Allelic and locus heterogeneity in inherited venous malformations

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Venous malformations are low-flow vascular lesions consisting of disorganized thin-walled vascular channels. These can occur sporadically but also as an autosomal dominant condition termed venous malformations, cutaneous and mucosal (VMCM; OMIM 600195). In two large unrelated kindreds mapping to chromosome 9, the identical R849W missense mutation was identified in the first kinase domain of Tie2, an endothelial cell-specific receptor tyrosine kinase. We report here the identification of four new kindreds with inherited venous malformations. Unlike the initial two families described, these four families demonstrate allelic and locus heterogeneity. In one of these families, the R849W mutation co-segregates with the disease phenotype. Three other families with venous malformations lack this mutation. One of these families is linked to markers near TIE2 on chromosome 9. In this family, we identified a novel mutation within the first kinase domain of Tie2 resulting in a Y897S change. Results from COS-1 cell transfections using expression constructs containing either the R849W or the Y897S mutation suggest that the receptors containing either mutation show ligand-independent hyperphosphorylation. These results suggest a gain-of-function mechanism for development of venous malformations in these families. Of the two remaining families, one excludes linkage to the TIE2 locus, establishing the existence of at least one additional locus for dominantly inherited venous malformations.

INTRODUCTION

The development of the vascular system is controlled through the interaction of two complex processes: vasculogenesis, the de novo formation of the vascular plexus, and angiogenesis, the outgrowth of new capillaries from pre-existing vessels (1,2). Much of what has been learned about the mechanisms and genes involved in vascular development comes through the use of the mouse as an experimental genetic model organism (3). Although this approach has elucidated much concerning the early stages of vascular development, the null mutations used in creating these mouse models often result in embryonic lethality. Thus, these models do not allow one to follow a transgenic organism into adulthood to study changes in the vasculature that may have a more subtle phenotype. As an alternative approach, important insights into normal vascular development can often be gained through the investigation of inherited diseases of the vasculature in humans. This strategy offers the opportunity to study affected organisms beyond the early stages of development.

Venous malformations, a common abnormality of the vasculature, usually occur sporadically but may also be inherited. Patients usually present with rubbery, compressible, bluish-purple nodules or a tumor-like vascular mass, often manifesting at birth (Fig. 1) (4–6). Lesions may also appear or enlarge throughout life, possibly influenced by physical trauma, hormonal fluctuations or hemodynamic changes (4–6). Venous malformations are often located in the skin and oral mucosa (5,6). Histologically, these lesions consist of haphazardly arranged dilated blood vessels. They show a significant lack of involvement of the normal surrounding smooth muscle and elastic tissue, resulting in vessels with a larger than normal luminal diameter lined with a single layer of endothelium and little to no supportive tissue (5,7).

An inherited form of venous malformations [venous malformations, cutaneous and mucosal (VMCM; OMIM 600195)] is a rare autosomal dominant disease that exhibits variable expressivity and high penetrance (4,8). Two unrelated VMCM families previously were linked to an 8 cM region of 9p21 (4,8) containing the receptor tyrosine kinase TIE2 (TEK) gene (7,9), a member of a novel family of receptor tyrosine kinases expressed almost exclusively on endothelial cells (10–15). An identical C2545T transition leading to an R849W substitution in the Tie2 receptor was shown to co-segregate with the affected phenotype in both families (7). This change, which is

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located in the first kinase domain of Tie2, appears to be a hyperphosphorylating mutation (7).

We report here the identification of four new kindreds with inherited venous malformations. Due to the lack of mucosal lesions in two of the families described here and the suggestion that these comprise a distinct genetic entity, we will refer to our families by the descriptive term, venous malformations (VM) families. Unlike the previously described families, these four families demonstrate allelic and locus heterogeneity. One of the families harbors the R849W mutation. This mutation is at a highly mutable CpG site, which might explain the occurrence of the same mutation in three unrelated families. We also describe the identification of a novel hyperphosphorylating Y897S mutation in the TIE2 gene in a new VM family. Two additional VM families show no evidence of linkage to 9p21. One of these families, which excludes linkage to the TIE2 locus, establishes the existence of at least one additional locus for inherited VM.

RESULTS

Clinical description of families

Family 90 has affected individuals in three generations in a pattern consistent with autosomal dominant inheritance. Affected individuals in this family have multiple superficial and deep venous malformations affecting the skin and oral mucosa. One family member has a large disfiguring lesion affecting one side of the face. No family member assessed has a history of intracranial vascular malformations. Only one has a history of gastrointestinal hemorrhage, which is thought to be secondary to intestinal polyps rather than vascular malformation. Lesions vary in size from a few millimeters to large lesions requiring surgical intervention. Age of onset varied in family members, and ranged from birth to teens or even later in life.

Family 105 has affected individuals in three generations in a pattern consistent with autosomal dominant inheritance. Affected individuals have multiple lesions occurring at various locations such as the legs, hands, neck and torso. Age of onset varied from the age of 18 months to 18 years. The proband presented with multiple raised blue lesions on her left leg posterolaterally below the knee and on the right side of her neck. These lesions had become more noticeable and painful over the 9 years since their first appearance. Gastrointestinal and mucosal lesions have not been observed in this family.

Family 137 has affected individuals in four generations in a pattern consistent with autosomal dominant inheritance. Venous malformations have been noted from the age of 2 years in one instance, but have also appeared for the first time in the first and second decade of life in individuals in this family. Some individuals describe additional lesions appearing in adult life. Age of onset is not consistent between parent and child. There is variation in severity and number of lesions between close family members (such as parent and child). Gastrointestinal and mucosal lesions have not been observed. There have been no episodes of gastrointestinal bleeding documented and no severe bleeding episodes from the skin lesions themselves. Lesions vary from <1 mm in width to those occupying most of the skin of the dorsal aspect of a forearm in a child. The majority of lesions are in the upper torso, for example in the axilla, between the scapulae and on the anterior chest wall, but lesions are also described on forearms, buttocks and legs. They occur less often on the face. The lesions typically are raised and deep blue in color. Some of the smaller lesions appear to be flat.

Family 154 has affected individuals in three generations in a pattern consistent with autosomal dominant inheritance. Age
of onset varied in family members and ranged from birth to teens or even later in life. There is variation in severity and number of lesions, with some family members having only two or three lesions and others having $>20$. Lesions varied in size from a few millimeters to several inches, and were present in most areas of the body including the arms, legs, torso, and head and neck regions. Mucosal lesions were noted on the tongue and in the oral cavity in several family members. Gastrointestinal lesions have not been described.

**Allelic heterogeneity for VM**

Blood samples from the probands of each of the four newly identified VM families were obtained and assayed for the known C2545T (R849W) mutation. Only the proband from family 154 possessed the mutation. An additional 18 members of family 154 were ascertained and collected (including 10 affected members). The mutation co-segregated perfectly with the disease phenotype (Fig. 2). Haplotype analysis confirmed that this family is unrelated to the two families previously described with this mutation (7). The occurrence of the identical mutation in three unrelated families may be due to the localization of the mutation at a hypermutable CpG site.

Additional members of the three other VM families were also ascertained and blood samples were collected. Genetic markers mapping near TIE2 were used in two-point linkage analyses for the three families lacking the R849W mutation. Only one family, family 90, appeared linked to the region using D9S169, the marker mapping closest to TIE2 (data not shown). DNA sequence analysis of the entire coding region of TIE2 identified a novel A2690C base change which creates a Y897S substitution within kinase region 1 (Fig. 3). The mutation co-segregated perfectly with the disease phenotype (13 family members; six affected individuals) (Fig. 4). This change was not seen in 162 individuals (324 alleles) of a matched control population.

**Ligand-independent hyperphosphorylation for R849W and Y897S mutants**

The R849W mutation previously was shown to be hyperphosphorylating in baculovirus transfections of Sf9 insect cells (7). To investigate the biochemical implication of the Y897S mutation, we used in vitro mutagenesis to incorporate either the Y897S or the R849W mutation into the full-length mouse Tie2 cDNA. These residues are conserved in both species, as is $>99\%$ of the kinase domain (one amino acid change in 272 amino acids). We also used a construct containing a K855R mutation which destroys the ATP-binding site resulting in a ‘kinase-dead’ mutant as a negative control (16). The corresponding residues in the mouse are R848W, Y896S and K854R. However, for simplicity, the mutations will be referred to by their human sequence counterparts.
The wild-type cDNA and the three mutant cDNAs were cloned into an expression vector and used to transiently transfect COS-1 cells. We performed duplicate transfections, one of which was stimulated with angiopoietin-1* (Ang1*) before harvesting (17,18). Vanadate was added before harvesting in order to inhibit any tyrosine phosphatases present (19). This has been shown to be required in order to demonstrate maximal ligand-induced phosphorylation of Tie2 (18,20). After harvesting, the cell lysates were immunoprecipitated with a Tie2 antibody. The Tie2 immunoprecipitates were run on a polyacrylamide gel and immunoblotted using a Tie2 antibody to show levels of protein expression. The blot was then stripped and reprobed with an anti-phosphotyrosine antibody to detect the level of phosphorylation of Tie2. Both VM mutations result in reproducible hyperphosphorylation of the Tie2 protein in the absence of ligand (Fig. 5). The R849W mutant shows an ~6-fold increase in ligand-independent autophosphorylation over wild-type, while the Y897S mutant shows an ~8-fold increase over wild-type. The data for the R849W mutant are consistent with those previously published using a different expression system (7). In contrast, little or no phosphorylation of wild-type Tie2 was observed in the absence of Ang1* stimulation. As expected, the K855R mutant remained unphosphorylated even in the presence of ligand, confirming its lack of kinase activity. Multiple bands are observed reproducibly only in the R849W and the Y897S mutant lanes, which may represent differentially processed forms of Tie2. Thus, both the R849W and Y897S VM mutations in Tie2 result in ligand-independent hyperphosphorylation of the receptor.

**Downstream interactors**

Tie2 can activate both phosphatidylinositol (PI) 3-kinase and Akt (20) to inhibit endothelial cell apoptosis (C. Kontos, S. Sankar, A. Wong and K. Peters, manuscript submitted for publication). Because this is the only signaling pathway downstream of Tie2 that has been linked to a specific cellular function, we sought to determine whether the Tie2 mutations seen in VM might alter this signaling pathway. As an initial approach, we evaluated the ability of the mutant kinases to bind p85, the regulatory subunit of PI 3-kinase, using the yeast two-hybrid system. Since Tie2 is phosphorylated when expressed in yeast, interactions with downstream signaling partners can be evaluated using this system (21). Consistent with previous results (20), the wild-type Tie2 kinase associated with p85 in a phosphotyrosine-dependent manner since no association was seen with the kinase-inactive mutant (Fig. 6). The C-terminal SH2 domain of p85 also bound to both the R849W and Y897S mutants, as demonstrated by activation of

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**Figure 3.** Identification of a novel Tie2 mutation in family 90. The entire TIE2 gene was RT–PCR amplified and sequenced from the proband of family 90. An A2690C change was the only difference identified. This results in a tyrosine to serine substitution (Y897S) within the first kinase domain.

**Figure 4.** Co-segregation of the Y897S mutation with VM in family 90. Genomic DNA from 13 family members was amplified using primers designed around the Y897S mutation. The A2690C base substitution creates a novel NlaIV restriction site. The mutation, as shown by the double bands, fully co-segregates with disease status within the family.
two separate reporter genes. This interaction appeared qualitatively similar to that observed with the wild-type kinase (Fig. 6). These findings demonstrate that the Tie2 mutations in VM do not disrupt association with PI 3-kinase. However, these mutations may affect this signaling pathway in some other way, either qualitatively or quantitatively.

**Histological analysis**

Most histological analyses of venous malformations come from sporadic cases, with the exception of those described in Vikkula et al. (23). In order to compare the histology of sporadic lesions with those present in VM families with the R849W and Y897S mutations, we obtained surgically resected lesions from one individual from each family for histological analyses. Sections showed skin and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina and subcutaneous tissue containing a poorly circumscribed, disorderly proliferation of blood vessels with dilated lumina. The histology of both lesions was consistent with previously published data (5,7).

We also examined the expression of Tie2 within the vascular lesion itself, since the biochemical data suggest an activating or gain-of-function mutation. Expression of Tie2 was detected both in normal, pre-existing vessels and in the vessels within the lesion.

**Locus heterogeneity for VM**

The remaining two probands of families 105 and 137 lacked both of the identified Tie2 mutations. We were unable to sequence the entire coding region of Tie2 in these two families due to lack of patient RNA for RT–PCR, and the fact that the complete genomic sequence of Tie2 has not been determined. The entire families were ascertained and blood samples collected for genetic linkage analysis (Fig. 8). Multipoint linkage analysis using markers flanking Tie2 excluded this gene in family 137 using the standard LOD score criteria of –2.0 in both the full pedigree and affecteds-only analyses, establishing the existence of an additional VM locus (Fig. 9). While no obligate recombinants for the distal flanking marker D9S161 were observed in family 137 due to its low informativeness in the family, several recombinants involving D9S161 were observed, allowing the VM locus to be excluded using standard LOD score criteria. In family 105, however, the multipoint lod scores were uninformative for the full pedigree analysis and slightly positive with a peak lod score of 1.20 within the interval for the affecteds-only analysis, as a common haplotype appeared to segregate to all affected individuals. However, the putative disease-associated haplotype is also transmitted to three of the five unaffected family members, suggesting that if family 105 does harbor a Tie2 mutation it may result in lower penetrance than the R849W and Y897S mutations.

**DISCUSSION**

The importance of Tie2 in angiogenesis during early development has been demonstrated using mouse transgenic and knockout technology. Tie2 gene expression in the mouse has been abrogated by homozygous inactivation of the gene (22,23) as well as expression of a dominant-negative transgene (22). In both cases, defects in vascular development and organization cause early embryonic lethality with localized hemorrhage (22,23). The blood vessels in these animals appear uniformly dilated, suggesting a role for Tie2 in vascular morphogenesis, and little or no capillary branching is observed (23). Significantly, the heterozygous Tie2 knockout mice appear normal (22,23).

Tie2 is also known to be up-regulated and activated during other angiogenic processes, such as wound healing (24), tumor angiogenesis (25–27) and during the female reproductive cycle.
For example, there are higher levels of Tie2 protein expressed in malignant breast tumors (25), and tumor angiogenesis and growth are halted when Tie2 activation is inhibited (26,27). Tie2 has been shown to be expressed and phosphorylated in a number of quiescent adult tissues as well. (24). These combined data suggest that in addition to its role in embryonic vascular development, Tie2 plays a role in maintenance and/or remodeling of the adult vasculature.

The mutation data from these VM kindreds support the hypothesis that Tie2 is involved in either vascular maintenance or remodeling. The venous malformations in affected individuals consist of dilated vessels that often lack or have a reduced smooth muscle layer. These lesions can appear congenitally, although others appear with increasing age. Thus, the Tie2 mutations observed in VM kindreds lead to a focal vascular alteration, rather than a global disruption of the vascular network.

Elucidation of the mechanism of activation and functions of Tie2 should aid our understanding of how these hyperphosphorylating mutations lead to the development of the vascular lesions observed in VM. The details of the Tie2 signaling pathway are just beginning to emerge. Tie2 can associate with p85, the regulatory subunit of PI 3-kinase, leading to activation of both PI 3-kinase and Akt (20). This pathway is involved in cell survival and anti-apoptosis, which might help to explain the role of Tie2 in normal vascular maintenance (C. Kontos, S. Sankar, A. Wong and K. Peters, manuscript submitted for publication). Although Tie2–p85 association appears not to be disrupted by the two activating mutations, it is unclear whether tonic activation of this pathway might alter the resultant biological responses of endothelial cells. For example, the oncogenic avian sarcoma virus, which encodes the catalytic subunit of PI 3-kinase, can transform cultured chicken embryo fibroblasts and induce focal hemangiosarcomas in chickens (28). In addition, the Tie2 mutations seen in venous malformations may not lead to simple on-or-off activation. For example, hyperphosphorylating mutations in the FGFR3 receptor tyrosine kinase observed in achondroplasia and thanatophoric dysplasia show a range of graded activation (29). Disease severity is correlated with degree of activation of the receptor by the mutation, and the same might hold true for the Tie2 mutations described here.

Several other potential downstream interactors for Tie2 have been identified, including GRB2, Shp2 and Dok-R (16,21). GRB2 and Shp2 are known to activate the RAS pathway (16,30) which is involved in cell proliferation and differentiation, while the Dok-R pathway is thought to be involved in cell motility and migration (21,30). Which of these processes, if any, is involved in the formation of venous malformations is uncertain. As endothelial cells grow and aggregate to form blood vessels, they release mesenchymal recruiting signals, possibly including platelet-derived growth factor, HB-epidermal growth factor and neuregulin (31–35). Given that the venous malformations display decreased surrounding supportive tissues, such as smooth

Figure 7. Representative views of three vascular lesions from two VM patients. (A–D) Patient 1 (family 154; R849W). (E and F) Patient 2 (family 90; Y897S). Routine histology of typical venous malformations. (A and E) A view of the dermis shows dilated, thin-walled vascular spaces, lacking surrounding smooth muscle layer. (B and F) Expression of Tie2 both in the endothelial cells lining the dilated vessels in the lesion (asterisks) and in the capillaries located in the adjacent dermis (arrows); note scattered pericytes also expressing Tie2. (C) Closer view of an area of thrombosis and recanalization close to a dilated vessel (empty space at the top of the picture). (D) The newly formed capillaries present in the areas of recanalization strongly express Tie2. (A, C and E) Hematoxylin and eosin: original magnification (A) and (E) ×40; (C) ×100. (B, D and F) Anti-Tie2. Diaminobenzidine with light hematoxylin: original magnification ×400.
Figure 8. Pedigrees show the genotypes for markers flanking the TIE2 locus on chromosome 9. (a) Family 105; (b) family 137.
muscle cells and pericytes, this pathway may be altered somehow. However, immunohistochemical analysis suggests that there is no gross alteration in Tie2 expression in these lesions.

Alternatively, the mechanisms of receptor activation and tyrosine phosphorylation by the hyperphosphorylating Tie2 mutations may be different from those following activation with Ang1. This may result in activation of a different subset of signaling proteins and subsequently lead to altered biological functions. The induction of the STAT signaling pathway is one such recently identified example (36). The wild-type Tie2 receptor activates only the STAT3 and STAT5 family members, whereas the R849W mutant can also activate STAT1 (36). Along with the inappropriate activation of STAT1, the R849W mutant retains the ability to activate STAT3 and STAT5, but to a greater degree than wild-type Tie2 (36). The novel Y897S may also result in aberrant signaling.

The activation of the JAK/STAT1 pathway results in inhibition of transforming growth factor-β (TGF-β) signaling (37). This suggests one potential model for vascular lesion formation, since one role of TGF-β is to stimulate matrix deposition and to induce differentiation of mesenchymal cells into smooth muscle cells and pericytes (2). The lack of smooth muscle cell support in the VM lesions could be explained by the lack of TGF-β signaling. The complexity of both the Tie2 and TGF-β signaling pathways suggests that several interacting events are required for vascular lesion formation. Further elucidation of the signaling pathways and cellular functions activated by the wild-type and mutant Tie2 receptors will be critical to understanding the pathophysiology of VM fully.

Inheritance of one of these TIE2 mutations most often predisposes an individual to the development of multiple venous malformations. As noted, these lesions are focal in nature and many develop later in life. Thus, a critical issue is the nature of the initiating factor for venous malformation development. This event could be triggered by local environmental or mechanical effects at the lumen of the vessel. For example, mechanical injury might lead to initial vascular changes (i.e. reduction in the number of smooth muscle cells), with subsequent development of the lesion due to hemodynamic changes in the vessel micro-environment. Another hypothesis is that lesion formation is initiated by mutation of the wild-type allele in a founder cell, leading to the development of a venous malformation. In a preliminary analysis of this hypothesis, we have obtained surgically resected venous malformations from one individual for each of the two mutations, but we have not observed loss of heterozygosity at the TIE2 locus for either of these lesions.

The four new VM kindreds described here demonstrate allelic and locus heterogeneity. The two mutations identified in TIE2 establish a role for the Tie2 signaling pathway in the formation of the vascular lesions. Another VM family, family 137, does not map to 9p21, which suggests that other genes are
also involved in VM. Family 105 is unable to be classified as linked or unlinked to the TIE2 locus, although it has been eliminated from harboring either of the two known mutations at this locus. These families will be used for genetic linkage analysis to identify additional loci contributing to venous malformations. The lack of mucosal lesions in families 105 and 137 suggests a locus-specific genotype–phenotype correlation when compared with the VMCM (TIE2) kindreds. However, additional families lacking mucosal lesions and mapping to a distinct locus are required to substantiate this.

Two potential candidate genes for the second locus might be the Tie2 ligands, Ang1 and Ang2. Genetic markers mapping near these loci were genotyped for these families, but no indication of linkage is evident (data not shown) (38). Identification of new loci may provide additional clues to the diverse signaling pathways that, when altered, lead to the formation of venous malformations.

MATERIALS AND METHODS

Family ascertainment

After obtaining informed consent, each participant completed informational questionnaires and family histories were taken. Affected status was assigned on the basis of two or more venous malformations. Blood samples were obtained from appropriate subjects and DNA was extracted using the Puregene kit (Gentra Systems, Minneapolis, MN). Some blood samples were collected and processed through Isocode DNA Sample Isolation Devices according to the manufacturer’s specifications (Schleicher & Schuell, Keene, NH).

Genotypes and linkage analysis

Analyses of simple repeat markers were performed as previously described (39). Two-point linkage analysis was also performed using age-dependent penetrance values based on four liability classes with age ranges 0–12 (penetrance 0.5), 12–21 (penetrance 0.75), 21–45 (penetrance 0.90) and >45 (penetrance 0.95) years as previously described (4). Disease allele frequency was set at 0.0001 (4). Allele frequencies for the disease allele classes with age ranges 0–12 (penetrance 0.5), 12–21 (penetrance 0.75), 21–45 (penetrance 0.90) and >45 (penetrance 0.95) years as previously described (4). Disease allele frequency was set at 0.0001 (4). Allele frequencies for the markers were taken from either GDB or the CEPH Genotype Database. Multipoint analysis was performed as previously described (40) using the map D9S259–2cM–D9S169–2cM–D9S161 (Whitehead Institute for Biomedical Research/MIT Center for Genome Research and the CEPH Genotype Database).

Mutation analysis

The Tie2-coding region was amplified by RT–PCR using RNA isolated from peripheral blood lymphocytes. Briefly, total cellular RNA was prepared from Ficoll-purified buffy coat of peripheral blood using RNA STAT-60 (Tel-Test, Friendswood, TX). Reverse transcription was performed on 2 µg of total RNA using either oligo(dT) primer or, in some cases, a Tie2-specific reverse strand primer. A minus reverse transcriptase control was included. An aliquot of the reverse transcription reaction was used directly in PCR amplification. The amplification products were then subjected to direct (cycle) sequencing using the Thermosequenase cycle sequencing kit (Amersham, Arlington Heights, IL). When a difference from the reported cDNA sequence was identified, a corresponding PCR assay was developed to amplify a small segment of genomic DNA to confirm the putative mutation.

The mutation assay for the R849W change has been described previously (7). Briefly, the primers used to amplify the region flanking the R849W mutation in family 154 were 5'-ATATTCTTTCATTCTTTTGTGGCAGCATC-3' and 5'-TCAGGGACTCTGGCCCTAAACAG-3'. PCR products were digested with MaelII, since the mutation destroys a MaelII restriction site. Primers used to amplify the region flanking the Y897S mutation in family 90 were 5'-TAGGCAATTTCCA-CAGCACATC-3' and 5'-TTTTGGCTCAAGTAGTCCATGCA-TGC-3'. PCR products were digested with NlaIV, since the mutation creates a NlaIV restriction site. Transfections

COS-1 cells, a gift of Dr B. Cullen (Duke University, Durham, NC), were cultured in Iscove’s medium + 5% fetal bovine serum (FBS) at 37°C in a 5% CO2 environment. The Tie2 polyclonal antibody (Tek C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phosphotyrosine antibody (PY20) was purchased from Transduction Laboratories (Lexington, KY). The full-length murine Tie2 cDNA in the PCRScript vector was obtained by RT–PCR from a mouse embryo cDNA library as described (16). This plasmid was used in site-directed mutagenesis according to the manufacturer’s specifications using either the Transformer Site-Directed Mutagenesis kit (Clontech, Palo Alto, CA) for R849W or the Quick-Change Mutagenesis kit (Stratagene, La Jolla, CA) for Y897S. Once the mutations were made, the entire cDNA sequence was verified by direct cycle sequencing. The full-length cDNAs containing the mutations and the normal Tie2 were then excised and cloned into the pcDNA expression vector. The K855R plasmid has been described previously, although it was designated as K854R due to a variant valine in the Tie2 sequence (16).

Immunoprecipitation and western blot

COS-1 cells were cultured in six-well plates to 70–80% confluence. The LipofectAMINE method was used for transfections according to the manufacturer’s instructions (Gibco BRL, Grand Island, NY). Briefly, cells were transfected with: (i) 2 µg of pcIneo vector alone; (ii) 2 µg of vector containing Tie2 cDNA with R849W; (iii) 2 µg of vector containing Tie2 cDNA with Y897S; or (iv) 2 µg of vector containing Tie2 cDNA with K855R. At 2 days post-transfection, cells were serum starved for 3 h. Vanadate (1 mM) was added to all the wells to reduce tyrosine phosphatase activity. After a 10 min incubation at room temperature, Ang1* ligand (250 ng) was added to half of the transfections and allowed to incubate for an additional 8 min. The Ang1* used was a gift from George Yancopoulos (Regeneron Pharmaceuticals, Tarrytown, NY) and is a recombinant form of Ang1 previously described (17). Ang1* is a genetically engineered form of Ang1 that consists of the first 73 amino acids of Ang2 fused to residues 77–498 of Ang1 with a cysteine to serine mutation at residue 256. Ang1* reportedly is easier to produce and retains the activity of wild-type Ang1 (17). All media were removed and the cells were lysed in 350 µl of lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na2VO4, 5 mM MgCl2, 0.5 mM dithiothreitol, 10 mM NaF, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na2VO4].
1 mM diethiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2.5 mg/ml leupeptin, 2.5 mg/ml aprotinin, 1 mM benzamidine, 10 mg/ml trypsin inhibitor] at 4°C for 30 min.

The lysate was then clarified by centrifugation in a microcentrifuge and 35 µl were stored for later analysis. The remaining cell lysate was pre-cleared with 50 µl of protein A–Sepharose [resolved in lysis buffer (w/v); Amersham Pharmacia Biotech, Piscataway, NJ] for 3 h. The pre-cleared lysate was incubated at 4°C with 10 µl of Tie2 antibody. After at least 3 h, 20 µl of protein A–Sepharose was added for an additional 3 h. The protein A–Sepharose–immunocomplex was then washed three times in 500 µl of lysis buffer and resolved in 15 µl of Laemmli buffer. The precipitated proteins were separated by SDS–PAGE (5%) and blotted to a nitrocellulose membrane (Hybond-P; Amersham Pharmacia Biotech) for 1 h, all at 45°C.

Immunodetection

Immunodetection was performed with the ECL western blotting analysis system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. NIH Image 1.6 was used for densitometry calculations.

Analysis of p85 binding in vitro

The interaction of Tie2 mutant proteins with the p85 subunit of PI 3-kinase was evaluated in the yeast two-hybrid system, as described previously and according to established protocols (20,41). Briefly, the kinase domain of Tie2 containing either the Y897S or the R849W mutation was subcloned into the yeast bait plasmid pJK202. The plasmids pJK-Tie2 (wild-type) and pJK-K855R (kinase-inactive mutant) were then transformed into yeast (strain EGY191) containing the lacZ reporter plasmid pJK103 were transformed sequentially with bait plasmids and a plasmid encoding the C-terminal SH2 domain of human p85 (amino acids 594–724). The resultant transformants, expressing the Tie2 kinase domains fused to the DNA-binding domain of LexA and p85 fused to the activation domain of B42, were tested for activation of the reporter genes LEU2 and lacZ as described.

Immunohistochemical technique

Biopsy material of three venous malformations from two VM patients was obtained from archival surgery specimens with informed consent. Tissue was fixed in formalin and embedded in paraffin following standard procedures. Briefly, 5 µm sections were deparaffinized, rehydrated and treated with H₂O₂. Phosphate-buffered saline was used for washes between steps. Pepsin was used as enzymatic pre-treatment. Slides were reacted with a polyclonal primary antibody (1:500) against Tie2 (Tek C-20 antibody; Santa Cruz Biotechnology) for 15 min at 45°C using a capillary gap technique. A secondary antibody was incubated for 10 min, followed by avidin–biotin complex (Vector, Burlingame, CA) or Histostain Plus (Zymed, South San Francisco, CA) for 10 min, all at 45°C. Diamobenzidine was used as the chromogen, with light hematoxylin as the counterstain.

REFERENCES

receptor, by secretion-trap expression cloning [see comments]. Cell, 87, 1161–1169.


