Production of Experimental Malignant Pleural Effusions Is Dependent on Invasion of the Pleura and Expression of Vascular Endothelial Growth Factor/Vascular Permeability Factor by Human Lung Cancer Cells

Seiji Yano, Hisashi Shinohara, Roy S. Herbst, Hiroki Kuniyasu, Corazon D. Bucana, Lee M. Ellis, and Isaiah J. Fidler

From the Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

We determined the molecular mechanisms that regulate the pathogenesis of malignant pleural effusion (PE) associated with advanced stage of human, non-small-cell lung cancer. Intravenous injection of human PC14 and PC14PE6 (adenocarcinoma) or H226 (squamous cell carcinoma) cells into nude mice yielded numerous lung lesions. PC14 and PC14PE6 lung lesions invaded the pleura and produced PE containing a high level of vascular endothelial growth factor (VEGF)-localized vascular hyperpermeability. Lung lesions produced by H226 cells were confined to the lung parenchyma with no PE. The level of expression of VEGF mRNA and protein by the cell lines directly correlated with extent of PE formation. Transfection of PC14PE6 cells with antisense VEGF165 gene did not inhibit invasion into the pleural space but reduced PE formation. H226 cells transfected with either sense VEGF 165 or sense VEGF 121 genes induced localized vascular hyperpermeability and produced PE only after direct implantation into the thoracic cavity. The production of PE was thus associated with the ability of tumor cells to invade the pleura, a property associated with expression of high levels of urokinase-type plasminogen activator and low levels of TIMP-2. Collectively, the data demonstrate that the production of malignant PE requires tumor cells to invade the pleura and express high levels of VEGF/VPF. (*Am J Pathol* 2000, 157:1893-1903)

More than 171,000 new cases of lung cancer are diagnosed annually in the United States, and its mortality rate of nearly 90% makes it the leading cause of cancer-related death.¹ Non-small-cell lung cancer (NSCLC) represents 70 to 80% of cases.² The end stage of NSCLC is associated with distant metastasis and the formation of

malignant pleural effusion (PE),^{3,4} leading to significant morbidity from progressive dyspnea, cough, and pain.^{5,6} The production of malignant PE correlates with impaired drainage of the pleural space due to obstruction of blood vessels and lymphatics of the lung and pleura, and increased formation of pleural fluid.⁷ Treatment of malignant PE consists of drainage by chest tube induction of pleural sclerosis by instillation of antibiotics, antiseptics, or antineoplastics.^{7,8} The results, unfortunately, are variable, as the procedure does not prolong survival.⁷ A better understanding of the molecular mechanisms that regulate the formation of malignant PE may offer ways to design novel effective therapies.

Considering its role in controlling cell permeability, vascular endothelial growth factor (VEGF) is a potent mediator. VEGF is a 35- to 43-kd dimeric polypeptide expressed in several isoforms (121, 165, 189, and 206 amino acids) resulting from alternative mRNA splicing of a single gene.⁹ Initially discovered because of its ability to increase vascular permeability, the molecule was first called vascular permeability factor (VPF).¹⁰ VEGF/VPF is a pivotal angiogenic factor mediating developmental, physiological, and pathological neovascularization.^{9,11,12} VEGF/VPF stimulates the proliferation and migration of endothelial cells and also induces expression of metalloproteinases and plasminogen activity by these cells.¹³⁻¹⁶ Recent reports indicate that VEGF/VPF plays a central role in formation of ascites in animal models.¹⁷⁻²⁰ Work from our laboratory showed that the expression level of VEGF/VPF by human ovarian cancer cells directly correlates with production of ascites.¹⁹ Increased permeability of blood vessels facilitates the extravasation of proteins and, thus, the formation of ascites.¹⁷ Similarly, the level of VEGF/VPF in human malignant PE correlated with the

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Address reprint requests to Isaiah J. Fidler, D.V.M., Ph.D., Department of Cancer Biology, Box 173, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. E-mail: ifidler@notes.mdacc.tmc.edu.

volume of PE. Whether expression of VEGF/VPF is solely responsible for formation of PE, however, remained unclear.

The purpose of this study was to determine the molecular mechanisms that regulate the pathogenesis of malignant PE by human NSCLC implanted orthotopically in nude mice. Using human lung adenocarcinoma and squamous carcinoma cells with different invasive properties and expression levels of VEGF/VPF, we demonstrate that PE formation requires both invasion of the pleural space by tumor cells and expression of VEGF/VPF.

Materials and Methods

Cell Lines

The human lung adenocarcinoma cell line PC14 was obtained from Dr. N. Saijo (National Cancer Center Research Institute, Tokyo, Japan).²¹ The selected PC14PE6 line was established from PE developed in a nude mouse injected intravenously (i.v.) with parental PC14 cells.²¹ Recent karyotypic analysis of the PC14PE6 cells ruled out contamination with murine cells (personal communication, Dr. S. Pathak, M. D. Anderson Cancer Center, Houston, TX). The human lung squamous cell carcinoma cell line H226 was obtained from Dr. J. D. Minna (University of Texas Southwestern Medical School, Dallas, TX).²² Tumor cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, twofold vitamin solution, and penicillin-streptomycin (CMEM; Flow Laboratories, Rockville, MD) and incubated at 37°C in 5% CO₂/95% air. The cultures were free of *Mycoplasma* and pathogenic murine viruses (assayed by Microbiological Associates, Bethesda, MD).

Reagents

Recombinant human (rh) VEGF165 and VEGF121 proteins and anti-human VEGF polyclonal Ab were purchased from R&D Systems (Minneapolis, MN).

Mice

Athymic Ncr-nu/nu male mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in a barrier-type facility approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Departments of Agriculture and Health and Human Services and the National Institutes of Health.

Model for PE

Monolayer cultures of PC14 and PC14PE6 cells were harvested by pipetting. H226 cells were harvested after a

2-minute exposure to a 0.25% trypsin-0.02% EDTA solution. The cells were washed twice with Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution (HBSS) and resuspended in HBSS. Cell viability was determined by the trypan blue dye exclusion test, and only single-cell suspensions of >90% viability were used for the *in vivo* studies. To produce lung lesions and PE, tumor cells (1 × 10⁶/300 μl HBSS) were injected into the lateral tail vein of unanesthetized nude mice.²¹ In some experiments, tumor cells were injected directly into the thoracic cavity (intrathoracic injection; i.t.).²¹ At different times after tumor cell injection, mice were euthanized, the subclavian artery was severed, and blood was harvested. The chest wall was carefully resected, and PE was harvested and measured using a 1-ml syringe. The blood and PE were centrifuged for 20 minutes (100 × g) at 4°C. The serum and supernatant of PE were stored at -70°C until an enzyme-linked immunosorbent assay (ELISA) was performed. The lungs were fixed in Bouin's solution, and the lung tumor lesions were counted under a dissecting microscope.

mRNA Analysis

Polyadenylated mRNA was extracted from 10⁷ to 10⁸ tumor cells growing under sparse (<100 cells/mm²) and confluent (>1200 cells/mm²) conditions using the Fast-Track mRNA isolation kit (Invitrogen, San Diego, CA). In some experiments, total RNA was extracted from 1 × 10⁶ tumor cells from confluent cultures using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). Total RNA (10 μg) or mRNA (3 μg) was electrophoresed on a 1% denaturing formaldehyde/agarose gel, transferred to a GeneScreen nylon membrane (DuPont Co., Boston, MA), and UV cross-linked with 120,000 μJ/cm² using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Hybridizations were performed as described previously.^{16,19} Nylon filters were washed twice at 60 to 65°C with 0.1% standard saline citrate (SSC) and 0.1% sodium dodecyl sulfate. The mRNA expression level was quantified by densitometry of autoradiographs with the use of Image Quant software (Molecular Dynamics, Sunnyvale, CA); the value for each sample was taken to be the ratio of the average of the areas for the specific mRNA transcripts to the area of the 1.3-kb GAPDH mRNA transcript in the linear range of the film.

Determination of VEGF/VPF, Basic Fibroblast Growth Factor (bFGF), and Interleukin-8 (IL-8) Protein Level

The protein level of VEGF/VPF, bFGF, and IL-8 in culture supernatants, mouse serum, and PE was determined using ELISA kits according to the manufacturer's instructions (R&D Systems). Preliminary experiments confirmed that the ELISA kit for VEGF/VPF detected both VEGF165 and VEGF121 proteins.

In Situ mRNA Hybridization Technique

Specific oligonucleotide DNA probes in the antisense orientation were designed complementary to the mRNA transcripts of the VEGF/VPF genes based on published reports of the cDNA sequence TGGTGATGTTGGACTC-CTCAGTGGGCU, 57.7% guanosine-cytosine (GC) content. The specificity of the oligonucleotide sequence was initially determined by a GenBank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (GCG, Madison, WI) based on the FastA algorithm. The specificity of the sequence was confirmed by Northern blot analysis. A poly d(T)₂₀ oligonucleotide was used to verify the integrity of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3' end via direct coupling, with the use of standard phosphoramidite chemistry (Research Genetics, Huntsville, AL). The staining for *in situ* mRNA hybridization was performed exactly as described previously.²³ A positive enzymatic reaction in this assay stained red. Known positive controls were used in each hybridization reaction. Controls for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and use of chromogen alone.

Histology and Immunohistochemistry

Lungs of nude mice harvested at necropsy were cut into 5-mm fragments and placed into either buffered 10% formalin solution or OCT compound (Miles Laboratories, Elkhart, IN) to be snap-frozen in liquid nitrogen. For VEGF/VPF staining, tissue sections (4 μ m thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, transferred to PBS, and treated with pepsin for 20 minutes at room temperature. For CD31 staining, frozen tissue sections (8 μ m thick) were fixed with cold acetone. The slides were rinsed twice with PBS and endogenous peroxidase was blocked by use of 3% hydrogen peroxide in PBS for 12 minutes. The samples were washed three times with PBS and incubated for 10 minutes at room temperature with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum. Excess blocking solution was drained, and the samples were incubated for 18 hours at 4°C with a 1:400 dilution of rabbit polyclonal anti-VEGF/VPF Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:100 dilution of rat monoclonal anti-CD31 Ab (Pharmin-gen, San Diego, CA). The samples were then rinsed four times with PBS and incubated for 60 minutes at room temperature with the appropriate dilution of peroxidase-conjugated anti-rabbit IgG or anti-rat IgG. The slides were rinsed with PBS and incubated for 5 minutes with diaminobenzidine (Research Genetics). The sections were then washed three times with distilled water and counterstained with Gill's hematoxylin. Sections (4 μ m thick) of formalin-fixed, paraffin-embedded tumors were also stained with hematoxylin-eosin for routine histological examination.

Zymography

Tumor cells were cultured for 24 hours in MEM containing 10% FBS. The cultures (30–50% confluence) were washed twice with PBS and incubated in serum-free MEM for another 24 hours, when the conditioned medium was harvested, spun at 1000 $\times g$ for 10 minutes, and used for substrate gel electrophoresis.²⁴ The samples were normalized by protein content and applied without reduction to a 7.5% polyacrylamide slab gel impregnated with 1 mg/ml gelatin (Sigma, St. Louis, MO). After electrophoresis, the gel was washed at room temperature for 30 minutes in washing buffer (50 mmol/L Tris-HCl, pH 7.5, 2.5% Triton X-100) and incubated overnight at 37°C with shaking in the developing buffer (50 mmol/L Tris-HCl, pH 7.5, 15 mmol/L CaCl₂, 1 μ mol/L ZnCl₂, 1% Triton X-100). The gel was stained with a solution of 0.5% Coomassie brilliant blue R-250. Clear zones against the blue background indicated the presence of gelatinolytic activity.

Vascular Density

The density of blood vessels in lung lesions was determined in tissue sections stained with anti-CD31 antibodies. Areas containing the highest number of capillaries and small venules were identified by scanning the tumor sections at low power (40 $\times$). Individual vessels were counted in multiple 100 $\times$ fields (10 $\times$ objective and 10 $\times$ ocular; 0.145-mm²/field). According to the criteria described by Weidner et al,²⁵ observation of a vessel lumen was not required for a structure to be classified as a vessel.

Vascular Permeability Assay

To evaluate permeability of vessels in the thoracic cavity, we injected mice that had lung tumors i.v. with 200 μ l of 0.5% Evans blue dye, which binds to endogenous serum albumin as a tracer.^{26–28} Thirty minutes later, the diaphragm was harvested and dye extravasation was evaluated and photographed using an Axioplan2 microscope (Carl Zeiss Inc., Thornwood, NY) equipped with an HBO 100 Hg lamp at excitation 546 λ and emission 590 λ .

To evaluate the bioactivity of VEGF/VPF secreted by tumor cells, we used the Miles assay adapted to the mouse skin.²⁷ To reduce any individual variation, nude mice without downy hair were kept apart during the assay. The mice were injected i.v. with 200 μ l of 0.5% Evans blue dye. Ten minutes later, serum-free culture supernatants of tumor cells (10⁶ cells/48 hours in MEM 50 μ l) were injected intradermally in several rows on the dorsal skin. Thirty minutes later, the mice were killed, the skin removed, and test sites were photographed. Each sample was tested in three different mice.

VEGF/VPF Isoforms

First-strand cDNA was synthesized from 1 μ g total RNA using a First Strand Synthesis Kit (Pharmacia, Piscataway, NJ) in 33 μ l of reaction mixture, according to the

Table 1. Production of Lung Lesions and PE by Human NSCLC Cells in Nude Mice

Route of injection	Cell line	Lung lesions			PE			Survival (days)
		Incidence	Number		Incidence	Volume (μl)		
			Median	Range		Median	Range	
i.v.	PC14	5/10	6	0–15	4/10	<20	<20–700	93–120
	PC14PE6	20/20	48	28–>150	20/20	790	250–1000	42–51
	H226	10/10	>150	107–>150	0/10	All <20		84–97
i.t.	PC14PE6	9/9	10	3–37	9/9	800	690–1040	14–24
	H226	10/10	11	9–24	0/10	All <20		42–45

Tumor cells (1×10^6) were injected intravenously (i.v.) or intrathoracically (i.t.) into nude mice. When the mice became moribund, they were killed, and development of lung lesions and PE were evaluated.

manufacturer's instructions. The synthesized first-strand cDNA (1μ l) was amplified by polymerase chain reaction (PCR) in a final volume of 50μ l containing 10 mmol/L Tris-HCl, 3 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.01% gelatin, 200 mmol/L dNTP, 50 pmol of each primer, and 2.5 U of AmpliTaq Gold *Taq* polymerase (Perkin Elmer, Foster City, CA). Sequences of VEGF/VPF primers used were sense, 5'-TCCAGGAGTACCCTGATGAG-3' and anti-sense, 5'-CTTTCCCTGGTGAGAGATCTGG-3' immediately flanking the region of the VEGF/VPF open reading frame involved in the alternative splicing of several exons. PCR amplification of VEGF/VPF cDNA was performed under the following conditions: 30 cycles of 1 minute at $94^\circ C$, 1 minute at $58^\circ C$, 1 minute at $72^\circ C$. Before the first cycle, an 8-minute denaturation step at $95^\circ C$ was included, and after 30 cycles the extension step was prolonged to 7 minutes at $72^\circ C$.²⁹

Subcloning of the VEGF165 Gene into pcDNA3 and Transfection Procedures

The full-length cDNA for VEGF165, a gift from Dr. Judith Abrahams (Scios Nova, Mountain View, CA), was subcloned into the *Bam*HI site of pcDNA3, a eukaryotic expression vector driven by the human cytomegalovirus promoter (Invitrogen). Subcloning into the *Bam*HI restriction site yielded an insert in the sense orientation. The orientation and proof of completeness of the insert were determined by DNA sequencing. Sense VEGF121 expression vector was obtained as described previously.²⁹

H226 cells plated at a density of 2×10^5 cells/100-mm dish were transfected and incubated for 12 hours with the sense VEGF165 or sense VEGF121 genes using a stable mammalian transfection kit (Stratagene, La Jolla, CA), and then washed and fed with fresh CMEM. Selection for resistance to neomycin was begun 48 hours after transfection by adding 250 μ g G418/ml (Life Technologies, Grand Island, NY). This medium was replaced every 3 days, and 3 weeks later, single G418-resistant colonies were transferred to individual wells of a 48-well plate. The expression of VEGF/VPF in individual clones was identified by Northern blot analysis.

Statistical Analysis

The significance of differences in microvessel density and the *in vitro* data was analyzed by the Student's *t*-test

(two-tailed). Differences in the number of lung lesions and in volume of PE were compared by using the Mann-Whitney *U* test.

Results

Formation of Lung Lesions and PE by Human NSCLC Cells

In the first set of experiments, human NSCLC cells were injected i.v. into nude mice. The production of lung lesions and induction of PE are summarized in Table 1. Five of 10 mice receiving an i.v. injection of PC14 cells had lung lesions measuring 1 to 4 mm in diameter (median survival, 110 days; range, 93–120 days). Four of the tumor-positive mice developed bloody PE ($0.5\text{--}1.6 \times 10^6$ RBC/ μ l PE). All 20 mice given an i.v. injection of the highly aggressive variant PC14PE6 cell line developed rapidly progressive lesions at the surface of the lung. Some of the lesions >5 mm in diameter invaded the pleural space. Moreover, all of the mice injected with the PC14PE6 cells developed bloody PE ($0.9\text{--}2.2 \times 10^6$ RBC/ μ l PE). Many of these mice had severe symptoms such as cyanosis and tachypnea. The median survival of the mice in this group was 45 days. All mice receiving an i.v. injection of H226 cells developed numerous 1- to 2-mm lesions within the lung parenchyma. No lesions extended into the pleural cavity. These mice had no PE and survived more than 12 weeks (median survival, 92 days; range, 84–97 days). The growth pattern of the PC14PE6 adenocarcinoma cells and the H226 squamous carcinoma cells recapitulated that found in patients. Lung squamous cell carcinomas tend to grow in the center of the lungs and are late to produce metastasis, whereas lung adenocarcinomas are usually located at the periphery of the lung and spread toward the pleural surface.²

To determine whether the absence of PE in mice with numerous H226 lung lesions was due to their inability to invade and grow in the pleural space, we injected H226 or PC14PE6 cells directly into the pleural space of nude mice. By 6 weeks after injection, the H226 cells produced lung lesions and solid tumors in the pleural space, but none of the mice developed PE (Table 1). In sharp contrast, 4 weeks after the i.t. injection of PC14PE6 cells, all mice had progressive tumor lesions in the lung and pleura with a large volume of PE (Table 1). These data

show that regardless of the route of injection, PC14PE6 cells produce PE, whereas H226 cells do not.

Expression of Matrix Metalloproteinases (MMPs), Tissue Inhibitors of Metalloproteinases (TIMPs), and Urokinase-Type Plasminogen Activator (uPA)

Since the invasive potential of tumor cells is regulated by the balance between MMPs and their inhibitors, TIMPs,³⁰ we evaluated the expression of MMP-2 and -9 in sparse and confluent cultures of PC14, PC14PE6, and H226 cells by gelatin zymography. The expression of uPA, TIMP-1, and TIMP-2 was determined by Northern blot analysis (Figure 1). PC14 and PC14PE6 cells constitutively expressed MMP-2 protein, whereas H226 cells expressed both MMP-2 and -9 proteins (Figure 2A). The highly invasive PC14 and PC14PE6 cells expressed high levels of uPA and TIMP-1, but not of TIMP-2, the inhibitor of MMP-2 (Figure 2B). In contrast, the H226 cells (low invasive potential) expressed low levels of TIMP-1, high levels of TIMP-2, and no uPA (Figure 2B).

In Vitro Expression of Angiogenic Cytokines by NSCLC Cells

We previously reported that the expression level of VEGF/VPF was directly associated with production of ascites by human ovarian cancer cells.¹⁹ Using Northern blot and ELISA, we next determined whether formation of PE in nude mice directly correlated with VEGF/VPF production by the NSCLC cells. Since expression of VEGF/VPF is cell density-dependent,³¹ the PC14, PC14PE6, and H226 cells were cultured under both sparse and confluent conditions. All three cell lines expressed higher levels of VEGF/VPF mRNA in confluent culture conditions than in sparse conditions (Figure 2A). Regardless of confluence, PC14 and PC14PE6 cells expressed higher levels of VEGF/VPF mRNA and protein than did H226 cells (Figure 2B). H226 cells expressed higher levels of bFGF mRNA or protein than did PC14 or PC14PE6 cells. No discernible differences in IL-8 expression were found among the three cell lines.

In Vivo Expression of Angiogenic Cytokines

We next measured the levels of VEGF/VPF, bFGF, and IL-8 in the serum of nude mice injected with PC14, PC14PE6, or H226 cells and the PE from mice with PC14 or PC14PE6 lung tumors (mice injected with H226 had no PE). Although VEGF/VPF was not detected in the serum of mice bearing PC14 or PC14PE6 lung tumors, the PE from these mice contained more than 30 ng/ml of VEGF/VPF (Table 2). Low levels of IL-8 and bFGF were detected in the serum of mice with PC14 or PC14PE6 lung tumors and tenfold higher levels of IL-8 and bFGF were detected in the bloody PE. These results suggest that angiogenic cytokines (VEGF/VPF, bFGF, and IL-8) were produced in the thoracic cavity, presumably by PC14 or PC14PE6

Figure 1. Expression of MMPs, uPA, and TIMPs by human NSCLC cells. **A:** Zymography. Tumor cells were cultured in serum-free medium to 50% confluence, and then the culture supernatants were harvested for gelatin zymography as described in Material and Methods. **B:** Northern blot analysis. Tumor cells cultured under sparse (S) and confluent (C) conditions were harvested and mRNA was extracted. Northern blot analysis was carried out as described in the Materials and Methods. Densitometric quantitation was derived from the ratio of the signal of the specific transcripts to that of the 1.3-kb GAPDH (internal control). N. D., not detected.

cells. No VEGF/VPF, bFGF, or IL-8 was detected in the serum of mice with actively growing H226 human squamous cell lung cancer.

The expression of VEGF/VPF, bFGF, and IL-8 was also examined in the lung tumor lesions using an *in situ* hybridization technique (to measure mRNA) and immunohistochemistry (to measure protein level). The integrity of the mRNA in the lesions was determined by poly d(T) reactivity (Figure 3). VEGF/VPF mRNA expression in PC14PE6 tumor cells was significantly higher than in H226 lung tumors. Consistent with the mRNA expression, more VEGF/VPF protein was detected in PC14PE6 tumors than in H226 tumors. Both PC14PE6 and H226 tumors expressed similar levels of bFGF and IL-8 mRNA

Figure 2. Angiogenic cytokine expression in NSCLC cells *in vitro*. **A:** Tumor cell lines were cultured under sparse (S) or confluent (C) conditions, mRNA was extracted, and a Northern blot was performed. Densitometric quantitation was derived from the ratio of the signal of the specific transcripts to that of the 1.3-kb GAPDH (internal control). **B:** Tumor cells were incubated for 24 hours under sparse (open columns) or confluent (solid columns) conditions, and culture supernatants were harvested. The concentration of angiogenic cytokines in the supernatants was determined by ELISA. At the end of the cell culture, the cells were counted and the number of cytokines produced by 10⁶ cells was calculated. N. D., not detected.

and proteins (data not shown). The PC14PE6 tumors contained a higher number of blood vessels than the H226 tumors (number of CD31-positive vessels/100× field = 43 ± 3 vs. 16 ± 4, respectively; *P* < 0.05).

Vascular Permeability in Mice with PE

Since VEGF/VPF produced by tumor cells can induce vascular hyperpermeability, and hence ascites,^{17,18} we next examined whether vascular hyperpermeability is associated with PE. Nude mice were injected i.v. with PC14PE6 or H226 cells (1 × 10⁶). After 6 weeks, 0.5% Evans blue dye (200 μl), which binds to endogenous albumin as a tracer,^{26–28} was injected i.v. into the mice. Thirty minutes later, the mice were killed, their diaphragms were carefully harvested, and the leaking of Evans blue dye from vessels lining the pleural surface was examined under fluorescent microscopy. Evans blue dye leaked from the vessels in the diaphragm of mice with PC14PE6 lung tumors and PE, but not in mice with the H226 lung tumors (Figure 3). These data clearly show that PE is associated with increased vascular permeability.

Transfection with the Sense or Antisense VEGF/VPF Gene

To provide definitive causal evidence for the role of VEGF/VPF in formation of PE, we transfected the sense VEGF/VPF gene into the H226 squamous cell carcinoma cells (VEGF/VPF low producing cells). Because VEGF/VPF consists of at least four isoforms (VEGF121, VEGF165, VEGF189, and VEGF206),⁹ we first determined by reverse transcriptase (RT)-PCR which isoform was predominantly expressed in the H226 and PC14PE6 cells. As shown in Figure 4A, VEGF121 and VEGF165 were the predominant isoforms expressed in the cell lines tested, although the magnitude of VEGF/VPF expression in the H226 cells was less than that in the PC14PE6 cells. For this reason, we elected to transfect the sense VEGF165 and sense VEGF121 genes into different H226 cells. Because the VEGF165 isoform contains all of the coding sequence of the VEGF121 isoform,³² PC14PE6 cells were transfected with only the antisense VEGF165 gene. Cells transfected with only pcDNA3 vector served as controls (Neo-control). Stable transfectants were obtained subsequent to selection and cloning in medium containing G418. The *in vitro* proliferation of the transfectants did not differ from parental or Neo-control cells (data not shown). Northern blot analysis showed that H226 cells transfected with the sense VEGF165 or sense VEGF121 genes (H226/V165 and H226/V121, respectively) expressed VEGF/VPF mRNA at 0.6 and 0.45 kb, respectively (Figure 4B), and the transfectants with antisense VEGF165 (PC14PE6/AS28 and PC14PE6/AS39) expressed the antisense VEGF/VPF mRNA (0.6 kb; Figure 4C). The expression of VEGF/VPF mRNA (3.7 and 1.4 kb) in the anti-sense-transfected clones was inhibited by 70 to 80% as compared to parental PC14PE6 or PC14PE6/Neo cells.

Table 2. Level of Angiogenic Cytokines in the Serum and PE of Tumor-Bearing Nude Mice

Cell line	VEGF/VPF (ng/ml)		bFGF (ng/ml)		IL-8 (ng/ml)	
	Serum	PE	Serum	PE	Serum	PE
PC14	<0.09	35.29 ± 24.23*	<0.01	0.09 ± 0.07	<0.03	5.24 ± 2.82
PC14PE6	<0.09	61.02 ± 36.41	0.02 ± 0.03	0.23 ± 0.17	0.27 ± 0.15	3.33 ± 1.09
H226	<0.09	N/A	<0.01	N/A	<0.03	N/A

The level of VEGF/VPF, bFGF, and IL-8 in the serum and PE of tumor-bearing mice was determined by ELISA. N/A, not available.
 *Mean ± S.D. of three mice.

H226/V165 and H226/V121 cells secreted 72.1 and 41.6 ng VEGF/VPF protein/ 10^6 cells/24 hours, respectively, whereas the H226 and H226/Neo cells secreted 0.8 and 0.6 ng VEGF/VPF protein/ 10^6 cells/24 hours. In a parallel experiment, PC14PE6/AS28 and PC14PE6/AS39 cells secreted 3.2 and 4.4 ng VEGF/VPF protein/ 10^6 cells/24 hours, respectively, whereas the PC14PE6 and PC14PE6/Neo cells secreted 15.6 and 16.4 ng VEGF/VPF

protein/ 10^6 cells/24 hours, respectively. The biological effect of the VEGF/VPF secreted by transfectants on vascular permeability was demonstrated using the Miles assay. As shown in Figure 5A, the culture supernatants from H226/V165 and H226/V121 cells, as well as rhVEGF165 and rhVEGF121, augmented vascular permeability, but those from H226 or H226/Neo cells did not. On the other hand, culture supernatants from PC14PE6 and PC14PE6/Neo cells increased vascular permeability, but those from PC14PE6/AS28 or PC14PE6/AS39 cells did not. Collectively, the results indicate that VEGF/VPF produced by sense VEGF/VPF gene transfectants was biologically active and that antisense VEGF/VPF gene transfection inhibited VEGF/VPF activity.

PE Formation by Tumor Cells Transfected with the Sense or Antisense VEGF/VPF Gene

Next, we injected control H226 cells transfected with either the sense VEGF165 or the sense VEGF121 gene i.v. into nude mice. H226/V165 and H226/V121 cells produced as many lung tumor colonies as the parental H226 or H226/Neo cells. Regardless of VEGF expression, none of the mice developed PE. In sharp contrast to lesions established by PC14PE6 cells, all H226 cell lines produced lesions in the lung parenchyma without invading the visceral pleura (Figure 4). To compensate for the lack of pleural invasion, we injected the H226 cells directly into the thoracic cavity of nude mice. The H226/V165 and H226/V121 cells produced a significant volume of PE in many of the injected mice, whereas the i.t. implanted parental H226 or H226/Neo cells did not (Table 3 and Figure 5B).

In a parallel experiment, PC14PE6 cells transfected with the antisense VEGF165 gene were injected i.v. or directly into the thoracic cavity of nude mice. When inoculated i.v., PC14PE6/AS28 and PC14PE6/AS39 cells produced significantly fewer experimental lung tumor lesions and a smaller volume of PE than did parental PC14PE6 or PC14PE6/Neo cells (Table 4). The antisense VEGF165 gene transfectants injected i.t. were tumorigenic but produced fewer lung lesions (corresponding to tumor progression) and lower incidence and volume of PE than did parental PC14PE6 or PC14PE6/Neo cells. Lung tumor lesions produced by antisense VEGF165 gene transfectants contained fewer blood vessels than did those produced by control cells. The number of CD31-positive vessels/100× field for PC14PE6/Neo and PC14PE6/AS39 cells was 46 ± 5 and 26 ± 5 , respectively ($P < 0.001$).

Figure 3. VEGF/VPF expression in lung metastasis by NSCLC cells and vascular permeability. Note that lung metastases produced by PC14PE6 cells expressed higher levels of VEGF/VPF than those produced by H226 cells. Leaking of Evans blue dye from vessels was observed in the diaphragm of mice bearing PC14PE6 cells but not H226 cells. Bar, 200 μ m.

Figure 4. Expression of VEGF/VPF isoforms by NSCLC cell lines and tumor cells transfected with the sense or antisense VEGF/VPF gene. **A:** Expression of VEGF/VPF isoforms determined by RT-PCR. RT-PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. The PCR products for the VEGF165 and VEGF121 transcripts are 457 and 303 bp, respectively. **B:** VEGF/VPF mRNA expression in H226 cells transfected with the sense VEGF/VPF genes. **C:** VEGF/VPF mRNA expression in PC14PE6 cells transfected with the antisense VEGF/VPF gene. Total RNA was extracted from confluent cultures, and Northern blot analysis was performed. The numbers in **C** are densitometric quantitation of the ratio of the area between the specific endogenous VEGF/VPF transcripts and the GAPDH transcripts with the value for parental cells defined as 1.0.

In the last set of experiments, we compared the expression level of VEGF/VPF in lung lesions produced by control tumor cells or cells transfected with sense or antisense VEGF/VPF gene with vascular permeability in mice with lung tumors. Lung lesions produced by H226/V165 (Figure 5B) and H226/V121 (data not shown) cells injected into the pleural space produced more VEGF/VPF protein than did lesions produced by control H226/Neo cells. Vessels in the diaphragm of mice with H226/V165 lung tumors were leaky, whereas those in mice with H226/Neo lung lesions were not, suggesting vascular hyperpermeability in mice bearing sense VEGF/VPF gene-transfected cells. Lung tumors produced by i.v. injection of cells transfected with an antisense VEGF165 gene produced a lower level of VEGF/VPF protein than did tumors produced by PC14PE6/Neo cells. Vessels in the diaphragm of mice with PC14PE6/Neo, but not PC14PE6/AS39 cells, leaked Evans blue dye.

Discussion

The present results provide evidence that the formation of PE is causally related to VEGF/VPF production by human NSCLC cells, provided they invade the pleural space. VEGF/VPF has been shown to induce interendothelial gaps and endothelial fenestration and to augment vascular permeability by activating cytoplasmic vesicular-vacuolar organelles in endothelial cells.^{33–35} In our study, vascular permeability measured by leakage of Evans blue dye was increased in mice with PE, suggesting that the primary mechanism by which VEGF/VPF induces PE is to cause vascular hyperpermeability.

The human lung squamous cell carcinoma H226 cells produced numerous tumor foci in the lung without PE, even when the cells were injected directly into the thoracic cavity. There are two causes of this failure. First, the cells do not invade the pleura. Second, the cells produce low levels of VEGF/VPF. We base this conclusion on the following evidence. First, low VEGF/VPF producing parental H226 cells injected directly into the thoracic cavity failed to develop PE even though they produced lesions in the lung and solid tumors in the thoracic cavity. Second, H226 cells transfected with the sense VEGF/VPF gene (VEGF/VPF high producing) injected i.v. produced numerous lung tumor lesions but no PE. Third, sense VEGF/VPF gene-transfected H226 cells injected directly into the thoracic cavity produced PE. Thus, the production of PE is associated with a high level of VEGF/VPF production in the thoracic cavity. VEGF/VPF was not detected in the serum of mice, even though the PE contained more than 30 ng/ml VEGF/VPF protein (Table 2), suggesting a short life of VEGF/VPF.

VEGF/VPF consists of at least four isoforms (VEGF121, VEGF165, VEGF189, and VEGF206) arising through alternative mRNA splicing of a single gene.^{9,36} The smaller VEGF121 and VEGF165 isoforms are the secreted forms, whereas the larger VEGF189 and VEGF206 isoforms tend to remain cell-associated,³⁶ with VEGF121 and VEGF165 isoforms more abundant and biologically potent.⁹ In this study, i.t. injection of H226 cells transfected with sense

Figure 5. Vascular permeability and PE formation by tumor cells transfected with the sense or antisense VEGF/VPF gene. **A:** Biological activity of VEGF/VPF secreted by tumor cells transfected with the sense or antisense VEGF/VPF gene on vascular permeability as measured by the Miles assay. Serum-free culture supernatants of tumor cells were harvested as test samples. Nude mice were injected i.v. with Evans blue dye. Ten minutes later, 50 μ l of medium, rhVEGF165 (50 ng/ml), rhVEGF121 (50 ng/ml), or test samples were injected intradermally. Thirty minutes later, test sites were photographed. **B:** PE formation, VEGF/VPF production, and vascular permeability. **White arrows** indicate pleural fluid (**black area**) produced in the thoracic cavity. Note that i.t. injection with H226/V165 cells, but not H226/Neo cells, induced the formation of PE. Intravenous injection with PC14PE6/Neo cells, but not PC14PE6/AS39 cells, induced PE formation. Lung lesions by H226/V165 or PC14PE6/Neo cells produced higher levels of VEGF/VPF protein than those by H226/Neo or PC14PE6/AS39 cells, respectively. Leaking of Evans blue dye from vessels in the diaphragm of mice bearing H226/V165 or PC14PE6/Neo cells, but not H226/Neo or PC14PE6/AS39 cells. Bar, 200 μ m.

Table 3. Production of Lung Lesions and PE by H226 Cells Transfected with the Sense VEGF/VPF Gene in Nude Mice

Route of injection	Cell line	Lung lesions			PE		
		Incidence	Number		Incidence	Volume (μ l)	
			Median	Range		Median	Range
i.v.*	H226	10/10	>150	34->150	0/10	All <20	
	H226/Neo	10/10	>150	42->150	0/10	All <20	
	H226/V165	10/10	>150	2->150	0/10	All <20	
	H226/V121	9/10	>150	0->150	0/10	All <20	
i.t.†	H226	9/9	13	9-21	0/9	All <20	
	H226/Neo	9/9	12	2-25	0/9	All <20	
	H226/V165	9/9	18	5-30	7/9	220	<20-1040
	H226/V121	9/9	16	4-50	3/9	<20	<20-40

*Nude mice injected i.v. with 1×10^6 tumor cells were killed on day 84.
†Five mice injected i.t. with 1×10^6 H226/V165 cells became moribund on day 42 and thus the experiment was terminated.

Table 4. Formation of Lung Lesions and PE by PC14PE6 Cells Transfected with the Antisense VEGF/VPF Gene

Route of injection	Cell line	Lung lesions			PE		
		Incidence	Number		Incidence	Volume (μ l)	
			Median	Range		Median	Range
i.v.	PC14PE6	10/10	32	5–72	9/10	410	<20–910
	PC14PE6/Neo	10/10	22	3–80	9/10	430	<20–630
	PC14PE6/AS28	10/10	8*	2–20	1/10	<20 [†]	<20–30
	PC14PE6/AS39	8/8	7*	3–25	1/10	<20 [†]	<20–320
i.t.	PC14PE6	8/8	22	10–37	8/8	690	120–1100
	PC14PE6/Neo	10/10	31	17–43	9/9	550	30–960
	PC14PE6/AS28	9/10	11*	0–30	2/10	<20 [†]	<20–690
	PC14PE6/AS39	10/10	6*	1–31	2/10	<20 [†]	<20–140

*Nude mice were injected with 1×10^6 cells and were killed on day 45.

[†] $P < 0.05$ as compared to control group (PC14PE6/Neo).

VEGF165 or sense VEGF121 genes induced 78% and 33% incidence of PE, respectively, and both rhVEGF165 and rhVEGF121 proteins augmented vascular hyperpermeability as measured by the Miles assay. Thus, both VEGF165 and VEGF121 can induce hyperpermeability and facilitate formation of PE. Consistent with a previous report²⁷ that antisense VEGF/VPF gene transfection into human melanoma cells inhibits experimental lung metastasis, we found that transfection of an antisense VEGF165 gene into PC14PE6 cells inhibited lung metastasis, in this case by suppressing vascularization. These findings suggest that VEGF/VPF is essential but not sufficient for lung metastasis formation of PC14 and PC14PE6 cells.

Although both H226 and PC14PE6 cells injected i.v. produced lesions in the lung parenchyma, only PC14PE6 cells invaded the pleura and caused pleuritis. Because tumor invasion is regulated by various molecules, including MMPs³⁷ and uPA,³⁸ we examined the expression of MMP-2, MMP-9, and uPA in the human NSCLC lines. The production of PE did not correlate with collagenase activity as measured by gelatin zymography. In culture, the noninvasive H226 cells produced both MMP-2 and -9, whereas PC14PE6 cells produced only MMP-2. The H226 cells, however, expressed high levels of TIMP-2 (inhibitor of MMP-2), whereas the PC14PE6 cells did not. The differences in uPA expression correlated with the biological behavior of the tumor cells. Consistent with a previous report showing that malignant PE contains high levels of uPA,³⁹ the PC14PE6 cells expressed a high level of uPA, whereas the H226 cells did not (Figure 3). Further determination of the role of uPA in invasion of lung cancer cells and formation of PE is underway.

Regardless of the level of VEGF/VPF production, the PC14PE6 cells developed malignant PE more rapidly and reproducibly than H226 cells transfected with the sense VEGF/VPF genes, suggesting that PE formation is multifactorial. Because PE formation is associated with impaired drainage of the pleural space due to obstruction of vessels and lymphatics of the lungs and pleura,⁴⁰ the degree of obstruction of vessels and lymphatics caused by H226 and PC14PE6 cells might be different. The other defined mechanism is increased pleural fluid formation,⁴⁰ the mechanism of which is the focus of our study. Our data suggest that VEGF/VPF is essential and sufficient for pleural fluid formation in the squamous H226 cell model;

however, the presence of other cofactors with VEGF/VPF might also increase pleural fluid formation additively or synergistically. One candidate is VEGF-C, a recently discovered member of the VEGF/VPF family. It has 30% homology to VEGF165⁴¹ and has been reported to stimulate the release of nitric oxide from endothelial cells and increase vascular permeability.⁴² Northern blot analysis failed, however, to demonstrate VEGF-C mRNA in any of the cell lines (unpublished data).

Lung cancer is the leading cause of malignant PE. At least 25% of all patients with lung cancer will develop PE at some time during the course of the disease.^{3,43} Standard treatment for PE is drainage followed by instillation of sclerosing agents, but the clinical efficacy of this treatment varies.⁷ In this study, we demonstrate that VEGF/VPF is responsible for PE formation by NSCLC cells in an animal model. Indeed, the level of VEGF/VPF in malignant PE of lung cancer patients is significantly higher than that in PE caused by nonmalignant diseases including heart failure and pulmonary tuberculosis.⁴⁴ Hence, targeting the production of VEGF/VPF and/or blocking the VEGF/VPF receptor may be a way to control malignant PE in lung cancer patients.

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