Plexin D1 Expression Is Induced on Tumor Vasculature and Tumor Cells: A Novel Target for Diagnosis and Therapy?

Ilse Roodink,1 Jos Raats,4 Bert van der Zwaag,2 Kiek Verrijp,1 Benno Kusters,1 Hans van Bokhoven,3 Marianne Linkels,1 Robert M.W. de Waal,1 and William P.J. Leenders1

Departments of Pathology, Neurology, and Human Genetics, Radboud University Nijmegen Medical Centre; and ModiQuest B.V. and Department of Biochemistry, Nijmegen Centre for Molecular Life Sciences, Nijmegen, the Netherlands

Abstract

We previously reported that during mouse embryogenesis, plexin D1 (plxnD1) is expressed on neuronal and endothelial cells. Endothelial cells gradually loose plxnD1 expression during development. Here we describe, using in situ hybridization, that endothelial plxnD1 expression is regained during tumor angiogenesis in a mouse model of brain metastasis. Importantly, we found PLXND1 expression also in a number of human brain tumors, both of primary and metastatic origin. Apart from the tumor vasculature, abundant expression was also found on tumor cells. Via panning of a phage display library, we isolated two phages that carry single-domain antibodies with specific affinity towards a PLXND1-specific peptide. Immunohistochemistry with these single-domain antibodies on the same tumors that were used for in situ hybridization confirmed PLXND1 expression on the protein level. Furthermore, both these phages and the derived antibodies specifically homed to vessels in brain lesions of angiogenic melanoma in mice after i.v. injection. These results show that PLXND1 is a clinically relevant marker of tumor vasculature that can be targeted via i.v. injections. (Cancer Res 2005; 65(18): 8317-23)

Introduction

It is commonly accepted that to grow beyond a size of 2 to 3 mm³, tumors have to recruit a neovasculature via angiogenesis (1, 2). Tumors accomplish this via expression of vascular endothelial growth factor-A (VEGF-A), either induced by hypoxia in the tumor center (3) or as a result of malfunctioning tumor suppressor gene products or activated proto-oncogenes (4, 5). A number of compounds that target the VEGF-A signaling pathway has been developed with the aim to inhibit angiogenesis and consequently tumor growth (6). Although such antiangiogenic therapies have been effective in animal tumor models (7, 8), translation to the clinical level has thus far proven to be less successful (9).

For this, there is a number of possible explanations. In clinically relevant situations, tumors may have been growing for months or even years at the time of diagnosis, and a significant proportion of the vasculature may be more or less mature and thus insensitive to angiogenesis inhibition. This situation is in sharp contrast to that in most animal models in which, as a rule, aggressive, fast-growing angiogenesis inhibition. This situation is in sharp contrast to that in most animal models in which, as a rule, aggressive, fast-growing tumors are studied. Furthermore, patients that are candidates for antiangiogenic therapy are typically patients with disseminated, uncontrollable cancer. We and others described that growth of metastases may not always be strictly dependent on angiogenesis (10–12). Because most metastases are blood borne, they grow out in organs with intrinsically high vessel densities like liver, lung, and brain, where they can grow in an angiogenesis-independent fashion by co-option of preexistent vessels (12–14). We have recently reported that an angiogenesis inhibitor that very effectively inhibits tumor growth in a number of s.c. tumor models (7) does not inhibit growth of infiltrative tumors in mouse brain. Moreover, upon treatment of mice carrying highly angiogenic brain tumors, angiogenesis inhibition resulted again not in tumor inhibition but rather in a phenotypic shift towards co-option and infiltration (15). These results imply that antiangiogenic therapy should ideally be supplemented by vascular targeting therapies in which the existing tumor vascular bed is attacked, resulting in secondary tumor cell death due to disruption of the tumor's blood supply (9).

To accomplish effective vascular targeting therapy, markers have to be identified that have specificity for tumor vasculature. Much effort has already been put in this but with varying success. Effective vascular tumor targeting has been accomplished using single-chain antibodies, directed against the fibronectin ED-B domain, which is selectively expressed and deposited in the extracellular matrix of newly formed vessels in angiogenic tumors (16, 17). Targeting of αvβ3-integrin (the expression of which is restricted to immature vessels) using Vaxitin yielded disappointing results (18), whereas endoglin expression was not specific for tumor blood vessels (19).

Plexins comprise a family of membrane proteins that are receptors for the semaphorins, a family of secreted and membrane-bound ligands (20). Class 3 semaphorins are potent axon repellants and are as such involved in morphogenesis of the nervous system (for review, see refs. 21, 22). These semaphorins activate plexins via binding of neuropilins in a multicomponent complex (23).

We previously identified and characterized plexin D1 (plxnD1) as a plexin that is not only expressed in neuronal cells but also in the vasculature during early stages of development (24). In adult vasculature, plxnD1 is absent. Recently, proof for a functional role of plxnD1 during vascular and cardiovascular morphogenesis was provided using zebrafish mutants and knockout mice (25, 26). PlxnD1 is a receptor for semaphorin 3E, and unlike other members of the plexin family, does not require neuropilins for Semaphorin 3E-mediated signaling (27).

The high expression levels of plxnD1 in angiogenic vessels during embryogenesis led us to investigate whether this protein is also expressed during tumor-associated angiogenesis. We found that plxnD1 was indeed expressed at high levels in tumor vessels of intracerebral Mel57-VEGF-A tumors (28) but not in unaffected brain vessels. Importantly, human tumors of different origin also expressed the protein in their vessels indicating that PLXND1
expression is not restricted to early stages of angiogenesis. Interestingly, tumor cells also frequently express PLXND1, making this protein a unique candidate for tumor-targeting therapies as it is expected to target both vessels and tumor cells. Lv. injection of M13 phages displaying anti-PLXND1 single-domain antibodies or the respective soluble single-domain antibodies, led to accumulation on tumor vessels but not on normal brain vessels. Thus, PLXND1 may be a promising target for antivascular and antitumor therapies.

**Materials and Methods**

**Phage display.** A phage display library was constructed by reverse transcription-PCR (RT-PCR) from Llama B lymphocytes essentially as described (29, 30). VH1/single-domain antibody fragments were ligated into phagemid vector pHELIXHis5SVS. resulting in a fusion product with 8\(^\text{His}\)-tag and VSG-tag at the COOH terminus (31). After electroporation in *Escherichia coli* TG1 cells, ampicillin-resistant colonies were collected and pooled. The library had a complexity of 8 \(\times\) 10\(^8\) clones. Eighty percent of plasmids contained full-length single-domain antibody insert as determined by PCR analysis and immunologic dot blot detection of the VSG-Tag in single-domain antibodies (see below). The phage library was propagated as phagemids in *E. coli* TG1 and TG1 Phage particles were rescued by infection with trypsin-sensitive helper phage M13K07 (32). Phages were purified and concentrated from the culture supernatant by precipitation with 20% polyethylene glycol/2.5 mol/L NaCl as described (33).

**Selection of PLXND1-binding phage/single-domain antibody.** Immunotubes (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 5 μg/mL keyhole limpet hemocyanin (KLH)-conjugated peptide (H\(_2\)N-ALEIQRRFPSPTPTNC-CONH\(_2\) corresponding to amino acids 1-16 of the mature human PLXND1 protein; Genbank accession no. AY116661) in 50 mmol/L NaHCO\(_3\) (pH 9.6). Of note, the glutamic acid on position 3 in this peptide is a lysine in the mouse sequence, the remaining amino acids are homologous to mouse plxnA1. After washing with PBS, 0.05% Tween 20 (PBST), nonspecific binding sites were blocked with 5% marvel in PBST (PBST, 1 hour at room temperature) and 10\(^{-5}\) phage particles from the library stock were incubated with the immobilized peptide for 90 minutes at room temperature. After rigorous washing with PBST and PBS, bound phages were eluted by trypsin treatment (10 mg/mL, 30 minutes at room temperature). After trypsin inactivation with 1% newborn calf serum, the eluate was used to infect log-phase TG1 cells to amplify PLXND1-binding phages and calculate number of binders.

To enrich for binding phages, four rounds of selection were done. From the second round on, selections were done against unconjugated peptides, immobilized on DNA-binding plates (Costar, Acton, MA) to prevent selection of KLH binders.

**Analysis of phage specificity by ELISA.** Individual PLXND1-binding phages with PCR-confirmed full-length single-domain antibody inserts were tested for specificity. Wells of DNA-binding plates or immunoplates (Nunc) were coated overnight at 4 °C with 50 µg/mL keyhole limpet hemocyanin (KLH)-conjugated peptide (H\(_2\)N-ALEIQRRFPSPTPTNC-CONH\(_2\) corresponding to amino acids 1-16 of the mature human PLXND1 protein; Genbank accession no. AY116661) in 50 mmol/L NaHCO\(_3\) (pH 9.6). Of note, the glutamic acid on position 3 in this peptide is a lysine in the mouse sequence, the remaining amino acids are homologous to mouse plxnA1. After washing with PBS, 0.05% Tween 20 (PBST), nonspecific binding sites were blocked with 5% marvel in PBST (PBST, 1 hour at room temperature) and 10\(^{-5}\) phage particles from the library stock were incubated with the immobilized peptide for 90 minutes at room temperature. After rigorous washing with PBST and PBS, bound phages were eluted by trypsin treatment (10 mg/mL, 30 minutes at room temperature). After trypsin inactivation with 1% newborn calf serum, the eluate was used to infect log-phase TG1 cells to amplify PLXND1-binding phages and calculate number of binders.

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**Analysis of phage specificity by ELISA.** Individual PLXND1-binding phages with PCR-confirmed full-length single-domain antibody inserts were tested for specificity. Wells of DNA-binding plates or immunoplates (Nunc) were coated overnight at 4 °C with 50 µg/mL PLXND1 peptide or an irrelevant peptide [1 µg per well in PBS/0.5 mol/L NaCl (pH 9.0), bovine serum albumin [BSA, 1 µg per well in 50 µg/mL NaHCO\(_3\) (pH 9.6)], or human IgG [1 µg per well in 50 µg/mL NaHCO\(_3\) (pH 9.6)]. After blocking nonspecific binding sites with MPBST, wells were incubated with phages in MPBST for 1 hour at room temperature and nonbound phages removed by rigorous washing. Bound phages were detected using horseradish peroxidase-conjugated anti-M13 (Amersham Pharmacia Biotech, Piscataway, NJ) and tetramethylbenzidine (BioMérieux B.V., Boxtel, the Netherlands). The reaction was terminated with 2 mol/L H\(_2\)SO\(_4\) and enzymatic activity quantified by measuring absorbance at 450 nm using an ELISA reader.

**Soluble single-domain antibody expression of plxnA1-specific clones.** Expression of soluble single-domain antibodies was induced in log-phase TG1 cells by culturing at 30 °C in 2% TYA medium/1 mmol/L isoprropyl-L-thio-B-d-galactopyranoside. Single-domain antibodies were collected by osmotic lysis using ice-cold TES buffer (200 mmol/L Tris-HCl, 0.5 mmol/L EDTA, 500 mmol/L sucrose) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Single-domain antibody concentrations were estimated via dot blot analysis using the mouse monoclonal anti-BSA-Tag, alkaline phosphatase–conjugated rabbit anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) and nitroblue tetrazolium/3-bromo-4-chloro-3-indolyl phosphate staining. Single-domain antibodies were tested in ELISA for PLXND1 peptide specificity.

**BIAcore analysis.** A BIAcore 2000 (Biacore AB, Uppsala, Sweden) biosensor was used to determine binding affinities of the single-domain antibodies. The sensor chip and protein coupling chemicals were purchased from Biacore AB. PLXND1 peptide/KLH conjugate (27 µg/mL in Na acetate, pH 4.0) was coupled to activated CM5 surfaces using N-ethyl-N′-(dimethylamino)propyl carbodiimide, N-hydroxysuccinimide, under conditions recommended by the manufacturer. Unreacted groups were inactivated by 1 mol/L ethanolamine (pH 8.5).

Kinetic measurements were done at 25°C with a flow rate of 10 µL/min in HBS-EP buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 3 mmol/L EDTA, and 0.005% surfactant P20].

Six concentrations of Ni affinity–purified single-domain antibodies (in the range of 1 mmol/L to 30 mmol/L) were used to determine the dissociation constants (K\(_d\)) of the interaction with the PLXND1 peptide. After each experiment, regeneration of the sensor surface was done with 10 mmol/L NaOH. Specific binding, defined by binding to a PLXND1 surface minus binding to a control BSA surface, was analyzed using the BIAevaluation 4.1 software and a 1:1 Langmuir binding model.

**In situ hybridization.** Generation of digoxigenin-labeled sense and antisense mouse *plxnA1* DNA probes was described before (24). A 600-bp human sense and antisense PLXND1 RNA probe, located in the 3′-untranslated region, was generated by transcription from a PCR product that was flanked by T7 and T3 promoters. Hybridizations were done using standard protocols.

**Immunohistochemistry.** Four-micrometer sections of archival, paraffin-embedded or frozen brain tumor tissue of different origin (glioblastoma multiforme and brain metastases of melanoma and sarcoma) were immunostained with anti-PLXND1 single-domain antibodies. In addition, cerebral mouse xenografts of the human melanoma cell line Me577-VEGF-A (28) were stained with these single-domain antibodies. Following deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.03% H\(_2\)O\(_2\). Antigen retrieval was done by treatment with Protease according to standard protocols. Subsequently, slides were preincubated with normal horse or goat serum (to block nonspecific binding sites in sections of human and mouse tissues, respectively) followed by incubation with single-domain antibodies for 1 hour. Single-domain antibodies were detected by sequential 1-hour incubations with a mouse or rabbit anti-VEGF-A antibody (Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands), biotinylated anti-mouse or anti-rabbit antibody as appropriate (Vector, Burlingame, CA), and avidin-biotin peroxidase complex (Vector). Finally, peroxidase was visualized by the 3-amin-9-ethylcarbazole (ScyTek, Logan, UT) peroxidase reaction with hematoxylin as counterstain. All steps were done at room temperature. The blood vessel origin of PLXND1 expression was confirmed by doing stainings on serial sections with single-domain antibodies and anti-human or anti-mouse (as appropriate) anti-CD31 antibody (DAKO, anti-human CD31; Hycult, Uden, The Netherlands, anti-mouse CD34).

**Animal experiments.** All experiments were approved by the Animal Experiment Committee of the Nijmegen University. The hematogenous brain metastasis protocol has been described previously (34). In short, 2 \(\times\) 10\(^5\) stably transfected Mel57 cells expressing the VEGF-A\(_{165}\) isoform were microsurgically injected into the right internal carotid artery of BALB/c nude mice. After 18 days, when animals showed neurologic symptoms, CE-MRI was routinely done to confirm presence of tumor (28). Mice were used 1 day later for iv. injections of phages or single-domain antibodies.

**In vivo homing of plxnA1-binding phages and corresponding single-domain antibodies.** PLXND1-binding phages (10\(^5\)) of clones A12, F8, or nonrelevant phages were injected in the tail vein of nude mice, carrying established Mel57-VEGF-A\(_{165}\) brain metastases (n = 2 for A12, n = 4 for F8, and

\(^5\) J. Raats, unpublished results.
n = 3 for control phage). In two other groups of mice, we i.v. injected 30 μg single-domain antibody F8 or a control single-domain antibody (n = 2 for each group). After 5 minutes, mice were anesthetized using isoflurane, the chests were opened, and nonbound phages were washed from the system by cardiac perfusion with 15 mL of PBS. Then, mice were sacrificed by cervical dislocation, and parts of brains, hearts, lungs, livers, spleens, and kidneys were snap frozen in liquid nitrogen. Other parts were fixed in formalin to be paraffin embedded. After short hematoxylin staining, tumors were dissected from 10-μm brain sections using laser capture dissection microscopy. Equivalent areas were dissected from unaffected brain, contralateral to the tumor. Subsequently, TG1 cells were infected with phages and eluted by trypsin treatment from dissected samples. Numbers of colony-forming phages were counted and used as a measure of tumor homing.

To qualitatively assess tumor homing by phages or single-domain antibodies, 4-μm sections were stained with anti-M13 p8 antibody (Abcam Ltd., Cambridge, United Kingdom) to detect bound phages, or anti-VSV-G antibodies (Sigma-Aldrich Chemie) to detect single-domain antibodies.

Results

PLXND1 RNA expression in animal and human tumors. To explore whether endothelial plxnD1 expression is a common feature of activated endothelial cells or is restricted to developing vasculature during embryogenesis as we described previously (24), we subjected brains of mice, carrying highly angiogenic Mel57-VEGF-A lesions, to in situ hybridization (ISH) analysis using a mouse plxnD1-specific probe. Vessels in these lesions, but not in unaffected brain tissue, expressed high levels of the transcript (Fig. 1A, inset, CD34 staining). To explore the clinical relevance of this finding, we subjected a number of different human brain tumors and normal brain to ISH using a human-specific PLXND1 probe. Vessels in brain metastasis of sarcoma (Fig. 1B) and melanoma (Fig. 1C), as well as a number of other tumor types (data not shown), expressed high levels of PLXND1 RNA. In normal human brain, no vessel-associated expression of PLXND1 was detected (Fig. 1D). ISH revealed that tumor cells themselves also were often strongly positive for PLXND1 (compare ISH profiles with the CD31 stainings in Fig. 1B and C, inset). In addition, primary brain tumors were found positive for PLXND1 both on blood vessels and tumor cells (data not shown).

Isolation of anti-PLXND1/single-domain antibodies. To confirm expression of PLXND1 on the protein level, we first selected PLXND1-binding VHH/single-domain antibodies from a pHENIXHis8VSV-vector-based Llama phage display library. During the four consecutive rounds of selection, a gradual enrichment of phages with PLXND1 affinity (up to 1,400-fold) was observed, indicating that specific binders were present (data not shown). Ultimately, 100 individual phages were analyzed for binding to unconjugated PLXND1 peptide in an ELISA-based assay. Seven independent clones, as revealed by BstNI fingerprinting (data not shown), showed specific affinity for the PLXND1 peptide. Phages A12 and F8 were chosen for further

![Figure 1](image-url)
analysis because the derived single-domain antibodies recognized PLXND1 in immunohistochemical stainings better than the other single-domain antibodies (see below). Sequence analysis revealed that A12 and F8 were independent clones, with homology in the CDR1 to CDR3 domains of ~40%. These phages but not an irrelevant single-domain antibody/phage or M13 helper phage bound specifically to PLXND1 peptide but not BSA, IgG, or an irrelevant peptide (Fig. 2A). Furthermore, soluble single-domain antibodies derived from these phages also bound specifically to the PLXND1 peptide (Fig. 2B).

The binding kinetics of the single-domain antibodies/PLXND1 peptide interactions were determined by BLACore analysis. Analysis of the binding data with the 1:1 Langmuir binding model showed a good fit for the single-domain antibody binding to the PLXND1 peptide consistent with a 1:1 binding interaction (data not shown). The $K_d$ values of the single-domain antibodies were found to be 2.4 x 10^{-13} and 3.9 x 10^{-8} mol/L for single-domain antibodies A12 and F8, respectively.

Immunohistochemical analysis. To confirm that single-domain antibodies A12 and F8 specifically recognize PLXND1 not only in vitro as a peptide but also in immunohistochemical stainings, we first stained mouse embryos of different developmental stages, in which plxnd1 expression was previously analyzed by ISH (24). ISH and immunohistochemistry grossly correlated to each other. Especially in the growth plate of trabecular bone, a site of active angiogenesis, blood vessels stained strongly positive both in ISH and immunohistochemistry using plxnd1-recognizing single-domain antibodies (Fig. 3A, inset, corresponding plxnd1-ISH).

We went on to use single-domain antibodies A12 and F8 for immunohistochemistry on different mouse and human tumors. Indeed, immunostainings with single-domain antibodies A12 and F8 confirmed the ISHs shown in Fig. 1. Vessels and, to a lesser extent, tumor cells in cerebral angiogenic Mel57 tumors stained positive (Fig. 3B; data not shown). In addition, in the human sarcoma and melanoma brain metastases (Fig. 3C and D), vessels as well as tumor cells stained positive with single-domain antibody A12. Interestingly, and consistent with the imperfect homology between the human peptide used for antibody selection and its mouse equivalent, single-domain antibodies seemed more efficient in detecting human than mouse plxnd1 (compare Fig. 3B with C and D). Furthermore, single-domain antibody F8 was more efficient than A12 in detecting mouse plxnd1. The positivity of the tumor cells in Mel57-VEGF-A tumors prompted us to investigate expression of PLXND1 in cultured Mel57-VEGF-A cells. Both by RT-PCR using PLXND1-specific primers and immunostainings with single-domain antibody A12, we found that tumor cells expressed the protein in vitro also (data not shown).

The presence of PLXND1 on tumor but not normal vessels suggests that this protein may be a suitable target for in vivo delivery of diagnostic and therapeutic compounds to tumors. To test this, we injected mice, carrying established Mel57-VEGF-A brain tumors, with 10^{12} colony forming units of phage A12, F8, or a nonrelevant phage in the tail vein and washed nonbound phages from the circulation by cardiac perfusion. After removal and snap freezing of part of the brains, areas containing tumor or unaffected brain tissue from the contralateral hemisphere were dissected using a laser capture dissection microscope and analyzed for phage content.

As illustrated in Fig. 4D, after i.v. injection of phage F8, significantly more phages were eluted from brain lesions than from comparable areas of normal brain tissue. Consistent with the apparently higher affinity of single-domain antibody F8 towards mouse plxnd1, more F8 than A12 phages were eluted from tumor. Immunostaining of frozen sections of the same brains with anti-M13 antibodies confirmed the presence of blood vessel–bound phages in tumor but not in normal brain (compare the anti-M13 staining in Fig. 4A with the anti-CD31 staining of a serial section in Fig. 4B, arrows point at a plxnd1-negative normal vessel). Upon i.v. injection of an irrelevant phage, no vessel-associated phages could be detected (Fig. 4A, inset).

Tumor targeting by phages is clinically less relevant. Therefore, we continued by testing whether purified single-domain antibodies are also able to target tumor vasculature in vivo. After i.v. injection of single-domain antibody F8, this antibody could be detected on tumor vessels by anti-VSV-G immunostaining (Fig. 4C). Importantly, no nonspecific homing to normal brain vessels could be observed (data not shown). Upon i.v. injection of an irrelevant single-domain antibody, no vessel-associated single-domain antibodies could be detected. However, we observed some staining in the interstitium of Mel57-VEGF-A tumors (Fig. 4C, inset). This is to be expected because the leaky tumor vessels are permeable to the small-sized single-domain antibodies.

Previous work already revealed that small amounts of the plxnd1 transcript can be detected in adult normal tissues, especially heart, lung, and liver. To investigate targeting of these organs
by single-domain antibody F8, we did anti-VSV-G immunostainings of frozen sections. No single-domain antibodies could be found associated with normal heart, kidney, liver, spleen, and lung (data not shown).

**Discussion**

In previous work, we described *plxnd1* expression in a subset of neuronal cell types and in endothelial cells during developmental angiogenesis (24). Very recently, *plxnd1* knockdown in mice and zebrafish has been described to result in maldevelopment of the vasculature (25, 26). These data suggest a role for *plxnd1* in vascular patterning, analogous to that of other plexin family members in axonal patterning (for review, see refs. 22, 35). Here we show that endothelial expression of *plxnd1* is not restricted to vasculature during development but also occurs in the vascular bed of tumors. Intriguingly, we found that *PLXND1* is also frequently expressed at high levels in tumor cells in a variety of tumor types. This expression profile makes *PLXND1* a potentially powerful target for diagnostic and therapeutic purposes in oncology. To our knowledge, this is the first example of a protein that is specifically expressed on both tumor vessels and tumor cells, allowing simultaneous targeting of different tumor compartments. Indeed, we could show that phages carrying single-domain antibodies against *PLXND1* on their surface effectively and specifically homed to cerebral melanoma metastases in mice after i.v. injection. These phages were all vessel associated.

When single-domain antibodies were injected, we found accumulation in the tumor vasculature and to a lesser extent also in the tumor interstitium. This interstitial localization could be partly attributed to nonspecific extravasation of the small-sized single-domain antibodies from leaky tumor vessels, because irrelevant control single-domain antibodies showed a similar localization. Importantly, the tumor vessel association of anti-PLXND1 single-domain antibodies was specific because control single-domain antibodies did not accumulate in vasculature.

Anti-PLXND1 antibodies may be suitable vehicles to deliver cytotoxic agents to tumors, especially because leaky tumor vessels will allow extravasation of targeting antibodies, thereby enabling a second wave of action against the *PLXND1*-expressing tumor cells themselves.

To be efficient tumor vessel–targeting vehicles, affinity of anti-PLXND1 antibodies should be as high as possible, whereas targeting of the tumor cell compartment may be more efficient with smaller, lower affinity antibodies with predicted high tumor penetrance (36). Whether the *K₅₀* values of our single-domain antibodies of 20 to 30 nmol/L allow therapeutic targeting of either tumor compartment is not known at the moment. It is however expected that mouse monoclonal targeting antibodies will be more successful for these purposes. These are currently under development.

The rather disappointing results with antiangiogenic compounds in clinical trials, which may be related to angiogenesis-independent tumor growth in vessel-dense organs (15), suggest...
that vascular targeting therapies may be more effective than antiangiogenic therapies. This stresses the importance of identifying molecular beacons that are specifically expressed in a wide range of tumors. Few of such molecules have been identified thus far. An alternatively spliced fibronectin molecule is deposited in the basement membrane of newly formed vessels, and single-chain antibodies against this protein have been successfully used to detect clinical tumors in whole body scintigraphs (16). RGD motif containing peptides have been used to target integrin $\alpha_v\beta_3$ (37, 38), resulting in endothelial apoptosis and antitumor activity in animal models, but expression of this integrin is restricted to early stages of angiogenesis. Because mature tumor vessels may therefore be less sensitive to such targeting, it may be more relevant to identify target markers that fulfill the prerequisite of being expressed in mature human tumors. PLXND1 seems such a marker. In a number of brain tumors, both primary tumors and metastases, the protein is abundantly expressed. In fact, we have not yet been able to identify a PLXND1-negative tumor. Nevertheless, a more extensive analysis of PLXND1 expression and distribution in a variety of human tumor types will be needed to make more rigorous statements about the usefulness of PLXND1 as a tumor marker in the clinic.

A functional role of PLXND1 in developmental angiogenesis is now well established (25, 26). Whether PLXND1 is functionally involved in vessel morphogenesis during tumor angiogenesis as well, is an intriguing question. If so, targeting of this protein may well lead to a functional blockade and consequently an antiangiogenic effect. This aspect is currently under investigation in our lab. Whether PLXND1 on tumor cells is functionally important, is also not known. Intriguingly, Gu et al. recently published that Semaphorin 3E is a ligand for PLXND1 (27). Because Semaphorin 3E has been identified in microarray experiments as a protein involved in tumor invasion and metastasis (39), this opens up the interesting possibility that a PLXND1/Semaphorin 3E loop is involved in aggressive tumor behaviour. In this context, neutralization of PLXND1 may directly inhibit tumor invasion and metastasis.

PLXND1 expression is not absolutely tumor specific. We and others have previously shown that low levels of the PLXND1 transcript can be found in normal adult heart, liver, and testis (24). Immunohistochemical analysis and ISH revealed that the cells that are responsible for these expression levels are mostly macrophages. Interestingly, these cells are characterized by their migratory potential. Taken together, this opens up the possibility that PLXND1 has a more general function in cell migration.
In conclusion, we have shown that plxnD1 is expressed in tumor but not normal vasculature as well as tumor cells in a wide range of tumor types. The expression patterns, and emerging data on PLXND1 function in vascular morphogenesis and possibly tumor cell behavior, highlights this protein as a potentially powerful tool for future diagnosis and therapy in oncology.

References


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