Elevated SP-1 Transcription Factor Expression and Activity Drives Basal and Hypoxia-induced Vascular Endothelial Growth Factor (VEGF) Expression in Non-Small Cell Lung Cancer

Karl Deacon, David Onion, Rajendra Kumari, Susan A. Watson, and Alan J. Knox

From the Centre for Respiratory Research and Division of Preclinical Oncology, University of Nottingham, Nottingham, NGS 1PB, United Kingdom

Background: VEGF is central to cancer angiogenesis; however, we have a poor understanding of how VEGF is regulated in lung tumors.

Results: High levels of SP-1 transcription factor expression amplify basal and hypoxia-induced VEGF expression.

Conclusion: SP-1 plays a key role in both genetic and hypoxic microenvironment regulation of VEGF in cancer.

Significance: Targeting of both VEGF and SP-1 may provide a more effective cancer therapy.

VEGF plays a central role in angiogenesis in cancer. Non-small cell lung cancer (NSCLC) tumors have increased microvascular density, localized hypoxia, and high VEGF expression levels; however, there is a lack of understanding of how oncogenic and tumor microenvironment changes such as hypoxia lead to greater VEGF expression in lung and other cancers. We show that NSCLC cells secreted higher levels of VEGF than normal airway epithelial cells. Actinomycin D inhibited all NSCLC VEGF secretion, and VEGF minimal promoter-luciferase reporter constructs were constitutively active until the last 85 base pairs before the transcription start site containing three SP-1 transcription factor-binding sites; mutation of these VEGF promoter SP-1-binding sites eliminated VEGF promoter activity. Furthermore, dominant negative SP-1, mithramycin A, and SP-1 shRNA decreased VEGF promoter activity, whereas overexpression of SP-1 increased VEGF promoter activity. Chromatin immunoprecipitation assays demonstrated SP-1, p300, and PCA/F histone acetyltransferase binding and histone H4 hyperacetylation at the VEGF promoter in NSCLC cells. Cultured NSCLC cells expressed higher levels of SP-1 protein than normal airway epithelial cells, and double-fluorescence immunohistochemistry showed a strong correlation between SP-1 and VEGF in human NSCLC tumors. In addition, hypoxia-driven VEGF expression in NSCLC cells was SP-1-dependent, with hypoxia increasing SP-1 activity and binding to the VEGF promoter. These studies are the first to demonstrate that overexpression of SP-1 plays a central role in hypoxia-induced VEGF secretion.

Unlimited tumor growth is dependent upon a combination of conditions, such as self-sufficiency in growth, limitless repli
cation, lack of anti-growth signals, lack of apoptosis, metastasis, suppression of immune surveillance, and deregulated angiogenesis (1). Rapid tumor growth causes localized hypoxia leading to angiogenesis, the formation of new blood vessels. Histological examination of lung tumors has established that non-small cell lung cancers (NSCLCs) have increased blood vessel formation (greater microvessel density), elevated levels of VEGF expression (2), and increased expression of cellular markers of hypoxia; all of these measurements are associated with decreased survival rates (2–6).

VEGF plays a central role in the angiogenic response to a diverse range of stimuli such as growth factors, prostanooids, hypoxia, and hypoglycemia (7–10). The VEGF family of four genes (A, B, C, and D) and their numerous splice variants exert their physiological role through the endothelial cell based receptor tyrosine kinases, VEGFR1 (Flt-1), VEGFR2 (KDR/Flk-1), and VEGFR3. The loss of the VEGF-A gene is lethal to the developing embryo (11, 12), and antibody blockade of the VEGF/VEGFR interactions both in vitro and in vivo or small molecular weight chemical inhibition of the VEGFRs prevents angiogenesis (13, 14).

Tumor cell control of VEGF expression is a combination of tumor microenvironment (hypoxia, hypoglycemia) and genetic/epigenetic factors (oncogenes), where VEGF-A gene expression can be controlled at transcription, post-transcription, and post-translation prior to secretion or integration into the extracellular matrix (15–17). Under normoxic conditions the proline residues of the transcription factor HIF-1α are hydroxylated by the prolyl-hydroxylase complex PHD1/2/3 creating a target for the ubiquitination of HIF-1α and consequent HIF-1α degradation. Rapid tumor growth past 2 mm in diameter leads to localized hypoxia (18). Hypoxia decreases PHD2 activity, leading to reduced HIF-1α hydroxylation/ubiquitination and increasing HIF-1α stability (19–21). Stable HIF-1α and Aryl Hydrocarbon

References:

1. This article contains supplemental Figs. S1 and S2.

2. The abbreviations used are: NSCLC, non-small cell lung cancer; HIF, hypoxia-inducible factor; QPCR, quantitative PCR; IP, immunoprecipitation; NHBE, normal human bronchial epithelial.
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Receptor Nuclear Translocator complexes bind to the hypoxia recognition element of the VEGF promoter leading to increased VEGF transcription. Hypoxia-induced VEGF expression can also be HIF-1α-independent with hypoxia-induced VEGF expression in colon cancer (22) and hypoxia activation of PGC-1 driving VEGF expression independent of HIF-1α in muscle cells (23). Tumor cells can increase VEGF expression by the oncogenic transformation associated with loss of cell cycle control, elevated p53 tumor suppressor, loss of the von Hippel-Lindau gene (24) and gain of function mutation of the GTPase Ras (25). In addition oncogene-driven growth factor overexpression, such as EGF (8) or endothelin-1 (26), led to elevated VEGF transcription, expression, and secretion.

SP-1 has been shown to drive VEGF secretion in some cancers, but its potential role in driving hypoxia-induced VEGF secretion has not been studied previously. Furthermore mechanisms responsible for elevated VEGF expression in NSCLC are unknown. Here we demonstrate in several NSCLC cell lines that under normoxic conditions constitutive VEGF expression was the result of increased SP-1 transcription factor expression, activity, and binding to the VEGF proximal promoter. In addition VEGF expression correlated with the levels of SP-1 overexpression in human NSCLC tumor tissue ex vivo. Furthermore, hypoxic induction of VEGF expression was also SP-1-dependent with increased SP-1 transcription factor activity and increased SP-1 binding to the VEGF promoter under hypoxic conditions with no hypoxic induction of the VEGF promoter when SP-1-binding sites were mutated, suggesting that these sites were critical. VEGF therefore joins a group of genes whose transcription control in response to hypoxia requires the SP-1 transcription factor (27–29). This is novel and has not been shown in any type of cancer cell previously. In conclusion, our studies suggest that SP-1 expression plays a central role in hypoxia-induced VEGF expression, angiogenesis, and the consequent progression of tumor growth.

EXPERIMENTAL PROCEDURES

Lung Tissue Samples—Human lung tumor samples were obtained from patients undergoing surgery at Queen's Medical Centre in Nottingham between June 2010 and April 2011 with informed patient consent and under ethical approval for this use from Nottingham Research Ethics Committee One (REC 08/H0403). The samples were obtained from the School of Clinical Science tissue bank (REC 10/H0405/6) approved by the Trent Research Ethics Committee. Samples obtained from the surgical resection of 30 patients were composed of 14 adenocarcinoma, 15 squamous cell carcinoma, and 1 adeno/squamous carcinoma.

Fluorescence Triple Stain Immunohistochemistry and Data Analysis—Sections were taken from embedded lung tissue samples for control (preimmune IgG) and test (SP-1 and VEGF IgG) immunohistochemistry. Antigen retrieval was performed by boiling sections in 10 mM citric acid (pH 6) for 20 min. Test sections were blocked then incubated with rabbit anti-SP-1 (SC-14027; Santa Cruz Biotechnologies, Santa Cruz, CA) (1 μg/ml) and mouse anti-VEGF (SC-729; Santa Cruz) (1 μg/ml) with 10 ng/ml DAPI. Control sections were incubated with rabbit IgG and mouse IgG (R & D Systems, Abingdon, UK) at 1 μg/ml each then goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594 (Invitrogen UK). The images were taken on a Nikon 90i microscope with FITC (465–495-nm excitation) and Texas Red (540–580-nm excitation) filters. Seven fields of view were recorded for each test slide at 20× magnification. Control slide fluorescence was subtracted from test slide fluorescence for each image to give total fluorescence units for VEGF (Texas Red) and SP-1 (FITC).

Cell Culture—Human NSCLC cell lines, A549, NCI-H460, and NCI-H1299 were obtained from the ATCC collection via LGC and MOR/P were obtained from Prof. Penella Wool (University of Sheffield). All cell lines were grown in RPMI 1640 medium with 10% fetal bovine serum (Harlan, UK), 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin-B, 4 mM l-glutamine (Sigma) at 37 °C, 5% CO₂, and 1% O₂ humidity. Cultures were grown to 100% confluence and plated to 24-well tissue culture dishes at a density of 5 × 10⁴ cells/ml for 18 h. Culture medium was removed and replaced with RPMI 1640 with 4 mM l-glutamine (serum-free) for 8 h prior to transfection or 24 h prior to further assay. Normal human bronchial epithelial cells were obtained from Lonza (Wokingham, UK) at passage 3 and were grown to passage 5 in BEGM medium (Lonza, UK). Hypoxic conditions were created in a nitrogen-fed incubator at 37 °C, 100% humidity, 5% CO₂, and 1% O₂ for either 4 or 20 h.

Luciferase Reporter Gene Transfection and Assays—Luciferase reporter gene transfection and assays were performed as published previously (30).

RNA Isolation and RT-QPCR—RNA extraction and first strand cDNA synthesis and quantitative real time PCR were performed as described previously (JBC endothelin-1). Quantitative real time PCR was performed with the following primers sets: for VEGF, VEGF165aBF GAGCAAGACACAAAA-TCCC and VEGF165/189AR CCTCGGCGTTGTCACATCTG; for SP-1, SP-1F GCATGCACCTGCCCCTACTGTAAAGAC and SP-1R CGTTTGTGCCTCTGTAGCTCATCC; for SP-2, 2-SP-2F GCCTGAAATGCACCTGCCCTACTGTAAAGAC and SP-2R CGATTTGGACACACTGTCAGGATCATC; for SP-3, SP-3F GCACAGACGATGACCCCTTCTG and SP-3R GCAGAA-TCTATACCGTCTGTACT for GAPDH, GAPDH CGAGTCGAGCAATTCGGATGGTGA and GAPDH CGAGTCGAGCAATTCGGATGGTGA for SP-3, 3-SP-3F GCAGTGACGTGACCCCTTCTG and SP-3R GCAGAAG-TCTATACCGTCTGTACT for GAPDH, GAPDH CGAGTCGAGCAATTCGGATGGTGA and GAPDH CGAGTCGAGCAATTCGGATGGTGA for 18S ribosomal RNA, 18 S RNAF CGGCTACCCCAATCCCGA-GAA and 18S rRNA GCCTGGAATTACCGCGGGCT. All gene specific quantification was calculated as ΔΔct (target ct–housekeeping Cct) relative to control or untreated cell experiment control to give a final ΔΔct (test/ΔΔct (basal)). All ct calculations were performed by Stratagene, MxPro 3.2.

Protein Isolation and Western Blot Analysis—Protein extraction and Western blot analysis of SP-1 were performed as described previously (31).

Enzyme-linked Immunosorbant Assay—Cell lines were plated to 24-well plates and grown to 100% confluence, and the medium was replaced with RPMI 1640 with l-glutamine only for 24 h. ELISA for VEGF-A (R & D Systems, Abingdon, UK) was performed according to the manufacturer’s protocol. All assay points were performed in triplicate on 24-well plates in a final medium
volume of 500 μl. All of the measurements were normalized to cell counts after supernatants were taken for assay.

**mRNA Stability**—The cell lines were grown to 100% confluence, with (test) or without (control) 5 μg/ml of actinomycin D (Sigma-Aldrich) for 24 h. Total RNA was extracted, and RT-QPCR was performed with either VEGF or SP-1 cDNA primers as detailed above. All gene-specific quantification were calculated as Δct (target ct − housekeeping ct) with the 18 S RNA “housekeeping” primer set. mRNA was quantified as percentage of the zero hour sample (100%) for both control and actinomycin D-treated cells. mRNA decay rates were calculated as the time taken to reach 50% of 0 h control (t_{1/2}).

**Chromatin Immunoprecipitation Assay**—Cell lines were grown to 100% confluence in 150-cm² flasks, and the medium
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(A) 
(B) 
(C) 
(D) 

EGR-1

-85 ccGGGGCGGGccGGCCGGGgtccggccGGGGCGGAG

SP-1

-50

EGR-1

SP1m ccGGTTCGGGccGTTCGGGGgtccggccGTTCGGG

EGRm ccGGGGCGGGGtaGGGGCGGGGgtccgttaGGGGCGGA

(F) 
(G) 

(H) 
(I)
was removed and replaced with serum-free RPMI 1640 for 24 h. Chromatin was prepared using the ChIP IT Express kit (Active Motif, Rixensart, Belgium) following the manufacturer’s instructions. Briefly, the cells were “fixed” with 1% formaldehyde; chromatin was sheared in 1 ml of shearing buffer at 35% amplitude for 10 min (30 s on, 60 s off) at 3 °C in an Active Motif EpiShear Sonicator. Immunoprecipitations consisted of 2 μg of antibody, 20 μg of chromatin, protein G magnetic beads in 200 μl incubated at 4 °C for 18 h on a bottle roller. IPs were washed and treated as per the manufacturer’s instructions (“output” samples). 10 μl of sheared chromatin provided the input DNA. Quantitative real time PCR was performed with the following conditions; 1 × KAPA SYBR FAST (Anachem, Luton, UK), 1 μl betaine, 250 nm forward and reverse primers. Primers for −85 to +50 relative to the VEGF gene transcription start site were as in Ref. 32. Quantitative real time PCR was performed in a Stratagene Mx3000P® real time PCR thermo-cycler with 1 cycle of 95 °C for 3 min and 55 cycles of 95 °C for 5 s and 62 °C for 1 min, with fluorescence quantification for product quantitation during the 62 °C segment. Dissociation curves were performed for each reaction set to confirm that ct values were derived from a single PCR product. IP PCR products were quantified as Δct (output/input) for each experiment. Control QPCRs were performed with the “input” and “output” samples with identical PCR conditions except primers designed +8000 bp from the VEGF transcription start site with the following sequences, VEGF 8000+ F, GCAGCCATGTTTGGCTCAG and VEGF 8000+ R, GGAAGGAGCAGATCACAGAG. These “OFF-Chip” reactions act as controls for nonpromoter region-specific immunoprecipitation. Antibodies used were as follows; SP-1 (17–601; Millipore), SP-3 (SC-644; Santa Cruz Biotechnologies), polyacetyl-histone H4 (H6–598; Millipore), polyacetyl-histone H3 (H6–599; Millipore), histone H3 (SC-10809; Santa Cruz Biotechnologies), histone H4 (SC-8657; Santa Cruz Biotechnologies), PCAF (SC-8999; Santa Cruz Biotechnologies), CBP (SC-583; Santa Cruz Biotechnologies), and normal rabbit IgG (12–370; Millipore).

**Plasmid Constructs**—VEGF promoter reporter constructs (33), including VEGF-135 with EGR and SP-1-binding site mutations (34) were a kind gift from Prof. Dieter Marmé (Institute of Molecular Oncology, Tumor Biology Centre, Freiburg, Germany). The SP-1 reporter constructs containing SP-1-binding sites (plasmid 194) and the control vector (plasmid 191) were a kind gift from Prof. Jeffrey E. Kudlow (School of Medicine, University of Alabama at Birmingham). The SP-1 DNA-binding domain construct, pEBGN–SP1, and empty vector pEBGN were kind gifts from Dr. Gerard Thiel (University of Saarland, Saarbrücken, Germany). The SP1 overexpression vector, pCMV–SP1 was a kind gift from Dr. Gilles Pages (Institut de biologie du développement et cancer, University of Nice, Nice, France). The shRNA construct (pURSP-1) targeting SP-1 was a kind gift from Prof. J McCormick (Carcinogenesis Laboratory, Michigan State University).

**Data Analysis**—All of the assays were performed in triplicate with data presented as the standard errors of the mean. Statistical analysis of correlation was tested with a Pearson’s correlation coefficient test using GraphPad Prism (GraphPad, San Diego, CA). The p values were scored as significant for 0.01–0.05 (*), 0.001–0.01 (**), and <0.001 (***)

**RESULTS**

**NSCLC Cell Lines Secreted Greater Amounts of VEGF-A than Normal Human Bronchial Epithelial (NHBE) cells, VEGF Protein Secretion Required Active VEGF mRNA Transcription, and the Last 33 Base Pairs of the VEGF Promoter Were Required for VEGF Promoter Reporter Activity**—Serum-free NSCLC cell lines secreted more VEGF-A than equivalent numbers of primary normal human bronchial epithelial cells over 24 h. Among the NSCLC cell lines, VEGF output was ranked highest to lowest as A549<H460<H199<MOR/P (Fig. 1A). The addition of the RNA polymerase II inhibitor, actinomycin D, for 24 h prior to VEGF ELISA analysis, caused 80% depletion of VEGF-A secretion from all four NSCLC lines under basal conditions (Fig. 1B). Parallel analysis of VEGF-A mRNA demonstrates that there was 90% depletion of VEGF mRNA within 24 h of actinomycin D treatment (Fig. 1C). Total mRNA in each NSCLC cell line was proportional to VEGF protein secretion (Fig. 1D) with A549 having the lowest VEGF mRNA level and MOR/P the greatest VEGF mRNA quantity, with the exception of H460 possessing a relatively higher mRNA than VEGF protein when compared with H1299. All VEGF mRNA had a short half-life of <2 h, with little difference between the cell lines (Fig. 1E), suggesting that altered VEGF mRNA stability did not explain the difference in mRNA accumulation between cell lines. All four NSCLC cell lines were transfected with VEGF promoter reporter constructs from positions +50 to −2018 (2068), +50 to −1286 (1340), +50 to −789 (840), +50 to −414 (465), +50 to −265 (318), +50 to −85 (135), and +50 to −52 (102) (all numbers are positions relative to the VEGF-A gene transcription start site) and assayed for luciferase activity after 24 h. 35 bp (from −85 to −50 bp prior to the transcription start site) of the VEGF promoter were required for promoter reporter activity in all four cell lines (Fig. 2, A–D).

**SP-1 Transcription Factor-Binding Sites Were Required for Constitutive VEGF Promoter Activity in NSCLC, SP-1 Transcription Factor Reporter Activity Correlated with Increased VEGF mRNA Expression and SP-1 Overexpression Can Drive VEGF Promoter Activity**—The last 855 bp of the VEGF promoter (from −85 to −0 bp prior to the transcription start site)
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(A) Overexpression of SP-1 increases VEGF levels.

(B) Comparison of VEGF levels in different cell lines.

(C) Effect of SP-1 on VEGF expression in Morris hepatoma cell line.

(D) VEGF levels in different conditions.

(E) Comparison of VEGF expression under various treatments.
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possesses a single AP-1 transcription factor-binding site, two EGR transcription factor-binding sites, and three SP1 transcription factor-binding sites (Fig. 2E). Site-directed mutagenesis of the two EGR binding sites (VEGF-135-EGRm) did not affect VEGF-135 activity in all four cell lines (Fig. 2, F–I), sitedirected mutagenesis of all three SP1-binding sites (VEGF-135-SP1m) completely eliminated VEGF promoter reporter activity in all four NSCLC cell lines (Fig. 2, F–I). We have established that the NSCLC cell lines produce differing quantities of VEGF-A protein and that the order of increased VEGF secretion (A549 < H460 < H199 < MOR/P) was paralleled by increasing VEGF transcript (Fig. 1D). Analysis of VEGF promoter constructs has established that the SP1 binding within the last 85 bp prior to the transcription start site was essential for VEGF promoter reporter activity. Transfection of the “194” SP1 activity reporter gene construct (in parallel with the same vector with all SP1 sites inactivated by mutation, “191”) demonstrated that there is greater SP1 transcription factor activity in the order A549 < H460 < H199 < MOR/P (Fig. 3A), an order of activity reflective of both VEGF mRNA accumulation and protein secretion for a 24-h period for each cell line. To establish that increased SP1 activity could drive VEGF promoter activity, the 2068-VEGF and 135-VEGF promoter reporters were co-transfected with a vector overexpressing the SP1 protein (Fig. 3, B–E). In all four cell lines, SP1 overexpression increases VEGF promoter reporter activity.

Mithramycin A Treatment and Either Dominant Negative SP1 or SP1 Ribozyme Construct Co-transfection Decreased Constitutive VEGF Promoter Activity—The pentacyclosantibiotic mithramycin A blocks SP1 binding to DNA by virtue of its ability to disrupt protein binding to GC-rich DNA (such as the SP1 interaction with GGGGGcGcG binding site) (35). Treatment of all four NSCLC cell lines with mithramycin A for 24 h after medium replacement decreased VEGF-A secretion (Fig. 4A) and inhibited VEGF mRNA accumulation (Fig. 4B). We extended this observation by co-transfecting the VEGF-2068 and VEGF-135 promoter luciferase reporter constructs with the pEBGN-ΔSP1 plasmid expressing the DNA-binding domain (amino acids 592–758) of SP1. The SP1 C-terminal protein can bind to the SP1 recognition motifs of promoter DNA but by virtue of its inactivity will displace native SP1 transcription factor activity. In each NSCLC cell line, pEBGN-ΔSP1 decreased VEGF promoter activity for both VEGF-2068 and VEGF-135 (Fig. 4, C–F). The GC-rich binding site recognized and bound by SP1 can bind a large family of SP1-related proteins, the SP1/Kruppel-like factors (36). The suppression of VEGF expression and secretion by mithramycin A and the reduction of VEGF promoter activity by a transactivation-inactive SP1 construct does not eliminate the possibility that both have displaced an SP1/Kruppel-like factor family member and not SP1 itself from the VEGF promoter. The SP1 shRNA/ribozyme construct pUR-SP1 provides a direct method of SP1 inhibition by decreasing SP1 expression (37). Co-expression of the pURSP1 construct with the VEGF-2068 luciferase reporter for 48 h decreased VEGF promoter activity in A549, H460, H1299, and MORP cell lines (Fig. 4, G–J).

NSCLC Have Elevated SP1 Protein Levels, and SP1 Protein Association with the Native VEGF Promoter Is Greater in Cell Lines Expressing More SP1 Protein, Both Correlating with Increased Chromatin Remodelling and Histone Acetyltransferase Binding—To assess SP1 protein levels in NSCLC cell lines, total protein extracts were analyzed by Western blot with a comparison with an equivalent protein loading from NHBE cells (Fig. 5A). The SP1 protein was visible as a doublet of 96 and 102kDa. The NSCLC cell lines had a far higher level of SP1 protein than that of NHBE, and there were greater amounts of SP1 in H1299 and MORP cell lines than that of the A549 and H460 cell lines. The level of SP1 protein was in the order NHBE [tlt] A549 = H460 < H1299 < MORP. With elevated SP1 protein expression correlating with increased SP1 transcription factor activity and the VEGF promoter demonstrating dependence on SP1-binding sites within VEGF-reporter gene constructs, we sought to confirm that the native VEGF promoter has a greater association with SP1. ChIP assays were performed in all four cell lines with SP1 antisera. QPCR was performed from −85 to +50 bp of the VEGF promoter (relative to the transcription start site) against total chromatin and the output IPs. The VEGF promoter from all four cell lines associated with the SP1 protein with increased binding in cell lines expressing greater quantities of SP1 protein (H1299 and MOR/P) (Fig. 5B). “Off ChIP” control QPCR at +8000 bp (data not shown) showed no nonspecific chromatin SP1-interaction. Of the family of Kruppel-like factor transcription factors, SP3 has the same site specificity and affinity as SP1 (38), and the SP3 protein is constitutively expressed at the same level in all four cell lines (data not shown). To provide a further control for the correlation between SP1 expression and SP1 VEGF promoter binding, we performed ChIP for SP3 and found a reduction in SP3/VEGF promoter binding as SP1 binding increases (Fig. 5C). With the high sequence homology between the SP1 and SP3 transcription factors and similar affinities for GC rich binding site on the VEGF promoter, ChIP for SP3 provides a control for the specificity of SP1 ChIP, and this in turn supports SP1 binding correlated with greater SP1 expression. By virtue of its ability to compete for the same binding site as SP1, SP3 may act as an inhibitor of SP1 transcription factor activity (39). Although we have not further investigated the role of SP3 in VEGF expression, high SP3 binding correlates with low SP1 binding (Fig. 5C), and the least VEGF transcription of all four cell lines was studied. Transcription initiation is dependent upon an open, remodelled, chromatin structure around the transcription start site. Acetylation of core histones such as

FIGURE 3. NSCLC cells have increasing SP1 transcription factor activity in parallel with elevated VEGF secretion, and SP1 overexpression can increase NSCLC VEGF promoter activity. NSCLC cell lines were transfected with 0.1 µg of the SP1 transcription factor reporter, plasmid 194, and the control SP1 reporter, with all SP1 sites functionally mutated (plasmid 191), for 24 h before assaying for luciferase activity. A, SP1 transcription factor activity is expressed as a ratio of reporter to negative control (194/191). B–F, overexpression of SP1 increased VEGF promoter-reporter construct activity in NSCLC cell lines A549 (B), H460 (C), H1299 (D), and MORP (E). NSCLC were co-transfected with 0.1 µg of VEGF-2068-luciferase or VEGF-135-luciferase plasmids with 1.0 µg of pCDNAIII empty vector or pCDNA-SP1 for 24 h followed by a luciferase assay. All of the measurements represent the means ± S.E. of three independent experiments. RLU, relative light units.
FIGURE 4. Inhibition of SP-1 DNA binding, introduction of a nonfunctional SP-1 protein (ΔSP-1) or an SP-1 shRNA construct will prevent VEGF secretion and transcription. A and B, NSCLC, VEGF secretion (A) and mRNA accumulation (B) are inhibited in by mithramycin A. NSCLC were treated with 1 · 10^{-6} M mithramycin A for 24 h, and the supernatants were assayed by ELISA for VEGF. Total RNA was extracted, and the levels of VEGF mRNA were assessed by RT-QPCR with reference to GAPDH as a fold over untreated control. Open bars, A549; gray bars, H460; lined bars, H1299; black bars, MORP. NSCLC were co-transfected with 0.1 µg of VEGF-2068-luciferase or VEGF-135-luciferase plasmids with 1.0 µg of SP-1 DNA-binding domain construct (ΔSP-1) for 24 h followed by a luciferase assay. C–F, A549 (C), H460 (D), H1299 (E), and MORP (F). NSCLC were co-transfected with 0.1 µg of VEGF-2068-luciferase (2068) with 0.25 or 0.5 µg of SP-1 shRNA construct (pURSP-1) for 48 h followed by a luciferase assay. G–J, A549 (G), H460 (H), H1299 (I), and MORP (J). All of the measurements represent the means ± S.E. of three independent experiments. RLU, relative light units.

FIGURE 5. NSCLC cells express higher levels of SP-1 total cell protein than normal human bronchial epithelial cells. Native SP-1 transcription factor binding to the VEGF promoter increases with VEGF expression as SP-3 transcription factor binding decreases. Increased SP-1 binding is paralleled by increased histone H4 acetylation and histone acetyltransferase binding to the VEGF promoter of NSCLC cells. A, 30 µg of total cellular protein from NHBE and A549 were Western blotted and probed with an anti-SP-1 antibody (left panel). 10 µg of total cellular protein from A549, H460, H1299, and MORP were Western blotted and probed with an anti-SP-1 antibody (right panel), and the blots were stripped and reprobed with an anti-GAPDH antibody. B and C, the images are representative blots from experiments repeated three times. ChIP was performed for transcription factors SP-1 (B) and SP-3 (C) from chromatin samples from NSCLC cells maintained in serum-free medium for 24 h. IP and input DNAs were subjected to QPCR for the VEGF promoter between −85 and + 50 bp relative to the transcription start site of the VEGF promoter. Control ChIP was performed with equivalent rabbit IgG for all chromatin samples. D, ChIP for identical samples and their relevant control IgGs (open bars) were performed for acetylated (black bars) and nonacetylated histone H4 (cross-hatched bars), acetylated (diagonal line bars), and nonacetylated histone H3 (horizontal line bars). E, p300 and histone acetyltransferase, F, P/CAF and histone acetyltransferase, G, CBP and histone acetyltransferase. All of the measurements represent the means ± S.E. of three independent experiments.
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Histone H4 and histone H3 by the histone acetyltransferase family (such as CBP, p300, and PCA/F) is a component of the remodeling process. We compared the ChIP products for acetyl-H4, acetyl-H3, total H4, and total H3 at the VEGF promoter (Fig. 5D). Active VEGF gene transcription and SP-1 binding was associated with an increase in histone H4 acetylation and a small increase in histone H3 acetylation. Increased histone acetylation was paralleled by increased histone acetyltransferase binding at the active promoter. We have found that there was increasing PCA/F (Fig. 5F) and CBP (Fig. 5G) binding across all four cell lines correlated with greater VEGF transcription and VEGF secretion. “Off-ChIP” controls confirmed that these binding events were localized to the proximal VEGF promoter (data not shown).

VEGF and SP-1 Protein Co-localize and Expression Levels Show a Strong Correlation in Human NSCLC Tumor Tissues—NSCLC tumor samples were analyzed for VEGF and SP-1 protein expression by dual fluorescence immunohistochemistry (Fig. 6, A and B). VEGF co-localizes with SP-1 in squamous cell carcinoma (Fig. 6A) and in adenocarcinoma (Fig. 6B). VEGF was cytoplasmic in distribution, and SP-1 is nuclear in distribution. Total fluorescence counts for Texas Red (VEGF) and FITC (SP-1) were taken, and the background counts for each slide were subtracted from parallel slice controls incubated with pre-immune IgG (seven fields of view for each patient sample). VEGF and SP-1 total fluorescence units were analyzed by Pearson’s correlation coefficient test, giving a Pearson R value of 0.503, p ≤ 0.0001, suggesting a highly significant correlation between VEGF and SP-1 protein expression levels in NSCLC tissue of both squamous and adenocarcinoma morphology (Fig. 6C).

The SP-1 Transcription Factor-binding Site of the Minimal VEGF Promoter Is Required for Hypoxia-induced VEGF Expression in NSCLC with SP-1 Transcription Factor Activity and SP-1 Binding, to the VEGF Promoter, Increased during NSCLC Hypoxia—NSCLC cell lines were incubated for 20 h under normoxic and hypoxic conditions with a VEGF ELISA performed on the resulting culture supernatants (Fig. 7A). The total hypoxia-stimulated VEGF output (hypoxia-normoxia) increased from A549 to MORP (Fig. 7B), and hypoxia increased VEGF mRNA accumulation in all four cell lines (Fig. 7C). With SP-1 central to basal VEGF transcription, we investigated the role of SP-1 in hypoxia-induced VEGF transcription. Pretreatment with mithramycin A decreased the hypoxic accumulation of VEGF mRNA in all four cell lines, implying a role for SP-1 in the hypoxic induction of VEGF mRNA accumulation (Fig. 7D). The minimal VEGF promoter contains three essential SP-1-binding sites (Fig. 2). NSCLC were transfected with the all of the VEGF promoter reporter constructs followed by 20 h of normoxia or hypoxia with subsequent luciferase assay for each cell line (Supplemental data S1), the minimal hypoxia-induced VEGF promoter was within the VEGF-135 construct. To establish what role SP-1 has in hypoxia-induced VEGF transcription the wild-type, SP-1 mutant, and EGR mutant VEGF-135-luciferase constructs were transfected into NSCLC cell lines, and the luciferase activity was measured after exposure to normoxic or hypoxic conditions for 20 h. The VEGF-135 construct had no hypoxic response without SP-1-binding sites (Fig. 7, E–H). To confirm that a cellular hypoxic response had taken place, the hypoxia response element luciferase plasmid (40) was transfected into each NSCLC cell line, and a luciferase assay was performed after 20 h of hypoxia (Supplemental data S2). If SP-1 plays a role in hypoxia-induced VEGF transcription, then the activity of SP-1 and binding of SP-1 to the VEGF promoter should increase under hypoxic conditions. NSCLC cells were transfected with the SP-1 reporter-luciferase construct (194) and its control vector (191) with 20 h of normoxia or hypoxia. In all four cell lines, hypoxia increased SP-1 reporter gene activity (Fig. 7, I–L). Chromatin immunoprecipitation assays were conducted for SP-1 (with control preimmune IgG) against the VEGF promoter with chromatin from normoxia or 4-h hypoxia-treated A549 or H1299 cell lines. The A549 cell line had the lowest SP-1 activity and SP-1 basal binding to the VEGF promoter (Figs. 3A and 5A) with the H1299 cell line the highest in both cases. Hypoxia induced a small but significant binding of SP-1 to the native VEGF promoter in A549 cells. In contrast there was a large increase in SP-1 binding to the native VEGF promoter in H1299 cells (Fig. 7M).

DISCUSSION

Immunohistochemical studies of non-small cell lung cancer tumors have established that there is a strong correlation between tumor-associated blood vessel formation, an increase in microvascular density, and the expression of VEGF-A protein (2–5). The aim of this study was to provide a molecular mechanism for the observed increased expression of VEGF in non-small cell lung cancer tumor and assess the role of this in the general mechanism of hypoxic regulation of VEGF expression. There are a number of novel features, some of which give greater insight into the mechanisms involved in VEGF secretion in lung cancer and some of which have more general novelty in respect to the role of SP-1 in hypoxic induction of VEGF.

With regard to fresh insight into the mechanisms involved in VEGF secretion in lung cancer, our study is the first to demonstrate that under basal conditions in NSCLC cells, the VEGF promoter is SP-1-dependent and that NSCLC cells express higher levels of active SP-1 protein than normal cells. We are also the first to establish in any cancer that increased SP-1 binding to the native VEGF promoter correlates with increased histone acetyltransferase binding and histone H4 acetylation. Furthermore, we are the first to demonstrate that there is a strong correlation between VEGF and SP-1 protein levels in human NSCLC tumor tissue.

**FIGURE 6. VEGF and SP-1 protein expression correlate and co-localize in human non-small cell lung cancer tumors.** Immunohistochemistry, 30 patient tumor samples were triple stained with anti-VEGF antibodies (Texas Red), anti-SP-1 antibodies (FITC), and DAPI. A, an example squamous cell carcinoma shows a combined triple image (upper left), DAPI (upper right), cytoplasmic VEGF (lower left, Texas Red filter), and nuclear SP-1 (lower right, FITC filter). B, an example adenocarcinoma shows a combined triple image (upper left), DAPI (upper right), cytoplasmic VEGF (lower left, Texas Red filter), and nuclear SP-1 (lower right, FITC filter). C, total VEGF and total SP-1 fluorescence measurements from 30 tumors (seven random fields of view, with preimmune antibody control fluorescence subtracted) were analyzed by a two-tailed Pearson correlation plot giving a Pearson R score of 0.503 and P score of <0.0001. TFU, total fluorescence units.
With regard to more generalizable biological novelty, we have shown for the first time a central role for SP-1 in hypoxia-induced VEGF expression. SP-1-binding sites were required for hypoxic induction of VEGF promoter reporter constructs, hypoxia-increased SP-1 activity, and hypoxic induction of SP-1 binding to the VEGF promoter. This is the first such observation of a role for SP-1 in hypoxia regulation of VEGF in any type of cancer.

Our observation of lower VEGF secretion from NHBE than NSCLC is from a single NHBE cell line. Although it could be argued that this reflects genetic variation, this is unlikely, because Lee et al. (41) and Takayama et al. (42) demonstrated that noncancerous airway epithelial cells secrete less VEGF than NSCLC cells.

In our study deletion analysis of the VEGF-A promoter defined constitutive activity in NSCLC cells in the last 85 bp prior to the transcription start site. Mutation of the three SP-1-binding sites, within this region, eliminates all promoter activity, and mutation of interdigitating EGR-1 binding sites did not affect VEGF promoter reporter activity. In a recent study Shimoyamada et al. (43) defined a similar region of the VEGF promoter when studying constitutive VEGF promoter activity in NSCLC cell lines (one of which, A549, forms part of this study). In their study all cells were cultured in 10% fetal calf serum, and the authors point out that growth factors such as EGF and PDGF (found in fetal calf serum) can induce EGR-1 activity (44–46). In contrast our study was conducted in serum-free conditions to remove environmental factors and better define any constitutive genetic factors responsible for VEGF expression. Under serum-deprived conditions, the SP-1-binding sites were essential for VEGF expression, and mutation of the EGR-1 binding site had no effect. Therefore it is likely that EGR-1 plays a role in VEGF expression in NSCLC, in response to growth factors present in the tumor microenvironment, whereas SP-1 overexpression and increased activity drives a high base-line VEGF expression in NSCLC tumor tissue. There has been only one previous study showing an increase in SP-1 expression in human lung tumors that showed that high SP-1 protein levels were correlated with CD147 expression (a cancer biomarker glycoprotein with a role in tumor metastasis) (47). The CD147 promoter possesses four SP-1-binding sites, and these studies showed that treatment with mithramycin or an SP-1 siRNA reduces CD147 RNA accumulation. Furthermore plasmid-driven SP-1 overexpression increased CD147 mRNA accumulation in a similar manner to our study of the VEGF promoter. Increased expression of the SP-1 correlates with a poor prognosis in multiple cancer types (48–52). In pancreatic and gastric carcinomas, there was a positive correlation between SP-1 and VEGF expression levels. High SP-1 expression was also found to correlate with components of cellular invasiveness in breast cancer and glioma. Thyroid cancer SP-1 expression levels are linked to expression of the sodium/iodide transporter (reducing the efficacy of radio-iodide therapy). The SP-1 transcription factor can interact with the promoters of many genes involved in self-sufficiency in growth, limitless replication, lack of anti-growth signals, lack of apoptosis, metastasis, deregulated angiogenesis, and suppression of immune surveillance (39); for this reason the consequences of SP-1 overexpression are far reaching in terms of malignant cell transformation. Lou and colleagues (37) overexpressed SP-1 in human fibroblasts leading to the creation of fibrosarcoma. Subsequent removal of SP-1 with an SP-1 ribozyme caused fibrosarcoma reversion to a nonmalignant state.

To date there is very little molecular detail of the epigenetic changes that take place at the VEGF promoter in NSCLC or other cancers. In this study we have shown that the VEGF promoter binds the histone acetyltransferases CBP and PCA/F. Localized histone polyacetylation is a required modification for active transcription; in this study we show that polyacetylation occurs at the VEGF promoter in all four NSCLC cell lines, specifically at histone H4 with polyacetylation between 5 and 25 times that of histone H3 at the VEGF promoter. This observation is promoter-specific because QPCR analysis of the same ChIP immunoprecipitations, at the SP-1 gene promoter (data not shown), have greater polyacetylation at histone H3 than at histone H4.

There is strong evidence, both histochemical and biochemical, for the occurrence of hypoxia in lung tumors (6). In this study, we have identified SP-1 overexpression as a cause of deregulated VEGF expression and have demonstrated that SP-1 plays a key role in hypoxia-induced VEGF expression from the same cell lines; however, we have not fully defined the mechanism of SP-1-dependent hypoxia-induced VEGF transcription. The study of colon cancer by Mizukami et al. (22) defined a region of the VEGF promoter capable of HIF-1α-independent hypoxic stimulation that contained all of the three SP-1-binding sites we have characterized, although further direct characterization of the role of SP-1 in hypoxia-induced VEGF expres-
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In summary, constitutive VEGF secretion in NSCLC cells is dependent upon the SP-1 transcription factor activity under normoxic and serum-free conditions in a panel of NSCLC cell lines. VEGF mRNA expression correlated with SP-1 transcription factor activity and SP-1 binding to the native VEGF promoter in NSCLC cells. NSCLC cells express high levels of the SP-1 protein when compared with the levels present in normal cells, and VEGF/SP-1 protein levels have a significant correlation in human non-small cell lung cancer tumors. Of far greater significance to general mechanisms of hypoxia-induced gene expression is our finding that hypoxia increased SP-1 activity, and binding to the VEGF promoter is required for hypoxia-induced VEGF expression. These studies suggest that SP-1 will be an important target in future cancer angiogenesis research.

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