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RhoA activation-mediated vascular permeability in capillary malformation-arteriovenous malformation syndrome: a hypothesis

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Capillary malformation-arteriovenous malformation (CM-AVM) syndrome is a class of capillary anomalies that are associated with arteriovenous malformations and arteriovenous fistulas, which carry a risk of hemorrhages. There are no broadly effective pharmacological therapies currently available. Most CM-AVMs are associated with a loss of RASA1, resulting in constitutive activation of RAS signaling. However, protein interaction analysis revealed that RASA1 forms a complex with Rho GTPase-activating protein (RhoGAP), a negative regulator of RhoA signaling. Herein, we propose that loss of RASA1 function results in constitutive activation of RhoA signaling in endothelial cells, resulting in enhanced vascular permeability. Therefore, strategies aimed at curtailing RhoA activity should be tested as an adjunctive therapeutic approach in cell culture studies and animal models of RASA1 deficiency.

CM-AVM: a rare RASopathy syndrome

CM-AVM syndrome is a recently identified class of multifocal capillary anomalies characterized by heterogeneous and mostly cutaneous spots on the face, neck, or limbs [1,2]. More serious vascular anomalies, such as arteriovenous malformations (AVMs) and arteriovenous fistulas (AVFs), are also reported in other tissues, such as the central nervous system (CNS) [3], where absence of an intermediate capillary bed linking arteries to veins can result in high-flow lesions that carry an increased risk of hemorrhage and neurological deterioration [4]. Currently, treatment of AVMs is predominantly limited to percutaneous endovascular embolization or surgical resection of lesions, depending on their location, accessibility, severity, and/or associated complications [5].

Most patients reported with CM-AVM present with a germline mutation in *RASA1*, which usually results in a nonfunctional gene product. Although some patients with CM-AVM harbor mutations in previously identified *RASA1* genomic loci, there is locus heterogeneity, because novel *RASA1* variants continue to be reported, and include nonsense, frameshift, splice site, or missense mutations [1,3,6]. *RASA1* encodes a p120-Ras GTPase-activating protein (RASGAP), which regulates the activity of the membrane-bound RAS, a highly conserved small GTP-binding protein (GTPase) that acts as a molecular switch by alternating between an active (GTP-bound) and inactive (GDP-bound) state (Fig. 1). By enhancing the hydrolysis of RAS-bound GTP, RASA1 switches the GTP-bound (active) RAS to the GDP-bound (inactive) state, thereby inhibiting its interaction with downstream signaling factors (Fig. 1). Therefore, it is predicted that loss of RASA1 function impairs the hydrolysis of RAS-bound GTP, resulting in enhanced RAS-mediated signaling processes. In agreement with this, a recent study showed evidence for brain AVMs associated with somatic activating RAS mutations in patients [7]. On balance, inhibition of the canonical RAS-MEK-ERK pathway has been proposed as the most direct therapeutic target for treatment of patients with CM-AVM harboring RASA1 loss-of-

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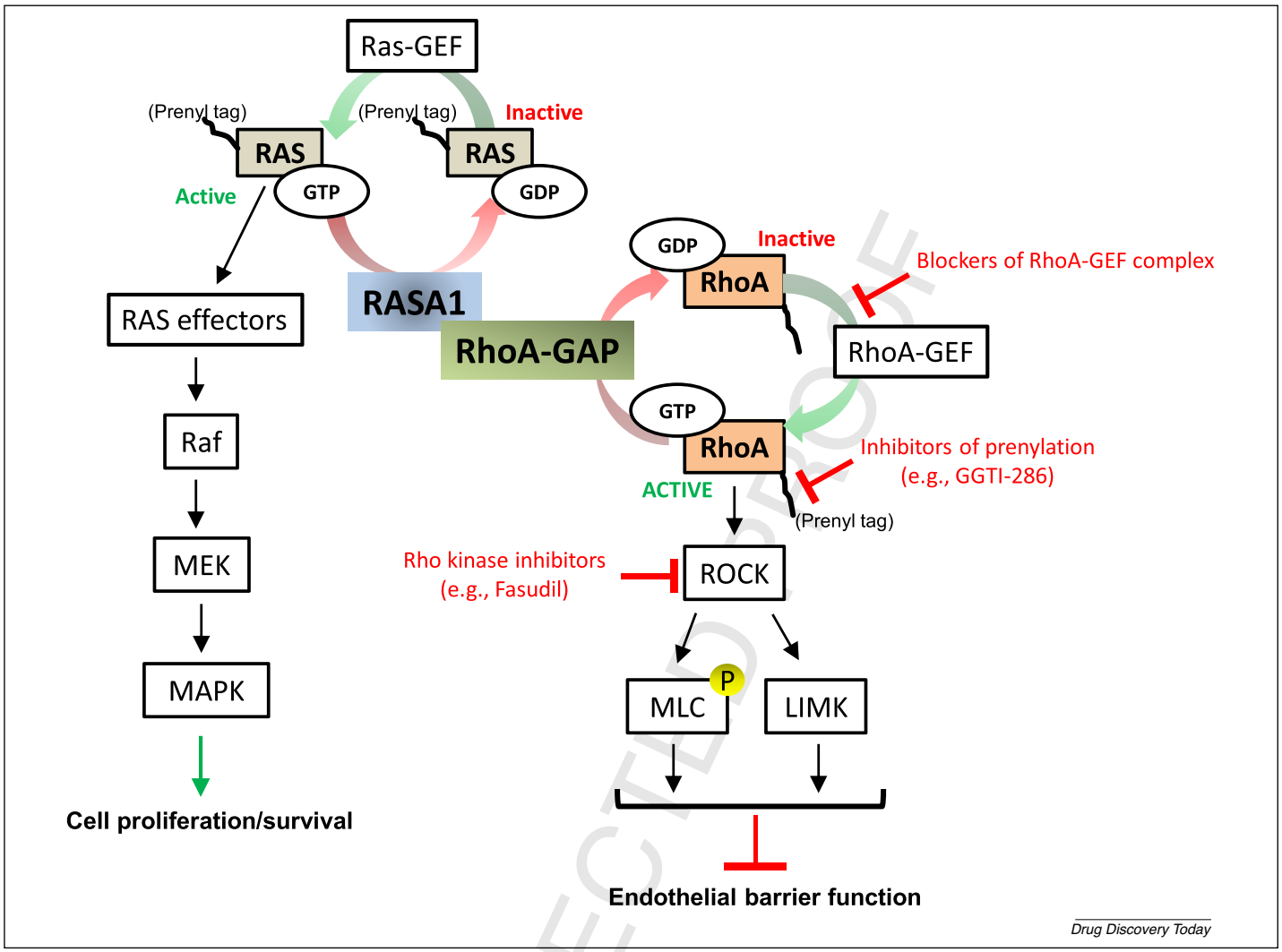

FIGURE 1

Summary of RAS and RhoA-mediated signal transduction pathways in endothelial cells. Both RAS and RhoA are prenylated small GTP-binding proteins belonging to the RAS superfamily of small GTPases [46]. Both RAS and RhoA alternate between a GTP-bound (active) and GDP-bound (inactive) state, mediated, in part, by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). RASA1 and RHOGAP form a complex [35]. RhoA activity, through its effector, Rho-associated protein kinase (ROCK), increases the phosphorylation of myosin light chain (MLC) of myosin II, resulting in loss of vascular stability. Examples of pharmacological inhibitors of RhoA signaling and their respective targets are depicted.

function. RAS (NRas, KRas, and HRas) is a component of multiple signal transduction pathways that regulate cell proliferation, cell cycle progression, and apoptosis and, accordingly, RAS genes constitute the most frequently mutated oncogene family [8–10]. In endothelial cells, it is speculated, based on animal studies, that enhanced RAS activation results in excessive endothelial cell proliferation, thereby, compromising the ability of endothelial cells to remain in a quiescent state and culminating in abnormal vascular morphogenesis and vascular leakage [11]. Similarly, in cultured human umbilical vein endothelial cells (HUVECs), loss of RASA1 expression is ensued by RAS hyperactivation and a concordant increase in endothelial cell proliferation, followed by neovascularization (as evidenced by increased tube formation) [12].

Herein, we present our hypothesis that, in addition to potential RAS-dependent effects on endothelial cell proliferative status that can result in aberrant angiogenesis, RAS-independent mechanisms might underlie at least some of these vascular manifestations of RASA1 loss-of-function in patients with a subtype of CM-AVM, giving rise to vessels that are both highly proliferative (as discussed earlier) and hemorrhage prone, because of the disruption of vascular stability.

Hypothesis: enhanced RhoA activation is a hallmark of RASA1 loss-of-function

Evidence from cell culture studies showed that RAS activity is coupled with that of another closely related molecular switch protein, RhoA, a member of the RAS superfamily of small GTPases. RhoA is a crucial regulator of actin dynamics [13]. In endothelial cells, activation of RhoA is implicated in enhanced *trans*-endothelial permeability through increased cell contraction, followed by junction protein remodeling [14,15]. RhoA hyperactivation is, in particular, a hallmark of several vascular pathologies typified by disruption of endothelial barrier function and vascular rupture, including cerebral cavernous malformations, aneurysmal subarachnoid hemorrhages, bacterial collagenase-induced brain hemorrhages, endotoxin-induced sepsis, edema, autoimmune disorders, and viral hemorrhagic fevers [16–22]. More specifically, RhoA signaling, mediated by the effector, Rho-associated protein kinase (ROCK), stimulates actomyosin contraction, which induces endothelial cell junction protein remo-

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deling and provokes increased vascular permeability [16] (Fig. 1). Intriguingly, both global and endothelial-specific loss of RASA1 were also associated with vascular ruptures and hemorrhages that resulted in lethality in embryonic mice [23,24]. Similarly, endothelial-specific induction of RAS in mice induces brain hemorrhages that are not rescued by inhibitors of the canonical RAS signaling [11]. However, to our knowledge, the involvement of RhoA signaling in mediating vascular permeability in CM-AVM models has not been directly addressed.

Protein interaction studies in growth-factor stimulated cells (derived from *Spodoptera fugiperda*) revealed that RASA1 forms a complex with a GTPase-activating protein (GAP) for GTP-bound RhoA, RhoGAP, a negative regulator of RhoA signaling [25] (Fig. 1). Subsequent studies revealed that the N terminus region of RASA1 forms a complex with RhoGAP, and this complex exhibits GAP activity against RhoA, as measured by enhanced hydrolysis of radioactively labeled and RhoA-bound GTP, suggesting an inhibitory role against active RhoA [26]. It was proposed that the complex formed between the N terminus of RASA1 and RhoGAP could provide RhoGAP better access to its substrates [26]. Both RASA1 and RhoGAP use the same catalytic arginine finger positioned in their phosphate-binding sites to facilitate GTP hydrolysis of their respective small GTPases, RAS and RhoA [27,28]. Co-immunoprecipitation studies established that the SH2 domains of RASA1 (found on the N terminus), which mediate protein–protein interactions, facilitate contact with the phosphotyrosine residues of RhoGAP in Sf9 insect cells and Hi5 cell lysates [29,30]. This association enables tyrosine phosphorylation and subsequent activation of RhoGAP and corresponding inhibition of RhoA activity [31,32]. Consistently, RhoGAP was shown to form a complex with RASA1 in human breast cancer cell lines [33] and in ectodermal cells during the gastrulation stage of *Xenopus* development, suggesting that coupling of RAS/MAPK and RhoA/ROCK signaling is instrumental for both cancer pathogenesis and mediating early aspects of embryogenesis [34]. More precise insights into the molecular basis for the interaction between RASA1 SH2 domain(s) and the phosphorylated tyrosines of RhoGAP have been shown through crystallographic structural analysis, revealing that the interaction is chiefly mediated by the direct binding of the N-terminal SH2 domain of RASA1 and the pTyr-1105 peptide of RhoGAP [35]. Accordingly, the physical interaction between RASA1 and RhoGAP suggests co-regulation or coupling of RAS/MAPK and RhoA/ROCK signaling in various cell types (insect cells versus cancer cells) and in different physiological contexts (e.g., embryonic development versus cancer pathogenesis) (Fig. 1). Consistently, loss-of-function studies revealed that miRNA-induced repression of RASA1 expression is ensued by activation of RhoA in pancreatic cancer cell lines, resulting in enhanced invasiveness and migratory behavior [36].

Additional insights from cancer biology revealed that the oncogenic potential of RAS (measured by formation of multilayered foci) is mediated, in part, by RhoA signaling [37]. The precise mechanism remains unknown but appears to be a RhoA-dependent augmentation of RAS activity [37]. Similarly, in mouse fibroblasts (NIH 3T3 cells), oncogenic RAS activation induced concomitant activation of RhoA, whereas pharmacological inhibition of the canonical RAS/MAPK signaling prevented RAS-mediated induction of RhoA activity [38]. The coupling of RASA1 and RhoGAP functions is further demonstrated by the fact that activation of RASA1 not only inhibits RAS signaling, but also suppresses RhoA signaling, both in vitro and in cell culture studies [39].

Concluding remarks

Taken together, emerging biochemical evidence derived mainly from nonendothelial cells reveals an interaction between RASA1 and RhoGAP [40], two GAPs. This interaction has been shown to result in interdependent and coordinated regulation of RAS and RhoA dynamics in various cell types and physiological contexts (Fig. 1). This suggests that the variable phenotypic manifestations of CM-AVMs arise from the intersection of RAS and RhoA-dependent signaling events on the endothelium. Intriguingly, there have been no studies, to our knowledge, that have directly investigated the involvement of RhoA hyperactivation in either patients with CM-AVM or endothelial cell culture or animal models of RASA1 deficiency or RAS induction. This is possibly because CM-AVM is a newly discovered vascular malformation for which we still lack sufficient insights into its etiological underpinnings. Nonetheless, evidence derived mostly from protein-interaction and cell culture studies compels us to hypothesize that RASA1 loss-of-function in endothelial cells is ensued by enhanced activation of RhoA signaling. Given the well-established involvement of RhoA in augmenting *trans*-endothelial permeability via cell junction protein remodeling in various vascular pathologies, we posit that increased RhoA activation could also account for the hemorrhage-prone vascular beds observed in murine models of RASA1 loss-of-function or endothelial-specific RAS induction [11,23,24].

Therefore, along with testing existing strategies aimed at curtailing RAS activation, we posit that targeting RhoA activity, using prevailing RhoA/Rho kinase (ROCK) inhibitors [15,41], could be a valid therapeutic strategy to test in animal models of CM-AVM. In particular, inhibitors of the RhoA effector, ROCK, such as the isoquinoline derivative, fasudil (HA-1077) (Fig. 1), have excellent safety profiles in clinical trials [42,43]. Additionally, because RhoA is a small GTPase that is prenylated at its CAAX terminus [44], inhibitors of prenylation, such as GGTI-286 (which target geranylgeranyltransferase I), can be tested for their efficacy to prevent post-translation modifications and membrane association of RhoA [45]. Moreover, because RhoA activation is catalyzed by specific guanine nucleotide exchange factors (GEFs), which facilitate the interaction of GTP-bound RhoA with its downstream effectors, small molecules that inhibit RhoA-GEF complex formation could be used to block the interaction of GTP-bound RhoA with its proximal downstream kinases [45] (Fig. 1). The efficacy and safety of inhibitors of RhoA signaling can readily be tested in endothelial cell culture models and small animal studies of RASA1 loss-of-function or RAS induction/over-expression, such as zebrafish or mice.

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