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Infantile hemangioma-derived stem cells and endothelial cells are inhibited by class 3 semaphorins

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ABSTRACT

Class 3 semaphorins were discovered as a family of axon guidance molecules, but are now known to be involved in diverse biologic processes. In this study, we investigated the anti-angiogenic potential of SEMA3E and SEMA3F (SEMA3E&F) in infantile hemangioma (IH). IH is a common vascular tumor that involves both vasculogenesis and angiogenesis. Our lab has identified and isolated hemangioma stem cells (HemSC), glucose transporter 1 positive (GLUT1^{+}) endothelial cells (designated as GLUT1^{sel} cells) based on anti-GLUT1 magnetic beads selection and GLUT1-negative endothelial cells (named HemEC). We have shown that these types of cells play important roles in hemangiogenesis. We report here that SEMA3E inhibited HemEC migration and proliferation while SEMA3F was able to suppress the migration and proliferation in all three types of cells. Confocal microscopy showed that stress fibers in HemEC were reduced by SEMA3E&F and that stress fibers in HemSC were decreased by SEMA3F, which led to cytoskeletal collapse and loss of cell motility in both cell types. Additionally, SEMA3E&F were able to inhibit vascular endothelial growth factor (VEGF)-induced sprouts in all three types of cells. Further, SEMA3E&F reduced the level of p-VEGFR2 and its downstream p-ERK in HemEC. These results demonstrate that SEMA3E&F inhibit IH cell proliferation and suppress the angiogenic activities of migration and sprout formation. SEMA3E&F may have therapeutic potential to treat or prevent growth of highly proliferative IH.

1. Introduction

Infantile hemangioma (IH) is the most common tumor of infancy [1,2]. It displays a unique life cycle that can be divided into three major phases: proliferating, involuting and involuted [3]. We have identified CD133-positive cells in the proliferating IH as hemangioma stem cells (HemSC) [4]. They are able to differentiate into endothelial cells (EC), pericytes and adipocytes *in vitro* and *in vivo*. When mixed in Matrigel and injected subcutaneously into nude mice, HemSCs form hemangioma-like blood vessels and recapitulate the life cycle of IH [3,4].

Glucose transporter 1 (GLUT1)-positive ECs are a hallmark of IH, and our recent data shows that these cells diminish as the tumor involutes. GLUT1-positive ECs in IH behave as facultative stem cells. They function as bona fide EC *in vivo* but when removed from the tumor and cultured *in vitro* as a purified cell population (called GLUT1^{sel} cells), they display stem cell-like properties. The GLUT1^{sel} cells are clonogenic and can undergo multi-lineage differentiation [5]. GLUT1-negative ECs, unlike from the GLUT1-positive ECs, consistently exhibit endothelial phenotype *in vitro* [5]. They were

Abbreviations: SEMA3, class 3 semaphorin; SEMA3E&F, semaphorin 3E and 3F; IH, infantile hemangioma; HemSC, hemangioma stem cells; GLUT1, glucose transporter 1; EC, endothelial cell; HemEC, hemangioma endothelial cell; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; NRP2, neuropilin 2.

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previously designated as hemangioma endothelial cells (HemEC) [6]. Noticeably, HemECs constitutively express phosphorylated vascular endothelial growth factor receptor 2 (VEGFR2) and low levels of VEGFR1 in comparison with normal EC from newborn foreskin [7].

Although benign, IH ranges in severity from cutaneous discolorations to massive, life-threatening lesions. Despite advances in treatments for children with IH, drugs such as corticosteroid and propranolol are accompanied by side effects [8,9]; for example, the hemangioma starts to regrow in 10–20% of cases when propranolol is stopped and not all patients respond well [10,11]. There is still a pressing need for improved therapies that will shorten the treatment duration or, ultimately, prevent problematic IH from forming.

Class 3 semaphorins (SEMA3s), a family of seven members of axon guidance molecules (SEMA3A-G), have been well studied in our lab [12,13]. They first were discovered as mediators of neuronal guidance during neuronal development. More recently, their critical roles in the vascular system and in tumor biology have been recognized [12,13]. To elicit the regulatory signaling in the cells, SEMA3s are required to bind to neuropilin receptors and to form a complex with Plexin family receptors. Semaphorin 3F (SEMA3F) negatively regulates tumor cell and EC migration *in vitro* and tumor angiogenesis and metastasis *in vivo* [14,15]. It binds to neuropilin 2 (NRP2) and Plexin A1 receptors and induces signaling that causes cytoskeletal collapse in tumor cells and ECs [15]. Semaphorin 3E (SEMA3E) can directly bind to Plexin D1 receptor without NRPs [16] and can also cause cytoskeletal collapse, thus inhibiting EC sprouting and adhesion [17,18].

In our previous studies, NRP2 and Plexin D1 were significantly upregulated during HemSC/GLUT1^{sel}-to-EC differentiation [5,19], suggesting SEMA3s may gain the ability to influence IH growth as endothelial differentiation occurs. Therefore, we set out to understand, for the first time, whether and how SEMA3s, particularly SEMA3E and SEMA3F (SEMA3E&F), affect HemSC, GLUT1^{sel} cell and HemEC behavior and thus modulate IH growth. We demonstrated that SEMA3E&F inhibit the angiogenic activity of HemSC, GLUT1^{sel} cells and HemEC, suppressing cell migration and proliferation as well as VEGF-A-induced sprouting. In addition, SEMA3E&F reduced actin stress fibers in IH cells, leading to cytoskeletal collapse and loss of cell motility. In particular, in HemEC, SEMA3E&F noticeably inhibited p-VEGFR2 and p-ERK. In summary, our data show that SEMA3E&F can suppress the angiogenic ability of IH-derived cells and thus may potentially inhibit IH growth, which suggests a therapeutic application of SEMA3E&F in the treatment and prevention of highly proliferative IH.

2. Materials and methods

2.1. Antibodies & ELISA

The following antibodies were purchased from Cell Signaling Technology: rabbit polyclonal anti-phospho-ERK1/2 antibody (#9101); mouse monoclonal anti-ERK1/2 antibody (#4696); rabbit polyclonal anti-Plexin A1 antibody (#3813); rabbit monoclonal anti-phospho-VEGFR2 antibody (#2478); rabbit monoclonal anti-VEGFR2 antibody (#2479). The goat polyclonal anti-Plexin D1 antibody (AF4160) was purchased from R&D Systems. The mouse monoclonal anti-NRP2 antibody (sc-13117) was purchased from Santa Cruz Biotechnology. The rabbit anti-VE-cadherin antibody (HPA030562) and mouse monoclonal anti-β-actin antibody (AC-15) were from Sigma–Aldrich. Human VEGF Quantikine ELISA Kit (DVE00) was obtained from R&D Systems.

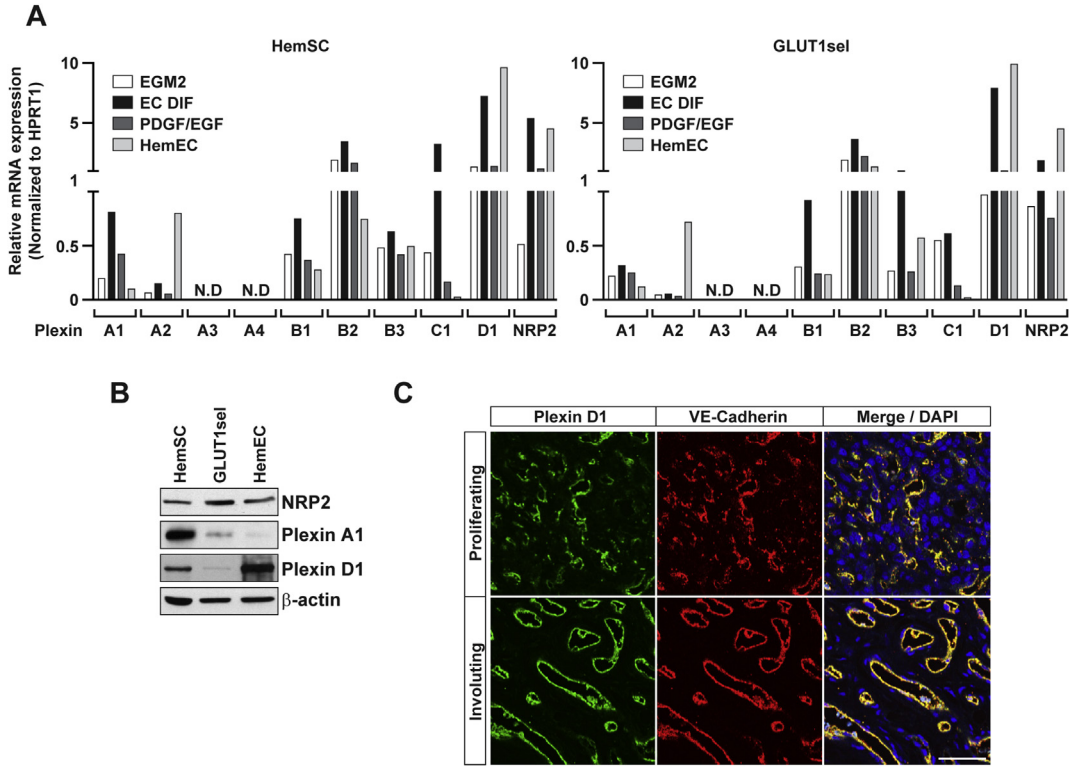

Fig. 1. Class 3 semaphorin receptors expressed on IH cells. A, HemSC and GLUT1^{sel} cells were cultured for 5 days in EGM-2, EC differentiation media or PDGF/EGF (negative control) media. Nine Plexins and NRP2 mRNA expression were analyzed by qPCR. mRNA levels were normalized to HPRT1 mRNA. N.D, not detected. B, Western blot for NRP2, Plexin A1 and Plexin D1 protein expression in HemSC, GLUT1^{sel} and HemEC. β-actin served as loading control. C, Expression of Plexin D1 in IH tissue. Immunofluorescence staining for Plexin D1 (green) and VE-cadherin (red) in proliferating (6 months) and involuting (31 months) IH tumor sections. Nuclei were counterstained with DAPI (blue). The scale bar indicates 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)