Secreted semaphorin 5A suppressed pancreatic tumour burden but increased metastasis and endothelial cell proliferation.

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BACKGROUND: Our earlier reports demonstrated that membrane-bound semaphorin 5A (SEMASA) is expressed in aggressive pancreatic cancer cells and tumours, and promotes tumour growth and metastasis. In this study, we examine whether (1) pancreatic cancer cells secrete SEMASA and (2) that secreted SEMASA modulates certain phenotypes associated with tumour progression, angiogenesis and metastasis through various other molecular factors and signalling proteins.

METHODS AND RESULTS: In this study, we show that human pancreatic cancer cell lines secrete the extracellular domain (ECD) of SEMASA (SEMA5A-ECD) and overexpression of mouse Sema5A-ECD in Panc1 cells (not expressing SEMASA; Panc1-Sema5A-ECD; control cells - Panc1-control) significantly increases their invasion in vitro via enhanced ERK phosphorylation. Interestingly, orthotopic injection of Panc1-Sema5A-ECD cells into athymic nude mice results in a lower primary tumour burden, but enhances the micrometastases to the liver as compared with Panc1-control cells. Furthermore, there is a significant increase in proliferation of endothelial cells treated with conditioned media (CM) from Panc1-Sema5A-ECD cells and a significant increase in microvessel density in Panc1-Sema5A-ECD orthotopic tumours compared with those from Panc1-control cells, suggesting that the increase in liver micrometastases is probably due to increased tumour angiogenesis. In addition, our data demonstrate that this increase in endothelial cell proliferation by Sema5A-ECD is mediated through the angiogenic molecules – interleukin-8 and vascular endothelial growth factor.

CONCLUSION: Taken together, these results suggest that a bioactive, secreted form of Sema5A-ECD has an intriguing and potentially important role in its ability to enhance pancreatic tumour invasiveness, angiogenesis and micrometastases.

Keywords: pancreatic cancer; secreted SEMASA; tumour burden and metastasis; semaphorin; reduced tumour burden; angiogenic factors
characterised by five thrombospondin-1-specific repeats in its extracellular domain (ECD) in addition to the semaphorin and plexin domains (Adams et al, 1996). Semaphorin 5A is known to induce inhibitory responses during optic nerve development (Oster et al, 2003) and also has major roles in innate immunity (Sugimoto et al, 2006) and idiopathic autism (Melin et al, 2006). For the first time, we observed the expression of SEMA5A in human pancreatic tumours, but not in normal pancreatic tissue. In addition, we showed that SEMA5A is expressed by pancreatic cancer cell lines established from metastases, but not those from primary tumours. We also demonstrated the membrane localisation of SEMA5A particularly to regions of cell–cell adhesion in aggressive pancreatic cancer cell lines. Overexpression of membrane-bound mouse SEMA5A in pancreatic cancer cells has been demonstrated to increase migration and aggregation in vitro and tumorigenesis and metastasis in vivo (Sadanandam et al, 2010b). These results suggest that membrane-bound SEMA5A functions as a paracrine molecule involved in pancreatic tumour growth and metastasis. However, different reports on various cancer types either endogenously expressing SEMA5A or treated with the ECD of mouse Sema5A have contrasting tumour phenotypes (Li and Lee, 2010; Lu et al, 2010; Pan et al, 2010).

Recently, others and we have demonstrated the interaction of Sema5A with Plexin B3 in different mouse tissues and various cell lines (ectopically expressing Plexin B3; Artigiani et al, 2004; Sadanandam et al, 2008). Moreover, we have demonstrated that Sema5A is a proangiogenic molecule that regulates various steps of angiogenesis including endothelial cell proliferation, survival and migration in vitro and sprouting of blood vessels in vivo through its functional receptor, Plexin B3 (Sadanandam et al, 2010a). However, SEMA4D, a membrane-bound protein and a close relative with structural similarity to SEMA5A (Sadanandam et al, 2007), has been shown to be secreted (Wang et al, 2001) and can regulate tumour angiogenesis and metastasis (Basile et al, 2006). In this report, we demonstrate that pancreatic cancer cells secrete ECD of SEMA5A (SEMA5A-ECD) and that secreted Sema5A enhances invasiveness and metastasis of tumour cells via ERK phosphorylation and proliferation of endothelial cells through upregulation of angiogenic factors.

MATERIALS AND METHODS

Cell culture

Human pancreatic cancer cell lines (Panc1 and T3M4) and immortalised human dermal microvascular endothelial cells (HMEC-1; obtained from the Centre for Disease Control and Prevention, Atlanta, GA, USA; Ades et al, 1992) were maintained in culture as an adherent monolayer with RPMI-1640/DMEM with 5% Foetal Calf Serum (Mediatech, Herndon, VA, USA) supplemented with 1X nonessential amino acids, 2mM l-glutamine and 1X vitamin solution.

Transfection of pancreatic cancer cells and purification of recombinant protein

Panc1 cells were stably transfected with either 1 μg of a cDNA construct containing the mouse Sema5A-ECD conjugated to human IgG Fc domain cloned in to the pcDNA3.1 vector (generous gift from Dr David Stretavan, University of California San Francisco at San Francisco, CA, USA) or pcDNA3.1 control vector (Stratagene, La Jolla, CA, USA) using LipofectAMINE (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. Panc1 cells transfected with Sema5A-ECD (Panc1-Sema5A-ECD) or control vector (Panc1-control) were selected and maintained with 400 μg ml⁻¹ G418 sulphate (Mediatech). Fc-tagged Sema5A-ECD was isolated by protein-A affinity chromatography from the supernatant of Panc1-Sema5A-ECD expressing cells and the protein purity was tested using silver staining (Fischer Chemical, Fairlawn, NJ, USA) as described previously (Sadanandam et al, 2010a).

Tumorigenic and metastatic assays

The tumorigenic and metastatic assays were performed using male athymic BALB/c nude mice (NCl-nu; 6–8 weeks old, purchased from the National Cancer Institute). In accordance with approval from the American Association of Laboratory Animal Care and current regulations and standards of the US Department of Agriculture, Department of Health and Human Services and National Institute of Health, the mice used for this study were maintained under specific pathogen-free conditions in facilities. All procedures performed in mice were in accordance with the institutional guidelines and were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

For tumour growth and metastasis, the preparation of cells and the orthotopic injections into the pancreas of the mice were performed as described in our previous study (Sadanandam et al, 2010b).

Immunohistochemistry (IHC)

Fixed paraffin-embedded tissue samples on slides were deparaffinised in a series of xylene and alcohol treatments (two times xylene, one time each in 100%, 95%, 80% and 70% alcohol) for 5 min each. The slides were washed with PBS for 5 min for three times and quenched for endogenous peroxidase activity by incubating with 3% H₂O₂ in PBS for 5 min. Slides were again washed thoroughly with PBS and blocked with blocking buffer (10% horse/donkey serum) for 30 min. Without further washing, the samples were incubated with primary antibody (anti-PCNA (5 μg ml⁻¹, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-SEMA5A (5 μg ml⁻¹; Sadanandam et al, 2010b), anti-interleukin (IL)-8 (2 μg ml⁻¹), anti-vascular endothelial growth factor (VEGF; 2 μg ml⁻¹; R&D System, Minneapolis, MN, USA) or anti-CD31 (1:100, Abcam, Cambridge, MA, USA) in blocking buffer for overnight at 4°C and then with biotinylated secondary antibody for 30 min to 1 h after washing with PBS. After washing the slides in PBS, the samples were incubated with ABC reagent (Vector Laboratories, Burlingame, CA, USA) for 15–30 min. Finally, DAB substrate (Vector Laboratories) was added until a brown colour developed and counter stained with hematoxylin. The quantitation of the number of cells stained for a particular antibody was determined by counting at least five different random fields in the same section at ×200 resolution using a Nikon microscope (Nikon Inc., Melville, NY, USA). The average number of positive cells was calculated.

mRNA analysis

Total cellular RNA isolation and reverse transcription based polymerase chain reaction (RT–PCR) specific to SEMA5A and β-actin was performed as described (Sadanandam et al, 2010b).

In vitro proliferation assay

For endothelial cell proliferation, cells (1 × 10⁴) were seeded in individual wells of 96-well flat-bottomed plates in triplicate and treated with serum-free supernatant from Panc1-Sema5A-ECD or Panc1-control cells. The proliferative activity of the cells after 72 h of incubation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using a microtiter plate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at 570 nm as described (Sadanandam et al, 2010a).

Invasion and migration assay

For invasion assay, cells (1 × 10⁴) were plated onto the upper chamber of transwell chambers and coated with matrigel (6.5 mm;
RESULTS

Secretion of SEMA5A in pancreatic cancer cells

To examine whether pancreatic cancer cells secrete SEMA5A, we performed Western blots for SEMA5A using serum-free supernatant (CM, see Materials and Methods for supernatant preparation) and protein lysates collected from T3M4, a pancreatic cancer cell line expressing high levels of SEMA5A (Sadanandam et al., 2010b). The results shown in Figure 1A demonstrate a band at ~110 kDa in the T3M4 CM whereas the protein lysate shows bands at ~135 kDa (membrane-bound SEMA5A) and ~110 kDa (equivalent to the molecular weight of its extracellular domain). These results suggest that SEMA5A is secreted in pancreatic adenocarcinoma cell lines.

Overexpression of Sema5A-ECD in pancreatic cancer cells increases their invasive potential

As the ~110-kDa band in T3M4 supernatant (Figure 1A) is predicted to be equivalent to Sema5A-ECD, we studied the role of Sema5A-ECD in pancreatic cancer. We stably transfected Panc1 cells, which do not express SEMA5A, with a cDNA construct containing mouse Sema5A (Panc1-Sema5A-ECD) fused with human IgG Fc region at the C-terminus or with empty vector (Panc1-control). Panc1-Sema5A-ECD cells expressed Sema5A mRNA whereas Panc1-control cells did not show detectable levels of Sema5A mRNA (Figure 1B). In addition, we performed immunoprecipitation followed by Western blot both using anti-Sema5A antibody and supernatant from Panc1-Sema5A-ECD and Panc1-control. We observed a band between ~180 and ~220 kDa that represents the Sema5A-ECD plus with human IgG Fc region (Figure 1B) similar to that shown by Oster et al. (2003).

Next, we examined whether expression of Sema5A-ECD in pancreatic cancer cells modulates their invasive potential. Significantly more Panc1-Sema5A-ECD cells invaded through Matrigel as compared with control cells (P < 0.05) in the absence of serum (Figure 1C). This result demonstrates that Sema5A-ECD modulates the invasive potential of pancreatic cancer cells.

Expression and phosphorylation of ERK in Sema5A treated Panc1 cells

Downregulation of D-Sema5c has been shown to decrease phosphorylation of ERK in a Drosophila model for metastasis (Woodhouse et al., 2003). To identify a signalling network mediating migration of pancreatic cancer cells by secreted Sema5A, we examined ERK phosphorylation after treatment with recombinant Sema5A-ECD. Increased levels of p42 ERK phosphorylation were observed in Panc1 cells treated with 10 ng ml \(^{-1}\) of Sema5A-ECD for 5 and 30 min compared with cells treated with media alone (Figures 1D and E) as well as a slight increase in the level of p44 ERK phosphorylation (Figure 1D and Supplementary Figure 1A). To verify if ERK signalling is important for Sema5A-ECD induced migration of pancreatic cancer cells, we performed transwell chamber migration assays by treating Panc1-Sema5A-ECD and Panc1-control cells with 10 \(\mu\)g concentration of MEK1 inhibitor (PD98059) or media alone. A significant decrease in migration of Panc1-Sema5A-ECD cells with MEK1 inhibitor was observed compared with the treatment with media alone whereas there was no change in the Panc1-control cells (Figure 1F). This data supports the hypothesis that Sema5A-dependent cellular migration is mediated through the increased ERK phosphorylation.

Sema5A-ECD overexpression enhances spontaneous metastasis

To examine the role of Sema5A-ECD in tumour cells in an orthotopic pancreatic microenvironment, we injected...
Panc1-Sema5A-ECD or Panc1-control (5 × 10^5) cells into the pancreas of athymic nude mice. Mice were killed after 10 weeks. The presence of primary tumour in the pancreas and metastatic cells in different organs was confirmed by H&E staining (data not shown). Surprisingly, no difference in tumour incidence was observed between mice injected with Panc1-Sema5A-ECD or Panc1-control cells (Figure 2A). However, in mice injected with Panc1-Sema5A-ECD cells, tumour burden was lower and yet there was an increased incidence of micrometastases to the liver (Figure 2A). Though there were no obvious macrometastases in either Panc1-Sema5A-ECD mice or control mice, there was a significant increase in number of micrometastatic cells to the liver (Figure 2A). Though there were no obvious macrometastases in either Panc1-Sema5A-ECD mice or control mice, there was a significant increase in number of micrometastatic cells to the liver (Figure 2A). Though there were no obvious macrometastases in either Panc1-Sema5A-ECD mice or control mice, there was a significant increase in number of micrometastatic cells to the liver (Figure 2A). Though there were no obvious macrometastases in either Panc1-Sema5A-ECD mice or control mice, there was a significant increase in number of micrometastatic cells to the liver (Figure 2A).

Next, we performed IHC for CD31 marker for microvessels in orthotopic tumours. Here, we identified a significant increase in microvessel density in orthotopic tumours expressing Sema5A-ECD as compared with control tumours (Figures 2C and D). These data suggest that Sema5A-ECD stimulates aggressiveness in pancreatic tumour cells leading to increased micrometastases via increased tumour angiogenesis, but with lower primary tumour burden.

**Sema5A-ECD increases endothelial cell proliferation and upregulates expression of pro-angiogenic factors**

To investigate whether Sema5A-ECD mediates increased metastasis by increasing endothelial cell proliferation (one of the steps involved in tumour angiogenesis), we performed MTT proliferation assays after treating HMEC-1 endothelial cells with conditioned serum-free supernatants from Panc1-Sema5A-ECD or Panc1-control cells (Figure 3A). Significantly increased proliferation was observed in HMEC-1 cells treated with serum-free conditioned supernatant from Panc1-Sema5A-ECD as compared with that from Panc1-control cells (Figure 3A). This result demonstrates that Sema5A-ECD increases proliferation of endothelial cells.

Next, we examined the expression of well-known pro-angiogenic regulators IL-8 and VEGF in the serum-free supernatants of Panc1-Sema5A-ECD or control cells *in vitro* by ELISA. Interleukin-8 and VEGF were significantly increased in the supernatant of Panc1-Sema5A-ECD cells as compared with control cells (Figures 3B and C). We further validated the regulation of IL-8 and VEGF expression by Sema5A-ECD by treating Panc1-Sema5A-ECD cells with neutralising antibody to Sema5A for 72 h and then measuring the expression levels of IL-8 and VEGF in the supernatant using ELISA. A significant decrease (P < 0.05) in IL-8 and VEGF protein expression was seen in the supernatant of Panc1-Sema5A-ECD treated with 10 ng of neutralising antibody to Sema5A (Figures 3D and E). Upregulation of IL-8 expression was also seen in...
Panc1-Sema5A-ECD orthotopic tumours as compared with control tumours by IHC \( (P < 0.05; \text{Figures 3F and G}) \). However, we did not observe a significant change in VEGF staining between orthotopic tumours from Panc1-Sema5A-ECD and Panc1-control (Supplementary Figure 1C). These results demonstrate that Sema5A-ECD increases the protein expression of IL-8 and to a certain extent VEGF in pancreatic cancer cells and tumours.

### DISCUSSION

Previously, we found that pancreatic tumours and aggressive pancreatic cancer cell lines express membrane-bound Sema5A, however, it is not expressed in normal pancreatic tissues or less aggressive pancreatic cancer cell lines (Sadanandam et al., 2007, 2010b). In this study, we sought to determine if pancreatic cancer cells secrete Sema5A and whether secreted Sema5A has a role in pancreatic tumour progression, metastasis and angiogenesis by regulating other molecules.

Many secreted forms of membrane-associated proteins such as CD44 (Stickeler et al., 2000) and transforming growth factor-β type III receptor (Bandyopadhyay et al., 2002) were reported to be involved in tumour metastasis. One transmembrane protein of the semaphorin family, SEMA4D, which displays structural similarity to Sema5A (Sadanandam et al., 2007), has also been reported to be released as a functional secreted protein and has been shown to be involved in tumorigenesis and metastasis (Wang et al., 2001; Basile et al., 2004, 2006). In this report, we detected Sema5A protein in the serum-free supernatant of a human pancreatic cancer cell line (T3M4) and the ~110-kD band was predicted to be similar to that of the Sema5A-ECD. To our knowledge, this is the first report showing that Sema5A is secreted.

In our previous study, we demonstrated that membrane-bound Sema5A increases pancreatic cancer invasion in vitro and metastasis in vivo (Sadanandam et al., 2010b). In this report, we studied the role of secreted Sema5A in pancreatic tumour progression and metastasis by overexpressing mouse Sema5A-ECD in Panc1 cells not expressing Sema5A. Sema5A-ECD expression in pancreatic cancer cells demonstrated increased invasion in vitro. This increase in invasion of pancreatic cancer cells induced by Sema5A-ECD especially in serum-free conditions suggests that this molecule is a motogenic factor secreted by tumour cells.

Previously, we have shown that orthotopic injections of pancreatic cancer cells overexpressing membrane-bound Sema5A in athymic nude mice are tumorigenic and metastatic (Sadanandam et al., 2010b). Here, we demonstrate that orthotopic injection of Sema5A-ECD-expressing cells resulted in reduced tumour burden, but enhanced micrometastases to the liver as compared with control transfected cells. It has already been reported that small primary tumours could lead to metastasis in different cancer types (Menz et al., 1985; Sho et al., 1998; Chiang

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**Table 1.**

| Cell lines | Average weight of mice in g | Average weight of tumour in g | Primary tumour | Micrometastases to liver |
|------------|-----------------------------|------------------------------|---------------|-------------------------|
| Panc1-control | 24.68 ± 3.34               | 0.77 ± 0.33                 | 100           | 60                      |
| Panc1-Sema5A-ECD | 24.966 ± 2.52             | 0.31 ± 0.15                 | 100           | 100                     |

**Figure 2.** Orthotopic assay, micrometastasis and microvessel density of Panc1 Sema5A-ECD. Athymic nude mice \( (n = 5) \) were injected orthotopically with Panc1-control or Panc1-Sema5A-ECD and were euthanised after 10 weeks. (A) Average weight of mice and tumour (tumour burden) as well as incidence of primary tumour and metastasis to the liver is shown. (B) There are a significantly enhanced number of micrometastatic cells per field of \( \times 200 \) resolution of the Nikon microscope in the liver of Panc1-Sema5A-ECD tumour-bearing mice as compared to control mice. (C) Immunohistochemistry using CD31 staining showing microvessels in Panc1-Sema5A-ECD and control orthotopic tumours. There is an increased angiogenesis in Panc1-Sema5A-ECD compared to the Panc1-control orthotopic tumours. (D) Densitometric quantitation of microvessel density from C. per field at \( \times 200 \) resolution of the Nikon microscope. The values are number of microvessels ± s.d. (bars) of five different areas per field or different slides and *a statistical significance of \( P < 0.05 \).
Figure 3  Sema5A-ECD stimulates endothelial cell proliferation and increases the expression of angiogenic factors. (A) MTT proliferation assay after 72 h using 1000 HMEC-1 cells per well that were treated with conditioned medium (no serum) from Panc1-Sema5A-ECD or Panc1-control. There is a significant (P < 0.05) increase in proliferation of HMEC-1 in the presence of supernatant from Panc1-Sema5A-ECD cells compared with control cells with no serum. Proliferation was measured as mean optical density (OD) at 570 nm (OD < 0.05) ± s.d. (bars) of triplicate culture and statistical significance of P < 0.05. Enzyme-linked immunosorbent assay for (B) IL-8 and (C) VEGF protein in the supernatant of Panc1-Sema5A-ECD and Panc1-control cells using appropriate antibodies. There is increased expression of IL-8 and VEGF in the supernatants of Panc1-Sema5A-ECD compared with control. Data are mean pg ml⁻¹ ± s.e.m. (bars) of triplicate samples and statistical significance of P < 0.05. Neutralisation of Sema5A-ECD in supernatants of Panc1-Sema5A-ECD using 10 ng ml⁻¹ of anti-Sema5A or rabbit-IgG antibody followed by ELISA for (D) IL-8 or (E) VEGF. There is a significant decrease in IL-8 and VEGF using 10 ng ml⁻¹ of anti-Sema5A antibody compared with rabbit-IgG. Data are mean pg OD ± s.d. (bars) of triplicate samples and statistical significance of P < 0.05. (F) Immunohistochemistry showing IL-8 staining in (a) Panc1-control and (b) Panc1-Sema5A-ECD orthotopic tumours using anti-IL-8 antibody. The magnification is × 200 resolution using a Nikon microscope. (G) The number of cells positive for IL-8 from F. The values are mean number of cells ± s.d. (bars) of three slides. *Statistical significant difference of P < 0.05.

et al, 2008). The mechanism by which secreted Sema5A induces increased metastasis with reduced tumour burden is intriguing. Based on our previous findings that Panc1 cells express Plexin B3, a functional receptor for SEMA5A (Sadanandam et al, 2008), we suggest that the secretion of SEMA5A in pancreatic cancer induces an autocrine mechanism via the ERK phosphorylation to metastasise to distant organs but with smaller primary tumour, unravelling this cell signalling mechanism might be the basis of our future studies.

We have previously shown that Sema5A can promote angiogenesis, in general (Sadanandam et al, 2010a). In this study, we show that Panc1-Sema5A-ECD is biologically active and also has the ability to promote tumour angiogenesis as shown by its ability to significantly increase in vitro proliferation of HMEC-1 endothelial cells and significantly increase microvessel density in orthotopic Panc1-Sema5A-ECD tumours compared with control tumours. In addition, supernatants from Panc1-Sema5A-ECD cells show increased expression of pro-angiogenic factors, IL-8 and VEGF. We speculate that secreted Sema5A derived from the membrane-bound protein could be the reason for the increased IL-8 and VEGF expression. The direct effect of Sema5A-ECD on endothelial cells to secrete IL-8 and VEGF was shown using neutralising antibody against Sema5A in endothelial cells. Overall, the increase in micrometastasis in Sema5A-ECD-expressing pancreatic cancer cells is due to increased tumour angiogenesis via IL-8 and VEGF.

In conclusion, in this study, for the first time we show that malignant pancreatic cancer cells secrete SEMA5A and that this secreted form demonstrates the ability to drive micrometastatic spread via the enhancement of angiogenesis and migration of pancreatic cancer cells via increased ERK signalling and expression of angiogenic factors, but with reduced primary tumour burden. These results suggest a potentially important and hitherto unsuspected role of the secreted form of SEMA5A in pancreatic cancer metastasis. Further study is warranted to examine the presence of secreted SEMA5A in pancreatic cancer patients and to understand the autocrine mechanism of its action.
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