Nuclear Localization of Vascular Endothelial Growth Factor-D and Regulation of c-Myc–Dependent Transcripts in Human Lung Fibroblasts

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Abstract

Lymphangiogenesis and angiogenesis are processes that are, in part, regulated by vascular endothelial growth factor (VEGF)-D. The formation of lymphatic structures has been implicated in multiple lung diseases, including pulmonary fibrosis. VEGF-D is a secreted protein produced by fibroblasts and macrophages, which induces lymphangiogenesis by signaling via VEGF receptor-3, and angiogenesis through VEGF receptor-2. VEGF-D contains a central VEGF homology domain, which is the biologically active domain, with flanking N- and C-terminal propeptides. Full-length VEGF-D (~ 50 kD) is proteolytically processed in the extracellular space, to generate VEGF homology domain that contains the VEGF-D receptor-binding sites. Here, we

report that, independent of its cell surface receptors, full-length VEGF-D accumulated in nuclei of fibroblasts, and that this process appears to increase with cell density. In nuclei, full-length VEGF-D associated with RNA polymerase II and c-Myc. In cells depleted of VEGF-D, the transcriptionally regulated genes appear to be modulated by c-Myc. These findings have potential clinical implications, as VEGF-D was found in fibroblast nuclei in idiopathic pulmonary fibrosis, a disease characterized by fibroblast proliferation. These findings are consistent with actions of full-length VEGF-D in cellular homeostasis in health and disease, independent of its receptors.

Keywords: vascular endothelial growth factor-D; nuclei; fibroblast; idiopathic pulmonary fibrosis; c-Myc

The vascular endothelial growth factor (VEGF) family of proteins plays important roles in endothelial cell proliferation, migration, survival, and permeability, resulting in regulation of vascular development, including angiogenesis and lymphangiogenesis (1, 2). VEGFs comprise a family of five polypeptides (VEGF-A, -B, -C, -D, and placental growth factor). These

growth factors are encoded by different genes, and the gene products are able to form homo- and heterodimers (3). This family of proteins contains eight cysteine residues forming a knot motif, which characterizes their biologically active domain and participates in protein folding and protein stability (4–7). The cysteines are part of the platelet-derived growth factor (PDGF)

domain that allows for dimerization and biological activity. The biologically active domain can be generated by alternative splicing and proteolytic processing of the mRNA or mature protein, respectively (3, 8).

VEGFs are primarily secreted proteins that initiate, through their receptors, signaling cascades, resulting in diverse cellular actions that involve transcriptional

(Received in original form September 27, 2013; accepted in final form December 28, 2013)

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This work was supported in part by the Intramural Research Programs, National Institutes of Health (NIH)/National Heart, Lung, and Blood Institute (NHLBI), and NIH/National Human Genome Research Institute, and NIH grant 1K22HL092223, NHLBI (S.E.-C.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 51, Iss 1, pp 34–42, Jul 2014

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Originally Published in Press as DOI: 10.1165/rcmb.2013-0417OC on January 22, 2014

Internet address: www.atsjournals.org

Clinical Relevance

Vascular endothelial growth factor (VEGF)-D is a lymphangiogenic and angiogenic growth factor that signals through receptors on the surface of endothelial cells. Here, we demonstrate the presence of VEGF-D in nuclei of human fibroblasts in culture. Furthermore, we show that, in lung tissue sections from patients with idiopathic pulmonary fibrosis, a disease characterized by fibroblast proliferation, nuclear localization of VEGF-D correlated with fibroblast density. We also demonstrate that VEGF-D localizes in cell nuclei independent of its known surface receptors. In cell nuclei, VEGF-D associates with RNA polymerase II and c-Myc, and, by knockdown and expression arrays, we show that VEGF-D modulates c-Myc transcriptional activity. Taken together, our data show novel intracellular functions for VEGF-D in fibroblasts.

and translational events (2). VEGF receptor (VEGFR) -1, -2, and -3 receptor tyrosine kinase can be activated by homo- or heterodimers of VEGFs resulting in phosphorylation of the receptor cytoplasmic tail, activation of downstream cytosolic transcription factors, and translocation of these transcription factors to the nucleus, to regulate specific target genes (2, 3, 8). Although VEGFs bind to specific canonical receptors, they are also able to interact with coreceptors, such as neuropilins (NRPs) (8). For instance, VEGF-D binds to VEGFR-3, VEGFR-2, and the transmembrane glycoprotein receptors, NRP-1 and -2 (9). Binding of VEGF to coreceptors induces internalization and enhances receptor activation of VEGF family proteins (reviewed in Ref. 10). The PDGF domain of these factors is usually flanked by residues of less-defined function. The prototypical member (VEGF-A) of this family contains sequence that allows binding to heparin sulfate and NRP near the C terminus. The elucidation of the functions of these factors is further complicated by formation of multiple products generated by alternative splicing (i.e., VEGFA, VEGFB, and placental growth factor), as well as post-translational processing of VEGF-D and VEGF-C.

VEGF-D is produced in multiple tissues, mainly by fibroblasts, macrophages, and smooth muscle cells (11, 12). Interestingly, fibroblasts play important roles in supporting organ-specific metabolism in health and disease (13). VEGF-D is a secreted protein comprising a central VEGF homology domain (PDGF/VHD), with flanking N- and C-terminal propeptides (11). Full-length VEGF-D (~ 50 kD) can be processed by two pathways in the extracellular space: (1) it is cleaved by the serine protease, plasmin, removing the N- and C-terminal ends to generate highly active VHD (14–16); or (2) it is proteolyzed by proprotein convertases (PCs). Furin and PC5 promote removal of both N- and C-terminal regions, whereas PC7 catalyzes the cleavage of only the C-terminal propeptide (17). *In vitro*, affinity of VHD for VEGFR-2 and VEGFR-3 has been observed to be much higher than that of full-length VEGF-D (16, 17). Furthermore, VEGF-D interaction with NRP-1 and -2 requires its partial processing and heparin (9). VEGF-D induces lymphangiogenesis through signaling via VEGFR-3, and angiogenesis via VEGFR-2, tyrosine kinase receptors present on the endothelial cell surface. Most of the roles of VEGF-D have been ascribed to cell signaling by binding to its receptor(s); however, very little is known about the distribution and potential role of the full-length protein.

We reported previously abnormal lymphangiogenesis in idiopathic pulmonary fibrosis (IPF), a rapidly progressive fatal lung disease (18). Here, we observed initially nuclear reactivity of anti-VEGF-D antibodies with IPF lung tissue sections, leading us to investigate a potential role of VEGF-D in transcription. Nuclear localization of VEGF-D was prominent, with increasing cell density and independent of its surface receptors. Full-length VEGF-D accumulated in fibroblast nuclei of both lung tissue and cultured cells. VEGF-D in nuclei associated with RNA polymerase II and c-Myc, and participated in the regulation of c-Myc-dependent transcripts, consistent with critical actions of VEGFs independent of their known receptors.

Materials and Methods

Additional information is available in the online supplement.

IPF Lung Tissue

Cells from subjects with IPF, enrolled in a protocol approved by the Institutional

Review Board of the National Human Genome Research Institute (04-HG-0211), and diagnosed according to published criteria (19), were used in selected experiments. IPF lungs were explanted organs from anonymous donors, and therefore clinical data are not available. Normal lungs were purchased from U.S. Biomax Inc. (Rockville, MD).

Cell Culture

IPF lung and normal lung fibroblasts were grown at 37°C, 5% CO₂/95% air in mesenchymal stem cell media (Lonza, Walkersville MD). For confocal microscopy, cells were plated at densities of 10,000, 20,000, 40,000, and 80,000 cells/well in four-well Lab-Tek II chamber slides (Nalge NUNC, Rochester, NY). All experiments were performed between passages 4 and 8.

Cell Fractionation, Western Blotting, and Immunoprecipitation

Fibroblasts were fractionated when grown at high density (20, 21). Proteins were separated by SDS-PAGE (Invitrogen, Carlsbad, CA), and transferred to nitrocellulose membranes, which were incubated with the indicated antibodies. Secondary antibodies were linked to horseradish peroxidase and reactivity was detected with SuperSignal chemiluminescent substrate (Pierce, Rockford, IL).

Confocal Microscopy and Image Analysis

Confocal images (Zeiss 510; Carl Zeiss Jena GmbH, Jena, Germany) were acquired sequentially for 4',6-diamidino-2-phenylindole (nuclei), FITC, and Texas red. High-resolution Z-series (100 nm/pixel) images were obtained throughout the cells. The three-dimensional datasets were deconvolved (Huygens software, SVI, Hilversum, The Netherlands) before three-dimensional reconstructions and analysis for colocalization. Quantification of colocalization was performed (Imaris 6.5 software package; Bitplane AG, Zürich, Switzerland). Fluorescence was quantified in 12-bit confocal images using Imaris Cell module v7.1 (Bitplane AG, Zurich, Switzerland). 4',6-Diamidino-2-phenylindole channel was used to identify and threshold the nuclei. Mean VEGF-D intensity per nucleus was measured. Fluorescence intensity is expressed as percentage of that of mock-transfected cells.

Figure 1. Nuclear localization of vascular endothelial growth factor (VEGF)-D in fibroblasts. (A) VEGF-D in predominantly nuclear distribution correlated with density of cultured fibroblasts. Single (left) and merged (right) pairs of images of VEGF-D (red) and 4',6-diamidino-2-phenylindole (DAPI; blue) fluorescence of representative cells at low (10,000 cells/well) and high (80,000 cells/well) cell density with marked differences in VEGF-D localization at low (predominantly cytoplasmic) and high (mostly nuclear) densities. (B) Semiquantitative representation of VEGF-D cytoplasmic–nuclear redistribution using line profile. Fluorescence intensities along the white line sectioning the cell through the nucleus are plotted for VEGF-D (red) and DAPI (blue); DAPI indicates the nucleus. Differences in the ratios of cytoplasmic/nuclear VEGF-D from mostly cytoplasmic (left) to mostly nuclear (right) with increasing fibroblast density are marked. All experiments were repeated at least three times. (C) Specificity of antibody reactivity to VEGF-D. Fibroblasts plated in four-well chamber slides were mock transfected, or treated with nontarget or VEGF-D small interference RNA (siRNA) before cell staining and image acquisition. Shown are VEGF-D (red), DAPI (blue), and differential interference contrast (DIC) images. These data confirm VEGF-D antibody specificity. (D) Full-length VEGF-D is enriched in nuclear fraction. Samples of proteins from lysate (TL; 2%), and postnuclear (PN; 10%) and crude nuclear (CN; 10%) fractions were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were reacted with antibodies against VEGF-D, histone H4 (nuclear marker), GRP-78 (ER marker), and GM130 (Golgi marker). Nuclear fraction was markedly enriched in full-length VEGF-D. (E) Overexpression of VEGF-D in normal lung fibroblasts. Normal lung fibroblasts 12 hours after transfection with plasmid encoding hemagglutinin (HA)-tagged VEGF-D. Cells were processed for immunofluorescence with anti-HA antibodies (red). Scale bar, 50 μm . Results show the presence of HA-tagged VEGF-D in cell nuclei.

Figure 2. Full-length VEGF-D is present in fibroblast nuclei and associates with RNA polymerase II (Pol II). (A) Localization of VEGF-D with RNA Pol II in nuclei of fibroblasts. Confocal microscopy images of immunolabeled confluent fibroblasts were analyzed for colocalization. Merged images (left) of VEGF-D (red) and proliferating cell nuclear antigen (green), or VEGF-D (green) and serine/arginine-rich splicing factor 2 (red), or VEGF-D (green) and RNA Pol II (red) show that the greatest colocalization (yellow) of VEGF-D is with RNA Pol II. Pixel scattergrams and colocalization coefficients (right) indicate approximately 33% of VEGF-D colocalized with RNA Pol II. (B) Coimmunoprecipitation of VEGF-D with antibodies against RNA Pol II. Immunoprecipitation (IP) of proteins from crude nuclei with nonimmune mouse IgG, or anti-RNA Pol II antibodies (Pol). (C) Colocalization of VEGF-D with 5-bromo-2'-deoxyuridine (BrdU) in fibroblast nuclei. Cultured fibroblasts were incubated with BrdU and reacted with anti-VEGF-D and anti-BrdU antibodies. Images of VEGF-D (green), BrdU (red), and merged fluorescence images show coincident presence of VEGF-D and BrdU in nuclear speckles; quantitative analysis (graph) indicates approximately 30% colocalization. No fluorescence was seen when BrdU was omitted. All experiments were repeated at least three times.

Cloning of pcDNA-HA-VEGF-D

Full-length VEGF-D cDNA was isolated from a normal human lung fibroblast cDNA library and cloned into pcDNA3.1 mammalian vector (Invitrogen, Carlsbad, CA), generating a hemagglutinin (HA)-tagged VEGF-D at its N-terminal end.

Experiments with Small Interference RNA

Lung fibroblasts were transfected with 100 nM VEGF-D, NRP-1, or control scrambled small interference RNA (siRNA) oligonucleotides (Dharmacon Research, Lafayette, CO). RNA was collected 48 hours after transfection using a Qiagen kit (Qiagen, Valencia, CA).

Affymetrix Oligonucleotide Microarray

The human U133_Plus_2 GeneChip (Affymetrix, Santa Clara, CA) was used for oligonucleotide microarray. The RNA for hybridization was prepared as described by Affymetrix. Affymetrix GCOS version 1.4 was used to calculate the signal intensity and the percent-present calls on the hybridized Affymetrix chip. Pathway analysis was

performed using Ingenuity Pathway Analysis (Ingenuity Systems Inc., Redwood City, CA).

Proliferation Assay

Fibroblasts were plated in 96-well plates (1,000 cells/well) and treated as indicated. Proliferation was measured with counting kit-8 (Dojindo Molecular Technologies, Rockville, MD).

Fluorescence-Activated Cell Sorting

Cell suspensions were incubated for 30 minutes with indicated antibodies and fluorescence-activated cell sorting (FACS) was performed as described previously (5).

Statistical Analysis

Experiments were repeated at least three times. When indicated, data are expressed as means ($\pm$ SD). Significance of difference between means was evaluated using *t* test or ANOVA, as appropriate. If a significant difference was found, a group-by-group comparison was done using Tukey. A *P* value less than 0.05 was considered significant. Analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

Results

VEGF-D Localizes in the Nuclei of Fibroblasts *In Vitro*

As fibroblasts are a source of VEGF-D (12), and this cell type could play a role in disease, the local function and action of VEGF-D in these cells was investigated. We first observed that VEGF-D in normal fibroblasts changed from largely cytoplasmic to predominantly nuclear with increasing cell density (Figures 1A and 1B). Nuclear signal is abolished with siRNA depletion of VEGF-D, confirming antibody specificity (Figure 1C). To identify the molecular form of nuclear VEGF-D, experiments were repeated using specific antibodies against the VHD and N- or C-terminal regions of VEGF-D (see Figure E1 in the online supplement). Results were similar with all of the antibodies, consistent with the presence of full-length VEGF-D in nuclei of lung fibroblasts in high-density cultures. This conclusion was confirmed by cell fractionation and Western blotting. Total cell lysates contained a weakly immunoreactive band, corresponding to the roughly 50 kD VEGF-D. The postnuclear fraction showed

a similar pattern, and the nuclear fraction was enriched with full-length VEGF-D (Figure 1D). Thus, full-length VEGF-D is necessary for its nuclear localization. To further confirm the ability of VEGF-D to traffic to the nucleus, HA-VEGF-D was transiently expressed in fibroblasts. Figure 1E shows that HA-VEGF-D is localized to the nucleus. Unsurprisingly, and consistent with prior observations (22), fibroblasts overexpressing VEGF-D detached from the plate within 6 hours of transfection.

VEGF-D Role and Function in Cell Nuclei

To explore the biological function of nuclear VEGF-D, we identified functional nuclear compartments with specific marker molecules, including proliferating cell nuclear antigen for sites of DNA replication (23), serine/arginine-rich splicing factor 2 for sites of RNA splicing (24), and RNA polymerase II for sites of transcription (25). By confocal microscopy, VEGF-D colocalized to a large extent with RNA polymerase II (33%). This contrasts with VEGF-D colocalization with proliferating cell nuclear antigen (1%) or SC35 (7.5%) (Figure 2A). Coimmunoprecipitation of VEGF-D with anti-RNA polymerase II antibody from crude nuclear extracts was likewise consistent with the existence of VEGF-D/RNA polymerase II complexes (Figure 2B). These data are consistent with a role for VEGF-D in RNA polymerase II-mediated processes.

To explore a functional association of VEGF-D with DNA in support of a role in transcriptional events, we incubated cells with 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog incorporated into newly synthesized DNA. We observed that VEGF-D colocalized with BrdU (29%) at sites of newly synthesized DNA (Figure 2C and Movie E1). Specificity of the anti-BrdU antibody was confirmed by the absence of immunofluorescence when BrdU was omitted. The staining pattern and the quantitative colocalization analysis appeared to closely mirror the distribution of RNA polymerase II, providing evidence that VEGF-D forms a complex with RNA polymerase II and DNA, and therefore may play a role in transcription.

A possible role of VEGF-D in DNA metabolism was further evaluated by examining the transcriptome of cells depleted of VEGF-D with siRNA (Figure 3A and Table E2). Because addition of the nontargeted siRNA resulted in changes in gene expression, we

Figure 3. Association of VEGF-D with c-Myc. Heat map of differentially expressed genes in lung fibroblasts treated with VEGF-D siRNA (siRNA), nontarget siRNA (control), or vehicle alone (mock). Data in (A) were obtained using Affymetrix human Genome HG-U133_Plus_2 GeneChip. The diagram represents 120 differentially expressed probes ($P < 0.01$) with at least twofold change of VEGF-D from both the mock- and control-treated cells. Each column represents a single experiment, and each row a single gene, with red for high expression levels and green for lower (scale at the bottom right). (B) Gene validation of microarray. Fibroblasts were grown to 90% confluence, then were mock transfected or treated with nontarget or VEGF-D siRNA. RNA was collected and quantitative real-time RT-PCR was performed for selected genes. Results show a decrease in VEGF-D, cyclin D1, and Lin7C levels ($P < 0.01$ for all genes), consistent with the array data. (C) Total lysates of lung fibroblasts were immunoprecipitated with nonspecific IgG (IgG) or anti-VEGF-D antibodies (VD). Samples (5%) of total lysate (Input) or immunoprecipitated proteins eluted from beads in 50 μ l of gel-loading buffer were separated by SDS-PAGE, and blots were divided for reaction with indicated antibodies. Experiments were repeated at least three times. (D) Confluent fibroblasts were double immunofluorescently labeled and confocal microscopy images were analyzed for colocalization. Merged images of VEGF-D (green) and c-Myc (red) display significant colocalization. Pixel scattergrams and colocalization coefficients indicate that roughly 20% of VEGF-D colocalizes with c-Myc. All experiments were repeated at least three times.

analyzed our data by comparing VEGF-D-depleted cells to mock and nontarget-treated cells. We identified 120 genes that were differentially expressed, and some transcripts were validated by quantitative real-time PCR (Figure 3B). Using MetaCore (GeneGo Inc., St. Joseph, MI), we identified metabolic pathways that were differentially regulated when VEGF-D was silenced (Table E3). These pathways are mostly related to cell cycle and not specific to angiogenesis and lymphangiogenesis. In addition, analysis of promoter sequences of differentially regulated genes suggested that c-Myc is a likely transcription factor binding those genes. To confirm an association between VEGF-D and c-Myc, we demonstrated that both proteins were immunoprecipitated with anti-VEGF-D antibodies (Figure 3C), and were colocalized in nuclei (20%) by confocal microscopy (Figure 3D). These data suggest

that VEGF-D could be a comodulator of c-Myc in lung fibroblasts.

VEGF-D Nuclear Localization Is Receptor Independent

To determine whether the nuclear localization of VEGF-D in fibroblasts is part of an autocrine or paracrine system involving VEGFR-2 or -3, we investigated the presence of these receptors on the surface of lung fibroblasts. FACS analysis for VEGFR-2 and -3 was performed using human umbilical vein endothelial cells and lymphatic endothelial cells known to express VEGFR-2 and -3, respectively, as positive controls. Although the cell surface marker, CD44, was present on fibroblasts (Figures 4A–4E), VEGFR-2 and -3 were not detected on lung fibroblasts by FACS analysis. NRP-1 and -2 are other surface receptors known to interact with VEGF-D. NRP-1, but not NRP-2, was present on

Figure 4. Lack of VEGFR-2 and -3 in lung fibroblasts (A–E). Flow cytometry analysis of human umbilical vein endothelial cells (HUVECs) (A) and lymphatic endothelial cells (LECs) (D) show reactivity with anti-VEGFR-2 and VEGFR-3 antibodies, respectively (red area in the scatterplot). Lung fibroblasts did not react with anti-VEGFR-2 (B) and anti-VEGFR-3 antibodies (E), but did react with antibodies to surface marker, CD44, (blue) (C). Black scatterplots, isotype (negative) control. Effects of VEGF-D on proliferation of lung fibroblasts (F). Serum-free cell culture medium was supplemented with VEGF-D at the indicated concentrations. Data show increased proliferation of lung fibroblasts with serum supplementation (* $P < 0.05$), but not with the addition of VEGF-D. Lack of effect of neuropilin (NRP)-1 depletion with siRNA on nuclear localization of VEGF-D. (G) Samples (30 μ g) of proteins from lysates of cells transfected with pools of four human NRP-1-specific or nontargeting (nontarget [NT]) siRNAs or with vehicle alone (Mock) were separated by SDS-PAGE before immunoblotting with indicated antibodies. Representative Western blot shows no effect of NRP-1 depletion on amounts of cellular VEGF-D. (H) Cells plated in four-well chamber slides were mock transfected, or treated with nontarget or NRP-1 siRNA, before acquisition of VEGF-D (red) and NRP-1 (green) images and quantification of fluorescence intensity of VEGF-D (red) over the nucleus using the Imaris Cell module of the software. (I) Data from three independent experiments expressed as a percentage of intensity of mock-transfected cells show no difference in VEGF-D nuclear localization ($P > 0.05$). Number of nuclei counted per condition for mock, 382 ± 105 ; NT, 335 ± 89 ; and NRP-1, 299 ± 40 .

fibroblast surfaces. Depletion of NRP-1 with siRNA did not affect amounts of cellular VEGF-D or its nuclear localization (Figures 4G–4I). Thus, VEGF-D localizes to the nucleus independently of canonical receptors, VEGFR-3 and -2, and coreceptors.

To examine further potential VEGF-D effects mediated by its cell surface receptors,

cell culture medium was supplemented with VEGF-D to assess effects on fibroblast proliferation. Increasing concentrations of VEGF-D did not alter cell proliferation (Figure 4F). Consistent with VEGF-D synthesis by fibroblasts, we also found low levels of VEGF-D in cell culture supernatant (0.1 pg/ μ l), suggesting that

VEGF-D can traffic to the nucleus and/or to the extracellular environment.

VEGF-D Nuclear Localization Is Transcription and Chromosome Region Maintenance-1 Dependent

An *in silico* analysis of the structure of VEGF-D showed that it does not contain a nuclear

localization signal. Our data indicate that VEGF-D was involved in transcription-related events. Proteins involved in transcription are known to shuttle to the nucleus in a transcription-dependent fashion (26). To assess transcription-dependent VEGF-D nuclear distribution, cells were treated with actinomycin D, a transcription inhibitor. Treatment with actinomycin D resulted in a decrease in nuclear VEGF-D (Figure 5, *middle panels*), consistent with a role for VEGF-D in transcription.

Chromosome region maintenance (CRM)-1 is an important receptor for protein export out of the nucleus (27). To examine the role of CRM-1 in VEGF-D nuclear accumulation, effects of CRM-1 inhibitor, leptomycin B, were investigated. VEGF-D nuclear accumulation was accentuated (Figure 5, *lower panels*) with leptomycin B treatment, consistent with a role for CRM-1 in VEGF-D nuclear export.

Figure 5. Effects of leptomycin B and actinomycin D on nuclear localization of VEGF-D. Confocal microscopy images of immunolabeled fibroblasts (40,000 cells/well) were analyzed for VEGF-D localization. Shown are images of VEGF-D (green) displayed alone (*left column*) or merged with DAPI (blue; *right column*) of cells that were untreated, or treated for 4 hours with leptomycin B (2.5 ng/ml) or with actinomycin D (5 μ g/ml). Results show that addition of leptomycin B results in accumulation of VEGF-D in the nucleus, and actinomycin D inhibits the nuclear localization of VEGF-D.

VEGF-D Localizes in the Nuclei of Fibroblasts in Pulmonary Fibrosis

To investigate whether these findings were also relevant *in vivo*, we used lung tissue sections from patients with IPF ($n = 4$), a disease characterized by abnormal fibroblast proliferation. We found that VEGF-D was concentrated in discrete areas of nuclei (Figure 6A). After incubation of IPF lung sections with antibodies against fibroblast surface protein-1 (28) and anti-VEGF-D, we observed a correlation between nuclear VEGF-D and fibroblast density, as evidenced by reaction with anti-fibroblast surface protein antibodies (Figure 6B). In the normal ($n = 4$) and in the IPF lungs, we found immunoreactivity with VEGF-D antibodies in alveolar macrophages and epithelial cells (Figures E2 and E3); VEGF-D, however, was not present in cell nuclei.

Discussion

Here, we show that the lymphangiogenic growth factor, VEGF-D, localized in the nucleus of fibroblasts *in vitro*, as well as in cells of lung sections from patients with IPF. This previously unknown property of VEGF-D is a function of the full-length protein and is surface receptor independent.

Our conclusion is supported by the following findings. First, fibroblasts in culture did not contain VEGFR-2 and -3. Second, the noncanonical VEGFR, NRP-1, was expressed, but its silencing did not alter VEGF-D nuclear localization. This is not surprising, as it was reported earlier that NRPs interact with the partially processed and not the full-length VEGF-D, and that the interaction requires the presence of heparin (9), which was not present in the culture medium. Third, HA-tagged VEGF-D is present in nuclei of transiently transfected fibroblasts, consistent with the conclusion that the full-length protein can localize in fibroblasts nuclei.

Although most of the effects of VEGF-D have been focused on endothelial cells, VEGF-D is produced by different cell types and secreted as a full-length protein; an intracellular role of the protein in producing cells has not been shown. The fact that no processed forms of the protein were found in the intracellular space supports this notion (16). In the extracellular space, VEGF-D is rapidly processed by initial cleavage of the C-terminal end. In the developing lung, only the fully processed and the C-terminal cleaved proteins were found, indicating that

the full-length, unprocessed protein is unlikely to be present in the extracellular space (16), and therefore it is unlikely that the full-length protein is internalized and translocated to the nuclei. In addition, and in support of our hypothesis of the intracellular trafficking of full-length VEGF-D to the nucleus, it has been determined that this form has lower affinity for cell surface receptors compared with the VHD (16, 17). Taken together, these data support our conclusion that the presence of full-length, unprocessed VEGF-D in cell nuclei results from an intracellular translocation independent of cell surface receptors. Growth factors are primarily secreted proteins that initiate, through their receptors, signaling cascades, resulting in diverse cellular actions, but they are not known to affect transcription by directly influencing RNA polymerase activity. However, epidermal growth factor (29) and fibroblast growth factor (FGF) (30), together with their receptors, can translocate from the cell surface to the nucleus, where they can bind DNA, activate RNA polymerase, and affect transcription. Both endogenous and exogenous FGF (basic FGF) have been shown to localize to the nucleus in a canonical receptor-independent manner, likely through heparan sulfate proteoglycans-mediated pathways (31). Interestingly, multiple isoforms of basic FGF have been found in cell nuclei, depending on the source (endogenous or exogenous protein); however, in the case of VEGF-D, we have found only the full-length, unprocessed protein.

In our studies, VEGF-D localized in cell nuclei to affect transcriptional activity, and its nuclear localization was decreased when transcription was inhibited. VEGF-D is known to play a critical role in angiogenesis and lymphangiogenesis through plasma membrane-associated receptors on endothelial cells; however, it is now evident that there is a VEGF-D function on nonendothelial cells. For instance, Mauceri and colleagues (32) have shown a critical role for VEGF-D/VEGFR-3-mediated signaling in axonal integrity and function in the hippocampus. It has also been shown that, in breast cancer cell lines (MCF-7 and MDA-MB-231 cells), full-length VEGF-D was antiapoptotic through nonreceptor-mediated pathways, resulting from an increase in the antiapoptotic protein, Bcl2 (33). Furthermore and consistent with a role for VEGF-D in fibroblast biology, Orlandini and colleagues (34) showed that fibroblasts overexpressing VEGF-D become more refractive, change

Figure 6. Nuclear localization of VEGF-D in fibroblasts from idiopathic pulmonary fibrosis (IPF). (A) Specificity of nuclear localization of VEGF-D in fibroblasts from IPF lung. Merged image of VEGF-D (red) and DAPI (blue) fluorescence staining of frozen sections of IPF tissue demonstrating nuclear localization of VEGF-D in fibroblasts at low (left panel) and high magnification (far right panel). VEGF-D nuclear immunofluorescence was not detected in controls where primary antibody was omitted (middle panel). (B) Nuclear localization of VEGF-D correlated with fibroblast density in IPF lung sections. Merged image of VEGF-D (green), fibroblast surface protein (FSP; red), and DAPI (blue) fluorescence of sections of IPF lung tissue shows lack of VEGF-D staining in fibroblast nuclei at low cell density (left) and nuclear localization of VEGF-D in fibroblasts at high cell density (center). Insets: higher magnification of indicated area (*). Graph (right) of relationship between FSP-positive cells and cell nuclei positive for VEGF-D per field ($n = 567$).

their spindle shape, and detach from the cell culture plate. Consistent with these findings, we found an increase in antiapoptotic pathways in fibroblasts when VEGF-D was silenced with siRNA. Intriguingly, pathways involved in cytochrome P450 regulation were differentially regulated by VEGF-D, suggesting perhaps a role for VEGF-D in drug-induced lung injury.

Multiple roles of VEGFs and their receptors are beginning to emerge. An earlier report described nuclear VEGF-A associated with the transcription factor, HuR, under hypoxic conditions (35), and, recently, another member of the VEGF family of proteins has been found in the regulation of the transcription factor, RUNX2, affecting cell differentiation (36). Interestingly, HuR is a transcriptional regulator of VEGF-A, suggesting a mechanism of self-regulation. More recently, Lee and colleagues (37) described an intracrine mechanism of VEGF signaling. In this case, intracellular VEGF binds to intracellular VEGFR-1, mediating survival in breast cancer cell lines. Although it is possible that similar mechanisms could be involved in VEGF-D nuclear localization, we

consider these pathways unlikely for VEGF-D, because fibroblasts did not contain mRNA for VEGFR-2, -3, and -1, and VEGF-D has extremely low affinity for VEGFR-1 (11).

Endogenous full-length VEGF-D accumulated in fibroblast nuclei and influenced the expression of Myc-regulated transcripts, but an obvious cellular phenotype was not detected at physiological levels. This fact is not surprising considering the observation that an unchallenged VEGF-D^{-/-} mouse has a mild decline in the number of lung lymphatics without functional consequences (38). The molecular interactions of VEGF-D required to affect transcription are unknown. We did not identify an obvious DNA-binding motif in VEGF-D, suggesting that VEGF-D effects on DNA metabolism are indirect. It is possible that VEGF-D indirectly modulates transcription by either regulating a transcriptional complex containing RNA polymerase II and c-Myc, or by complexing with c-Myc or other transcription factors.

Our findings provide strong evidence for an intracellular action of the

lymphangiogenic growth factor, VEGF-D, in cell nuclei, independent of its known receptor-mediated function. Density-dependent nuclear accumulation of VEGF-D, similar to that in cultured cells, was observed with fibroblasts in IPF, suggesting that nuclear VEGF-D plays an important role in fibroblast homeostasis *in vivo*, particularly, perhaps, in fibrotic lung disease. The understanding of VEGF-D nuclear trafficking might lead to potential clinical application in pulmonary fibrosis. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Martha Vaughan for helpful discussions and critical review of the manuscript, Dr. Zu-Xi Yu (National Heart, Lung, and Blood Institute [NHLBI] pathology core) for assistance with pathology experiments, Drs. Nalini Raghavachari and Xiuli Xu (NHLBI genomics core) for assistance with the gene array experiments, Dr. J. Phillip McCoy and Ms. Ann Williams for assistance with the flow cytometry experiments (NHLBI flow cytometry core), and Mr. Kevin Frischmann (Bitplane AG, Zürich, Switzerland) for assistance with Imaris Cell.

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