Vascular Endothelial Growth Factor Receptor-1 Activation Mediates Epithelial to Mesenchymal Transition in Human Pancreatic Carcinoma Cells

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Abstract

Our laboratory has shown that vascular endothelial growth factor receptor-1 (VEGFR-1) expression on human pancreatic cancer cell lines mediates cell migration and invasion. Because epithelial to mesenchymal transition (EMT) also plays a role in cell motility by altering the cell phenotype and morphology, we hypothesized that VEGFR-1 activation induces molecular alterations that mediate EMT. Our treatment of the human pancreatic cancer cell line L3.6pl with the VEGF-1 ligands VEGF-A and VEGF-B led to morphologic changes characteristic of EMT, including loss of polarity, increased intercellular separation, and the presence of pseudopodia. Immunofluorescent staining with antibodies to E-cadherin and β-catenin showed that VEGFR-1 activation led to translocation of E-cadherin and β-catenin from their usual cell membrane-bound location to the cytoplasm and nucleus, respectively. Western blotting showed that VEGFR-1 activation led to decreased expression of the epithelial markers E-cadherin and plakoglobin, increased expression of the mesenchymal markers vimentin and N-cadherin, and increased nuclear expression of β-catenin. Pretreatment of tumor cells with a VEGFR-1 blocking antibody inhibited the VEGFR-1-induced immunohistochemical and molecular changes in E-cadherin. VEGFR-1 activation led to an increase in expression of the EMT-associated transcription factors Snail, Twist, and Slug. The changes mediated by VEGFR-1 in this pancreatic carcinoma cell line are highly consistent with the changes characteristic of EMT. Given our previous finding of VEGFR-1-mediated tumor cell invasion and migration in pancreatic carcinoma cells, we hypothesize that VEGFR-1 plays a role in tumor progression in pancreatic cancer through the induction of EMT. (Cancer Res 2006; 66(1): 46-51)

Introduction

Pancreatic cancer is an aggressive disease, with few patients surviving 2 years after diagnosis. Current therapies for locally advanced or metastatic disease have little effect on the natural history of this malignancy. Understanding the molecular mechanisms underlying the progression of pancreatic cancer may provide insight for the development of novel antineoplastic therapies.

Vascular endothelial growth factor receptor-1 (VEGFR-1 or Flt-1), one of three tyrosine kinase receptors for vascular endothelial growth factor (VEGF), is a cell membrane-bound tyrosine kinase receptor that binds VEGF (or VEGF-A). VEGFR-1 also serves as the sole tyrosine kinase receptor for VEGF-B and placental growth factor. VEGFR-1 was initially thought to play a minor role in VEGF-mediated signal transduction. However, knockout studies targeting VEGFR-1 have implicated VEGFR-1 as a critical mediator of both developmental and physiologic angiogenesis (1). Although VEGFR-1 was originally described as being exclusively expressed on endothelial cells, our laboratory recently published data describing VEGFR-1 expression on tumor cells in several pancreatic and colorectal cancer cell lines (2, 3). In these two tumor systems, VEGFR-1 was found to be functional and its activation led to increased invasion and migration.

The epithelial to mesenchymal transition (EMT) is a process initially observed in embryonic development in which cells lose epithelial characteristics and gain mesenchymal properties such as increased motility and invasion (4). Growth factors including hepatocyte growth factor, transforming growth factor-β, and epidermal growth factor have been found to induce EMT (5). Recent research suggests that EMT is also important in tumor progression (4, 6). Bates et al. previously established a link between VEGFR-1 and EMT in colon carcinoma cell organoids (7). We have extended this observation to human pancreatic cancer cells where our studies were intended to comprehensively analyze the molecular and morphologic changes associated with EMT following VEGFR-1 activation in human pancreatic cancer cells.

In this study, we hypothesized that VEGFR-1 increases the invasion and migration of pancreatic cancer cells by mediating EMT. To test this hypothesis, cells from human pancreatic cancer cell lines were treated with the VEGF-1 ligands, VEGF-A and VEGF-B. Gross cell morphology, immunohistochemical localization of the cellular components involved in EMT, and expression of the molecular markers of EMT were assessed for changes after VEGFR-1 activation. Finally, to determine if VEGFR-1 activation was sufficient to induce EMT, a monoclonal blocking antibody to VEGFR-1 was used to determine if the observed EMT changes could be inhibited.

Materials and Methods

Cell lines and culture conditions. The L3.6pl metastatic pancreatic cancer cell line derived from the FG human pancreatic cancer cell line was kindly provided by I. J. Fidler, DVM, Ph.D. (The University of Texas M.D. Anderson Cancer Center, Houston, TX). The PANC-1 human pancreatic cancer cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured and maintained in minimal essential...
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Reagents. Recombinant human VEGF-A^{165} and VEGF-B^{167} were purchased from R&D Systems, Inc. (Minneapolis, MN) and used at concentrations of 10 and 50 ng/mL, respectively, for all experiments. The monoclonal VEGFR-1 blocking antibody 18F1 was provided by ImClone Systems (New York, NY). In the VEGFR-1 blocking experiments, cells were pretreated with 18F1 1 hour before being treated with VEGF-B. Human KLI-H IgG (ImClone Systems), a nonspecific human antibody, was used as a control. To focus these studies, we used only VEGF-B because this is a more specific ligand for VEGFR-1 than VEGF-A. In addition, our laboratory has shown that VEGFR-2 (KDR) is not expressed in pancreatic cancer cells (2).

Antibodies used for immunofluorescent and Western blot analyses were as follows: rabbit anti-actin (Sigma-Aldrich, Co., St. Louis, MO), rabbit anti-ZO-1 (Invitrogen Corp., Carlsbad, CA), mouse anti-E-cadherin (Invitrogen), rabbit anti-β-catenin (Chemicon International, Inc., Temecula, CA), mouse anti-plakoglobin (Chemicon International), mouse anti-N-cadherin (Invitrogen), goat anti-vimentin (Upstate Chemical, Inc., Temecula, CA), goat anti-Snail (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-Twist (Santa Cruz Biotechnology), goat anti-Slug (Santa Cruz Biotechnology), rabbit anti-lamin B1 (Active Motif, Carlsbad, CA), rabbit anti-Cu/Zn superoxide dismutase (Stressgen, Inc., Vancouver, BC, Canada).

Morphologic analysis. Cells were grown to 40% to 50% confluence in MEM + 10% FBS and were then serum-deprived overnight in MEM + 1% FBS. Cells were then treated with VEGF-A and VEGF-B in MEM + 1% FBS and visualized with a Nikon light microscope at 10× magnification and imaged every 24 hours with digital photography. Two observers blinded to the treatment conditions assessed the images for the presence or absence of morphologic characteristics consistent with EMT: spindle-shaped cells (loss of polarity), increases in intercellular separation, and pseudopodia. The observers ranked images in order of increasing presence of morphologic changes consistent with EMT.

Fluorescent immunohistochemistry. L3.6pl cells were grown on poly-

l-lysine-coated glass coverslips (BD Biosciences, San Jose, CA) to 40% to 50% confluence, serum-deprived overnight (MEM + 1% FBS), and then treated with VEGF-A or VEGF-B in the presence or absence of pretreatment for 1 hour with VEGF-1 blocking antibody or nonspecific control IgG. After being fixed with acetone, cells were permeabilized in 0.5% Triton X-100 (Sigma-Aldrich), and blocked with normal horse and goat serum in PBS. Cells were incubated with primary antibodies (E-cadherin or β-catenin) overnight at 4°C. Slides were then washed and incubated with the appropriate FITC-conjugated secondary antibody. Cells were then incubated with Hoechst 33342 (Invitrogen) for nuclear staining and mounted with propyl gallate under glass coverslips. For actin staining, cells were plated on glass coverslips and fixed, incubated with Alexa Fluor 488 phalloidin (Molecular Probes, Inc., Eugene, OR) overnight at 4°C, and then mounted with propyl gallate. Cells were then visualized for immunofluorescence with a laser scanning Olympus microscope at ≥20 magnification or a Confocal LSM510 microscope (Carl Zeiss MicroImaging, Inc., Grand Island, NY) at ≥63 magnification.

Western blot analysis. L3.6pl cells at 50% to 70% confluence were treated with VEGF-A or VEGF-B in the presence or absence of VEGF-1–blocking antibody after overnight serum deprivation in MEM + 1% FBS. For whole cell protein extraction, cells were lysed with radioimmunoprecipitation assay buffer "B" protein lysis buffer. Nuclear protein was extracted with a commercially available kit (ActiveMotif). The isolated protein was quantified by a commercially available modified Bradford assay (Bio-Rad Laboratories, Hercules, CA). Western blot protein samples were prepared by boiling isolated proteins with denaturing sample buffer. The protein was then separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). The membranes were then blocked with 5% nonfat dry milk in TBS and 0.1% Tween 20 for 1 hour and probed with the appropriate primary antibody overnight at 4°C. The membranes were then washed and incubated with the appropriate horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) for 1 hour at room temperature. Membranes were then washed and protein bands were visualized by using a commercially available enhanced chemiluminescence kit (Amersham Biosciences). To verify the accuracy of loading of protein isolated from whole-cell lysate, membranes were incubated in stripping solution for 30 minutes at 65°C, washed, and reprobed with anti-β-actin antibody as a loading control. The purity and loading accuracy of nuclear lysates was confirmed by probing of isolated nuclear fractions with anti-lamin B1 antibody (exclusively nuclear protein) and anti-Cu/Zn superoxide dismutase antibody (exclusively cytoplasmic protein). Confirmation of expression level changes of E-cadherin following VEGF-A and VEGF-B stimulation was done in a second pancreatic cancer cell line, PANc-1, in an identical manner.

Statistical analysis. Statistical analysis was done with InStat Statistical Software V2.03 (GraphPad Software, San Diego, CA). Statistical significance was defined as P < 0.05.

Results

VEGFR-1 activation induces morphologic changes consistent with EMT in L3.6pl cells. The first indication that EMT induced by VEGFR-1 activation might mediate the increase we previously observed in migration and invasion in pancreatic cancer cells was changes in the morphology of L3.6pl cells that had been treated with VEGF-A and VEGF-B. At 48 hours after the initiation of treatment, blinded investigators observed differences in the gross appearance of VEGF-treated cells as compared with untreated cells. The phenotypic changes observed included loss of cell polarity causing a spindle-cell morphology, increased intercellular separation signifying loss of intercellular adhesion, and increased formation of pseudopodia observed emanating from the cell membrane (Fig. 1). We confirmed the loss of polarity by immunofluorescent staining for ZO-1. The loss/decrease of ZO-1 following VEGF-A and VEGF-B treatment indicates that VEGFR-1 activation leads to loss of tight junctions associated with organizing membrane polarity (8). In addition, VEGFR-1 activation led to reorganization of actin from a predominantly membrane-bound location to newly formed pseudopodia that we observed via light microscopy. Actin was also observed to form stress fibers throughout the cytoplasm following treatment with VEGF-A and VEGF-B. These changes are typical of cells with a mesenchymal phenotype rather than the usual epithelial phenotype of L3.6pl cells, indicating that the cells were undergoing EMT after VEGFR-1 activation. Approximately 30% of the cells in culture were observed to be undergoing changes consistent with EMT by both direct visualization and fluorescent microscopy. Of note, all morphologic studies were done on plastic culture containers, whereas all immunofluorescent studies were done on glass slides, which may explain the subtle differences in the appearance between morphologic and immunohistochemical studies.

VEGFR-1 activation induces changes in the localization of cellular EMT markers in L3.6pl cells. One of the hallmarks of EMT is the breakdown of the cytoplasmic-cell adhesion complex (9). The breakdown of this complex causes a change in the localization of E-cadherin and β-catenin from their usual membrane-bound location. To determine if activation of VEGFR-1 led to changes consistent with EMT, immunofluorescent staining

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was done on L3.6pl cells treated with VEGF-A or VEGF-B. VEGF-treated cells exhibited a change in E-cadherin from an organized, membrane-bound structure to a disorganized state that was dispersed throughout the cytoplasm (Fig. 2A). The maximal changes in E-cadherin were observed 6 hours after treatment of cells with VEGF-A and 24 hours after treatment of cells with VEGF-B. The change in E-cadherin localization after VEGF-A treatment also persisted, albeit to a lesser extent, at 24 hours (data not shown).

h-Catenin was observed to translocate to the nucleus from its usual membrane-bound location 24 hours after treatment with either VEGF-A or VEGF-B (Fig. 2A). Nuclear h-catenin acts as a transcription factor to induce further EMT changes (10). Pretreatment with VEGFR-1 blocking antibody inhibited the changes in localization of E-cadherin and h-catenin seen with VEGF treatment (Fig. 2B).

**VEGFR-1 activation is sufficient to cause molecular changes consistent with EMT in L3.6pl cells.** To determine if VEGFR-1 activation on L3.6pl cells induced the specific molecular changes consistent with EMT, Western blotting was done on cell lysates and nuclear extracts from cells treated with VEGF-A and VEGF-B. Again, changes consistent with breakdown of the cytoplasmic cell adhesion complex, decreased cell adhesion, and the switch to a mesenchymal cell phenotype were detected (Fig. 3A). Expression of the epithelial adhesion molecules E-cadherin and plakoglobin were both observed to decrease 48 hours after VEGF-A or VEGF-B treatment. Because E-cadherin is a central mediator of EMT, we confirmed that the decrease in E-cadherin expression was not a cell line–specific phenomenon. Western blotting of protein lysates of the PANC-1 pancreatic cancer cell line treated with VEGF-A and VEGF-B showed a decrease in E-cadherin protein levels (data not shown). Conversely, an increase in the expression of the mesenchymal cell markers N-cadherin and vimentin were maximally observed at 72 and 48 hours after VEGF-A and VEGF-B treatment, respectively. The increase in N-cadherin was observed as early as 48 hours after treatment. Furthermore, in correlation with our findings from the immunofluorescent experiments, the nuclear expression of h-catenin was observed to increase 24 hours after treatment with VEGF-A or VEGF-B. Similar to the immunofluorescent studies, pretreatment of L3.6pl cells with VEGFR-1 blocking antibody inhibited the decrease in E-cadherin expression observed when cells were treated with VEGF-B (Fig. 3B). Furthermore, the increases in N-cadherin and vimentin expression were also blocked by the VEGFR-1 antibody when cells were treated with VEGF-B (Fig. 3B). The ability of the VEGFR-1 blocking antibody to inhibit the changes in E-cadherin localization and expression indicates that VEGFR-1 activation alone is sufficient to

Figure 1. VEGFR-1 activation induces morphologic changes consistent with EMT in L3.6pl pancreatic cancer cells. L3.6pl cells treated with VEGF-A or VEGF-B were assessed by observers blinded to the treatment conditions for morphologic changes consistent with EMT. At 48 hours, the presence of spindle-shaped cells with loss of polarity (red arrows), increased intercellular separation (black arrows), and pseudopodia (white arrows) were noted in treated cells but not in untreated cells. Fluorescent confocal microscopy for ZO-1 confirmed the loss of polarity by revealing the loss of ZO-1-associated tight junctions. Alterations in the cytoskeleton by VEGFR-1 activation were confirmed by the reorganization of actin into pseudopodia and stress fibers.
induce EMT in L3.6pl cells. All of the expression changes in epithelial adhesion markers and mesenchymal markers have been extensively described to occur during the EMT as cells switch to a more motile mesenchymal phenotype (9, 11).

**EMT changes induced by VEGFR-1 activation are transcriptionally mediated.** The transcription factors Snail, Twist, and Slug have previously been shown to play an important role in mediating the changes observed with EMT (11, 12). To determine if Snail, Twist, or Slug have roles in EMT induced by VEGFR-1 in L3.6pl cells, we used Western blotting of nuclear extracts from L3.6pl cells treated with VEGF-A or VEGF-B (Fig. 4). At 6 hours after treatment with VEGF-A or VEGF-B, increases in the expression of Snail, Slug, and Twist in the nucleus were observed at varying degrees, all of which were consistent with EMT. We examined the effect of the VEGFR-1-blocking antibody on expression of the transcription factor that had the greatest increase in expression after VEGF-B treatment, Twist, as proof of principle that this change was mediated by VEGFR-1. Pretreatment with the VEGFR-1 antibody blocked the increase in nuclear expression of Twist observed after treatment of cells with VEGF-B (data not shown).

**Discussion**

Recent studies have implicated EMT in cancer progression by noting that epithelial-derived tumor cells could switch their phenotype to a more primitive mesenchymal phenotype that

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**Figure 2.** VEGFR-1 activation induces changes in the localization of cellular EMT markers in L3.6pl cells. Immunofluorescent staining for E-cadherin and β-catenin was done on L3.6pl cells treated with VEGF-A or VEGF-B. VEGF-treated cells showed changes in localization of both E-cadherin and β-catenin from their usual cell membrane–associated site. **A,** cells treated with VEGF-A or VEGF-B exhibited E-cadherin in a disorganized, diffuse distribution in the cytoplasm, whereas β-catenin translocated to the nucleus. **B,** pretreatment of cells with VEGFR-1 inhibitor prevented the observed changes in localization of E-cadherin and β-catenin.
facilitates motility and invasion (4). A few studies have also examined the possible role of EMT in pancreatic cancer (13, 14) but, to our knowledge, no studies have described VEGFR-1 as a possible mediator of EMT in pancreatic cancer. We hypothesized that EMT was the likely mechanism by which VEGFR-1 increased pancreatic cancer cell migration and invasion.

In this series of experiments, we showed that activation of VEGFR-1 in pancreatic cancer cells leads to morphologic and molecular alterations consistent with EMT that are likely to facilitate the induction of migration and invasion that we observed in our previous studies (2). We first showed that activation of VEGFR-1 on L3.6pl pancreatic cancer cells by its ligands VEGF-A and VEGF-B induced morphologic changes consistent with EMT. Specifically, the cells became more spindle-shaped, developed pseudopodia, and exhibited increased separation between cells. Then, using immunofluorescent staining, we showed that VEGFR-1 activation induced breakdown of the cytoplasmic-cell adhesion complex, with relocation of E-cadherin to the cytoplasm and

![Image: Figure 3. VEGFR-1 activation induces molecular changes consistent with EMT in L3.6pl cells. Cell lysates or nuclear extracts from L3.6pl cells treated with VEGF-A or VEGF-B were subjected to Western blotting. A, expression of the epithelial cellular adhesion molecules E-cadherin and plakoglobin was decreased at 48 hours. Increased expression of β-catenin in the nuclear extracts was noted at 24 hours. Expression of the mesenchymal markers N-cadherin and vimentin was increased at 72 and 48 hours, respectively. B, pretreatment of L3.6pl cells with VEGFR-1-blocking antibody before VEGF-B treatment inhibited the decrease in E-cadherin and increases in N-cadherin and vimentin observed with treatment with VEGF-B alone, indicating that VEGFR-1 activation is sufficient to induce EMT.]
translocation of β-catenin to the nucleus from their usual membrane-bound locations. Decreased expression of E-cadherin and that of another epithelial marker, plakoglobin, paralleled these immunohistochemical changes on the molecular level. Further evidence for the role of VEGFR-1-induced EMT in pancreatic cancer cells was supported by the increased expression of the mesenchymal markers N-cadherin and vimentin after treatment with VEGF-A and VEGF-B. Using a monoclonal blocking antibody to VEGF-1, we showed that blockade of VEGF-1 activation was sufficient to block EMT induction by VEGF-B. These studies confirmed that these changes were mediated through VEGF-1 and were not the aberrant expression of another VEGF tyrosine kinase receptor or due to the activation of neuropilin-1. Finally, we observed that VEGF-1 activation led to the up-regulation of the nuclear expression of the transcriptional regulators of EMT Snail, Slug, and Twist. Snail, Slug, and Twist seemed to be differentially regulated by VEGF-A and VEGF-B activation of VEGF-1. The differences in this signaling may be due to differences in the binding affinity of VEGF-A and VEGF-B for VEGFRs, although this is only a hypothesis at this time. Whereas down-regulation of E-cadherin in other tumor types (15) has been found to be mediated by promoter hypermethylation, we found no evidence of hypermethylation of the E-cadherin promoter after VEGF-1 activation by pyrosequencing (data not shown).

Our studies show that VEGF-1 on tumor cells may contribute to the aggressive behavior of pancreatic cancer cells by inducing EMT. In addition to demonstrating functional significance, EMT has been associated with tumor progression and poor prognosis in patients (16–18). Further investigation of the functions of tumor cell–associated VEGF-1 and its mediation of EMT is important given the potential efficacy of anti-VEGF therapy for patients with cancer, including pancreatic cancer (19–21). Targeting downstream mediators of EMT such as E-cadherin and N-cadherin are currently being investigated in some model systems, including one study in pancreatic cancer (22). Targeting VEGF-1 and downstream mediators of EMT may provide the foundation for the development of novel therapeutic approaches for this morbid and lethal disease.

Acknowledgments

Received 8/29/2005; revised 10/11/2005; accepted 10/24/2005.

Grant support: NIH grants T-32 09599 (A.D. Yang, E.R. Camp, and T.W. Bauer) and ROI CA112900 (L.M. Ellis) and the Lockton Fund for Pancreatic Cancer Research (M.J. Gray and L.M. Ellis).

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We thank Dr. Jean-Pierre Issa, for his contribution to the E-cadherin promoter methylation studies; Dr. Corazon D. Bucana, for her contribution to the immunohistochemical imaging studies; and Christine Wogan, Department of Scientific Publications, and Rita Hernandez, Department of Surgical Oncology, for editorial assistance.

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