Both Antiangiogenesis- and Angiogenesis-Independent Effects Are Responsible for Hepatocellular Carcinoma Growth Arrest by Tyrosine Kinase Inhibitor PTK787/ZK222584

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Abstract

Vascular endothelial growth factor (VEGF) plays an important role in tumor angiogenesis of hepatocellular carcinoma. Inhibition of VEGF receptors could theoretically reduce angiogenesis and tumor growth in hepatocellular carcinoma, but this remains to be proven with an experimental study. This study examined the angiogenesis-dependent and angiogenesis-independent activities of PTK787/ZK222584 (PTK787), a tyrosine kinase inhibitor of VEGF receptors, in nude mice bearing human hepatocellular carcinoma xenografts. The in vitro effects of PTK787 on proliferation, apoptosis, and cell cycle distribution in human hepatocellular carcinoma cell lines were also studied. Oral administration of PTK787 resulted in a significant reduction in tumor volume and microvessel formation of hepatocellular carcinoma xenografts in nude mice. PTK787 inhibited tumor cell proliferation in a dose-dependent manner and also induced tumour cells to undergo apoptosis both in vivo and in vitro. The proapoptotic response was associated with down-regulation of Bcl-2 and Bcl-x1 expression and induction of cleavage of caspase-3. In addition, PTK787 induced growth arrest in hepatocellular carcinoma cells, which was associated with G1 arrest and partial G2-M block. This effect correlated with an increase in p21WAF1/CIP1 (p21) and p27KIP1 (p27) protein expression. In conclusion, this study showed that PTK787 is a potentiator of tumor growth in hepatocellular carcinoma by both angiogenic effect and direct effects on tumor cell proliferation and apoptosis. Our data suggest that blockage of VEGF receptors may provide an effective therapeutic approach for human hepatocellular carcinoma. (Cancer Res 2005; 65(9): 3691-9)

Introduction

Hepatocellular carcinoma is one of the most common malignancies in the world. Surgery offers a chance of cure, but the prognosis of hepatocellular carcinoma patients remains poor (1). Recent studies have indicated that tumor angiogenesis plays a significant role in the progression of hepatocellular carcinoma (2).

A number of angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor are expressed in hepatocellular carcinoma (3, 4). Of these factors, VEGF is one of the most potent angiogenic factors involved in neovascularization (5). Recently, our group has shown that tumor expression of VEGF correlated positively with venous invasion in hepatocellular carcinoma (6, 7). The effects of VEGF are mediated mainly through two structurally related, high-affinity tyrosine kinase receptors, VEGF receptor 1 (Flt-1) and VEGF receptor 2 (Flk-1/KDR; ref. 8). Flk-1/KDR is regarded as a key signaling receptor required for the full spectrum of VEGF responses, including endothelial cell proliferation, migration, differentiation, and induction of vascular permeability (9, 10). Recent data indicate that Flt-1 is also important in angiogenic signaling (11, 12). Both Flt-1 and Flk-1/KDR are expressed primarily on endothelial cells and are up-regulated at sites of active angiogenesis (13). VEGF receptors are overexpressed in many human cancers, including hepatocellular carcinoma, suggesting that the role of the VEGF signaling pathway extends beyond angiogenesis in solid tumors (14–16). Because of the key roles of VEGF and its receptors in tumor angiogenesis and growth, inhibiting VEGF signal transduction provides an opportunity for therapeutic intervention. One approach currently under clinical trials in patients with advanced cancer is to use recombinant antibody to neutralize VEGF (17). An alternative approach aims to generate small molecule inhibitors of VEGF receptor tyrosine kinase domain. PTK787 is a potent tyrosine kinase inhibitor, binding directly to the ATP-binding sites of VEGF receptors (18). It inhibits both Flt-1 and Flk-1/KDR, but it also inhibits other class III receptor tyrosine kinases with less potency, such as platelet-derived growth factor receptor β (PDGFR-β), Flt-4, c-kit, and c-fms (18). PTK787 inhibits endothelial cell migration and proliferation, but it does not have any cytotoxic or antiproliferative effects on cells that do not express VEGF receptors (18). PTK787 has been shown to inhibit the growth of several human xenograft tumors including epidermoid, colon, prostate, renal, and thyroid carcinomas (18–21). In the present study, we examined the effects of PTK787 on tumor growth and angiogenesis of hepatocellular carcinoma xenografts in nude mice. Direct effects of PTK787 on hepatocellular carcinoma cell lines were also investigated. Furthermore, the molecular mechanisms of PTK787 on hepatocellular carcinoma were studied by investigating the expression of cell cycle–related proteins p21 and p27 and apoptosis-related proteins Bcl-2, Bcl-x1, and caspase-3.

Materials and Methods

Cell lines. The hepatocellular carcinoma cell lines PLC and Hep3B were obtained from American Type Culture Collection (Manassas, VA). HuH 7 was provided by Dr. H. Nakabayashi (Hokkaido University School of Medicine, Hokkaido, Japan). Cell lines were cultured in DMEM containing supplements (10% FCS, penicillin/streptomycin, and l-glutamine). Immortalized human hepatocyte cell line MIHA was provided by Dr. Roy Chowdhury (Albert Einstein College of Medicine, New York, NY) and cultured in Waymouth’s MB 752/1 medium with supplements.

Drug. PTK787 was provided by Novartis Pharmaceutical Ltd. (Basel, Switzerland).
Ectopic and orthotopic human hepatocellular carcinoma models in nude mice. Male BALB/c athymic (nu/nu) mice (4-5 weeks old) were purchased from Laboratory Animal Unit, The University of Hong Kong, Hong Kong. The research protocol was approved by the Institutional Ethics Committee on the Use of Live Animals for Teaching and Research. PLC and Hep3B tumors were initially established by s.c. injection of 5 × 10⁶ cells in PBS, and cubic tumor fragments of 2 to 3 mm³ size were implanted s.c. for the therapeutic experiments in mice. Mice were randomized into three groups (n = 10 in each group) as follows: (a) daily oral administration of vehicle solution (water) for PTK787 (control group); (b) daily oral administration of 50 mg per kg per day PT7K87 for 4 weeks; and (c) daily oral administration of 100 mg per kg per day PTK787 for 4 weeks. Drug treatment was initiated when tumor volumes reached 25 to 50 mm³ and tumor growth was monitored weekly. For orthotopic tumor model, a piece of tumor fragment of 2 mm³ was implanted into the liver tissue of the left lobe. PTK787 administration at 50 mg per kg per day started on day 5 after tumor implantation. After 4 weeks, both untreated and treated nude mice were sacrificed. Tumor volume was calculated according to the formula [tumor volume = largest diameter × (perpendicular² / 2)]. The orthotopic tumor model was used to show the effect of PTK787 on tumor growth in the liver compared with the ectopic tumor model. All the other studies were done in ectopic xenografts.

Histologic and immunohistochemical studies. Mice were killed and tumors were excised for histologic study. For detection of VEGFR expression in hepatocellular carcinoma xenografts, tumor sections were incubated with rabbit polyclonal antibodies at a 1:100 dilution for Flk-1/KDR or Flt-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Nonimmune rabbit serum was included as a negative control. Immunostaining was carried out using DAKO EnVision Plus System, peroxidase (3,3'-diaminobenzidine; DakoCytomation California, Inc., Carpinteria, CA). For evaluation of tumor angiogenesis, paraffin sections were immunostained with anti-CD31 monoclonal antibody (Santa Cruz Biotechnology), and microvessel density was evaluated as described previously (2). At low power field (>40), the tissue sections were screened, and five areas with the most intense neovascularization were selected. Microvessel counts of these areas were done at high-power field (HF, ×200) and the mean microvessel count of the five most vascular areas was taken as the microvessel density, which was expressed as the absolute number of microvessels per HF.

Expression of Flk-1/KDR and Flt-1 on cell lines by flow cytometry analysis. Cells lines were incubated with Flk-1/KDR or Flt-1 antibodies (Santa Cruz Biotechnology) for 45 minutes at 4°C, washed with ice-cold PBS, and incubated with anti-rabbit FITC (BD PharMingen, San Diego, CA) for 30 minutes. Cells were washed and subjected to flow cytometry analysis by FACS Calibur (Becton Dickinson, San Jose, CA). Rabbit immunoglobulin G (Zymed Laboratories, South San Francisco, CA) was included as a negative control.

Hepatocellular carcinoma cell proliferation assays. Proliferation of hepatocellular carcinoma cell lines was measured by bromodeoxyuridine (BrdUrd) incorporation using BrdUrd labeling and detection kit (Roche Diagnostics Co., Indianapolis, IN). Cells were plated at a density of 5 × 10⁴ cells per well within 96-well plates and cultured overnight followed by washing cells with PBS twice and replacing growth medium with medium (0.1% FCS) containing recombinant human VEGF (0.1-100 ng/mL; R&D Systems, Minneapolis, MN). BrdUrd labeling solution was added after 24 hours. Cells were incubated for another 16 hours before fixation, addition of nucleases, anti-BrdUrd-PoD, and peroxidase substrate. The absorbance at 405 nm (with a reference wavelength at 490 nm) was measured using an ELISA plate reader (Molecular Devices Co., Sunnyvale, CA).

Effects of PTK787 on hepatocyte and hepatocellular carcinoma cell line proliferation. Cells were seeded into 96-well plates and incubated overnight. PTK787 was added in serial dilutions in the medium containing 1% FCS and the plates were incubated for another 72 hours. Cell proliferation was done using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay by adding 20 μL of CellTiter 96 Aqueous solution (Promega Co., Madison, WI) into each well containing 100 μL culture medium, and cells were incubated for 4 hours at 37°C. The absorbance at 490 nm was measured using an ELISA plate reader (Molecular Devices).

Detection of cell cycle by flow cytometry analysis. Hepatocellular carcinoma cell lines were seeded in 24-well plates and treated with PTK787 at different concentrations in the medium containing 1% FCS for 24 hours. Cells were washed, fixed with ice-cold 70% ethanol and incubated in 800 μL PBS, 100 μL RNase (1 mg/mL; Sigma, St. Louis, MO), and 20 μL propidium iodide (PI: 2 mg/mL; Sigma) for 30 minutes at 37°C followed by flow cytometry analysis using FACSCalibur (Becton Dickinson). The percentage of cells in the G0-G1 and G2-M phases was assessed by ModFit LT software (Verity Software House, Topsham, ME).

Detection of cell apoptosis by flow cytometry analysis. Hepatocellular carcinoma cell lines were seeded in 24-well plates and treated with PTK787 at different concentrations for 24 or 48 hours in the medium containing 1% FCS. Cells were harvested and resuspended in binding buffer [10 mMol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L CaCl₂] at a concentration of 1 × 10⁶ cells/mL. Five microliters of Annexin V-FITC (BD PharMingen) and 10 μL of PI (50 μg/mL; Sigma) were added to 100 μL of resuspended cells. Cells were gently mixed and incubated for 15 minutes at room temperature in the dark and analyzed within 1 hour by FACSCalibur (Becton Dickinson).

Terminal deoxynucleotidyl transferase–mediated nick-end labeling assay for apoptosis in hepatocellular carcinoma xenografts. Terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assay was done using In situ Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer’s instructions. Five areas were selected under microscope and apoptosis of these areas were counted at HF (×400).

Measurement of vascular endothelial growth factor levels in the supernatants of hepatocellular carcinoma cell culture in plasma and tumor of nude mice. Tumor tissue and blood were collected from nude mice with xenografts after the last PTK787 treatment. Tumor cytosol was obtained by homogenization of tissues as described (22). Homogenates were centrifuged at 20,000 × g at 4°C for 10 minutes. The supernatants were collected for assay of the tumor cytosolic VEGF concentration, and the total protein concentration was determined using Bio-Rad total protein assay system (Bradford, Hercules, CA). Three-day hepatocellular carcinoma cell culture supernatants were collected, centrifuged, and stored at −80°C for further analysis. Levels of VEGF protein in the hepatocellular carcinoma cell culture supernatants, plasma, and tumor cytosol were measured by Quantikine Human VEGF Immunoassay (R&D Systems) according to the manufacturer’s instructions.

Western blot analysis for Flk-1/KDR and Flt-1 in cell lines. Bcl-2, Bcl-xL, caspase-3, p21, and p27 in both hepatocellular carcinoma xenografts and cell lines. Cell lines were lysed in lysis buffer (Cell Signaling, Beverly, MA) for 20 minutes at 4°C. For analysis of the expression of bcl-2, bcl-xL, caspase-3, p21, and p27, hepatocellular carcinoma cell lines were treated with PTK787 at various concentrations in the medium with 1% FCS for 24 hours, washed twice with PBS before lysis. For tumor samples from nude mice, tissues were homogenized and lysed using lysis buffer. The lysates were centrifuged at 15 minutes, 12,000 × g, 4°C and equal amounts of solubilized proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked with TBST [20 mmol/L Tris (pH 7.6), 135 mmol/L NaCl, and 0.1% Tween 20] containing 5% nonfat milk and immunoblotted with the following antibodies: Flk/KDR (1:1,000), Flt (1:1,000), Bcl-2 (1:1,000), Bcl-xL (1:1,000; Santa Cruz Biotechnology), caspase-3 (1:1,000), p21 (1:2,000; Cell Signaling), and p27 (1:2,000; Transduction Laboratory, Lexington, KY) for 18 hours at 4°C followed by detection using horseradish peroxidase–conjugated secondary antibody (1:1,000; Santa Cruz Biotechnology; 1 hour, room temperature). Immunoreactive protein bands were visualized by the enhanced chemiluminescence system (Amersham Biosciences).

Statistical analysis. Continuous data were expressed as mean ± SE. One-way ANOVA or two-tailed Student’s t test was used where appropriate. P < 0.05 was considered statistically significant.
Figure 1. A, effects of PTK787 on the ectopic tumor growth of human PLC and Hep3B xenografts. PTK787 administration p.o. daily at 50 or 100 mg per kg per day was started when tumor size reached 25 to 50 mm³. Points, mean; bars, ± SE. *P < 0.05 compared with control for treatment with PTK787 at both doses. B, effect of PTK787 on orthotopic PLC tumor growth. PTK787 administration at 50 mg per kg per day was initiated on day 5 after tumor implantation into the left lobe of liver. Representative photos show that the tumors (black arrows) of untreated and treated groups. Columns, mean; bars, ± SE. *P < 0.01 compared with control for treatment with PTK787. C, effect of PTK787 on angiogenesis was measured by tumor microvessel density using anti-CD34 monoclonal antibody staining. At low-power field (×40), the tissue sections were screened and five areas with the most intense neovascularization were selected. Microvessel counts of these areas were performed at HPF (×200). Representative photos show that PTK787 treatment at 50 mg per kg per day for 28 days resulted in significantly decreased CD34-positive cells (brown color). D, in all tissues collected from PLC and Hep3B tumor xenografts, the density of microvessels was significantly lower after 14, 21, and 28 days of PTK787 administration, compared with the untreated control groups. Columns, mean; bars, ± SE. *, *P < 0.05 when compared with controls.
Results

**Effect of PTK787 on growth of hepatocellular carcinoma xenografts in nude mice.** PTK787 induced dose- and time-dependent inhibition of growth of PLC and Hep3B tumors after daily oral administration at doses of 50 or 100 mg per kg per day for 4 weeks (Fig. 1A). Oral administration of PTK787 at a dose of 50 mg per kg per day to nude mice implanted ectopically with PLC cells resulted in a significant reduction of 56%, 58%, and 60% in tumor volumes in nude mice after 14, 21, and 28 days, respectively, whereas PTK787 at a dose of 100 mg per kg per day resulted in an even more dramatic decrease of 78%, 83%, and 86% in tumor volumes after 14, 21, and 28 days, respectively. PTK787 reduced Hep3B ectopic tumor growth by 37%, 50%, and 58% at a dose of 50 mg per kg per day and by 57%, 68%, and 69% at a dose of 100 mg per kg per day, after 14, 21, and 28 days of administration, respectively. To further illustrate the effect of PTK787 on hepatocellular carcinoma, we established orthotopic tumor model by implantation of PLC tumor fragment in the liver. Compared with the untreated group, PTK787 treatment at 50 mg per kg per day for 28 days resulted in dramatic reduction of tumor volume by 82% (from average 141.6 ± 36.4 to 26.1 ± 12.3 mm³; Fig. 1B). PTK787 was well tolerated by the mice at both dosages and no significant effects on body weight or general well-being of the nude mice were observed. On histologic examination by H&E staining, necrosis of tumor tissues was observed in the groups treated with PTK787 for 14 to 28 days but not in the untreated controls (data not shown).

**Effect of PTK787 on tumor angiogenesis.** After 14 days of treatment with PTK787 at a dose of 50 or 100 mg per kg per day, a significant decrease in tumor microvessel density was observed in the PTK787 groups compared with the control groups in association with reduced tumor growth, and the tumor microvessel density remained significantly lower in the PTK787 groups after 21 and 28 days of treatment (Fig. 1C and D).

**Expression and function of Flk-1/KDR and Flt-1 on hepatocellular carcinoma cell lines.** Flow cytometry data showed that hepatocellular carcinoma cell lines PLC, Hep3B, and HuH 7 overexpressed both Flk-1/KDR and Flt-1 determined by both percentage of VEGF receptor–positive cells and VEGF receptor expression levels (mean channel fluorescence). MIHA also expressed both VEGF receptors but at low level (Fig. 2A).

Using Western blot analysis, we also detected both Flk-1/KDR and Flt-1 in hepatocellular carcinoma xenografts. Representative results of immunohistochemistry staining for Flk-1/KDR and Flt-1 in tumor cells. Rabbit serum replaced VEGFR antibodies and served as a negative control (×200).

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**Figure 2.** A, surface expression of Flk-1/KDR and Flt-1 in hepatocellular carcinoma cell lines. MIHA, PLC, Hep3B, and HuH7 were labeled with anti-Flk1/KDR or anti-Flt-1, followed by antirabbit FITC and analyzed by flow cytometry. Expression of Flk1/KDR and Flt-1 was presented as percentages of cells positive for the receptors and as mean channel fluorescence (expression levels) on each cell line. Columns, mean; bars, ±SE. B, expression of Flk-1/KDR and Flt-1 in total cell extracts was determined by Western blot analysis. Results of a representative study. Two additional experiments yielded similar results. C, effect of VEGF on hepatocellular carcinoma cell proliferation. Cells were incubated VEGF at various concentrations for 24 hours followed by addition of BrdUrd. Each experiment was done in quadruplicate. Columns, mean of six experiments; bars, ±SE. D, expression of Flk-1/KDR and Flt-1 in hepatocellular carcinoma xenografts. Representative results of immunohistochemistry staining for Flk-1/KDR and Flt-1 in tumor cells. Rabbit serum replaced VEGFR antibodies and served as a negative control (×200).
(M, 150,000) and Flt-1 (M, 180,000) in hepatocellular carcinoma cell lines (Fig. 2B). To determine the functionality of VEGF receptors on hepatocellular carcinoma cell lines, we evaluated the proliferation of hepatocellular carcinoma cells in the presence of exogenous VEGF using BrdUrd incorporation assays. VEGF stimulation led to an increase in cell proliferation (Fig. 2C). To verify VEGF receptor expression in tumor xenografts, we analyzed tumor sections by immunohistochemistry. Both Flk-1/KDR and Flt-1 staining was observed in tumor cells (Fig. 2D).

**Direct effects of PTK787 on hepatocellular carcinoma cell lines.** As shown in Fig. 3, PTK787 inhibited proliferation of hepatocellular carcinoma cell lines in a dose-dependent manner. The average IC₅₀ values of PLC, HuH7, and Hep3B from three experiments were 3.2, 6.0, and 11.1 μmol/L, respectively. In contrast, immortalized human hepatocytes were not affected by PTK787 with IC₅₀ of 30 μmol/L. This stimulatory effect by VEGF at concentration of 50 ng/mL could be inhibited by PTK787, and IC₅₀ values of PLC, HuH7, and Hep3B from three experiments were 4.1, 7.3, and 12.4 μmol/L, respectively. Changes in the cell cycle profile induced by PTK787 were also studied. As shown in Fig. 3B, PTK787 triggered a significant increase in the number of cells in G₀-G₁ phase, with a corresponding decrease in the number of cells in G₂-M phase.
Furthermore, PTK787 induced tumor cell apoptosis in a dose- and time-dependent manner (Fig. 3C).

**Effect of PTK787 on tumor cell apoptosis in hepatocellular carcinoma xenografts.** After 7 days of PTK787 treatment at an oral dose of 50 mg/kg daily, the mean number of TUNEL-positive cells was significantly increased compared with the untreated controls (P < 0.010, Fig. 4).

**Effects of PTK787 on vascular endothelial growth factor expression in cell lines and hepatocellular carcinoma xenografts.** All three hepatocellular carcinoma cell lines produced VEGF, and the release of VEGF was inhibited by PTK787 in a dose-dependent manner (Fig. 5A). In addition, PTK787 treatment resulted in a reduction of VEGF levels in both the plasma and tumor tissues from the hepatocellular carcinoma xenografts (Fig. 5B and C). However, when the VEGF level was corrected for the reduction in cell density in the cell culture treated by PTK787, there was no significant reduction in the VEGF level on a per cell basis.

**Effects of PTK787 on expression of Bcl-2, Bcl-xL, caspase-3, p21, and p27 in hepatocellular carcinoma xenografts and cell lines.** The effects of PTK787 on target proteins Bcl-2, Bcl-xL, and caspase-3, both in vitro and in vivo, were evaluated by Western blot analysis. Figure 6A showed that PTK787 markedly down-regulated the expression of Bcl-2 and Bcl-xL after 7 and 14 days of PTK787 administration in nude mice hepatocellular carcinoma xenografts. Cleavage of caspase-3 protein was also significantly increased after 7 and 14 days of PTK787 treatment, but there was no significant change of the total caspase-3 protein. Reduced Bcl-2 and Bcl-xL protein expression was also observed in hepatocellular carcinoma cell lines after PTK787 pretreatment (Fig. 6B).

To elucidate the molecular basis of G1 arrest induced by PTK787, we also studied the expression of cyclin-dependent kinase (CDK) inhibitors p21 and p27 on both tumor tissues and tumor cell lines of hepatocellular carcinoma. Oral administration of PTK787 at a dose of 50 mg/kg daily for 21 or 28 days resulted in a significant increase of p21 and p27 expression (Fig. 6C). We also detected significantly increased p21 and p27 protein expression in hepatocellular carcinoma cell lines pretreated with PTK787 at 5 or 10 μmol/L (Fig. 6D).

**Discussion**

The inhibition of tumor angiogenesis is a promising novel approach of anticancer therapy. Among the most potent inhibitors of angiogenesis currently available is PTK787, a very potent inhibitor to Flk-1/KDR (IC50, 0.037 μmol/L) and Flt-1 (IC50 0.077 μmol/L) in vitro but is also active against other tyrosine kinases at higher concentrations. A previous study has shown the good bioavailability by oral administration of PTK787, and its pharmacologic properties have been described elsewhere (18).
Recently, it has been reported that angiogenic response increased during hepatocarcinogenesis, and antibodies targeting VEGF receptors significantly attenuated hepatocellular carcinoma development and lung metastasis in addition to suppressing neovascularization (23). Therefore, VEGF and its receptors may provide potential targets for a novel therapeutic strategy against hepatocellular carcinoma.

Oral administration of PTK787 at a dose of 25 to 100 mg per kg per day was previously shown to inhibit tumor growth in some xenografts of human cancers (18). The effect of PTK787 on hepatocellular carcinoma, which is one of the most vascular tumors, has not been investigated before. Our study showed that daily treatment with PTK787 at a dose of 50 or 100 mg per kg per day effectively inhibited ectopic and orthotopic tumor growth in nude mice bearing established human hepatocellular carcinoma xenografts. Immunohistochemical study of the tumors revealed that PTK787 significantly reduced tumor microvessel formation. This is compatible with the key effect of PTK787 as an antiangiogenic agent (18).

Administration of PTK787 was found to impart antitumor activity in diverse types of human solid cancer xenograft models (18–21). However, in previous studies, the antitumor activity of PTK787 was attributed to the inhibition of VEGF signaling in the tumor vasculature, and a direct antiproliferative effect on solid tumor cells was either not studied (19–21) or not observed (18). A previous study has shown that PTK787 could inhibit the growth of multiple myeloma cells (24). A similar direct effect on cancer cells may occur in the antitumor activity of PTK787 in solid cancers. Previous reports on the expression of VEGF receptors in the tumor cells of several human solid cancers (14, 25–29) led us to investigate possible angiogenesis-independent antitumor effect of PTK787 on human hepatocellular carcinoma. We observed surface expression of both Flk-1/KDR and Flt-1 in all hepatocellular carcinoma cell lines. By employing Western blot analysis as a second independent approach, we also detected the expression of both VEGF receptors in human hepatocellular carcinoma cell lines. Immunohistochemical analysis showed that both VEGF receptors were also expressed in the tumor tissues from hepatocellular carcinoma xenografts. We found that treatment with exogenous VEGF resulted in a significant increase in cell proliferation in hepatocellular carcinoma cell lines. We evaluated the direct effects of PTK787 on hepatocellular carcinoma cell lines and showed that PTK787 could inhibit hepatocellular carcinoma cell proliferation in vitro. The stimulatory effect by VEGF could also be inhibited by PTK787. Furthermore, we found that VEGF was expressed and secreted by all hepatocellular carcinoma cell lines, and treatment with PTK787 contributed to a dose-dependent inhibition of VEGF production by hepatocellular carcinoma cell lines. However, PTK787 inhibited hepatocellular carcinoma cell proliferation. Hence, the reduction of VEGF level in the PTK787-treated cell lines may be related to a reduction in the density of the hepatocellular carcinoma cells. In fact, by normalizing VEGF production on a per cell basis, there was no significant reduction of VEGF secretion among PTK787 treatment groups and control.

Although VEGF signaling is thought to occur primarily in endothelial cells, numerous studies have suggested that VEGF may act in an autocrine loop fashion in a variety of malignant tumor cells (14–16, 25–28). Our results implied for the first time the biological relevance of VEGF as an autocrine growth factor in human hepatocellular carcinoma, and PTK787 might exert an angiogenesis-independent inhibitory effect on tumor growth through blocking the autocrine loop of VEGF and its receptors. Indeed, PTK787 has been reported to inhibit Flt-1 expressing multiple myeloma cell proliferation and VEGF-induced tyrosine phosphorylation of Flt-1 (24). In addition, we also found that PTK787 induced hepatocellular carcinoma cells to undergo apoptosis both in vitro and in vivo.

In this study, we observed reduction in both plasma and tumor cytosol VEGF levels in nude mice bearing hepatocellular carcinoma xenografts after PTK787 treatment. The effect of PTK787 administration on tumor VEGF expression was not evaluated in most of the previous studies on PTK787 in animal tumor models. The reduced plasma VEGF level upon PTK787 treatment might be partly attributable to the reduced tumor volume, as the production of VEGF was dependent upon tumor cell mass. The decrease of tumor cytosol VEGF level might be
Recently, Bcl-2 and Bcl-xL have been regarded as potent therapeutic targets of cancer therapy based on their ability to disrupt apoptosis. Cancer Res 2005; 65: (9). May 1, 2005 3698 www.aacrjournals.org

Reduced expression of p27 has been reported to be associated with decreased after PTK787 treatment both in vivo and in vitro. Our study showed for the first time that PTK787 induced expression of the key regulators of apoptosis, Bcl-2 and Bcl-xL, both in vivo and in vitro. Our study provided the first data demonstrating that the expression levels of both Bcl-2 and Bcl-xL significantly decreased after PTK787 treatment both in vivo and in vitro. Recently, Bcl-2 and Bcl-xL have been regarded as potent therapeutic targets of cancer therapy based on their ability to disrupt apoptosis and confer resistance to chemotherapy and radiotherapy in cancer cells including hepatocellular carcinoma (30–32). We also detected activation of caspase-3 after PTK787 administration. In addition, our study showed for the first time that PTK787 induced expression of the CDK inhibitors p21 and p27 and accumulation of cells in G1 phase. In human hepatocellular carcinoma, reduced p21 expression was previously shown and it might play a role in hepatocarcinogenesis (33). Therefore, up-regulation of p21 by PTK787 may be an important mechanism of its anticancer effect. It has been well documented that p21 expression is regulated by at least two alternative mechanisms, p53 dependent and p53 independent. Although we did not measure p53 expression after PTK787 treatment, PTK787 induced p21 up-regulation in p53-deleted Hep3B cells, suggesting a p53-independent mechanism for PTK787-mediated modulation of p21 expression. Furthermore, we also detected increased p21 expression in Hep3B tumor tissues (data not shown). Recent studies have highlighted the relevance of p27 in the progression of various human malignancies (34, 35). Reduced expression of p27 has been reported to be associated with portal vein invasion, intrahepatic metastasis, and shorter disease-free survival in hepatocellular carcinoma (36). In this study, we have found a strong induction of p27 expression by the administration of PTK787 both in vitro and in vivo, which may contribute to the antitumor effect of the drug.

A previous report revealed that PTK787 also exhibited activity against PDGFR-β (18) and induced dose-dependent inhibition of the PDGF response at a higher dose range (18). Because hepatocellular carcinoma expressed a high level of PDGFR-β (37), PTK787 could also act through a PDGF pathway. However, this needs to be clarified with further studies.

In humans, PTK787 is currently studied in phase III trials in combination with standard chemotherapy for first- and second-line treatment in patients with colorectal cancer (38). Phase I/II studies showed that PTK787 was well tolerated in cancer patients and contributed to a reduction of tumor perfusion and vascular permeability measured by dynamic contrast-enhanced magnetic resonance imaging (39). Thomas et al. have reported an impressive stabilization in patients with advanced cancer (40). The findings in our study provide the rationale for testing PTK787 in patients with advanced hepatocellular carcinoma. The search for a new therapeutic agent that can be tested clinically for the treatment of advanced hepatocellular carcinoma is particularly important because of the current lack of effective systemic chemotherapy for hepatocellular carcinoma (41, 42).

In conclusion, this study shows that inhibition of VEGF receptors by oral administration of PTK787 is an effective approach to inhibit the growth of hepatocellular carcinoma, both by its antiangiogenic effect and direct antiproliferative effect on hepatocellular carcinoma. To our knowledge, this is the first report providing evidence that PTK787 is capable of inhibiting angiogenesis and blocking autocrine regulation of hepatocellular carcinoma growth. Furthermore, PTK787 may involve multiple pathways related to growth arrest and apoptosis induction of tumor cells. Our study shows the therapeutic potential of

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**Figure 6.** Effect of PTK787 treatment on Bcl-2, Bcl-xL, caspase-3, p21, and p27 protein expression in PLC tumor xenografts and PLC and Hep3B cell lines. Western blot analysis was performed on total lysates from tumor specimens of mice untreated (control) or treated with PTK787 50 mg per kg per day. Bcl-2, Bcl-xL, caspase-3 (A), p21 and p27 (C) protein expression was analyzed by Western blot analysis. Expression of Bcl-2 and Bcl-xL (B), p21 and p27 (D) in PLC and Hep3B cells untreated or treated with PTK787 at different concentrations for 24 hours was analyzed by Western blot as described in Materials and Methods. Results of a representative study. Two additional experiments yielded similar results.
PTK787 for hepatocellular carcinoma and provides the basis for clinical trials on the use of PTK787 alone or in combination with conventional antitumor therapies for the treatment of hepatocellular carcinoma.

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