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The Cardiovascular Triad of Dysfunctional Angiogenesis

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Abstract

Cerebral cavernous malformation is a clinically well-defined microvascular disorder predisposing to stroke; however, the major phenotype observed in zebrafish is the cardiac defect, specifically an enlarged heart. Less effort has been made to explore this phenotypic discrepancy between human and zebrafish. Given the fact that the gene products from Ccm1/Ccm2 are nearly identical between the two species, the common sense has dictated that the zebrafish animal model would provide a great opportunity to dissect the detailed molecular function of Ccm1/Ccm2 during angiogenesis. We recently reported on the cellular role of the Ccm1 gene in biochemical processes that permit proper angiogenic microvascular development in the zebrafish model. In the course of this experimentation, we encountered a vast amount of recent research on the relationship between dysfunctional angiogenesis and cardiovascular defects in zebrafish. Here we compile the findings of our research with the most recent contributions in this field and glean conclusions about the effect of defective angiogenesis on the developing cardiovascular system. Our conclusion also serves as a bridge for the phenotypic discrepancy between humans and animal models, which might provide some insights into future translational research on human stroke.

Keywords

Cerebral cavernous malformation; Stroke; Microvascular malformation; Cardiovascular triad; Angiogenesis; Cardiovascular defects; Animal models; Zebrafish

Introduction

With the advent of zebrafish transgenesis and imaging technology, any single vascular endotholial cell along the vasculature can be analyzed in the zebrafish. Endothelium-specific GFP-labeled zebrafish lines provide a powerful tool to dissect angiogenic events during embryonic development. Therefore, these transgenic zebrafish lines have been considered as the potential model to study human strokes. However, on the mutant zebrafish model of cerebral cavernous malformation, one clinical type of the human stroke, there is obvious phenotypic discrepancy between humans and animal models. Although the zebrafish models still provide great details in studying angiogenesis, understanding the etiology of the

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phenotypic discrepancy between humans and zebrafish models would greatly enhance our future translational research on human stroke.

Cerebral cavernous malformations (CCMs), also known as cavernous angiomas or cavernomas, are vascular malformations in the brain with a characteristic MRI appearance. CCMs account for 10–15% of all vascular malformations of the CNS and are the second most common type of vascular malformation following venous angiomas [1]. CCM patients present with seizures, intracranial hemorrhage, and stroke; however, nearly half of the cases can be clinically asymptomatic [1]. Pathohistological observations indicate that CCM lesions comprise endothelium-lined sinusoids that lack the mature vascular structure, such as tight junctions with little or no intervening neural tissue, resulting in an incompetent blood–brain barrier, and thus potentially causing stroke [2-4]. Hereditary CCM has been found to be caused by a loss-of-function mutation in one of the three CCM genes: KRIT1 (CCM1), MGC4607 (CCM2), and PDCD10 (CCM3). On the molecular level, CCM1 interacts with integrin cytoplasmic domain associated protein-1-alpha, suggesting a role in β1 integrin-mediated signaling during angiogenesis [5-7]. Researchers have established that all three CCM gene products interact with each other and form a CCM complex [8-11] that modulates signal transduction pathways [7,12,13]. While the precise molecular etiology connecting the perturbed CCM protein complex to microvascular malformation has remained elusive, the complex's critical importance is evidenced by the fact that at least one of its components is depleted in most human CCMs. Therefore, much attention has recently been directed toward the roles played by the CCM complex in vasculogenesis and/or angiogenesis.

Surprisingly, in contrast to humans, major cardiovascular defects have been observed in Ccm animal models [14-16]. Enlarged heart chambers and major blood vessel changes have been described in Ccm1 (*santa*, *san*) and Ccm2 (*valentine*, *vtn*) mutant zebrafish lines, respectively [14-17]. It has been postulated that both Ccm1 and Ccm2 regulate the concentric growth of the myocardium without changing the fate of the cardiomyocyte or endothelial cells in zebrafish [16]. In mice, Ccm1 knockout has been reported to induce dilation of heart chambers and large arteries (such as the aorta) and to preferentially narrow certain arteries, leading to the hypothesis that Ccm1 is essential for vasculogenesis, the de novo formation of the blood vessel backbone. Associated heart defects have been presumed secondary to vascular malformation [18]. Two other studies in zebrafish embryos presented similar findings that Ccm1 and Ccm2 mutations cause the dilation of major vessels (especially primitive veins) and the progressive thinning of endothelial cells lining these primitive vessels. Thus, Ccm1 proteins regulate the genesis of vascular endothelial cells, particularly vascular tubular formation during vasculogenesis, further supporting the perturbed vasculogenesis hypothesis found in mouse models [17-19]. In relation to the reported pathohistological presentations, other studies in mouse and zebrafish further emphasized defective endothelial association and barrier function [20,21]. However, up to date, no attempt has been made to bridge the phenotypic discrepancy between stroke in humans and cardiovascular defects in animal models, which is the major focus of this review.

With the transgenic/mutagenic technology, much progress has been made in understanding vascular development. As a primitive vertebrate, zebrafish possess vascular anatomy, function, and spatiotemporal gene expression patterns that are largely shared across vertebrates. Such similarities suggest an evolutionarily conserved mechanism of vascular development [22-24]. For this reason, much microvascular research has been performed using this species. As with other vertebrates, the development of the embryonic vascular system in zebrafish involves two processes: vasculogenesis and angiogenesis. At the beginning of embryonic development, vasculogenesis is the construction of a primitive

circulation backbone of large vessels. In zebrafish, vasculogenesis occurs with the migration of angioblasts from the lateral posterior mesoderm to form two major axial vessels, the primitive aorta and primitive vein. Angiogenesis is the emergence of new, smaller vessels from the vascular skeleton created in vasculogenesis.

The contribution of gene-knockout zebrafish to cardiovascular research cannot be overstated. However, the nature of this technology requires that we take an occasional step back to grasp the bigger picture. Zebrafish allow for isolation of the effect of different genes one at a time. This piecemeal focus—a knockout here, a knockout there—can make grasping the overarching themes emerging from such research difficult. In the course of our research on Ccm1 gene function and zebrafish microvascular development, a study of existing literature yielded several themes regarding the effect of dysfunctional angiogenesis on the cardiovascular system. Specifically, the knockout of genes involved in proper angiogenesis in zebrafish results in a relatively consistent, defective cardiovascular phenotype of enlarged heart, major vessel dilation, and blood stasis. Although each of this cardiovascular triad could be heterogeneous in the underlying etiology, many knockout fish lines with dysfunctional angiogenesis share the same phenotypic triad. The Ccm1 knockout zebrafish presented in our research [25], which feature dysfunctional angiogenesis, exhibit this phenotypic triad as well [25,26]. Since zebrafish has much distinctively primitive vasculature system from mammals, enlarged heart phenotype will not be termed as or inter-exchanged with similar mammalian pathological defects such as cardiomegaly, pericardial edema, or cardiac hyperplasia in this review to avoid any confusion.

Enlarged Heart

Enlarged heart has been reported in zebrafish with knockouts of various genes, and these enlarged heart phenotypes vary in heart size, heart shape, and etiology. Early hemodynamic flow influences heart size and shape during zebrafish embryogenesis [27]. Cardiac endothelial cells, primordial cells that control the development of the heart, change shape during embryogenesis when subjected to shear forces. Early in vivo experiments have demonstrated that during zebrafish embryogenesis, the shear forces of blood flowing in the heart are strong enough to reshape the developing heart [28]. As such, a high level of shear force can induce an enlarged heart. Cardiomyopathies have been found to result in an enlarged heart [29,30]. An enlarged heart also can be induced by failed tube formation during vasculogenesis, such as in disruption of axial vessel formation [19,31,32], failure of posterior cardinal vein (PCV) formation [33], and fusion of the dorsal aorta (DA) and PCV [34-37].

However, it is now clear that most cases of enlarged heart seen in knockout zebrafish are secondary to defective microvasculature created during zebrafish embryogenic angiogenesis. The physiology underlying this etiology is an increase in circulatory resistance due to the absence of sufficient, patent microvascular vessels and space. The resulting blood flow obstruction and backup of blood in the arterial system leads to an enlarged heart during embryogenesis. For instance, regressing intersegmental vessels (ISVs) have been found to result in an enlarged heart in a host of mutant lines [38-44]. Furthermore, cardiomegalies caused by defective microvessels have been described in mutants where a downstream component of Rac1-Cdc42 signaling is deficient, causing defective angiogenesis [45]. Enlarged heart phenotypes also have been observed in mutant lines with defective ISV sprouting from major vessels [46-49]. Many failed angiogenic ISVs causing cardiomegalies have been attributed to notochord and somite defects in different mutants [50-58]. Likewise, we observed that a Ccm1 mutation zebrafish also exhibit +enlarged heart due to defective ISVs [25]. The responsibility of defective ISVs for the enlarged heart observed in zebrafish

mutants has been further substantiated by an antiangiogenic experiment in which inhibition of ISV development resulted in an enlarged heart [59].

Malformed Major Vasculature

The malformation of major vessels during embryogenesis is another common theme of zebrafish vascular research. These defects can be attributed to developmental flaws in either vasculogenesis or angiogenesis. When vasculogenesis is perturbed, vessel malformation in either the DA [60,61], PCV [62,63], or both [19] can be observed. A few reports have shown that perturbed vasculogenesis leads to disrupted angiogenesis, including studies showing that the absence of the PCV results in the malformation of existing intersegmental arteries that sprout from the DA [33,64].

However, most research has shown that perturbed angiogenesis is the primary cause of major axial vessel malformation. Without the successful formation of a network of ISVs connecting the arterial and venous vessels, blood backs up in the arterial system, unable to reach the veins. In turn, the blood backs up further into the heart and then the major veins. This situation is exacerbated by an increase in blood volume that occurs as part of angiogenesis. Faced with increasing pressure, the major vessels dilate. This dilation is a common feature seen in zebrafish with disrupted angiogenesis, although the location and the degree of dilation among major vessels vary among different mutant zebrafish lines. Specifically, PCV dilation is a major characteristic phenotype [19,29,44,65,66]. Likewise, the dilation of the caudal vein (CV) also appears to be secondary to defective ISV circulation [56,67-71]. Malformation along the entire axial vessels due to defective angiogenesis has been reported [58]. Our Ccm1 knockout zebrafish study, which features successful ISV formation but failed vacuole and lumen formation during angiogenesis, also had dilated axial vessels [25]. The timing of major vessel dilation suggests defective angiogenesis and not vasculogenesis to be the etiology of this dilation. Specifically, PCV dilation in Ccm1 mutant embryos is not observed at 32 hpf (prior to angiogenesis but after vasculogenesis) [25] but is present at 60–80 hpf (after angiogenesis is complete) [17].

Blood Stasis

The third effect of defective angiogenesis on the embryological development of the cardiovascular system is blood stasis. Without numerous patent intersegmental vessels, blood is unable to flow from the arterial system to the venous system, causing blood pooling. Evidence has been published that obstructed vessels formed in defective vasculogenesis or angiogenesis can result in such blood pooling [34-37]. Blood circulation was found to be diminished or absent in zebrafish mutant lines with defective angiogenesis that resulted in defective ISVs [48,49,55,56,58,60,72,73]. Additional research has found that slow blood flow is associated with blood cell accumulation in heart chambers and nearby axial vessels. Trapping of blood cells in the heart is another reproducible endpoint for microvessel defects [74]. Among mutant zebrafish lines with defective ISVs, static blood in heart chambers [43,55,73], the PCV [72], the aortic arch and the DA or PCV [49,56], and the CV [44] has been reported. The same finding of blood stasis due to dysfunctional angiogenesis was also reported in our Ccm1 mutant zebrafish research [25]. It needs to be emphasized here that the same phenotype of blood stasis may be rooted from the different causes. Our data clearly showed that although no circulation was found in the ISV, normal circulation was observed in primitive vasculature, such as DA, PCV, and CV during embryonic angiogenesis in Ccm1 mutant zebrafish, indicating that unpatented intersegmental vessels are one of the primary causes of blood stasis [25].

Conclusion

In this review, we discuss the phenotypic discrepancy of CCM mutations between humans and zebrafish and describe the cardiovascular triad of dysfunctional angiogenesis in zebrafish based on the ongoing research and collective points of view regarding the phenotypic presentation of perturbed angiogenesis during zebrafish embryonic development. We also briefly discuss the reciprocal relationship among each of three cardiovascular phenotypes during the embryonic development. It is important to emphasize that the most obvious and noticeable phenotype, which is usually first observed, might not be the initial event during the consequential pathogenesis of the entire vasculature development. Perturbed angiogenesis can eventually result in the pathogenic phenotypes of the heart and/or major primitive vasculatures, in a reverse manner, during embryonic development.

For our hypothesis, an argument had been made that there are a large number of mutants that display loss of circulation as a result of cardiac contractile defects (such as myosin, cardiac troponin T [silent heart] mutants, etc.); however, most of them do not display the same sort of heart defect as seen in the Ccm1/Ccm2 mutants. We think this argument provides further supportive evidence for our hypothesis. As we mentioned before, the combination of three major components during zebrafish embryonic angiogenesis (the strong contraction, dramatically increased blood volume, and relatively unchanged hemo-space) would dramatically enhance contractile force and hemodynamic resistance in cardiac chambers, which eventually results in the enlarged heart phenotype. Of course, our hypothesis does not reject the fact that some cardiac contractile or structure component defects could generate dilated or hypertrophic heart phenotypes, which also show an enlarged heart, comparable to human condition. The point is that a single cardiac phenotype, such as an enlarged heart, can be caused by a profoundly different mechanism. Another argument has been raised that it is very hard to believe that the enlarged heart phenotype is secondary to the vascular defect during this early stage of embryonic development. This notion is probably true in the vasculogenesis stage where the heart just starts to initiate its function. However, we think this argument also indirectly supports our hypothesis in the angiogenesis stage where the heart starts to generate blood flow. The blood flow certainly contributes to normal microvessel formation in some aspects as evidenced in a *silent heart* mutant, but not the sole cue as presented in our Ccm1 observation. The zebrafish heart during this stage of embryonic growth consists of a single endocardial layer surrounded by a few layers of myocardium and perhaps some fibroblasts that normally function to generate strong flow dynamic force necessary for the circulation during rapid development and growth of vasculature. However, it is still rapidly growing by itself to accommodate the continually expanding circulation system, and the vulnerability of the structure and strength of the heart is apparently undoubted in this stage. Any perturbation of the circulation would result in the negative impact on the maturation of heart, which could result in destructive force for the heart structure and eventually leads to enlarged heart phenotype. Although the shape and degree of the enlargement may vary due to the complexity of the circulation system, possibly in temporal and spatial-dependent manner during this stage of development.

In some cited references in this review, there is actually much less discussion or even no mention of enlarged heart or malformed primitive vasculature phenotype due to the subjective interest and focus of the research. The enlarged heart phenotypes in those references were identified through screening and examining the published cardiovasculature images, in combination with Zfin data, despite of the difficulty frequently encountered due to the often distorted shape of the heart in the images. Furthermore, when we referred to a specific mutant, it usually includes either null or deficient gene function. The spectrum of heart enlargement was also varied among the described mutants, such that the enlarged heart phenotype in some mutants is much subtle compared to Ccm1/Ccm2 mutants. Our effort

here is not to discriminate the difference among these similar phenotypes, but to establish a new hypothesis (or paradigm) to explain the common cause for the phenotype. Furthermore, because of the rapid development pace in zebrafish, significant morphological changes in the vasculature can be observed in the time interval of an hour, and timely and more precise observation is the key to address many unanswered questions in the future.

Our findings underscore not only the importance of patent, functioning angiogenic ISVs on the cardiovascular performance, but also the temporal and spatial locality of microvasculatures on the integrity of the entire dynamic cardiovascular system of zebrafish developing embryos. This conclusion appears to be well supported by the evidence we have reported in our Ccm1 study. Our research demonstrated that Ccm1 is required for vacuole and lumen formation during the creation of ISVs in angiogenesis [25]. Ccm1 deficiency sets in motion a cascade of vascular pathology. Failed lumen formation in angiogenic vessels results in an obstructed ISV network. At the same time, blood volume is increasing during angiogenesis. This combination of obstruction and larger blood volume results in increased cardiac afterload (higher arterial pressure), increased cardiac preload (higher atrial and ventricle volumes), and consequent enlarged heart. Due to the simple two-chamber heart structure, the enlargement of the atrium and ventricle allows blood flow back to the veins in lieu of efficient ejection through the aorta. This increased venous blood volume causes enlargement of the PCV [25]. This pathology and the resulting defective cardiovascular system are shared with other zebrafish with different mutations affecting angiogenesis. Enlarged heart [16], dilated axial primitive vessels [17,19], and blood stasis in and around the heart are all different expressions of the same root problem—obstructed microvasculature.

Inconsistent phenotypes reported in Ccm animal models, major cardiovascular defects, are all unrelated to the human condition of distended, thin-walled capillaries and arterioles (large artery and veins are not affected) in the brain. This constellation of distinctive pathologic findings in animal models [14-19,75] has made understanding CCM pathophysiology difficult. Consequently, uncovering the molecular etiology of CCM and linking the pathogenesis in humans and animal models have proven daunting. In earlier research, we proposed that the CCM complex was involved in microvascular angiogenesis through integrin-mediated signaling [7]. Seeking support for this hypothesis, we examined a variety of endothelial cell activities that can be assessed in cultured cells in vitro and that are thought to contribute to the formation of new blood vessels in vivo. These include VEC migration, lumen formation, and angiogenic ability. Our in vivo results showed that angiogenic lumen formation did not occur in *san* embryos despite the well-developed axial vessels and normal early process of angiogenesis [25]. These results were corroborated by our in vitro data [7,11,25]. In summary, our review further affirms that microvascular angiogenesis depends on a series of temporally and spatially coordinated events, and diversified phenotypes observed in the same mutation reflect the perturbation of angiogenic events in temporal and spatial manner. Understanding the etiology of the phenotypic discrepancy between humans and zebrafish models would greatly enhance our translational research on human stroke.

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