The angiostatic molecule Multimerin 2 is processed by MMP-9 to allow sprouting angiogenesis

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Abstract

Angiogenesis is a crucial process occurring under physiological and pathological conditions, including cancer. The development of blood vessels is tightly regulated by a plethora of cytokines, endothelial cell (EC) receptors and extracellular matrix (ECM) components. In this context, we have shown that Multimerin 2 (MMRN2), an ECM molecule specifically secreted by ECs, exerts angiostatic functions by binding VEGFA and other pro-angiogenic cytokines. Here, we demonstrate that during angiogenic stimuli *MMRN2* mRNA levels significantly decrease. Furthermore, we provide evidence that MMRN2 is processed by matrix metalloproteinases (MMPs) including MMP-9 and, to a lesser degree, by MMP-2. This proteolytic cleavage correlates with an increased migration of ECs. Accordingly, MMRN2 down-regulation is associated with an increased number of EC pseudopodia at the migrating front and this effect is attenuated using specific MMP-9 inhibitors. The down-modulation of MMRN2 occurs also in the context of tumor-associated angiogenesis. Immunofluorescence performed on tumor sections indicate a broad co-localization of MMP-9 and MMRN2, suggesting that the molecule may be extensively remodeled during tumor angiogenesis. Given the altered expression in tumors and the key role of MMRN2 in blood vessel function, we postulate that analyses of its expression may serve as a marker to predict the efficacy of the treatments. In conclusion, these data further support the role of MMRN2 as a key molecule regulating EC function and sprouting angiogenesis.

Introduction

Angiogenesis, the formation of new blood vessels from preexisting vessels, is a key process pertinent for reproduction, development, and wound repair [1,2]. The development of new vessels also plays a critical role in the onset and progress of many diseases, including cancer [3]. Pathological angiogenesis is a hallmark of cancer [4]. It is widely accepted that tumor growth is angiogenesis-dependent and blocking this process is regarded as a promising therapeutic approach to impair tumor growth [5,6]. Since the proposition of this groundbreaking hypothesis, many anti-angiogenic agents have been developed and are currently being utilized in the clinic. However, monotherapies with antiangiogenic drugs were not as effective as initially expected [7–12]. Fortunately, improvements have been achieved by combining anti-angiogenic treatments and standard chemotherapy with benefits in progression-free and overall survival

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[13,14]. Nevertheless, detrimental drawbacks of these approaches include resistance, reduced delivery of the drugs within tumors, and increased tumor hypoxia, which in turn may positively affect the development of metastasis [15,16]. Unfortunately, a significant fraction of patients does not receive benefit from the anti-angiogenic therapies and identification of clinical biomarkers is the focus of intense research [13,17–19].

The formation of blood vessels is tightly regulated and involves the coordinated interaction among a multitude of different cell types, cytokines, growth factors, and extracellular matrix (ECM) constituents [3,20–27]. ECM remodeling allows endothelial cell (EC) sprouting and is favored by activating several proteases. Matrix metalloproteinases (MMPs) [28] and in particular MMP-2, MMP-9, and MMP-14 play a key role in this context and are implicated in the local release of VEGF [29–31]. The MMPs encompass a large family of cation-dependent metallo-endo- or metallo-exo-peptidases responsible for coodinating a number of biological processes implicated in development and pathology [32–37].

Among the ECM molecules affecting angiogenesis, we have recently demonstrated that Multimerin 2 (MMRN2) halts angiogenic sprouting by sequestering VEGFA and impinging on the VEGF/VEGFR2 signaling axis [26,27]. MMRN2 belongs to the EDEN (EMI Domain ENdowed) protein family [38–40]. MMRN2 displays similar molecular architecture; although, unlike other EDEN family members, its expression is confined in tight juxtaposition with the EC surface [41]. Given the strategic pan-endothelial localization of MMRN2, we hypothesized that, following an angiogenic stimulus, it could be degraded to allow efficient vessel sprouting. The results of this investigation demonstrate that MMRN2 cleavage favors EC motility and sprouting angiogenesis and further clarifies how this EC-specific molecule is regulated during angiogenesis.

Results

Down-regulation of MMRN2 expression promotes endothelial cell migration

Corroborating the angiostatic role of MMRN2, we depleted endogenous expression in HUVEC (Fig. 1A) with knockdown achieving a >95% inhibition of the protein. The depletion of MMRN2 from HUVEC induced a significant increase of their motility in scratch test assays (Fig. 1B). Similar results were obtained in random motility assays where speed and distance covered by HUVEC were increased (Fig. 1C). Accordingly, the addition of recombinant MMRN2 (rMMRN2) reverted the effect (Fig. 1 C), further supporting the angiostatic role of MMRN2. We next analyzed the morphology of ECs in relation to MMRN2 expression by introducing a wound into the EC monolayer. Upon MMRN2 knockdown, ECs displayed a prominent motile phenotype with characteristic elongated shapes and an increased number of pseudopodia (Fig. 1D). In addition, MMRN2 expression was underrepresented at the EC migratory front and was particularly low along the pseudopodia (Fig. 1D,E). To verify if this phenomenon could be observed during vessel sprouting, we performed aortic ring assays. Importantly, MMRN2 expression was reduced in the tips of the sprouting vessels, whereas it was associated with that of CD31 in pre-existing vessels (Fig. 1F).

MMRN2 is down-regulated and degraded in response to angiogenic stimuli

The above evidence, as well as our previous studies, suggest that MMRN2 is deposited in the late stages of vessel development and exerts a homeostatic role for ECs. Thus, it is possible that, once the

Fig. 1. Depletion of MMRN2 leads to increased EC pseudopodia and migration. (A). Western blot of MMRN2 expression in HUVEC following transduction with control (siScr) or MMRN2 specific (siMMRN2) siRNA adenoviral constructs. β-Actin was used for normalization. (B). Representative images of scratch tests following depletion of MMRN2 and evaluation of migration (%) and distance (μm) covered by ECs after 6 h post-scratch. Scale bar = 160 μm. Statistics were obtained using the paired Student's *t*-test. (C) Graphs representing random motility of HUVEC cells transduced with the control (siScr) or MMRN2 specific (siMMRN2) adenoviral constructs in the presence of recombinant MMRN2 (rMMRN2); and evaluation of the total and euclid distance and speed of ECs. Statistics were obtained via one-way ANOVA. (D) Representative images of HUVEC transduced with control (siScr) or MMRN2 specific (siMMRN2) adenoviral constructs following scratch. Cells were stained with anti-MMRN2 antibody (green), phalloidin (red) and nuclei with TO-PRO® 3 (blue); cell pseudopodia are highlighted with an asterisk. The panels on the right include magnified details in which white arrowheads indicate cell pseudopodia. Scale bar = 50 μm. Right: graph reporting the number of cell pseudopodia per cells as evaluated by counting. Statistics were obtained using the paired Student's *t*-test. (E) Left graph: quantification of MMRN2 staining normalized to CD31 in the whole cell monolayer respect to the migration front. Right graph: evaluation of MMRN2 staining in the cells of the migration front with respect to their pseudopodia. Data assessed with Volocity software. Statistics were obtained using the paired Student's *t*-test. (F) Representative images of whole mounted aortic rings embedded in collagen type I and stained with anti-MMRN2 (green) and anti-CD31 (red) antibodies. Nuclei were stained with TO-PRO® 3 (blue). White arrowheads highlight tips of the sprouting neovessels devoid of MMRN2 staining. Scale bar = 150 μm. Right graph: evaluation of the staining for MMRN2 normalized to that of CD31 in pre-existing vessels (PEV) compared to newly formed vessels (NFV). Statistics were obtained using the paired Student's *t*-test. The experiments were repeated at least three times and represent the mean ± SD.

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