ORIGINAL ARTICLE

Paul N. Morris · Benjamin J. Dunmore · Amir Tadros ·
Douglas A. Marchuk · Diane C. Darland ·
Patricia A. D'Amore · Nicholas P. J. Brindle

Functional analysis of a mutant form of the receptor tyrosine kinase Tie2 causing venous malformations

Abstract Tie2 is expressed predominantly in endothelial cells and is required for blood vessel formation and maintenance. A missense mutation resulting in an R to W substitution in the kinase domain of Tie2 co-segregates with an autosomal dominantly inherited form of vascular dysmorphogenesis, venous malformation (VM). The mechanism by which this activating mutation leads to vessel dysmorphogenesis in VM is not known. Here we examined Tie2 activation status in VM and found activated receptor in lesional and non-lesional vessels. To gain insight into functional effects of VM mutant Tie2, wild-type and R849W mutant receptor were expressed in cultured human venous endothelial cells. Mutant Tie2 was constitutively phosphorylated in endothelial cells in vivo and caused a marked suppression of apoptosis. The anti-apoptotic kinase Akt was constitutively activated in cells expressing mutant receptor. Dominant-negative Akt inhibited the pro-survival activity of mutant Tie2. Migration of smooth muscle cells induced by conditioned medium from cells expressing mutant receptor was similar to that from cells expressing wild-type receptor. These data suggest that a primary effect of R849W Tie2

PAUL N. MORRIS graduated in medicine from the University of Leicester in 1993. He obtained his surgical fellowship from Edinburgh and proceeded to train as a Plastic Surgeon. With a specific interest in vascular anomalies, he completed a research M.D. studying the molecular basis of venous malformations. On completion of his training in plastic surgery he intends to specialize in cleft lip and palate surgical reconstruction.

NICHOLAS P.J. BRINDLE received his Ph.D. degree in Biochemistry from the University of Manchester, UK. He is presently Reader in Vascular Cell Biology at the Department of Cardiovascular Sciences, University of Leicester. His research interests include receptor tyrosine kinase signalling in vascular development and maintenance.

P. N. Morris · A. Tadros · N. P. J. Brindle (✉)
Department of Cardiovascular Sciences,
University of Leicester,
RKCSB, P.O. Box 65, Leicester, LE2 7LX, UK
e-mail: npjb1@le.ac.uk
Tel.: +44-116-2525802, Fax: +44-116-2523179

B. J. Dunmore
Department of Obstetrics and Gynaecology,
University of Cambridge,
Cambridge, CB2 2SW, UK

D. A. Marchuk
Department of Molecular Genetics and Microbiology,
Duke University Medical Center,
Box 3175, Durham, NC 27710, USA

D. C. Darland · P. A. D'Amore
Schepens Eye Research Institute
and Departments of Opthalmology and Pathology,
Harvard Medical School,
20 Staniford Street, Boston, MA 02114, USA

in VM is to allow survival of mural cell poor vessels via ligand-independent Tie2 activation of Akt and endothelial survival, rather than to directly induce formation of dysmorphogenic vessels.

Keywords Endothelial cell · Apoptosis · Tie2

Abbreviation *VM:* Venous malformation

Introduction

Vascular development is initiated by the process of vasculogenesis, in which endothelial precursor cells differentiate and organize into primitive vascular tubes in situ.

Sprouting from these tubes via angiogenesis, involving endothelial cell migration and proliferation, in combination with intussusception, leads to growth and organization of the vascular tree [1, 2]. These processes are tightly controlled by a complex network of soluble and non-diffusible factors, including vascular endothelial growth factor and the angiopoietins, extracellular matrix proteins and angiostatic molecules [3, 4]. Although there have been major advances in uncovering critical controlling aspects of vasculogenesis and angiogenesis, understanding the process of vessel assembly and maintenance still remains a significant challenge. In particular, the signalling pathways controlling vessel stability, maintenance and remodelling in postnatal vasculature are poorly understood.

Inherited diseases of the vasculature can provide insights into the process of vessel formation. Importantly, where these anomalies are not developmentally lethal, they can also illuminate mechanisms involved in postnatal vascular maintenance and remodelling. Venous malformations (VM) are common vascular anomalies manifesting as a vascular mass composed of dilated channels lined by endothelial cells [5, 6]. Vessels of VM have an abnormal ratio of endothelial to smooth muscle cells, with very few supporting smooth muscle cells [5, 7] leading to a functionally low resistance vessel. The majority of VM are sporadic, although the anomaly can be inherited [5, 6]. An autosomal dominantly inherited form of VM (designated venous malformations, multiple cutaneous and mucosal; OMIM number 600195) has been shown to segregate with the same C2545T transition in the coding sequence of the receptor tyrosine kinase Tie2 in two unrelated families [7]. Another independent study of inherited VM found a third unrelated family in which the disease co-segregated with the same mutation [8], making this transition the single most common mutation detected in inherited VM.

Tie2 is expressed predominantly by endothelial cells and is essential for vessel formation where it is involved in microvessel sprouting, integrity and maturation [9]. Amongst other effects, Tie2 activation in cultured cells promotes monolayer integrity and inhibits endothelial apoptosis [10, 11, 12, 13]. A family of ligands, designated the angiopoietins, have been identified for Tie2. The best characterized of these are Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2). Ang-1 is a stimulatory ligand expressed by mesenchymal cells and smooth muscle cells associated with vessels [14, 15], whereas Ang-2 can act as a naturally occurring antagonist [16].

The C2545T transition in the Tie2 coding sequence, which has been linked to VM, results in substitution of an arginine at position 849 for a tryptophan (R849W) in the kinase domain of the receptor. The effects of this mutant Tie2 on endothelial function are not known. Analysis of the VM mutant R849W Tie2 expressed in insect cells revealed it to be hyper-phosphorylated and have elevated tyrosine kinase activity compared with wild-type receptor [7]. If and how this results in the development of VM is yet to be defined, although the relative lack of abluminal support cells has led to the suggestion that endothelial expression of the mutant receptor leads to defective recruitment of and/or interaction with mural cells [7].

Materials and methods

Materials

cDNA encoding human Tie2 and R849W VM mutant Tie2 were obtained from the American Tissue Culture Collection and subcloned into the expression vector pCR3 (Invitrogen Life Technologies, Paisley, UK). An expression vector encoding dominant-negative Akt, K179A/T308A/S473A, was kindly provided by Dr. B.A. Hemmings [17]. Antisera against the extracellular domain of Tie2 was from R & D Systems (Abingdon, UK), anti-phosphotyrosine antibodies were from BD Transduction Laboratories (San Jose, Calif., USA) phospho-Akt (S473/T308) and Akt antibodies were from Cell Signaling Technology (Beverly, Mass., USA) and anti-phospho-Tie2 (Y1094/1102) was from Oncogene Research Products (San Diego, Calif., USA). Human umbilical vein endothelial cells were cultured and transfected as detailed previously [18]. Transfection efficiency, as judged by expression of green fluorescent protein was routinely greater than 40%. Human saphenous vein smooth muscle cells were obtained and cultured as described previously [19].

Immunohistochemistry

Biopsy material from VM was obtained with informed consent from one of the patients described previously with the mutation giving rise to R849W Tie2 [8]. Immunohistochemistry was performed on 5-μm sections essentially as detailed previously [20]. Primary antibodies were detected with biotin-conjugated secondary antibodies and the Chemmate/Envsion Detection System (Dako, Cambridge UK). Negative controls with non-specific primary antibodies were included.

Apoptosis and cell survival assays

Assessment of survival and apoptosis was performed in transfected cells as described previously [21]. Briefly, cells were transfected with control vector, wild-type Tie2 or R849W Tie2, together with green fluorescent protein, to identify transfected cells. Apoptotic index was determined in transfected cells following staining with 4′6-diamidino-2-phenylindole at 0.2 μg/ml. In parallel cultures cell survival was determined by counting numbers of viable transfected cells in ten designated areas in gridded tissue culture wells before and after growth factor deprivation. Cell viability was routinely determined by exclusion of 0.2% trypan blue.

Immunoprecipitation and western blotting

Immunoprecipitation and western blotting was performed as previously described [22]. Briefly, endothelial cells were washed in PBS and lysed at 4°C in 0.5 ml of lysis buffer (50 mM Tris, pH 7.4, containing 50 mM NaCl, 1% Triton X100, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EGTA and Complete protease inhibitor cocktail). For immunoprecipitation lysates were cleared by centrifugation (13,000 g for 10 min at 4°C). Immunoprecipitations were performed by adding 0.4 μg/ml antibody to the lysate and incubating with rotation at 4°C for 2 h. Pre-washed protein A-Sepharose was added to the protein-antibody complex and incubated for a further 2 h. Immunoprecipitates were washed four times with lysis buffer. Proteins were eluted by boiling in Laemmli sample buffer containing 100 mM dithiothreitol and resolved by sodium dodecyl sulphate gel electrophoresis. Proteins were electrophoretically transferred onto nitrocellulose membranes

and probed with appropriate antibodies. Immunoreactive proteins were visualized with a peroxidase conjugated secondary antibody and a chemiluminescent detection system [23].

Smooth muscle cell migration

Monolayers of endothelial cells transfected with empty vector, wild-type Tie2 or R849W Tie2 were washed extensively with PBS and incubated for 48 h in serum-free Dulbecco's modified Eagle's medium containing 1 mg/ml BSA. Conditioned media and identical media that had not been conditioned were centrifuged at 2500 g for 15 min to remove any particulate material. Media was placed in the lower chamber of Transwell tissue culture wells containing 8-μm pore size inserts. Smooth muscle cells (1×10^5) suspended in serum-free Dulbecco's modified Eagle's medium were placed in the top chamber of the inserts and cells were allowed to migrate for 6 h at 37°C. Smooth muscle cells on both sides of the insert membrane were fixed in 70% ethanol at −25°C and cells stained with haematoxylin/eosin. Filters were mounted in glycerol and the numbers of cells migrating through the porous membrane were counted at ×400 magnification in ten high-power fields on the underside of each insert membrane.

Results

Tie2 is activated in VM lesional and non-lesional vessels

VM associated with R849W mutant Tie2 are characterized by enlarged and dilated vascular channels with a relative lack of mural cell support compared with non-lesional vessels (Fig. 1A, E). Segments of the lesional vessels are devoid of mural cells whereas in other areas mural cells are disorganized. The R849W mutant Tie2 has constitutively elevated kinase activity and increased receptor autophosphorylation in vitro which has been implicated in the pathogenesis of the vessel defects that characterize VM [7]. To examine the activation status of Tie2 in dysmorphogenic and non-dysmorphogenic vessels, sections were probed with an antibody that recognizes phosphorylated Tie2. Surprisingly, phospho-Tie2 was detected in the endothelial cells of both dysmorphogenic vessels and normal vessels and staining was of similar intensity in both normal and pathological vessels (Fig. 1B, F). Both Normal and VM vessels had similar levels of Tie2 immunopositivity (Fig. 1G, H). These observations suggest that the all-or-none activation of Tie2 as a result of the R849W mutation in VM vessels is not the basis of the vascular dysmorphogenesis.

R849W Tie2 constitutively suppresses endothelial cell death

To gain insight into the mechanisms by which mutation of Tie2 contributes to VM we investigated the functional impact of R849W mutant Tie2 in endothelial cells. Expression of R849W mutant Tie2 in human umbilical vein endothelial cells led to constitutively elevated receptor tyrosine phosphorylation (Fig. 2A). In comparison, over-expressed wild-type Tie2 showed relatively low levels of phosphorylation. Densitometric scanning of blots from
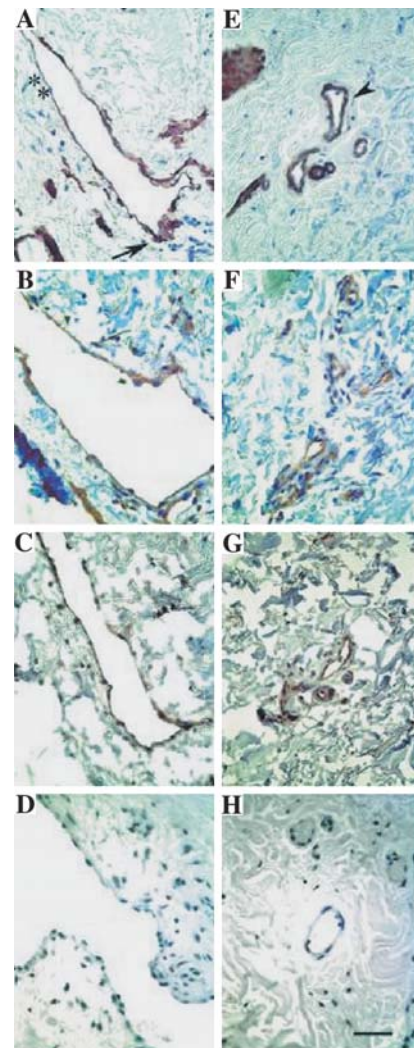

Fig. 1 Dysmorphogenic vessels in a patient with VM. Representative views are shown of dysmorphogenic (**A**–**D**) and non-dysmorphogenic (**E**–**H**) vessels from a patient with the mutation resulting in R848 W Tie2. Staining for the presence of smooth muscle cell actin reveals areas in which smooth muscle cells are disorganized (*arrow*) and areas with poor smooth muscle cover (*asterisk*) in the dilated lesional vessels (**A**) compared with uniform smooth muscle cell distribution (*arrowhead*) in non-dysmorphogenic vessels (**E**). Tyrosine phosphorylated Tie2 (**B**, **F**) and total Tie2 (**C**, **G**) is seen in both types of vessel. **D**, **H** Negative control sections. *Scale bar* 50 μm

three independent experiments and normalization of tyrosine phosphorylated Tie2 to Tie2 expression level revealed that R849W Tie2 exhibited a 4.4±0.24-fold higher level of tyrosine phosphorylation than wild-type Tie2. These results are consistent with previous studies in COS and Hek293 cells [8, 24] but demonstrate the effect in a more physiologically relevant endothelial background. In normal vessels Tie2 is maintained in an activated, phosphorylated, state by Ang-1 produced by mural cells [12, 25]. The present data suggest that even in the absence of mural cells to provide Ang-1 tyrosine phosphorylation of R849W mutant Tie2 would be able to occur.
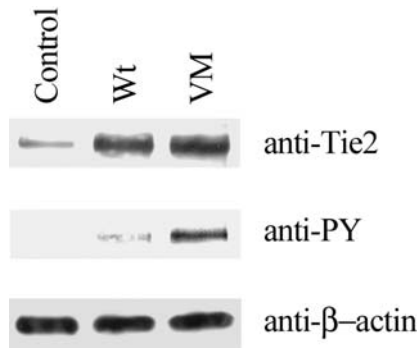
Fig. 2 VM mutant R849W Tie2 is tyrosine phosphorylated in endothelial cells. Human umbilical vein endothelial cells were transfected with plasmids encoding wild-type Tie2 (*Wt*), R849W mutant Tie2 (*VM*) or empty plasmid (*Control*). Cell lysates were resolved by sodium dodecyl sulphate gel electrophoresis and immunoblotted with antibodies recognizing phosphotyrosine. Membranes were stripped and Tie2 was detected by re-probing with antibodies recognizing Tie2 and β-actin as indicated. VM mutant Tie2 has increased levels of tyrosine phosphorylation

During new vessel formation nascent vessels require association with mural cells for survival; newly formed vessels that lack mural cell investment regress via endothelial apoptosis [26, 27]. Ang-1, expressed by the periendothelial cells, is thought to contribute to vessel stability via activation of Tie2 and suppression of endothelial apoptosis [12]. Thus it is surprising that although vessels of VM have a paucity of mural cells, the vessels do not regress. Therefore we hypothesized that the constitutive signalling that occurs as a result of the R849W mutation in Tie2 leads to endothelial survival in the absence of ligand. To examine this possibility endothelial cells were co-transfected with vectors encoding R849W Tie2 and green fluorescent protein. Two days post-transfection cells were switched to media lacking growth factors for 18 h, and the apoptotic index of the transfected cells was determined. In order to distinguish between the effects of mutant Tie2 and those of increased Tie2 expression per se parallel cultures of endothelial cells were transfected with wild-type receptor and green fluorescent protein and analysed similarly. Expression of R849W Tie2 significantly inhibited endothelial apoptosis (Fig. 3A), thus leading to markedly increased endothelial survival (Fig. 3B).

We also addressed the possibility that the R849W Tie2 leads to lack of mural cell investment as a result of decreased ability of endothelial cells to recruit smooth muscle cells. This was tested by examining the ability of serum-free medium conditioned for 48 h by endothelial cells expressing R849W Tie2 or wild-type Tie2 to induce migration of smooth muscle cells. We could find no difference in smooth muscle cell migration induced by medium conditioned by endothelial cells expressing R849W or wild-type Tie2 (data not shown).
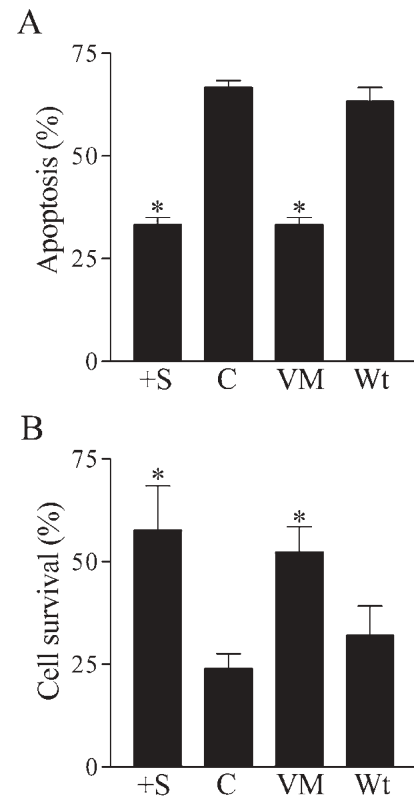

Fig. 3 VM mutant R849W Tie2 inhibits endothelial cell death. Human umbilical vein endothelial cells were transfected with plasmids containing no insert (*C*), R849W VM mutant Tie2 (*VM*) or wild-type Tie2 (*Wt*). After 48 h cells were changed to serum-free medium for 18 h. The percentage of apoptotic cells and cell survival were determined in parallel cultures of transfected cells as described in the text. For comparison control transfected cells cultured with 10% serum (*+S*) are also shown. Data are presented as mean and SEM for at least three independent experiments. *$P<0.05$ (Student's *t* test) vs. C. Expression of R849W VM mutant Tie2 significantly inhibits endothelial apoptosis and promotes endothelial survival

R849W Tie2 signals via Akt to suppress endothelial death

Suppression of endothelial apoptosis following Ang-1 activation of wild-type Tie2 has been shown to involve the anti-apoptotic serine/threonine kinase Akt [12, 28]. The activation state of Akt was therefore examined in endothelial cells expressing R849W Tie2. Akt activation, as determined by immunodetection of phospho-S473/T308-Akt, was constitutively elevated in endothelial cells expressing the mutant receptor (Fig. 4). To test whether Akt is involved in the anti-apoptotic effect of R849W Tie2 a dominant-negative form of the kinase was co-expressed with the mutant receptor. Co-expression of the K179A/T308A/S473A dominant-negative Akt inhibited the anti-apoptotic and pro-survival effects of R849W Tie2 (Fig. 5A, B). These data indicate that R849W Tie2 constitutively activates Akt in endothelial cells and suggests that this serine/threonine kinase is required for the persistent pro-survival activity of the mutant receptor.
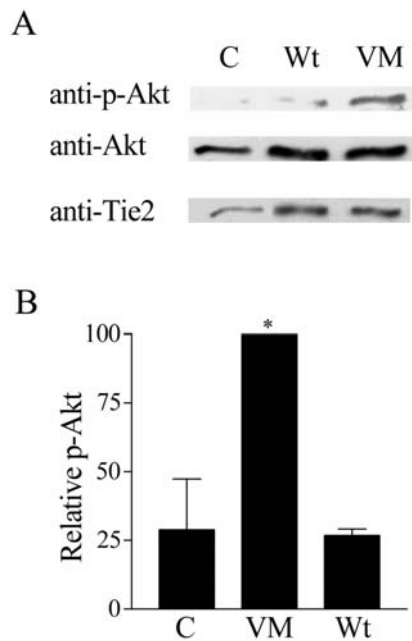
Fig. 4 VM mutant R849W Tie2 activates Akt. HUVEC were transfected with plasmids containing no insert (*C*), wild-type Tie2 (*Wt*) or R849W VM mutant Tie2 (*VM*). After 48 h cells were changed to serum-free medium for 18 h and whole-cell lysates were prepared. Cellular proteins were resolved by sodium dodecyl sulphate gel electrophoresis and immunoblotted with antibodies recognizing phospho-S473/T308-Akt (*P-Akt*). Membranes were stripped and probed with antibodies recognizing and total Akt and Tie2. **A** Representative blot showing increased phospho-Akt in cells expressing R849W Tie2. **B** The relative levels of p-Akt/Akt were determined by densitometric scanning of blots from three independent experiments. The relative P-Akt/Akt for VM expressing cells is arbitrarily presented as 100. Data are shown as means and SEM for three independent experiments. *$P<0.02$ (Student's t test) vs. control transfected cells. Activated phospho-Akt was significantly higher in endothelial cells expressing R849W VM mutant Tie2 compared with those expressing wild-type Tie2 and control transfected cells

Fig. 5 Akt is required for the anti-apoptotic activity of VM mutant Tie2 in endothelial cells. Human umbilical vein endothelial cells were transfected with R849W mutant Tie2 (*VM*) and either control vector (*CV*) or K179A/T308A/S473A dominant-negative Akt (*dn-Akt*). After 48 h cells were changed to serum-free medium for 18 h. The percentage of apoptotic cells and cell survival were determined in parallel cultures as described in the text. For comparison the percentage apoptosis and cell survival of serum-deprived control transfected cells (*C*) are also shown. Data are presented as mean and SEM for at least three independent experiments. *$P<0.05$ (Student's t test) vs. CV+VM. Expression of dominant-negative Akt inhibited the anti-apoptotic and pro-survival activity of R849W VM mutant Tie2

Discussion

In this study we have begun to explore the impact of mutant Tie2 (R849W) on endothelial functions with an emphasis on its potential role in the pathogenesis of VM. Although immunohistochemical analyses cannot detect subtle differences in the levels of expression or phosphorylation, our data indicate that Tie2 receptors are expressed and phosphorylated to similar levels in the endothelium of both normal and VM vessels. This suggests that receptor phosphorylation alone does not lead to the observed vessel defects. However, it should be remembered that immunohistochemical analysis was performed with an antibody recognizing Tie2 phsophorylated on tyrosines 1094 and 1102. As Tie2 undergoes phosphorylation on multiple tyrosine residues, it remains a possibility that the mutant receptor has increased phosphorylation on tyrosine residues other than those to which the immunohistochemical analyses was directed.

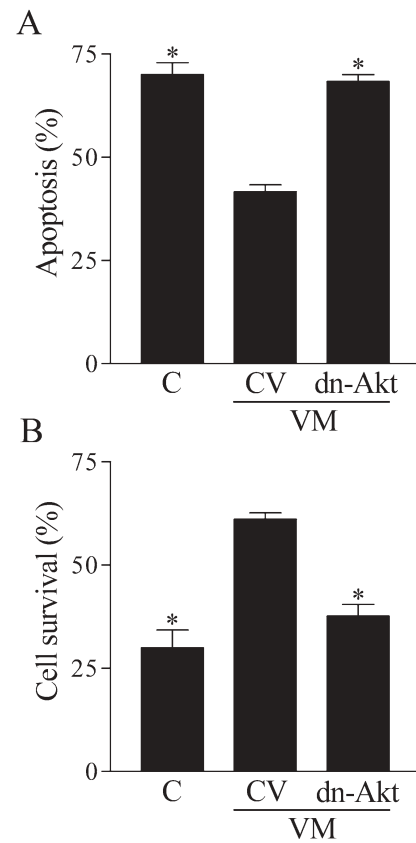Based on our present observations we speculate that the mutant receptor circumvents the normal requirement for mural cell investment in vessel stabilization. Whereas mural cell-derived Ang-1 is needed to activate Tie2 and prevent apoptosis in endothelial cells of normal vessels, the mutant receptor is constitutively activated. The mutant receptor signals via Akt to suppress endothelial apoptosis. Thus the survival advantage conferred to endothelial cells expressing this mutant receptor may allow VM vessels to resist regression despite their lack of mural cell support. The lack of effect of R849W Tie2 on the ability of endothelial cells to chemoattract smooth muscle cells suggests that the relative lack of mural cells in VM vessels does not result from defects in recruitment of mural cells. In light of these data we speculate that the primary effect of R849W Tie2 is to allow the VM vessels to escape regression rather than to be directly responsible for the vascular defects. If this model is correct, an additional stimulus defect would be required for the initial formation of mural cell poor vessels. These vessels, if allowed to

persist, would then develop into dilated vascular channels due to the lack of regulatory influence on size and structure normally mediated by mural cell interaction. The potential involvement of an additional stimulus defect may explain why in inherited VM the lesions are focal and often develop later in life. Finally, our observations also suggest that interference with the pro-survival activity of mutant Tie2, at the receptor level or downstream, may be a strategy for inducing regression of established VMs.

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