The regulatory effect of semaphorin 7A on proliferation and migration in human umbilical vein endothelial cells

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Abstract

Semaphorin 7A (SEMA 7A), a factor originally identified as regulating axon growth, has recently been implicated as a pro-angiogenic factor. The molecular mechanisms for this ability to stimulate angiogenesis have not been identified. This study examines if SEMA 7A can have a direct effect on vascular endothelial cells or whether it indirectly induces angiogenesis through stimulation and recruitment of macrophages as has been suggested. Using human umbilical vein endothelial cells (HUVECs), the ability of SEMA 7A to affect proliferation and migration was examined. HUVECs were exposed to SEMA 7A directly or to conditioned media collected from macrophages exposed to SEMA 7A and a cell proliferation assay was performed. Additionally, the ability of the cells to migrate was also measured using a transwell and a scratch assay. Direct exposure of HUVECs to SEMA 7A resulted in a significant decrease in cell proliferation. Preliminary results also suggest that direct exposure also results in a slight inhibitory effect on the migration of HUVECs. SEMA 7A treatment of macrophages did not result in the production of factors that stimulate HUVECs to proliferate. Additionally, our results suggest that macrophages exhibited a slight stimulation of migration in response to SEMA 7A.

Introduction

Angiogenesis is the process by which new blood vessels are produced from existing vessels (reviewed in Blanco and Gerhardt, 2013). Many different factors work together to regulate this very important process including growth factors such as members of the vascular endothelial growth factor family (VEGF). Another family of molecules that has recently been identified as having both pro- and anti- angiogenic characteristics, are the semaphorins (Neufeld et al., 2012) which were originally identified as factors that regulate axon path finding in both a positive and negative manner. Studies suggest that in vivo expression of exogenous semaphorin 7A (SEMA 7A) resulted in both angiogenesis (Ghanem et al., 2011) and macrophage recruitment (Holmes et al., 2002). The molecular mechanisms of this pro-angiogenic effect of SEMA 7A is not fully known and it is not clear whether this activity can be attributed to a direct effect on the vascular endothelial cells or was indirectly due to the ability to recruit macrophages to the area. Interestingly, SEMA 7A is a membrane bound molecule that has an RGD binding domain, which can bind to beta-1 integrin. In axons, SEMA 7A has been shown to bind to beta-1 integrin and stimulate the ERK pathway (Pasterkamp et al., 2003). Activation of the ERK pathway has been shown to stimulate proliferation and migration, which are key factors in angiogenesis. Beta-1 integrins are present on endothelial cells, and have been shown to support VEGF ERK signaling (Senger et at., 2002) (Stupack and Cheresh, 2004). This suggests that SEMA 7A may stimulate angiogenesis through direct stimulation of the ERK pathway in endothelial cells rather than through indirectly recruiting macrophages to the area to release pro-angiogenic factors.
Using an in vitro tissue culture model, the ability of SEMA 7A to stimulate angiogenesis directly (figure 1A) in human umbilical vein endothelial cells (HUVECs) or indirectly (figure 1B) through macrophage stimulation were examined. Upon exposure to SEMA 7A, early events in angiogenesis were measured through proliferation and migration assays.

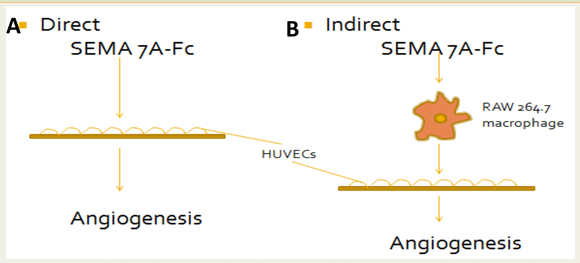


Figure 1 Diagram of the direct and indirect models examined in this study. In this study, an *in vitro* tissue culture system was used to determine whether SEMA 7A can (A) directly act on HUVECs to induce angiogenesis or whether it can (B) indirectly promote angiogenesis by inducing macrophages (RAW 264.7 cells) to release pro-angiogenic factors.

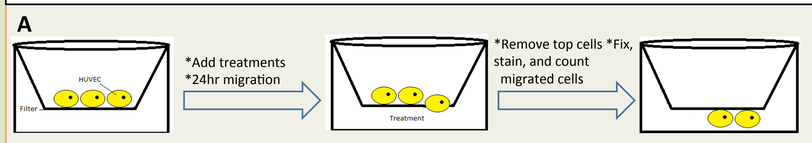
Direct Effects of SEMA 7A on Proliferation

Figure 2 Semaphorin 7A directly inhibits growth of HUVECs. Equal numbers of HUVECs were plated in growth media under the following conditions: growth media control (GM), VEGFA, soluble semaphorin 7A (SEMA 7A), both VEGFA and soluble SEMA 7A, SEMA 7A-coated well (SEMACt), or SEMA 7A-coated well in the presence of VEGFA. After 24 hours, a cell proliferation assay was performed using a salt that is reduced by dehydrogenases in the cells producing an orange colored formazan dye, which can be detected by measuring the absorbance at 450 nm using a microplate reader. Six assays were performed in duplicate and the average of the ratio of absorbance relative to the control was determined. Exposure of the HUVECs to bound SEMA 7A results in a significant decrease in proliferation relative to the control as revealed by a student's t-test ($p < 0.0001$). This decrease was relieved by the addition of VEGFA. VEGFA alone significantly increased the number of cells ($p=0.0242$).

Treatment	Proliferation
GM	1.00 ± 0.000
VEGFA	1.06 ± .0174*
Sema7A	1.00 ± .0168
Sema7A/VEGFA	1.06 ± .0254*
Sema7Act	0.96 ± .0137*
Sema7Act/VegfA	1.09 ± .0727

Figure 1: Shows the amount of proliferation increase or decrease relative to normal growth media (GM) without VEGFA. Values are based off reaction with a dye which reacts with living cells to produce an orange color, and color intensity was read by a Mircoplate reader. An * represents data that is significantly different from growth media using a Student's T-test for P<.05.

Direct Effects of SEMA 7A on Migration



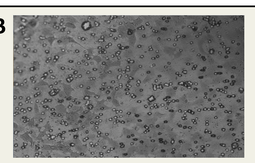
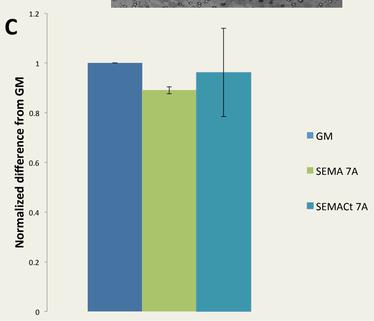


Figure 3 Transwell migration assay results suggest that SEMA 7A inhibits HUVEC migration. A. Schematic diagram of transwell migration assay in which equal numbers of HUVECs are plated on a porous filter separating the upper and lower chamber under the following conditions: growth media (GM) alone, soluble SEMA 7A (SEMA 7A), or SEMA 7A bound to the filter (SEMACt 7A). 24 hours later, cells remaining on the top of the filter were removed, cells that migrated to the bottom of the filter were fixed and stained with 1% crystal violet and counted. **B.** Photomicrograph of underside of coated filter following migration, fixation and staining show cells that have migrated to the underside of the filter. **C.** Graph of the average number of cells that have migrated to the bottom chamber under the various conditions for two different assays done in duplicate. Results suggest that exposure to SEMA 7A in the bottom chamber perturbs the ability of the HUVECs to migrate through the pores towards the lower chamber.



Indirect Effects on Proliferation

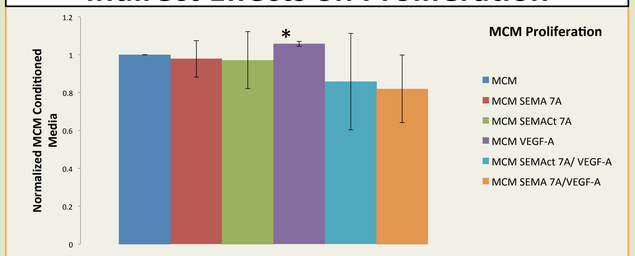
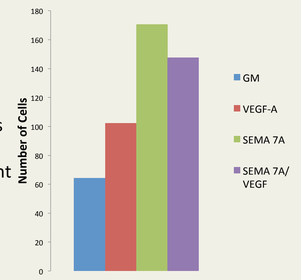


Figure 4 Effects of macrophage conditioned media on HUVEC proliferation. RAW 264.7 cells, a murine macrophage cell line, were grown in the presence or absence of either soluble SEMA 7A or bound SEMA 7A, and media was collected after 24 hours to yield the following conditioned media: macrophage conditioned media alone (MCM), and macrophages treated with soluble SEMA 7A (MCM SEMA) or bound SEMA 7A (MCM SEMACt). HUVECs were then incubated in the conditioned media in the presence or absence of VEGFA for 24 hours followed by a proliferation assay as described in figure 2. Treatment of RAW 264.7 cells with SEMA 7A does not result in the production of factors by these macrophages that induce proliferation in HUVECs.

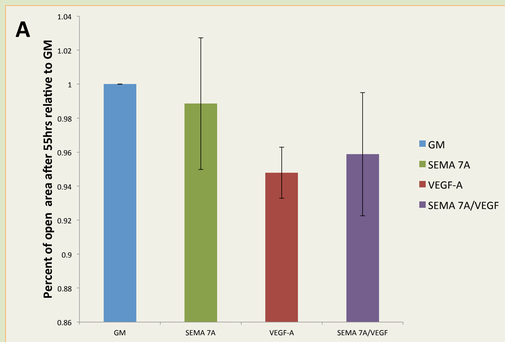
Macrophage Transwell Migration

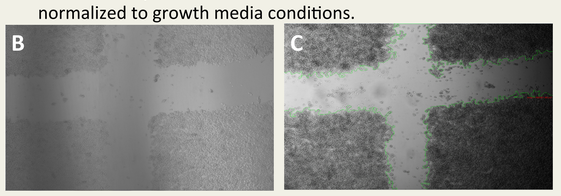
Figure 5 VEGF and SEMA stimulates migration of macrophages. Using RAW 264.7 macrophage cells, a transwell migration assay was performed as in figure 3. The results suggest that, consistent with the literature, soluble SEMA 7A, when placed in the lower chamber, stimulates chemotaxis.



Macrophage Scratch Migration

Figure 7 Scratch assay used to examine the effect of SEMA 7A and VEGF-A on macrophage proliferation and migration. Raw 264.7 macrophages were grown to a confluent monolayer, and then scratched or wounded in a "+" pattern using a 200μL pipette tip (example in B). Immediately following the scratch, cells were treated with either SEMA 7A, VEGF-A, or SEMA 7A/VEGF. Pictures were taken at 0hr and 55hrs. The area of the open wound was determined using Nikon Camera programming (example in C). The graph (A) represents the degree of migration, which was calculated by determining the percentage of open wound at 55 hours as compared to the area at 0 hours. Results were normalized to growth media conditions.





Conclusions

1. Exposure of HUVECs to bound semaphorin 7A significantly inhibits proliferation.
2. Preliminary results suggest that exposure of HUVECs to soluble semaphorin 7A has a slight inhibitory effect on migration.
3. Semaphorin 7A does not stimulate macrophages to produce factors which inhibit or promote HUVEC proliferation.
4. Preliminary results suggest that semapharin 7A may stimulate migration in macrophages as is consistent with the literature.

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Acknowledgments

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