Treatment for Malignant Pleural Effusion of Human Lung Adenocarcinoma by Inhibition of Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Phosphorylation[1]

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ABSTRACT

Malignant pleural effusion (PE) is associated with advanced human lung cancer. We found recently, using a nude mouse model, that vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is responsible for PE induced by non-small cell human lung carcinoma cells. The purpose of this study was to determine the therapeutic potential of a VEGF/VPF receptor tyrosine kinase phosphorylation inhibitor, PTK 787, against PE formed by human lung adenocarcinoma (PC14PE6) cells. PTK 787 did not affect the *in vitro* proliferation of PC14PE6 cells, whereas it specifically inhibited proliferation of human dermal microvascular endothelial cells stimulated by VEGF/VPF. A specific platelet-derived growth factor receptor tyrosine kinase inhibitor, CGP57148 (used as a control because PTK 787 also inhibits platelet-derived growth factor receptor tyrosine kinases), had no effect on proliferation of PC14PE6 or human dermal microvascular endothelial cells. i.v. injection of PC14PE6 cells into nude mice produced lung lesions and a large volume of PE containing a high level of VEGF/VPF. Oral treatment with CGP57148 had no effect on PE or lung metastasis. In contrast, oral treatment with PTK 787 significantly reduced the formation of PE but not the number of lung lesions. Furthermore, treatment with PTK 787 significantly suppressed vascular hyperpermeability of PE-bearing mice but did not affect the VEGF/VPF level in PE or expression of VEGF/VPF protein and mRNA in the lung tumors of PC14PE6 cells *in vivo*. These findings indicate that PTK 787 reduced PE formation mainly by inhibiting vascular permeability, suggesting that this VEGF/VPF receptor tyrosine kinase inhibitor could be useful for the control of malignant PE.

INTRODUCTION

Malignant PE[3] is associated with highly symptomatic, advanced-stage lung cancer. Most patients with PE present with progressive dyspnea, cough, or chest pain that compromises their quality of life (1). Malignant PE is most often caused by lung adenocarcinoma, because this type often forms a primary tumor in the periphery of the lung and invades the pleural cavity (1). Malignant PE has consistently been shown to indicate a poor prognosis in advanced lung cancer patients, being associated with high morbidity and mortality (2, 3). Previous reports demonstrate that drainage followed by instillation of sclerosing agents is useful for controlling PE and improving the quality of life of patients. However, the efficacy of this treatment is variable and does not extend the survival of lung cancer patients (1, 4). Clearly, a more effective therapy for malignant PE is needed.

Among the possible targets for PE treatment is VEGF, also called VPF (5), an important multifunctional cytokine that promotes developmental, physiological, and pathological neovascularization (6–8). VEGF/VPF consists of at least four isoforms (VEGF121, VEGF165, VEGF189, and VEGF206), arising through alternate splicing of mRNA from a single gene (6). It can be produced by various cell types, including many tumor cells and activated macrophages (6). VEGF/VPF has been shown to stimulate the proliferation and migration of endothelial cells and to induce the expression of metalloproteinases and plasminogen activity by these cells (6, 9–12). The cytokine is also a powerful inducer of vascular hyperpermeability. Through this property, the molecule plays a central role in ascites fluid formation by murine tumors and human ovarian cancer cell lines in animal models (13–17).

[3] The abbreviations used are: PE, pleural effusion; VEGF/VPF, vascular endothelial growth factor/vascular permeability factor; Flt-1, fms-like tyrosine kinase; Flk-1, fetal liver kinase; KDR, kinase insert domain-containing receptor; FBS, fetal bovine serum; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; IL, interleukin; rh, recombinant human; ISH, *in situ* hybridization; IHC, immunohistochemistry; HDMEC, human dermal microvascular endothelial cell; PTK 787/ZK232394, 1-[4-chloroanilino]-4-[pyridylmethyl] phthalazine dihydrochloride.

Recently, we developed a model for PE by human lung adenocarcinoma cells (PC14 and its highly metastatic variant, PC14PE6) in nude and severe combined immunodeficiency mice (18) and clarified the role of VEGF/VPF in PE formation. The i.v. injection of PC14 and PC14PE6 cells, expressing high levels of VEGF/VPF, produced lung lesions that in turn produced large-volume PE in mice. On the other hand, i.v. or intrathoracic injection of H226 cells (VEGF/VPF low-expressing human lung squamous cell carcinoma) produced lung lesions without detectable PE. Interestingly, *VEGF/VPF* gene transfection into H226 cells resulted in induction of PE when tumor cells were injected intrathoracicly.[4] This evidence suggests that VEGF/VPF plays a crucial role in PE formation by human non-small cell carcinoma cells.

Two VEGF/VPF receptors have been identified: Flt-1 and the Flk-1/kinase insert domain-containing receptor (KDR), are high-affinity VEGF/VPF receptors with an extracellular domain containing seven immunoglobulin-like domains and a split tyrosine kinase intracellular domain (6). Flk-1 has 85% homology with the human homologue, KDR. Both Flt-1 and Flk-1/KDR have been shown to be important regulatory systems for vasculogenesis and physiological angiogenesis (19–24). However, the interaction of VEGF/VPF with Flk-1/KDR is thought to be the more important interaction for tumor angiogenesis because it is essential for induction of the full spectrum of VEGF/VPF functions (6). In fact, many compounds and molecules developed to block VEGF/VPF activities mediated by Flk-1/KDR have been shown to have antiangiogenic activity in animal models (25–27).

One such molecule is an inhibitor of tyrosine kinase phosphorylation of Flk-1/KDR and Flt-1, called PTK 787 (28). PTK 787 directly inhibits phosphorylation of the VEGF/VPF receptor tyrosine kinases and suppresses angiogenesis induced by VEGF/VPF. At slightly higher doses, it also inhibits PDGF receptor tyrosine kinase phosphorylation (28). It can be given p.o., is well tolerated, and has been demonstrated to inhibit the growth of several carcinomas in nude mice (28).

In this study, we examined the therapeutic efficiency of PTK 787 against malignant PE caused by human lung adenocarcinoma cells (PC14PE6) established in nude mice. PTK 787 specifically inhibited VEGF/VPF-induced proliferation of human dermal endothelial cells and had no direct effect against PC14PE6 cells. We found that oral feeding with PTK 787 suppressed the formation of malignant PE by inhibiting vascular permeability. These findings suggest that therapy with the VEGF/VPF receptor tyrosine kinase phosphorylation inhibitor PTK 787 is worthy of study in clinical trials.

MATERIALS AND METHODS

Cell Lines. The human lung adenocarcinoma cell line PC14PE6 was maintained in Eagle's minimal essential medium

supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, 2-fold vitamin solution, and penicillin-streptomycin (Flow Laboratories, Rockville, MD) in a 10-cm dish and incubated in 5% CO_2-95% air at 37°C. PC14PE6 cells were free of *Mycoplasma* and pathogenic murine viruses (assayed by Microbiological Associates, Bethesda, MD). Cultures were maintained for no longer than 6 weeks after recovery from frozen stocks. HDMECs (Cascade Biologicals, Portland, OR) were cultured in Medium 131 with Microvascular Growth Supplement (Cascade Biologicals). For proliferation assays, HDMECs were used at passages 2–5.

Reagents. rh VEGF165, rhbFGF, and antihuman VEGF polyclonal antibody were purchased from R&D Systems (Minneapolis, MN). PTK 787/ZK232394 was discovered and synthesized in the Department of Oncology Research, Novartis Pharmaceuticals (Basel, Switzerland) and was profiled in collaboration with the Institute of Molecular Medicine (Tumor Biology Center, Freiburg, Germany), as well as the Oncology Research Laboratories of Schering AG (Berlin, Germany). The studies described in this report were performed with either a dihydrochloride or succinate salt. For *in vitro* assays, a stock solution of 10 mM of PTK787/ZK 222584 was prepared in DMSO. This was diluted further in buffer or medium so that the concentration of DMSO in assay systems did not exceed 0.1%. For *in vivo* studies, the vehicle for the dihydrochloride salt was distilled water. The succinate salt was suspended in vehicle containing 5% DMSO and 0.5% Tween 80 in distilled water (28). The PDGF receptor tyrosine kinase phosphorylation inhibitor CGP57148 (28, 29) was from Novartis.

Cell Proliferation Assay. HDMECs (5×10^3/well) plated in triplicate in 96-well plates precoated with 1.5% gelatin were incubated overnight in supplemented M131 medium. PC14PE6 cells (2×10^3/well) plated in triplicate in 96-well plates were incubated in MEM containing 5% FBS. The cells were then washed and incubated for 72 h with test samples in fresh MEM containing 5% FBS. The proliferative activity was determined by the 3-(4,4-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide assay using an MR-5000 96-well microtiter plate reader set at 570 nm (12).

Animals. Male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 6–8 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH.

Model for Lung Metastasis and PE. Cultured PC14PE6 cells were harvested by pipetting. The cells were washed twice and resuspended in Ca^{2+}- and Mg^{2+}-free HBSS. Cell viability was determined by a trypan blue exclusion test, and only single-cell suspensions of >90% viability were used. Tumor cells (1×10^6/300 μl of HBSS) were injected into the lateral tail vein of unanesthetized nude mice (18). After the indicated periods, mice were euthanized by methoxyflurane, the subclavian artery was severed, and blood was harvested. The chest wall was then cut carefully with a pair of scissors, PE was harvested using a 1-ml

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[4] S. Yano, H. Shinohara, R. S. Herbst, H. Kuniyasu, C. D. Bucana, L. M. Ellis, and I. J. Fidler. Production of 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, submitted for publication.

syringe, and the volume of PE was measured using the syringe. The blood and PE harvested were centrifuged for 20 min (100 × g) at 4°C. The serum and supernatant of PE were stored at −70°C until the ELISA was performed. The lungs were fixed in Bouin's solution, and the number of lung lesions was determined with the aid of a dissecting microscope.

Although this model does not entirely mimic all steps for PE formation in humans, this is one model in which PE is reproducibly developed, as reported previously (18).

Assays of VEGF/VPF, bFGF, and IL-8 Protein Levels. Levels of VEGF/VPF, bFGF, and IL-8 protein in culture supernatants, mouse serum, and PE were determined using ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Oligonucleotide Probes. We designed antisense oligonucleotide DNA probes complementary to the mRNA transcripts of the *VEGF/VPF* genes based on published reports of the cDNA sequence (15) TGGTGATGTTGGACTCCTCAGT-GGGCU, 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 (Madison, WI) based on the FastA algorithm. A poly(dT)$_{20}$ oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3′ end via direct coupling using standard phosphoramidite chemistry (Research Genetics, Huntsville, AL).

ISH. ISH was performed as described previously (30). Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific Co.). The slides were placed in the Microprobe slide holder (Fisher Scientific Co.), dewaxed, and rehydrated with Autodewaxer and Autoalcohol (Research Genetics), digested with pepsin, and then hybridized by use of the Microprobe manual staining system (Fisher Scientific Co.). The probes were hybridized for 45 min at 45°C, and the samples were then washed three times for 2 min each time with 2× SSC (1× SSC = 0.15 M NaCl, 0.15 M sodium citrate) at 45°C. RNase-free water was used to make up Tris buffer and 2× SSC solutions. The samples were then incubated with alkaline phosphate-labeled avidin for 30 min at 45°C, rinsed in 50 nM Tris buffer (pH 7.6), rinsed with alkaline phosphate enhancer for 1 min, and incubated with a chromogen substrate for 20 min at 45°C. Additional incubation with fresh chromogen was done if it was necessary to enhance a weak reaction. A positive enzymatic reaction in this assay stained red. Known positive controls were used in each hybridization reaction. Controls for endogenous alkaline phosphate included treatment of the sample in the absence of the biotinylated probe and use of chromogen alone.

Histology and IHC. The lungs of nude mice were harvested at autopsy, cut into 5-mm thickness, and placed in 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 μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, rehydrated in graded alcohol, transferred to PBS, and treated with pepsin for 20 min at room temperature. For CD31

staining, frozen tissue sections (8 μm 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 min. The samples were washed three times with PBS and incubated for 10 min 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 h at 4°C with a 1:400 dilution of rabbit polyclonal anti-VEGF/VPF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:100 dilution of monoclonal rat anti-CD31 antibody (PharMingen, San Diego, CA). The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of peroxidase-conjugated antirabbit IgG or antirat IgG. The slides were rinsed with PBS and incubated for 5 min with diaminobenzidine (Research Genetics, Huntsville, AL). The sections were then washed three times with distilled water, counterstained with Gill's hematoxylin, washed once with distilled water and once with PBS, and rinsed again with distilled water. The slides were mounted with a Universal mount (Research Genetics) and examined using a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm. Sections (4 μm thick) of formalin-fixed, paraffin-embedded tumors were also stained with H&E for routine histological examination.

Vascular Density. Blood vessels in solid tumors growing in the lungs of nude mice were counted under light microscope after immune staining of sections with anti-CD31 antibody. Areas containing the highest number of capillaries and small venules were identified by scanning the tumor sections at low power (×40). After the areas of high vascular density were identified, individual vessels were counted in ×100 fields [×10 objective and ×10 ocular (0.145 mm^2)/field]. On the basis of criteria described by Weidner *et al.* (31), observation of a vessel lumen was not required for a structure to be classified as a vessel.

Permeability Assay (Miles Assay). The Miles assay uses intradermal injection of test substances and intravascular injection of Evans blue dye (which binds to endogenous serum albumin) as a tracer to assay permeability in peripheral vessels. The assay was performed essentially as described (32, 33) with minor modification. To reduce individual variation, nude mice without downy hair were carefully chosen, and each mouse was kept separately during the assay. Nude mice were injected i.v. with 200 μl of 0.5% Evans blue dye (Sigma Chemical Co., St. Louis, MO). Ten min later, 50-μl of samples were injected intradermally in rows on the dorsal skin. Thirty min after the injection with samples, the mice were killed, and the skin was removed. Wheals (5 mm in diameter) were resected and incubated in 500 μl of formamide at 37°C for 48 h to extract Evans blue dye. The absorbance of the extracts was read at 630 nm in a spectrophotometer.

Statistical Analysis. The significance of differences in data of *in vitro* experiments, the Miles assay, and vessel density were analyzed using Student's t test (two-tailed). The remaining *in vivo* data were analyzed using the Mann-Whitney U test or χ^2 test.
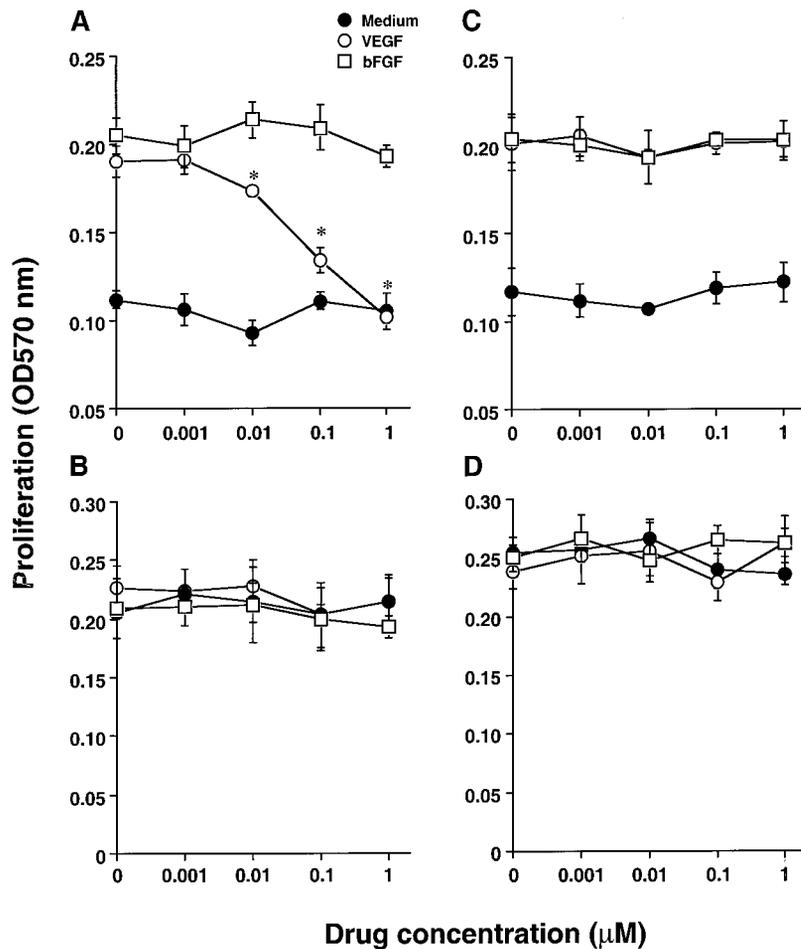
Fig. 1 Effects of PTK 787 and CGP 57148 on *in vitro* proliferation of HDMECs and PC14PE6 cells. HDMECs (5×10^3/well; *A* and *C*) plated in 96-well plates were incubated overnight in supplemented M131 medium. PC14PE6 cells (2×10^3/well; *B* and *D*) were plated in triplicate in 96-well plates and were incubated overnight in MEM containing 5% FBS. The cells were then washed and incubated with different doses of PTK 787 (*A* and *B*) or CGP57148 (*C* and *D*), in the presence or absence of rhVEGF165 (20 ng/ml) or rhbFGF (20 ng/ml) in fresh MEM containing 5% FBS. The 3-(4,4-dimethylthiazol-2-yl)-2.5-diphenyl-tetrazolium bromide assay was performed after 72 h, as described in "Materials and Methods." The values shown represent the means of triplicate cultures; *bars,* SD. The results shown are representative of four independent experiments with similar results. ∗, $P < 0.05$, compared with the respective control.

RESULTS

PKT 787 Inhibited Proliferation of Endothelial Cells but not Lung Cancer Cells. In the first set of experiments, we examined the effect of PTK 787 on the proliferation of HDMECs and PC14PE6 cells *in vitro*. PTK 787 did not affect proliferation of HDMECs incubated in medium alone (Fig. 1*A*). Addition of rhVEGF/VPF or rhbFGF to the medium significantly stimulated HDMEC proliferation, suggesting that HDMECs express receptors for VEGF/VPF and bFGF, as is the case with most endothelial cell lines. Under these experimental conditions, PTK 787 inhibited proliferation of HDMECs stimulated by rhVEGF165 but not rhbFGF, indicating the specificity of PTK 787 to VEGF/VPF receptors (Fig. 1*A*). In contrast, PTK 787 did not affect proliferation of PC14PE6 cells, irrespective of the presence of rhVEGF165 or rhbFGF (Fig. 1*B*). Moreover, a PDGF-receptor tyrosine kinase phosphorylation inhibitor, CGP57148, used as a control did not affect the proliferation of HDMECs or PC14PE6 cells, irrespective of the presence of rhVEGF165 or rhbFGF (Fig. 1, *C* and *D*).

Treatment with PTK 787 Inhibited PE Formation. We next examined the therapeutic effects of PTK 787 on PE formation by PC14PE6 cells. The effective dose of PTK 787

was chosen according to a previous report (28). PC14PE6 cells (1×10^6) were injected i.v. into nude mice. Oral feeding with PTK 787 commenced 14 days after tumor injection (because at this time, the PC14PE6 cells progress to micrometastases in the lung) and continued until mice were killed. In the first experiment, all mice in the control group developed lung metastases and PE, and treatment with 10 mg/kg PTK 787 had no effect on lung metastasis, lung weight (represents total tumor volume), or PE formation (Table 1). Treatment with 100 mg/kg PTK 787 inhibited lung weight, although the reduction in the number of lung metastases did not reach significance. In addition, PE formation (both incidence and volume) was remarkably inhibited in this group. In experiment 2, we examined the effect of a lower dose of PTK 787 (50 mg/kg) on formation of PE and lung metastasis. All mice of the control group developed lung metastasis and PE, and treatment with 10 mg/kg PTK 787 again had no effect, consistent with the results in experiment 1. The number of lung metastases or lung weight was not inhibited significantly by treatment with 50 mg/kg PTK 787; however, this treatment remarkably inhibited PE formation (both incidence and volume). In experiment 3, the effect of oral treatment with the PDGF receptor tyrosine kinase inhibitor CGP57148 was examined using the same model. Although therapy with

Table 1 Effect of VEGF/VPF receptor tyrosine kinase phosphorylation inhibitor on the formation of lung metastasis and PE by PC14PE6 cells in nude mice

PC14PE6 cells (1×10^6) were injected i.v. in nude mice on day 0. The mice were given oral feedings with distilled water (control), PTK 787 (started on day 14), or CGP57148 (started on day 7). Daily therapy continued until day 48. The mice were killed on day 49, and lung metastasis, lung weight, and PE were evaluated as described in "Materials and Methods."

| Experiment | Treatment | Lung metastasis | | | Lung weight (mg) | | PE | | |
| | | Incidence | Median | Range | Median | Range | Incidence | Volume (µl) | |
								Median	Range
1	Control	10/10	64	26–115	600	302–680	10/10	630	20–900
	PTK 787 10 mg/kg	10/10	54	2–97	548	251–994	9/10	650	<20–1060
	PTK 787 100 mg/kg	9/9	44	4–76	286[a]	218–620	4/9[b]	<20[a]	<20–700
2	Control	10/10	53	6–>150	637	290–910	10/10	803	30–990
	PTK 787 10 mg/kg	9/9	44	1–>150	420	195–718	7/9	780	<20–1000
	PTK 787 50 mg/kg	7/9	26	0–103	405	212–600	5/9[b]	20[a]	<20–799
3	Control	10/10	59	38–139	573	241–944	10/10	690	150–1050
	CGP57148 10 mg/kg	7/8	58	4–>150	559	185–796	6/8	550	<20–1010
	CGP57148 50 mg/kg	10/10	48	3–>150	543	208–661	8/10	500	<20–1430

[a] Significantly different from control group ($P < 0.05$; Mann-Whitney U test).
[b] Significantly different from control group ($P < 0.01$; χ^2 test).

Table 2 Effect of treatment with PTK787 on the levels of angiogenic cytokines in the serum and PE of PC14PE6 cell-bearing nude mice
  Data shown represent mean ± SD of three mice.

| Treatment | VEGF/VPF (ng/ml) | | bFGF (ng/ml) | | IL-8 (ng/ml) | |
	Serum	PE	Serum	PE	Serum	PE
Control	<0.09	60.99 ± 36.36	0.02 ± 0.03	0.23 ± 0.17	0.07 ± 0.15	3.33 ± 1.09
PTK 787 10 mg/kg	<0.09	63.85 ± 17.10	0.03 ± 0.06	0.07 ± 0.03	0.52 ± 0.01	2.99 ± 0.34
PTK 787 50 mg/kg	<0.09	73.70 ± 21.54	0.04 ± 0.07	0.39 ± 0.33	0.73 ± 0.32	4.10 ± 0.56

CGP57148 was started earlier (on day 7) than therapy with PTK 787, it had no significant therapeutic effects on the formation of PE or lung metastasis. These results suggest that PTK 787 specifically inhibits PE formation produced by PC14PE6 cells. It also inhibited lung weight (total tumor volume of lung metastases) at the highest dose (100 mg/kg) tested. On the basis of these results, we chose to use 50 mg/kg PTK 787 in the following experiments.

Treatment with PTK 787 Inhibited Tumor Vascularization but not VEGF/VPF Expression. To better determine the mechanism by which treatment with PTK 787 inhibited PE formation, we examined the effect of treatment with PTK 787 on VEGF/VPF production by PC14PE6 cells *in vivo*. We first measured the levels of VEGF/VPF, as well as bFGF and IL-8, in both the serum and the PE that developed in PTK 787-treated, PC14PE6-bearing mice treated and untreated controls. As shown in Table 2, treatment with PTK 787 did not suppress the levels of VEGF/VPF, bFGF, or IL-8 in the serum or PE. Next, we examined the effect of PTK 787 treatment on VEGF/VPF expression in lung tumors by ISH and IHC. Treatment with PTK 787 did not affect VEGF/VPF expression in lung metastasis produced by PC14PE6 cells (Fig. 2). We also found that production of bFGF or IL-8 in lung metastases was not affected by treatment with PTK 787 *in vivo* (data not shown). However, angiogenesis (one of the two major phenomena induced by VEGF/VPF) quantitated in lung lesions of PTK 787-treated mice was inhibited compared with that of control mice (number of CD31 positive cells ×100 field; 34 ± 7 *versus* 52 ± 5; $P < 0.05$). This effect, in the absence of decreased cytokine levels, suggests that treatment with PTK 787 might exert its effect on endothelial cells as opposed to tumor cells.

Treatment with PTK 787 Inhibited Vascular Permeability. The other major activity of VEGF/VPF is thought to be the induction of vascular hyperpermeability (13). Therefore, we examined the effect of treatment with PTK 787 on vascular permeability. Nude mice were treated with or without 50 mg/kg PTK 787 or CGP57148 for 3 days, and then a skin permeability assay (Miles assay) was performed. As shown in Fig. 3*A,* rhVEGF165 and PE (containing 30 ng/ml VEGF/VPF) caused by PC14PE6 cells induced vascular hyperpermeability in control and CGP57148-treated mice. However, neither rhVEGF165 nor PE produced *in vivo* by PC14PE6 cells significantly enhanced permeability in mice treated with PTK 787. These findings indicate that treatment with PTK 787 can inhibit induction of hyperpermeability caused by VEGF/VPF, presumably by blocking VEGF/VPF receptor function. We further explored how many treatments with PTK 787 were necessary for inhibition of vascular permeability. Results shown in Fig. 3*B* indicate that a 2–3-day treatment with PTK 787 was enough to inhibit the induction of vascular permeability in this assay.

Finally, we investigated the effect of treatment with PTK 787 on vascular permeability of PE-bearing mice. Nude mice were injected with PC14PE6 cells. Five weeks later, the mice were given oral feeding with or without PTK 787 (50 mg/kg)
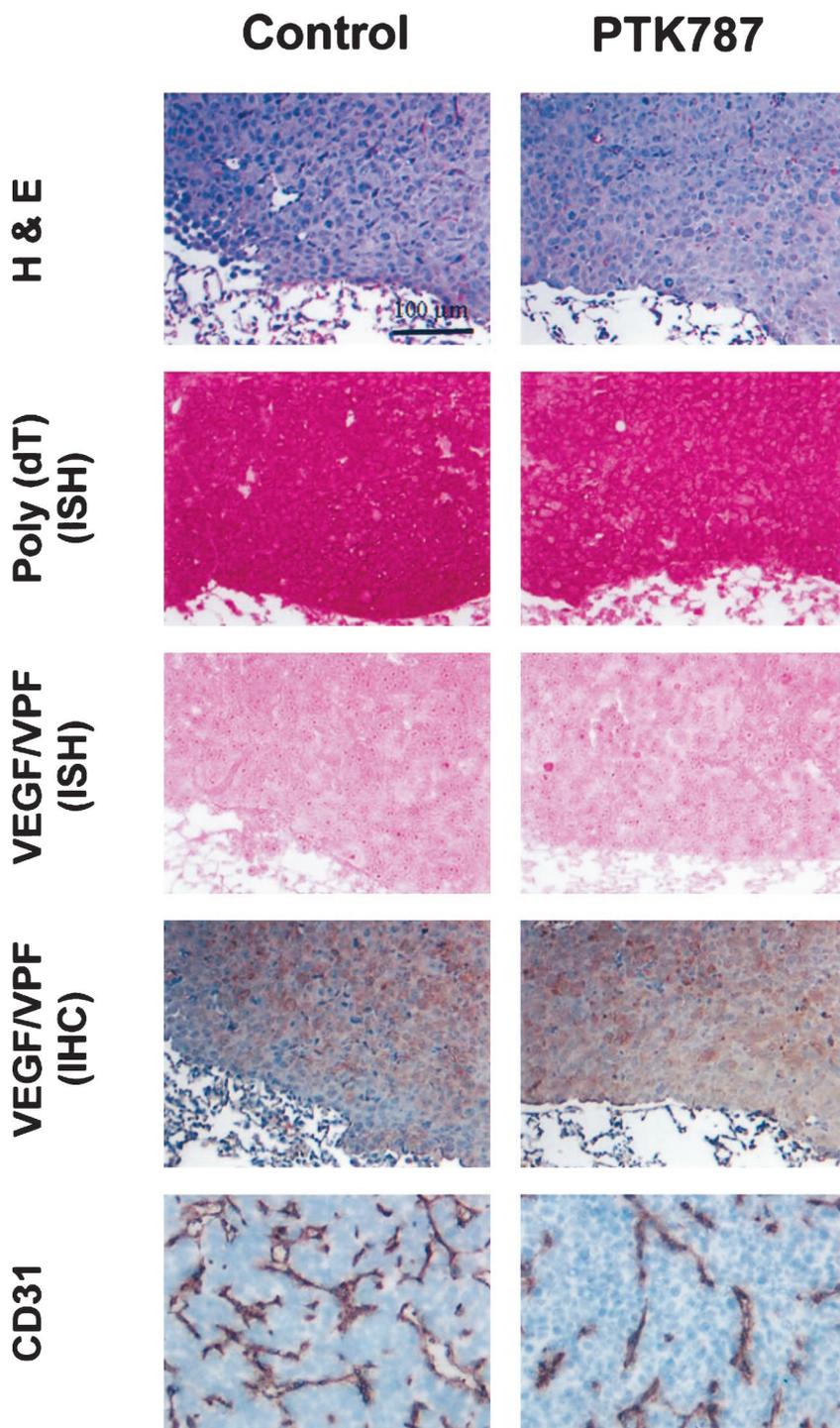
Fig. 2 Effect of oral treatment with PTK 787 on VEGF/VPF expression and vascularization in lung tumors produced by PC14PE6 cells. PC14PE6 cells (1×10^6) were injected i.v. into nude mice. Oral feeding with distilled water (control) or PTK 787 (50 mg/kg) was started 14 days after tumor injection and continued until the mice were killed. VEGF/VPF expression in lung tumors was examined by ISH and IHC. Vascularization was examined after the staining with anti-CD31 antibody, as described in "Materials and Methods."

for 6 days. Two h after the last oral feeding, the mice were injected with Evans blue dye. Fifteen min later, the mice were killed, and PE was carefully harvested. After the centrifugation of PE, the absorbance of the supernatant from the PE was measured at 630 nm. In the control group (without PTK787 treatment), $A_{630 \text{ nm}}$ (mean $\pm$ SE) of PE with or without Evans

blue dye injection was 0.465 ± 0.081 and 0.094 ± 0.014, respectively. These results showed that Evans blue dye-bound endogenous albumin had leaked into PE, indicating increased vascular permeability of PE-bearing mice (33). Under these experimental conditions, treatment of PE-bearing mice with PTK 787 significantly inhibited leaking of
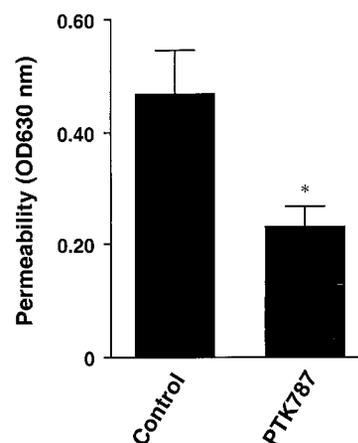
A

B
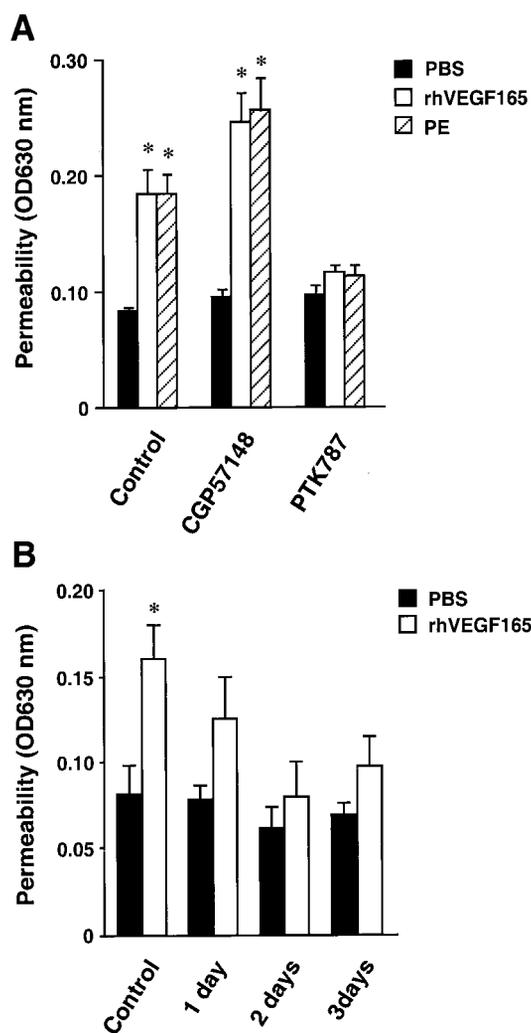
Fig. 3 Effect of treatment with PTK 787 and CGP 57148 on vascular permeability in the skin measured by the Miles assay. *A,* nude mice were given oral feeding with distilled water (*Control*), CGP 57148 (50 mg/kg), or PTK 787 (50 mg/kg) for 3 days. Two h after the last oral feeding, 50 μl of PBS, rhVEGF165 (30 ng/ml), or PE (containing 30 ng/ml VEGF) were injected intradermally into nude mice preinjected with 0.5% Evans blue dye (200 μl). Permeability was determined by the Miles assay as described in "Materials and Methods" and quantitated by measuring the absorbance at 630 nm. Data represent the means for groups of five mice; *bars,* SE. ∗, $P < 0.05$. *B,* nude mice were given oral feeding with distilled water (*Control*) or PTK 787 (50 mg/kg) for 1, 2, or 3 days. Two h after the last oral feeding, 50 μl of PBS or rhVEGF165 (30 ng/ml) were injected intradermally into nude mice preinjected with 0.5% Evans blue dye (200 μl). Permeability was determined by the absorbance at 630 nm. The data represent the means for groups of five mice; *bars,* SE. ∗, $P < 0.05$.

Evans blue dye into PE ($A_{630 \, nm}$: 0.229 ± 0.038; Fig. 4). These findings further suggest that treatment with PTK 787 inhibited vascular permeability of PE-bearing mice.

DISCUSSION

Recently, we developed an animal model for PE (18) and found that VEGF/VPF is responsible for PE formation produced

Fig. 4 Effect of treatment with PTK 787 on vascular permeability of PE-bearing mice. PC14PE6 cells (1×10^6) were injected i.v. into nude mice on day 0. The mice were given oral feedings with distilled water (*Control*) or PTK 787 (50 mg/kg) on days 35–40 daily. Two h after the last oral feeding, the mice were injected with 0.5% Evans blue dye (100 μl). Fifteen min later, the mice were killed, and PE was carefully harvested and then centrifuged. The $A_{630 \, nm}$ of the supernatants of PE was measured. The data represent the means for groups of four mice; *bars,* SE. ∗, $P < 0.05$.

by non-small cell lung carcinoma cells in this model. Here, we demonstrate that treatment with a VEGF/VPF receptor tyrosine kinase phosphorylation inhibitor, PTK 787, can inhibit PE formation by human lung adenocarcinoma cells in this model through the inhibition of vascular permeability.

The interaction of VEGF/VPF and its receptors (Flt-1 and Flk-1/KDR) has been shown to play an important role in angiogenesis of malignant diseases (25–27). Therefore, VEGF/VPF and its receptors (especially Flk-1/KDR) represent ideal targets for antiangiogenesis therapy. PTK 787 is a selective inhibitor of VEGF/VPF receptor tyrosine kinase phosphorylation and has been shown to inhibit VEGF/VPF-mediated responses *in vitro* and *in vivo* (28).

In this study, we found that PTK 787 did not affect the *in vitro* proliferation of PC14PE6 cells, whereas it specifically inhibited the proliferation of HDMECs (presumably expressing VEGF/VPF receptors) stimulated with VEGF/VPF. In addition, treatment with PTK 787 inhibited vascularization in lung tumors produced by PC14PE6 cells, although it did not affect VEGF/VPF expression in tumors formed by PC14PE6 cells. These findings strongly suggest that treatment with PTK 787 directly inhibits endothelial cell function but not tumor cell function.

The two major functions of VEGF/VPF are induction of angiogenesis and vascular hyperpermeability, both of which are thought to be mediated mainly by Flk-1/KDR (6, 33). In this study, we found that treatment with PTK 787 inhibited the two major functions of VEGF/VPF. Treatment with the highest dose (100 mg/kg) of PTK 787 inhibited vascularization in the lung metastasis and it inhibited total tumor volume, represented by lung weight. However, this treatment did not significantly reduce the number of lung metastases, and there was no significant correlation between the number of lung metastases and the volume of PE (Fig. 5). This is not unexpected because angio-
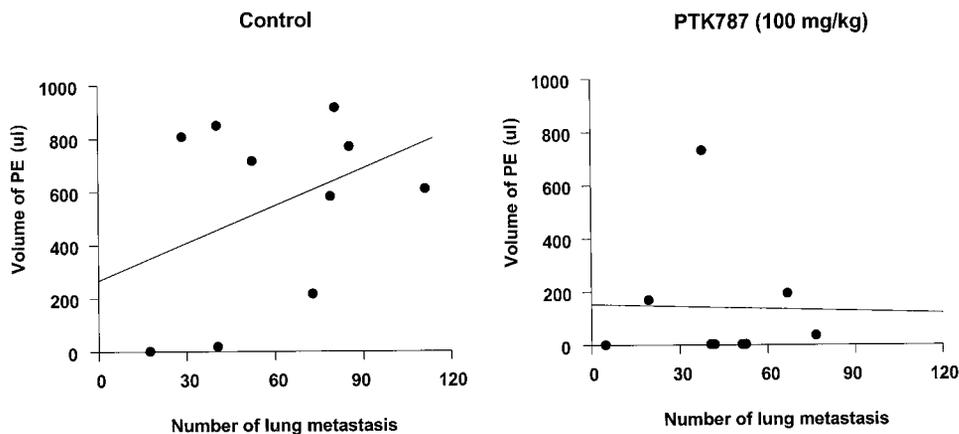
Fig. 5 Lack of correlation between the number of visible lung metastases and volume of malignant PE. PC14PE6 cells (1×10^6) were injected i.v. into nude mice on day 0. The mice ($n = 10$) were given oral feedings with distilled water (control) or PTK 787 ($n = 10$; starting on day 14). Daily oral treatments continued until day 48. The mice were killed on day 49, when lung metastasis and PE were evaluated as described in "Materials and Methods."

genesis is not necessary for small tumors (<1–2 mm in diameter; Ref. 34). On the other hand, skin vascular permeability induced by rhVEGF/VPF or PE (containing VEGF/VPF) was significantly inhibited by treatment of the mice with PTK 787 as shown in Fig. 3. Moreover, treatment with 50 mg/kg PTK 787 for 6 days significantly inhibited the vascular permeability of PE-bearing mice (Fig. 4). Collectively, these findings suggest that a primary mechanism by which treatment with PTK 787 inhibited PE was suppression of vascular permeability.

Lung cancer is the leading cause of malignant PE (1), and at least 25% of all of the patients with lung cancer will develop PE at some time during the course of the disease (35). The standard treatment for PE, drainage followed by instillation of sclerosing agents, produces variable results (1, 4, 36). We found recently that in a nude mouse model for non-small cell lung carcinoma cells, PE formation directly correlates with expression of VEGF/VPF by the tumor cells. Moreover, the levels of VEGF/VPF in malignant PE of lung cancer patients are much higher than that in PE caused by benign diseases, including heart failure and pulmonary tuberculosis (37). In this study, we showed the therapeutic potential of PTK 787 against malignant PE caused by human lung adenocarcinoma cells in an animal model.

Recently, various compounds that inhibit the function of VEGF/VPF and VEGF/VPF receptors, including humanized neutralization antibody for VEGF/VPF (38), dominant-negative VEGF/VPF (39), soluble VEGF/VPF receptors (26, 40), and low molecular weight compounds that inhibit VEGF/VPF receptor tyrosine kinases (27) have been developed, and their antiangiogenic activities have been demonstrated. The main advantages of PTK 787 over these compounds are as follows: (*a*) PTK 787 is a smaller compound with a low molecular weight and is easier to synthesize; (*b*) PTK 787 can be administered p.o. and hence may improve patient compliance. PTK 787 was very effective in this animal model. The drug administered daily for at least 35 days did not produce undesirable side effects. To confirm its efficacy, it will be necessary to evaluate the ability of PTK 787 to control malignant PE with a high level of VEGF/VPF in lung cancer patients receiving long-term treatment.

In summary, we demonstrate that the p.o.-administered VEGF/VPF receptor tyrosine kinase inhibitor PTK 787 inhibits the formation of malignant PE by human lung adenocarcinoma cells, through the inhibition of vascular permeability. Therefore, PTK 787 could be useful for the control of malignant PE in lung cancer patients, and clinical trials are warranted.

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