogeneity (not shown). Possibly, the combination of extensive vasodilation and hyperpermeability induced by $VEGF_{165}$ led to more severe edema in the surrounding brain tissue than that induced by the other isoforms. We have not examined this in detail.

In all VEGF-expressing tumors, irrespective of the isoform, the

[4] Manuscript in preparation.

blood-brain barrier marker Glut-1 was absent or only partially expressed on vessels. Whether VEGF-A actively down-regulates Glut-1 expression on pre-existing brain vessels, or newly formed endothelium simply does not express this brain endothelium-specific protein, is not completely clear. The fact that in $VEGF_{165}$ lesions intratumoral vessels were completely negative for Glut-1, whereas at the tumor rim mosaic vessels were found, is in favor of the second option. At the tumor rim, pre-existing vessels become gradually influenced by tumor-derived VEGF-A, leading to a mixture of mother endothelial cells (Glut-1 positive) and daughter cells (Glut-1 negative). As endothelial cell proliferation continues, Glut-1-negative endothelial cells become more and more abundant until in the intratumoral vessels, they prevail.

Depending on which VEGF isoform was expressed, tumors had distinct patterns of contrast enhancement in MRI experiments. Tumors that expressed $VEGF_{121}$ characteristically showed a circumferential enhancement, because of a peritumorally located, dilated, and leaky vasculature, combined with low intratumoral vessel density. The larger isoform-expressing tumors showed a more homogeneous, bulky enhancement. These MR images were in good agreement with our morphological findings. Peritumoral Gd-DTPA enhancement in the $VEGF_{121}$-expressing tumors indicated the presence of leaky vessels outside of the main tumor mass, from which the contrast agent could extravasate and accumulate in the surrounding tissue, whereas the strong signal drop after USPIO injection was in accordance with the high density of dilated vessels, causing a high local vascular volume. The relatively low intratumoral vessel density accordingly led to low levels of enhancement of the lesion itself, both with Gd-DTPA and USPIOs. The bulky enhancement seen in $VEGF_{165}$ and $VEGF_{189}$ lesions was also in accordance with the high vascular volume of these tumors. Whether CE-MR images of human tumors can be correlated to VEGF isoform expression is not known. If so, it may helpful for prognosis and possibly for selection of patients as candidates for anti-VEGF therapy. In a preliminary study, we found that the heterogeneity in expression levels of the different VEGF isoforms in human tumors makes this question difficult to answer.

Differential effects of the various VEGF-A isoforms have only recently received attention in the literature. Via targeted disruption of specific VEGF exons, Maes *et al.* (36) and Zelzer *et al.* (37) have generated mice that selectively express $VEGF_{120}$. $VEGF_{120}$ could not substitute for the larger isoforms because during development, these mice developed severe angiogenesis-related abnormalities (36, 37). These defects might in fact be related to vasculogenesis rather than angiogenesis, because $VEGF_{121}$ and $VEGF_{165}$ are equally effective in inducing angiogenesis in the chick chorioallantoic membrane angiogenesis assay and collateral formation in ischemic rabbit hindlegs (16). In a recent report, $VEGF_{120}$ was described to be relatively incapable of inducing branching of blood vessels in various organs during development (38).

In tumors, controversial observations have been reported regarding VEGF isoform activities. VEGF-null fibroblasts have been generated and used to establish fibrosarcomas expressing each individual VEGF isoform (39). Analysis of s.c. tumors grown from these cell lines revealed that only $VEGF_{164}$ could compensate for the lack of the endogenous VEGF gene, whereas $VEGF_{120}$ had a negligible angiogenic effect. In this model, $VEGF_{188}$ expression led to highly vascularized, yet small, tumors. From these data the hypothesis emerged that $VEGF_{120}$ diffuses away from the tumor and recruits vasculature from surrounding tissue, whereas the extracellular matrix-binding isoform $VEGF_{188}$ induces local branching and sprouting angiogenesis. In this model, the $VEGF_{164}$ isoform has intermediate activity. This model is consistent with our current findings; whereas $VEGF_{121}$ failed to induce an intratumoral neovascular bed, $VEGF_{189}$ expression in our brain model led to highly vascularized and relatively large tumors. This largely confirms results from other groups (23, 24) and might be explained by the fact that in the vessel-dense brain, there is no need for extra recruitment of blood vessels from the surroundings (26).

The difference between $VEGF_{121}$ and $VEGF_{165}$ is the presence of the exon 7-encoded domain in $VEGF_{165}$, which is responsible for neuropilin and HSPG binding. Neuropilin 1 potentiates the activity of the VEGF-A/VEGFR2 complex (40), and HSPGs can act as chaperones for $VEGF_{165}$ (41) or even transmit $VEGF_{165}$ signals (42). In this light, it was a remarkable observation that $VEGF_{121}$ induced extensive endothelial cell proliferation, a VEGFR2-mediated response, in pre-existing vessels to an extent that at least equaled that induced by the larger VEGF isoforms. This suggests that neuropilin 1-enhanced VEGFR2 binding cannot account for the differences between the *in vivo* activities of the 121 and 165 isoforms. A second argument against a role for neuropilin and HSPGs in the $VEGF_{165}$-induced tumor phenotype can now be found in our previous study in which we used a $VEGF_{165}$ mutant (see above; Ref. 26). This mutant did contain the intact exon 7 sequence and therefore had intact neuropilin and HSPG affinity. Nevertheless, it did not induce sprouting angiogenesis but led to a $VEGF_{121}$-like vascular phenotype.

An interesting observation was that $VEGF_{121}$ was an efficient inducer of endothelial cell proliferation in the absence of sprouting angiogenesis. This shows that activation of VEGFR2, the receptor that is responsible for proliferation, occurred but was not sufficient for angiogenesis in this model, contrary to what is generally believed. Obviously, endothelial proliferation via VEGFR2 activation is a critical step in the initial stages of angiogenesis but is not sufficient for completion of this process; migration, adhesion, and protease activities are obviously necessary as well. Remarkably, in a recent report it was shown that $VEGF_{121}$, in contrast to the larger VEGF-A isoforms, was unable to mediate endothelial migration *in vitro* (43), possibly because extracellular matrix-bound VEGF-A is needed for migration. Whether this concept also explains our *in vivo* results remains to be established. Alternatively, certain signals induced by $VEGF_{165}$ and $VEGF_{189}$ may fail to occur in the $VEGF_{121}$-induced signal transduction cascade, thereby rendering $VEGF_{121}$ unable to induce sprouting angiogenesis. It would be interesting to know the nature of these signals and the receptors from which they emerge, because apparently the differences between $VEGF_{121}$ and $VEGF_{165}$ activities cannot be solely attributed to differential binding to neuropilins, VEGFR2, and HSPGs. These issues are currently under investigation in our laboratory.

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