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The RON tyrosine kinase receptor regulates VEGF production in pancreatic cancer cells

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Abstract

Objectives—The RON receptor mediates tumorigenic phenotypes in pancreatic cancer (PC) but no investigations currently have implicated RON signaling as a regulator of angiogenesis in PC. Angiogenesis is vital to oncogenesis and vascular endothelial growth factor (VEGF) is the most well-characterized angiogenic protein. This study sought to determine the effect of RON stimulation on *in vitro* angiogenesis and VEGF production in PC cell lines.

Methods—VEGF levels from conditioned media of HGFL-stimulated BxPC-3 and FG cells were quantitated via ELISA and likewise interrogated in the presence and absence of MAPK and PI3K/AKT inhibitors. To determine *in vitro* angiogenesis, HMVEC cells were subsequently exposed to the same conditioned media to assay for microtubule formation.

Results—RON signaling resulted in a 52% and 34% increase in VEGF levels in BxPC-3 and FG cells, respectively. VEGF secretion was inhibited with MAPK or PI3K blockade in BxPC-3 cells but only MAPK inhibition resulted in decreased VEGF production in FG cells. BxPC-3 conditioned media induced tubule formation in HMVEC cells, which was abrogated by RON inhibition.

Conclusions—RON signaling results in MAPK-mediated VEGF secretion by PC cells and promotion of microtubule formation. These findings suggest another mechanism by which RON signaling may promote PC progression.

Keywords

RON; Pancreatic cancer; Angiogenesis; VEGF

Introduction

Tumor growth beyond 100–200 μ m necessitates angiogenesis, the in-growth and development of new vasculature, as beyond this size tumor metabolism requires a greater oxygen and nutrient supply than can be delivered by simple diffusion alone.^{1–4} In order for angiogenesis to proceed, a complex array of signaling events must occur to stimulate the activation, proliferation, and

migration of nearby vascular endothelial cells into the tumor microenvironment. Perhaps the most potent and well-studied angiogenic factor is vascular endothelial growth factor (VEGF). Overexpression of VEGF has been shown to correlate with tumor progression and portend a poor prognosis in a variety of gastrointestinal malignancies including colon^{5, 6}, gastric^{7, 8}, and pancreatic cancer.^{9–11} The VEGF family contains six glycoprotein members with the most well-studied member being VEGF-A, referred to simply as VEGF. Although hypoxia seems to be the most potent stimulus for VEGF production via a hypoxia inducible factor (HIF-1) dependent pathway^{12, 13}, several receptor tyrosine kinases (RTKs) have been shown to upregulate VEGF expression in cancer cells via a HIF-1 independent pathway.^{14, 15} The epidermal growth factor receptor (EGFR)^{16, 17}, insulin-like growth factor receptor^{14, 18}, and c-Met^{19–21} are three such RTKs that have been shown to induce VEGF expression upon activation. The receptor d'origine nantais (RON) tyrosine kinase receptor has recently been shown to be overexpressed in pancreatic cancer^{22–24}, the fourth leading cause of cancer death in the United States.^{24, 25} The RON receptor is homologous to the known proto-oncogene, c-Met²⁶, and is activated by a single known ligand, hepatocyte growth factor-like protein (HGFL).^{27, 28} The RON receptor is a transmembrane glycoprotein that is composed of two disulfide linked heterodimer subunits (150 and 35 kDa).^{26, 27, 29} Upon activation by HGFL, RON undergoes autophosphorylation which results in the phenotype described as “invasive growth” characterized by increased proliferation, migration, invasiveness, and apoptotic resistance.^{22, 30–32} To date, however, there has been no direct evidence demonstrating a role for RON signaling in angiogenesis and VEGF production. In this report, we demonstrate that activation of RON signaling by HGFL induces VEGF production in murine and human pancreatic cancer cells and that conditioned media from these cells results in tubule formation in human microvascular endothelial cells. We further demonstrate that RON-induced VEGF production is dependent on MAPK signaling. These data suggest another mechanism by which RON signaling may promote pancreatic cancer progression and lend additional support to the development of RON-directed therapies.

Materials and Methods

Cell Lines and Maintenance

The mouse pancreatic intraepithelial neoplasia (PanIN) cell line was derived as previously described and maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.^{22, 33} The human pancreatic cancer cell line BxPC-3 was acquired from American Type Culture Collection (Manassas, VA) and FG cells were a kind gift provided by Dr. David Cheresch. Both were maintained in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin. The human microvascular endothelial cell line, HMVEC-L, was acquired from Cambrex (Walkersville, MD) and maintained in EBM-2 basal medium supplemented with EGM-2 SingleQuot Kit containing supplements and growth factors according to recommendations with a final FBS concentration of 2%. All cells were grown in a humidified incubator at 37°C and 5% CO₂.

Affymetrix Gene Chip

PanIN cells plated in 6-well cell culture plates were grown to approximately 60–70% confluency to ensure maximal RNA isolation at later steps. At this time, the media was removed and complete media supplemented with 400ng/ml HGFL (R&D Systems, Minneapolis, MN) was added and the PanIN cells were stimulated for variable time points (0 hr, 30 min, and 12 hr). After the designated time point, the cells were washed three times with PBS and lysed with Trizol (Invitrogen, Carlsbad, CA) for RNA isolation per manufacturer specifications. Samples were then purified with RNeasy purification columns (Qiagen, Valencia, CA) with on-column DNase digestion according to manufacturer specifications. Purified RNA was then analyzed utilizing the Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Assay kit to determine

RNA concentration and purity (Agilent Technologies, Inc., Santa Clara, CA). Biotinylated cRNA from each sample was then hybridized to an Affymetrix GeneChip mouse genome 430 v2.0 array and stained using standard procedures (Affymetrix, Santa Clara, CA). The 430 v2.0 array contains 45,000 probe sets representing over 34,000 mouse genes.

Immunoblot and Immunoprecipitation

Cells were plated into 60mm cell culture plates (BxPC-3 at 1.5×10^6 cells/plate and FG at 1.1×10^6 cells/plate). Once cells reached 70–80% confluency, they were serum starved overnight. The cells were then treated with either mouse anti-human RON antibody at 8 μ g/ml (R&D Systems, Minneapolis, MN), the PI3 Kinase inhibitor LY294002 at 20 μ M (InvivoGen, San Diego, CA), or the MEK1/2 inhibitor U0126 at 25 μ M (InvivoGen) under serum-free conditions for one hour at 37°C, 5% CO₂. The media in the control plates were likewise changed to serum-free media alone. After the incubation phase, all media was again removed and replaced with fresh serum-free media with or without inhibitors $\pm$ 200ng/ml HGFL (R&D Systems) for 8 hours at 37°C, 5% CO₂. All experiments were performed four separate times. Supernatant was removed from the cultures and stored at –20°C for use on VEGF ELISA. Cells were washed two times in cold PBS and lysed in 250 μ l RIPA Buffer (20mM Tris pH8.0, 5mM EDTA, 150mM NaCl, 1% Triton X-100) containing complete protease inhibitors and PhosSTOP phosphatase inhibitors (Roche Applied Science)). The lysates were placed on ice for 30 minutes followed by centrifugation at 15,000g for 15 minutes and supernatants collected. Protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce, Inc., Rockford, IL). Immunoblotting was performed using 30 μ g of lysate and separated on 4–12% NuPAGE Bis-Tris gels (Invitrogen), transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA), and subsequently probed. For immunoprecipitation experiments, 500 μ g of cell lysates were incubated with 1 μ g of RON C-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 minutes on ice followed by the addition of Protein A/G UltraLink Resin (Pierce, Inc.) for 1 hour at 4°C with rotation. The beads were collected and washed for two 15 minute intervals in RIPA buffer at 4°C with rotation. After removal of the final wash, the beads were resuspended in 1X NuPAGE LDS sample buffer (Invitrogen) containing 1X NuPAGE sample Reducing Agent (Invitrogen) and incubated at 60°C for 30 minutes to disassociate the protein from the beads. Samples subsequently underwent immunoblotting as described above. Antibodies used were rabbit RON β (C-20) at 1:500 (Santa Cruz Biotechnology, Inc.); mouse anti-phosphotyrosine mAb 4G10 at 0.6 μ g/ml (Millipore); rabbit polyclonal phospho-Akt (Ser473) at 1:1000; rabbit polyclonal phospho-p44/42 MAPKinase at 1:1000; rabbit polyclonal Akt at 1:1000; rabbit polyclonal p44/42 MAPKinase at 1:1000 (Cell Signaling, Danvers, MA). Goat anti-mouse-HRP (Millipore) and goat anti-rabbit-HRP (Santa Cruz Biotechnology, Inc.) were both used as the secondary antibodies at 1:5000. The reaction was developed with ECL Plus reagent (GE Healthcare, Piscataway, NJ).

RON Activation and Enzyme-Linked ImmunoSorbent Assay (ELISA)

Supernatant removed from the cultures under immunoblot and immunoprecipitation procedure was spun to remove particulates, diluted 1:2 in calibrator diluent, supplied in the VEGF Quantikine ELISA kit (R&D Systems), and run in duplicate per the manufacture recommendations. The optical density (OD) of each ELISA well was measured using the SpectraMAX 190 microplate reader (Molecular Devices, Sunnyvale, CA) set at 450nm with wavelength correction set at 570nm. Data tabulation and standard curve generation was performed by the Softmax Pro 2.21 software. A new standard curve was generated utilizing the included VEGF standard for each replicate sample assayed. After subtracting the background OD from empty microplate wells, the mean OD was plotted against the known VEGF values supplied with the kit and linear regression analysis was performed to create the standard curve. The experimental VEGF values were thus calculated from this standard curve

and multiplied by the dilution factor. Final values for each treatment category were averaged and standard error of the mean obtained.

Microtubule Formation

HMVEC cells were used in this *in vitro* assay of angiogenesis as described previously.^{34, 35} Briefly, growth factor reduced Matrigel (BD Biosciences, Bedford, MA) was diluted 1:1 with sterile PBS for a total volume of 60 μ l and placed into each well of a 96-well tissue culture plate. The fresh admixture was allowed to gel in a humidified incubator at 37°C and 5% CO₂. At the same time, conditioned media from BxPC-3 stimulated with HGFL, as described above, was collected and cell debris removed by spinning at 6000 RPM for 1 minute at 4 °C. The supernatant was then recovered and placed into a Cetricon YM-3 concentrator (Millipore) and spun at 4500 RPM for 45 minutes after which the concentrator tube was flipped and the concentrate was collected by spinning for 5 minutes at 2000 RPM according to manufacture suggestions. All centrifugation steps were performed at 4 °C and yielded a final volume of 200 μ l. Each aliquot of conditioned media was then warmed to 37 °C, 1*10⁴ HMVEC cells were added to each sample, and aliquoted into the previously prepared 96-well Matrigel plate. HMVEC cells plated with RPMI + 1% FBS served as a positive control while those plated in fresh PBS served as a negative control. The HMVEC cells were then allowed to adhere for 6 hours at which time the Axiovert 100 microscope with 100x objective and AxioCam MRC5 camera were used to take pictures of each well. AxioVision (v4.5) software was used to measure indicators of tubule formation including tubule length, branch points, enclosed tubule area, and perimeter of enclosed tubules.

Statistics

All experiments were repeated at least in triplicate and evaluation of photomicrographs performed for the microtubule experiments were performed in a blinded fashion. GraphPad Prism v3.03 software (GraphPad Software, San Diego, CA) was used for statistical analysis and comparison between treatment groups was performed using ANOVA with Dunnett's multiple comparison post-test analysis. A value of $p \leq 0.05$ was considered statistically significant.

Results

RON signaling induces VEGF secretion by pancreatic cancer cells

We previously described RON receptor expression in both murine and human PanIN specimens as well as the fact that RON expression progressively increases with progression of PanIN grade.²² Utilizing an Affymetrix Gene Chip to interrogate the transcriptome activated by RON signaling, we identified a 225% increase in VEGF mRNA expression in cells derived from murine PanIN at 12 hours post-HGFL administration ($p < 0.01$, Figure 1A). In order to further validate these findings, we examined VEGF expression in two human pancreatic cancer cell lines, BxPC-3 (wildtype K-ras) and FG (mutant K-ras). Stimulation of BxPC-3 cells with 200 ng/ml of HGFL resulted in a 51% increase in VEGF protein levels when compared to control (769.7 pg/ml vs. 380 pg/ml, $p < 0.001$, respectively; Figure 1B). When FG cells were stimulated with HGFL as described, a 34% increase in VEGF production was noted when compared to untreated controls (502.6 pg/ml vs. 331.7 pg/ml, $p < 0.001$, respectively; Figure 1C). In addition, when compared to cells treated with HGFL, co-incubation with an inhibitory RON antibody at the time of HGFL stimulation abrogated this increase in BxPC-3 cells demonstrating the effect was specific to RON (769.7 pg/ml vs. 605 pg/ml, $p < 0.05$, respectively). In FG cells,, RON antibody inhibited VEGF secretion, though this did not quite reach statistical significance (502.6 pg/ml vs. 471.5 pg/ml, $p > 0.05$, respectively). These experiments reveal that RON receptor signaling induces VEGF secretion in both murine and human pancreatic cancer cells..

VEGF Production in BxPC-3 and FG pancreatic cancer cell lines is regulated by the MAPK and PI3K pathways

After having demonstrated that HGFL induces VEGF expression by pancreatic cancer cells, we next sought to identify the oncogenic signaling pathways downstream of RON which were critical to this process. We previously demonstrated that the PI3K/Akt and MAPK pathways are activated by RON signaling in pancreatic cancer cells. Since both of these signaling pathways have also been shown to regulate VEGF, we examined their role in RON-mediated VEGF expression.³⁶ The incubation of BxPC-3 cells with the PI3K inhibitor, LY294002, resulted in a decrease in baseline VEGF production when compared to untreated controls, although this was not statistically significant (264.1 pg/ml vs. 380 pg/ml, $p>0.05$, respectively). The addition of LY294002 to HGFL treated BxPC-3 cells resulted in a 53% reduction in VEGF production compared to HGFL treated cells alone, demonstrating that PI3K signaling can mediate HGFL-induced VEGF expression (352.1 pg/ml vs. 769.7 pg/ml, $p<0.001$, respectively). Likewise, concurrent MAPK inhibition with U0126 during HGFL stimulation resulted in a 85% decrease in VEGF production when compared to HGFL treated cells alone (115.9 pg/ml vs. 769.7 pg/ml, $p<0.001$, respectively; Figure 2A). In contrast, VEGF expression by FG cells did not appear to require PI3K signaling as we observed no significant decrease in VEGF production when HGFL treated FG cells were concurrently treated with LY294002 when compared to FG cells stimulated with HGFL alone (477.2 pg/ml vs. 502.6 pg/ml, respectively, $p>0.05$). Interestingly, when the MAPK pathway was inhibited in FG cells, there was a 23.6% decrease ($p<0.05$) in VEGF production when compared to FG cells stimulated with HGFL alone (384.1 pg/ml vs. 502.6 pg/ml, respectively; Figure 2B). These data demonstrate that RON-dependent VEGF production in BxPC-3 cells is contingent on intact PI3K and MAPK pathways whereas in FG cells it is reliant on an intact MAPK, but not PI3K, pathway.

Conditioned media from RON-stimulated pancreatic cancer cells augments microtubule formation

After demonstrating that RON signaling promotes VEGF expression in pancreatic cancer cells, we next evaluated whether conditioned media from RON activated BxPC-3 cells could promote microtubule formation in HMVEC cells, an *in vitro* indicator of angiogenesis. Microtubule formation was quantified by measuring the length of microtubule formation, microtubule branch points, total microtubule area, and microtubule perimeter in a blinded fashion. The later two parameters involve the measurement of microtubules that form an enclosed area within them. HMVEC cells grown in conditioned media from HGFL-stimulated BxPC-3 cells demonstrated abundant tubule formation, consistent with an *in vitro* angiogenic phenotype (Figure 3A–D). When compared to untreated controls, the HMVEC cells grown in conditioned media demonstrated increased microtubule formation as manifested by a 32% increase in microtubule length (4703.6 μm vs. 6215 μm , respectively), 284% increase in enclosed microtubule area (6121.6 μm^2 vs. 23505.5 μm^2 , respectively), 198% increase in microtubule perimeter (181.3 μm vs. 540.4 μm , respectively), and 135.5% increase in number of branching points (27.6 vs. 64.9, respectively; Figure 4A–D). Microtubule formation was completely abrogated when BxPC-3 cells were co-incubated with an anti-RON antibody again providing evidence that the effects are dependent on RON signaling. These data suggest that not only does activation of RON signaling in BxPC-3 cells result in upregulation of VEGF expression, but that the VEGF secretion by pancreatic cancer cells is able to direct the formation of microtubules in HMVEC cells.

Discussion

Pancreatic cancer is the fourth leading cause of death from cancer in the United States with over 34000 deaths estimated in 2009.²⁵ Sadly, there has been little progress in early detection

of the disease and most patients continue to be diagnosed at an advanced stage. Thus, much energy has been directed at the identification of novel chemo- and bio-therapeutics for the treatment of pancreatic cancer. Multiple Phase III trials have failed to demonstrate a benefit to traditional chemotherapy doublets and triplets, further encouraging the investigation of, biologic agents including those that inhibit angiogenesis. Unfortunately, a recent study of gemcitabine plus the VEGF monoclonal antibody, bevacuzimab (Cancer and Leukemia Group B (CALGB) 80303) failed to demonstrate benefit over gemcitabine alone in patients with advanced pancreatic cancer.³⁷ These results may be explained in numerous ways, but certainly suggest our understanding of the role and regulation of angiogenesis in pancreatic cancer remains limited.

In this report, we demonstrate that RON, a tyrosine kinase receptor that is upregulated in pancreatic cancer and capable of inducing invasive growth, can also positively regulate the production of the potent angiogenic mediator, VEGF in pancreatic cancer cells. We observe this in both murine PanIN cells as well as the human pancreatic cancer cell lines BxPC-3 and FG, independent of K-ras mutation status since BxPC-3 possesses wildtype K-ras unlike the other two cell lines. Because we were interested in the role of RON signaling in pancreatic cancer progression, our initial studies were performed in murine PanIN cells. It is noteworthy that these cells respond with abundant upregulation of VEGF mRNA in response to RON ligand. This supports the hypothesis that RON may function as an important angiogenic signal during the evolution of PanIN to invasive pancreatic cancer. Stimulation of the RON receptor in the human pancreatic cancer cell lines BxPC-3 and FG similarly resulted in an increase in VEGF production and the conditioned media from these cells stimulated RON-dependent tubule formation. It is certainly not surprising that RON regulates VEGF in pancreatic cancer cells. Other receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR), Erb, platelet-derived growth factor receptor, and the RON homologue, c-Met, have been shown to regulate VEGF expression.^{15–18, 21} Each of these receptors can activate the PI3K and MAPK pathways which we demonstrate are important downstream regulators of RON-mediated VEGF production in pancreatic cancer cells. We did note a difference between the FG and BxPC-3 cell lines with respect to their sensitivity to MAPK and PI3K inhibition, perhaps reflecting underlying differences in mutant K-ras mediated signaling. Conflicting data exists regarding K-ras mutational status and subsequent activation of the MAPK and PI3K pathways, as the presence of oncogenic K-ras does not equate into differential MAPK or PI3K signaling and ultimately VEGF production.^{38–40} In addition, evidence exists that hepatocyte growth factor, the ligand for the RON homologue c-Met, can stimulate angiogenesis independent of VEGF production perhaps by direct stimulation of the MAPK and PI3K cascades and induction of other angiogenic factors.⁴¹ Abundant evidence exists for the presence of multiple salvage pathways to maintain expression of angiogenic mediators in the absence of VEGF, arguing for a therapeutic approach directed at multiple levels of inhibition.^{42–44} Such a strategy is currently being assessed in the AVITA/BO17706 phase III clinical trial. This trial is evaluating the effect of gemcitabine and erlotinib (an EGFR inhibitor), versus gemcitabine, erlotinib, and bevacizumab (a VEGF inhibitor), and has finished patient accrual and awaiting data analysis. It will be of great interest to compare the results of this study with CALGB 80303 to determine added benefits of EGFR inhibition. This may offer insight into the potential of combined RON/VEGF inhibition in pancreatic cancer.⁴⁵

Our data suggests that, both PanIN and pancreatic cancer cells express VEGF in response to RON signaling. Our data therefore supports a role for RON in the process of angiogenesis and suggest this may occur at the earliest stages of pancreatic carcinogenesis. Despite the results of recent clinical trials, we believe that there are many questions that must be answered before discounting anti-angiogenic therapies as a treatment option for pancreatic cancer. Clearly, *in vivo* studies are required to further interrogate the significance of the role RON may play in regulating pancreatic cancer angiogenesis. Such investigations will be necessary to determine

if RON-directed therapy would form a logical part of combination therapy directed at angiogenic signaling in pancreatic cancer.

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Figure 1. RON receptor signaling results in increased VEGF production in PanIN, BxPC-3, and FG pancreatic cancer cells

(A) Murine PanIN cells were assayed for gene expression following exposure to RON ligand using Affimetrix Gene Chips. mRNA was isolated at 0 hour, 30 minute, and 12 hours post-stimulation with 400 ng/ml HGFL. This revealed a 225% increase in VEGF mRNA at 12 hrs post-stimulation. (B) RON-dependent VEGF production was examined in BxPC-3 cells. Following exposure to HGFL, ELISA of conditioned media from HGFL-stimulated BxPC-3 cells was evaluated. A 51% increase in VEGF levels was observed, as compared to control, a finding which was abrogated by co-incubation with an inhibitory RON antibody at the time of HGFL stimulation. (C) FG human pancreatic cancer cells demonstrated a 34% increase in

VEGF production when compared to untreated controls. (When compared to untreated controls: # = $p < 0.05$, * = $p < 0.01$, ** = $p < 0.001$)

Figure 2. RON-mediated VEGF production is dependent on the PI3K and MAPK signaling
 (A) After incubation of BxPC-3 cells with either LY294002 or U0126 with and without concomitant HGFL stimulation, an ELISA for VEGF in the conditioned supernatant was performed. This demonstrated a 53% reduction in VEGF production when the PI3K pathway was inhibited with LY294002 compared to HGFL stimulated BxPC-3 cells alone. In addition, MAPK inhibition with U0126 during HGFL stimulation resulted in a 85% decrease in VEGF production when compared to HGFL treated cells alone. (B) In contrast to BxPC-3 cells, FG cells demonstrated a 23.6% decrease in VEGF production when cells were coincubated with HGFL and U0126, a MAPK inhibitor, compared to those cells treated with HGFL alone. However, VEGF production was independent on PI3K signaling in FG cells. (When compared to untreated controls: # = $p < 0.05$, * = $p < 0.01$, ** = $p < 0.001$)

Figure 3. Conditioned media from RON-stimulated BxPC-3 pancreatic cancer cells results in HMVEC microtubule formation

(A) HMVEC cells grown in PBS do not form microtubules whereas the HMVEC cells grown in 1% FBS, as a positive control, (B) initiate microtubule formation. Conditioned media from HGFL treated BxPC-3 cells (C) initiates a significant increase in microtubule formation which is abrogated by the addition of an inhibitory RON antibody (D).

Figure 4. HMVEC cells grown in conditioned media from RON-stimulated BxPC-3 pancreatic cancer cells increased microtubule size and area

AxioVision (v4.5) software was used to quantify HMVEC microtubule formation. Comparing the HGFL treatment group to control, there was a 32% increase in microtubule length (A), 284% increase in enclosed tubule area (B), 198% increase in the perimeter of enclosed tubules (C), and 135.5% increase in the number of branch points (D). In all cases, conditioned media from RON-stimulated BxPC-3 cells resulted in an increased microtubule size and area which was reversed by antibody-mediated blockade of the RON receptor.