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Mechanisms of vascular stability and the relationship to human disease

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Abstract

Purpose of review—The genetic basis for a variety of vascular malformation syndromes have been described, with an increasing functional understanding of the associated genes.

Recent findings—Genes responsible for familial vascular malformation syndromes have increasingly been shown to be involved in the control of vascular stability. Summary Genes involved in vascular stability pathways are good candidates for causing vascular malformation syndromes. Although these findings confirm the biologic importance of the involved pathways, further explanations are required to describe the focal nature of disease.

Keywords

cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy; cerebral cavernous malformation; cavernous malformation-arteriovenous malformation; vascular stability; cutaneomucosal venous malformation

Introduction

The vascular system provides an essential network allowing blood to flow throughout the body. Endothelial cells line all blood vessels and form a semi-permeable membrane that regulates the passage of fluid, nutrients, and leukocytes out of the blood and into the interstitial space [1,2]. In order to regulate barrier function, adjacent endothelial cells utilize adherens and tight junctions to maintain strong cell–cell contacts [3]. Further maturation and stabilization of the capillary occurs through tight endothelial associations with pericytes [4]. Under normal conditions, the endothelium of a mature capillary is quiescent, stable, and limits vascular leak. Many pathologic conditions cause a destabilization of the vascular network resulting in endothelial hyperpermeability, excessive vascular sprouting, and angiogenesis. Systems that promote these destabilizing pathways have received considerable attention, with vascular endothelial growth factor (VEGF) and its receptor tyrosine kinases being a prototypical example [5]. Recently, pathways that promote vascular stability have also been described. The best characterized of these systems is angiopoietin-1 and its interactions with the receptor, Tie-2. It was thus interesting to note that activating mutations in Tie-2 result in a rare vascular malformation syndrome, cutaneomucosal venous malformations (VMCMs) [6]. Rare genetic disease syndromes have the potential to provide in-vivo biologic insights into pathways that are important for normal health and homeostasis

[7]. There are a number of vascular malformation syndromes with genetic basis. As more is known about the mechanisms underlying vascular malformations, it becomes apparent that these pathways are involved in the control of vascular stability. In this review, we will explore emerging data regarding vascular stability pathways associated with vascular malformation syndromes.

Vascular stability includes the control of endothelial cell cytoskeleton and junction proteins and the interaction of endothelial cells with mural cells. Vascular malformation syndromes result from abnormal control of each of these components.

Cutaneomucosal venous malformations: Tie2-Ang1 signaling

Venous malformations are the most common of vascular malformations and account for over 50% of individuals reporting to vascular malformation centers. Venous malformations are the result of local aberrations in angiogenesis occurring in fetal development and can range in clinical spectrum from benign cosmetic varicosities to multifocal lesions in multiple vital organs [8,9]. The majority of vascular malformations have no known genetic component and are sporadic in nature. However, families with dominant inheritance of venous malformations have been identified and linkage analysis has localized mutations in the TEK gene on chromosome 9p21. This gene codes for the angiopoietin-1 receptor protein Tie2 and the majority of mutations identified thus far have been in or near the N-terminal-most of two kinase domains within the intracellular portion of the protein [9]. These families suffer from autosomal-dominant venous malformations, termed VMCM, which tend to involve small multifocal mucocutaneous lesions in addition to the possibility of other venous malformations (Fig. 1). Although they only make up nearly 2% of reported venous malformations, the understanding of the genetic factors leading to VMCM have lead to the partial understanding of the much more common sporadic venous malformations (reviewed in [10•]).

To date, 17 families have been reported with mutations in or near the kinase domain of Tie2, roughly 60% of these are missense mutations causing an arginine–tryptophan substitution (R849W) [11••]. When exogenously expressed in human umbilical vein endothelial cells (HUVECs), this mutation had no effect on migration or cell proliferation when compared with its wild-type counterpart. However, Tie2-R849W expressing cells were significantly more resistant to apoptosis by serum withdrawal and form unstable tubes [12], suggesting a possible mechanism for the propagation of vascular malformations that possess a paucity of smooth muscle or pericyte support that would normally induce endothelial apoptosis [13]. Additionally, Hu *et al.* [12] determined that Tie2-R849W was hyperautophosphorylated and, unlike its wild-type counterpart, activated the signal transducer and activator of transcription-1 (STAT1) transcription factor in an Ang1-independent but Janus Kinase (JAK)-dependent manner. Intriguingly though, STAT1 activation has actually been shown to downregulate angiogenesis *in vivo* [14], suggesting that further evaluation of STAT1 signaling in VMCM patients is warranted and this pathway could be a potential therapeutic target in treating vascular malformations.

Another insight into the understanding of venous malformations has come from the discovery of a ‘second hit’ within the VMCM of an individual with the inherited R849W mutation [11••]. Under the hypothesis of a paradominant mode of inheritance for VMCM, they found a Tie2 deletion mutation in the vascular lesion of a single patient that did not correspond to the genotype of DNA isolated from the blood of this patient. This finding prompted the authors to sequence DNA from vascular malformations of other patients whose venous malformations were inconsistent with the dominant inherited VMCM. In screening 62 venous malformations from 57 patients for mutations in TEK (Tie2), the

authors found 30 mutations in 28 individuals. Upon further characterization of the most prominent of these mutations, a leucine–phenylalanine substitution (L914F) within the same kinase domain as the inherited R849W mutation, they determined that this mutation also conferred hyperautophosphorylation of the Tie2 receptor, suggesting a similar mechanism for the development of sporadic venous malformations as to that of VMCM [11••].

Together, these recent findings suggest new targets for the treatment of VMCM and more importantly venous malformations collectively. They also underscore the importance of research into the mechanisms of ‘rare’ inherited conditions, as these studies are a distinct example of how this research can lead to a better understanding of the mechanism of related conditions.

Capillary malformation–arteriovenous malformation and cerebral cavernous malformations: control of GTPase pathways

Small GTPases are molecular switches that are well positioned to act as regulators of endothelial cell–cell interactions, junctional proteins, and the cellular cytoskeleton. Ras is a GTPase known to be important in linking signals from receptor tyrosine kinases (RTKs) at the cell surface to mitogen-activated protein kinase (MAPK) pathways in the cell to regulate growth, differentiation, and proliferation [15]. The importance of Ras *in vivo* is confirmed by the identification of increased levels of activated Ras in cancer cells. As the identification of Ras, over 100 further GTPases have been identified and characterized in separate families of the Ras ‘superfamily’ of GTPases based on structural and functional characteristics. The Rho (Ras homolog) family GTPases were originally identified on the basis of homology to Ras and have been shown to control the cellular cytoskeleton and influence cell–cell interactions in a number of cell types including endothelial cells [16].

These proteins are activated when bound to GTP and then become inactivated as they hydrolyze GTP to GDP. The dynamics of GTP binding and hydrolysis, as well as the subcellular localization of GTPases, contribute to the biologic effects of these systems. A variety of associated proteins modulate GTPase activity by promoting GDP–GTP exchange (guanine–nucleotide exchange factors or GEFs) or by promoting GTP–GDP hydrolysis (GTPase-activating proteins or GAPs). Scaffold proteins contribute to GTPase regulation by bringing the GTPase switch into proximity with downstream signaling effectors [17], and posttranslational isoprenylation allows GTPases to correctly localize with membranes. The potential complexity of GTPase signaling is staggering, and *in-vitro* studies cannot confirm the importance of individual pathways *in vivo*. Human vascular malformation syndromes have highlighted GTPase-associated pathways that are of particular importance to vascular stability *in vivo*.

Capillary malformation–arteriovenous malformation

The importance of GTPase pathways in vascular stability was highlighted by the finding of mutations in RASA1 in a rare vascular malformation syndrome, capillary malformation–arteriovenous malformation (CM-AVM) [18]. Affected families have manifestations ranging from isolated capillary malformations (Fig. 2), to underlying high-flow arteriovenous malformations of the brain, limbs, face, or spinal cord [19], to Parkes Weber syndrome with arteriovenous fistulas underlying the capillary malformation and associated with soft tissue and skeletal hypertrophy. These lesions segregated with loss of function mutations in RASA1, which encodes the GAP p120-RasGAP. As GTPase activity leads to hydrolysis of GTP to GDP and inactivity of signaling, the loss of p120-RasGAP opens the potential for increased Ras activation. RASA1 has homology with neurofibromin (NF1), the gene mutated in cases of neurofibromatosis, yet NF1 expression is not sufficient to compensate

for loss of RASA1 in families with CM-AVM [20]. The importance of RASA1 relative to NF1 or two other homologous genes, RASA2 and RASAL, in vascular stability would not have been predicted on the basis of in-vitro studies and was clearly shown by human genetic studies.

In addition to its function as a Ras-GAP, p120-RasGAP has been shown to associate with p190-RhoGAP, where it may influence Rho kinases to modulate the cellular cytoskeleton [21,22]. Further, p120-RasGAP associates with Rap1a [23], a GTPase often associated with cellular adhesion and cell–interactions, and the bait used to initially identify KREV1/RAP1a interaction trapped-1 (KRIT1) [24], a gene subsequently found to be mutated in a subset of families with cerebral cavernous malformations (CCMs) [25,26].

Cerebral cavernous malformations

CCMs are common vascular malformations described predominantly in the central nervous system (Fig. 3). Lesions consist of dilated endothelial channels or caverns lacking smooth muscle support and filled with blood or thrombus [27]. Occasionally, CCMs rupture leading to hemorrhagic stroke or death. Even prior to any clinically apparent hemorrhage, all lesions are associated with hemosiderin, a blood breakdown product in the surrounding brain parenchyma. Ultrastructural studies suggest absent or diminished tight junctions in close association with this hemosiderin, implying localized loss of the blood–brain barrier and loss of vascular stability [28].

CCM lesions are found with a prevalence of one in 200 people [29,30]. Approximately, 20% of patients have familial disease, following an autosomal-dominant pattern of inheritance. CCMs have been linked to loss of function mutations in the genes encoding any of three structurally distinct proteins, KRIT1 (a.k.a. CCM1) [25,26], CCM2 (a.k.a. Osmosensing Scaffold for MEKK3–OSM, Malcavernin, or MGC4607) [31,32], or Programmed cell death 10 (PDCD10, a.k.a. CCM3) [33]. For a more complete review of CCM genetics, see [34••]. The three CCM disease genes are structurally unrelated intracellular proteins that lack catalytic domains and have been found to associate with one another and influence a variety of signaling pathways. This topic was recently reviewed in greater detail by Faurobert [35••].

Initial evidence that CCMs might result from abnormal GTPase activity came with the identification of KRIT1 mutations in families with CCM and linkage to CCM1. Further proof that the KRIT1–RAP1a interaction is functionally important and came from studies in endothelial cells *in vitro* [36] and Krit1 heterozygous knockout mice *in vivo* [37]. KRIT1 and RAP1a interact to influence endothelial cell junctional integrity through downstream signaling cascades such as beta-catenin signaling. Krit1 has also been shown to interact genetically with the related GTPase, Rap1b [38].

CCM2 was identified simultaneously as a gene associated with familial CCM and as a scaffold protein to facilitate stress signaling from the small GTPase Rac1 to p38 MAPK [39]. CCM2 also associates with RhoA and its leads to increased RhoA activity in the endothelium [40•]. Increased RhoA activity is associated with vascular instability. Favorable effects of RhoA inhibition in CCM2 deficiency are seen both *in vitro* and *in vivo*, suggesting a possible therapeutic strategy for CCM disease [40•].

A question that arises in each of these genetic vascular malformation syndromes is how the ubiquitous presence of the genetic mutation results in disease and vascular derangement in discrete vascular territories, sparing other vascular beds. A ‘two-hit’ model has also been proposed to explain lesion formation in CCM. In this model, biallelic germline and somatic mutations combine as the stimulus for lesion formation. Evidence for a second genetic hit

was initially found for KRIT1 [41] and has since been identified for all three CCM genes [42•,43•,44•]. Mouse models with mutations in *Krit1* [45] and *Ccm2* [40•,46,47] have been developed, yet despite the presence of heterozygous loss of function mutations in analogy to human disease, these mice do not develop CCM lesions. To test the ‘two-hit’ hypothesis, these mice were mated onto a p53 knockout background predisposed to spontaneous loss of heterozygosity to provide the second hit. Although this model introduces confounding comorbidities, these mice frequently develop CCM lesions in support of the ‘two-hit’ theory [48•]. It is hoped that further work with conditional knockout alleles for the CCM genes will address the issue more completely.

Another theory for the CNS selectivity of CCM lesions takes into account the expression patterns for the CCM proteins. Although mouse and fish studies have confirmed an essential role for CCM proteins in the endothelium [40•,47,49], the proteins are highly expressed in neuronal cells [50–53]. Recently, CCM2 was shown to play an important role in regulating cell death in neuronal cells [54]. As both endothelial and neuronal functions for CCM2 have been demonstrated, it remains to be confirmed whether CCM lesions form as a result of impaired mechanisms intrinsically in endothelial cells, impaired signaling from adjacent neuronal cells, or a combination of both.

Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leucoencephalopathy: delta-Notch signaling

The necessity of delta-Notch signaling in the endothelium is well exemplified by loss of a single copy of delta-like ligand 4 (*Dll4*) or deletion of *Notch1* causing vascular defects and embryonic lethality [55,56]. When *Dll4* activates *Notch1*, a portion of the intracellular domain of *Notch1* (*NotchICD*) is cleaved through a γ -secretase-dependent mechanism. *NotchICD* then translocates to the nucleus and regulates transcription [57]. Notch signaling plays a well defined role in cell fate decisions through a process known as lateral inhibition [58]. VEGF induces endothelial cells at the leading edge of a vascular sprout to adopt a tip cell phenotype by mechanisms recently described. These studies demonstrated that *Dll4* on tip cells signals through Notch on the trailing adjacent stalk cells to ensure that these stalk cells do not also become tip cells. During vascular patterning, this allows for the correct number of endothelial tip cells and vascular sprouts. Using a γ -secretase inhibitor to inhibit Notch signaling, Hellstrom *et al.* [59] found increased filopodial protrusions in endothelial tip cells of the mouse retina. Notch inhibition, using the γ -secretase inhibitor or using *Dll4*^{+/–} mice resulted in increased vascular sprouting, density, and disrupted normal vascular patterning. Furthermore, activation of Notch signaling reduced tip cell filopodia and vascular density [59].

The delta-Notch signaling paradigm has recently become more interesting, as it has been shown that another Notch ligand, Jagged, antagonistically interferes with *Dll4*–Notch interactions in endothelial cells [60••]. As opposed to *Dll4*, which is expressed in endothelial tip cells and some endothelial stalk cells, Jagged1 is primarily expressed in endothelial stalk cells and functions in a pro-angiogenic fashion to downregulate *Dll4*–Notch signaling from stalk cells to tip cells [60••]. Thus, preventing the tip cell from being converted back into a stalk cell. These interactions are mediated by the differential expression of the Notch-glycosyltransferase family Fringe; when Fringe is expressed, it modifies Notch in a manner that converts Jagged1 from an agonist to a *Dll4* antagonist [60••]. This addition to the pathway helps explain the tightly controlled balance between tip and stalk cell in angiogenesis.

The clinical relevance of delta/Jagged-Notch signaling is apparent in the cerebral small vessel disease: cerebral autosomal-dominant arteriopathy with subcortical infarcts and

leukoencephalopathy (CADASIL), now recognized as the most common cause of inherited stroke and vascular cognitive impairment in adults [61••] (Fig. 4). CADASIL is an autosomal-dominant disorder resulting from mutations in NOTCH3. The precise mechanism of the development of CADASIL is unknown; however, it is clear that there is excess accumulation of the Notch extracellular domain (NotchECD) within smooth muscle of cerebral small vessels [62•]. It also seems apparent that the disease mechanism is a result of gain of novel function in the protein, as Notch signaling appears intact and neither upregulated nor downregulated in cells possessing mutated Notch protein [61••]. Until recently, mouse models attempting to mimic CADASIL have been unsuccessful [63,64]. Joutel *et al.* [62•] have recently created a transgenic mouse overexpressing the mutated Notch3 (TgN3R169C) specifically in smooth muscle. Although the authors made no attempt at determining the molecular mechanism for the disease, they did document similar pathological aspects of CADASIL including white matter degeneration and the accumulation of NotchECD aggregates. They also demonstrated that the white matter degeneration was preceded by microcirculatory disappearance and reduction in microvessel diameter [62•], both of which would increase the vascular resistance of the brain parenchyma, suggesting a physiologic mechanism for symptoms of CADASIL.

Conclusion

Recent work characterizing genetic vascular malformation syndromes has identified numerous disease genes that are associated with pathways controlling vascular stability. In this review, we have highlighted the convergence between these lines of investigation. It is clear that genes involved in pathways controlling vascular stability are good candidates as disease genes in vascular malformation syndromes. Conversely, genes associated with vascular malformations are likely to function in pathways controlling vascular stability. The ability to show such functions for disease genes may depend on our ability to answer the lingering questions highlighted by these syndromes, such as, ‘Why do lesions occur in discrete locations in the presence of ubiquitous, germline mutations?’ and ‘What are the determinants of variability in disease manifestations and severity?’ Further investigations exploring a ‘two-hit’ mechanism for lesion genesis and characterizing the role of environmental factors in disease phenotype should provide important insights into these questions. A better understanding of these factors should also allow for the generation of animal models that more faithfully reproduce disease pathology for use in mechanistic and preclinical studies.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000–000).

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Figure 1. Cutaneomucosal venous malformations

Patients affected with cutaneomucosal venous malformations associated with germline activating mutations of Tie-2. Reproduced with permission from [10].

Figure 2. Capillary malformation: arteriovenous malformation
Characteristic capillary malformations in patients with capillary malformation: arteriovenous malformation associated with loss of function mutations in *RASA1*. Reproduced with permission from [18].

Figure 3. Cerebral cavernous malformation

MRI images taken from a patient with familial cerebral cavernous malformation (CCM) and history of multiple hemorrhagic infarcts. (a) T2 weighted images demonstrate multiple lesions of varying sizes (arrows). (b) Gradient-echo (GRE) MRI detects iron found in the hemosiderin deposits of CCM lesions. These imaging sequences are recommended in the evaluation for CCM owing to the greatly increased sensitivity in comparison to T1-weighted or T2-weighted images. (c) Signal-weighted intensity (SWI) images represent the latest modality in the MRI evaluation of CCM, demonstrating even greater sensitivity for smaller lesions.

Figure 4. Cerebral autosomal-dominant arteriopathy with subcortical infarcts and leucoencephalopathy

MRI images taken from a patient with cerebral autosomal-dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) associated with NOTCH3 mutation. (a) T1-weighted images demonstrate lacunar infarcts in a patient with CADASIL. (b) T2-FLAIR imaging demonstrates deep infarcts as well as white matter changes (leucoencephalopathy). (c) Gradient-echo (GRE) sequences detect hypointense signals from previous small hemorrhages in the thalamus and the brainstem. Reproduced with permission from [61••].