Blockade of the Epidermal Growth Factor Receptor Signaling by a Novel Tyrosine Kinase Inhibitor Leads to Apoptosis of Endothelial Cells and Therapy of Human Pancreatic Carcinoma

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ABSTRACT

We determined whether down-regulation of the epidermal growth factor-receptor (EGF-R) signaling pathway by oral administration of a novel EGF-R tyrosine kinase inhibitor (PKI166) alone or in combination with gemcitabine (administered i.p.) can inhibit growth and metastasis of human pancreatic carcinoma cells implanted into the pancreas of nude mice. Therapy beginning 7 days after orthotopic injection of L3.6pl human pancreatic cancer cells reduced the volume of pancreatic tumors by 59% in mice treated with gemcitabine only, by 45% in those treated with PKI166 only, and by 85% in those given both drugs. The combination therapy also significantly inhibited lymph node and liver metastasis, which led to a significant increase in overall survival. EGF-R activation was significantly blocked by therapy with PKI166 and was associated with significant reduction in tumor cell production of VEGF and IL-8, which in turn correlated with a significant decrease in microvessel density and an increase in apoptotic endothelial cells. Collectively, our results demonstrate that oral administration of an EGF-R tyrosine kinase inhibitor decreased growth and metastasis of human pancreatic cancer growing orthotopically in nude mice and increased survival. The therapeutic effects were mediated in part by inhibition of tumor-induced angiogenesis attributable to a decrease in production of proangiogenic molecules by tumor cells and increased apoptosis of tumor-associated endothelial cells.

INTRODUCTION

Cancer of the exocrine pancreas is characterized by extensive local invasion and early lymphatic and hematogenous metastasis (1, 2). At the time of diagnosis, >80% of patients present with either locally advanced or metastatic disease (1–3). The inability to detect pancreatic cancer at an early stage, its aggressiveness, and lack of effective systemic therapy are responsible for rapid death from this disease. In fact, only 1–4% of all patients with adenocarcinoma of the pancreas survive 5 years after diagnosis (4, 5). For patients with advanced pancreatic cancer, even the recent introduction of the deoxycytidine analogue gemcitabine does not extend median survival beyond 6 months (6). Clearly, novel approaches to human pancreatic carcinoma therapy are needed.

Recent advances in the understanding of the biology of this disease may now offer new approaches to its therapy. Research efforts using archival human pancreatic tumor tissue or human pancreatic cancer cell lines have identified a number of characteristic biochemical and genetic abnormalities. These include point mutations at codon 12 of the K-ras oncogene in 75–90% of pancreatic adenocarcinoma specimens (7, 8) and homozygous deletions involving the cyclin-dependent kinase-inhibitory p16 gene, found in 85% of human pancreatic cancer xenografts (9). Mutation or homozygous deletion of Smad4 (DPC4), a signal transduction molecule mediating the antiproliferative effects of TGF-β are found in >50% of tumors (10, 11) as are mutations in p53 (8, 12). Other investigators have also demonstrated that receptor protein tyrosine kinases, such as the EGF-R (13), c-erbB2 (14), insulin-like growth factor I (15), and fibroblast growth factor receptor (16), are highly expressed in human pancreatic cancer tissues or pancreatic cancer cell lines.

Although pancreatic cancer cells are characterized by growth-promoting effects of genetic and biochemical changes, extensive interaction with normal host cells is necessary for progressive growth and metastasis of tumors. A critical tumor-host interaction necessary for local growth and metastasis is the neovascularization of growing tumors (22, 23). The extent of angiogenesis depends on the balance between proangiogenic and antiangiogenic factors released by tumor cells and host cells.

Human pancreatic cancer cells secrete the proangiogenic molecules VEGF, IL-8, and basic fibroblast growth factor (24, 25). VEGF, currently regarded as the major proangiogenic factor for most types of human cancer (26), is strongly induced by EGF and TGF-α (27, 28). Thus, both EGF-R-mediated proliferation and angiogenesis are fundamental to the progressive growth of human pancreatic carcinoma and have been independently evaluated as targets for therapy (20–31). The purpose of the present study was to evaluate whether down-regulation of EGF-R signaling pathways by a novel oral EGF-R tyrosine kinase inhibitor, PKI166, inhibits growth and metastasis of human pancreatic cancer implanted into the pancreas of nude mice. We show that daily oral administration of PKI166 combined with weekly injections of gemcitabine leads to significant therapeutic effects mediated in part by induction of apoptosis in tumor-associated endothelial cells.

3 The abbreviations used are: TGF, tumor growth factor; EGF-R, epidermal growth factor receptor; PBS, fetal bovine serum; MAb, monoclonal antibody; IHC, immunohis-tochemistry; DAB, 3,3′-diaminobenzidine; IL, interleukin; PCNA, proliferating cell nuclear antigen; MTT, tetrazolium salt; MVD, microvessel density; PKI, protein tyrosine inhibitor; PKI166, 4-[(R)-phenethylamino-6-(hydroxyl) phenyl]-7H-pyrrolo[2,3-d]-pyrimidine; ICD, intracellular domain; VEGF, vascular endothelial growth factor; PECAM, platelet/endothelial cell adhesion molecule; TUNEL, terminal deoxynucleotidyltrans-ferase-mediated nick end labeling; PTK, protein tyrosine kinase.

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MATERIALS AND METHODS

Pancreatic Cancer Cell Lines and Culture Conditions. For these studies, we used a highly metastatic cell line (L3.6pl) isolated after successive cycles of selections in nude mice (24). The original COLO 357 human pancreatic cancer cell line established from a celiac lymph node metastasis of well-differentiated, mucin-containing pancreatic cancer cells (32) was injected by Vezeridis et al. (33) into the spleen of nude mice to isolate liver metastases designated as the L3.3 line. We injected the L3.3 cells into the pancreas of nude mice and isolated liver metastases, which we designated as L3.6pl (pancreas-liver). After two additional orthotopic injection-selection cycles, we isolated the cells designated as L3.6pl, which produced spontaneous liver metastasis at a significantly higher incidence than the original COLO 357 cells (24). The highly metastatic human pancreatic cancer L3.6pl cells were maintained in DMEM, supplemented with 5% FBS, sodium pyruvate, nonessential amino acids, l-galactosamine, 2-mercaptoethanol, and 10 mM MnCl2, 10 mM NaN3, 1 mM DTT, 0.1 μCi/lassay (30 μl) [γ-32P]ATP, 5 μM ATP, 30 μg/ml poly(Ala-Glu-Lys-Tyr) (6:2:5:1; Sigma P1152), 1% DMSO, and 50 ng of c-AbI enzyme. Src kinase (60 μg/ml) was preactivated with 50 μM ATP for 10 min at room temperature, and kinase inhibition was measured in 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 μM NaN3, 1 mM DTT, 0.1 μCi/lassay (30 μl) [γ-32P]ATP, 20 μM ATP, 25 μg/ml poly(Glu/Tyr, 4:1), 1% DMSO, and 10 ng of c-Src enzyme.

GST-fused kinase domains of KDR, Flt-1, Flk, Tek, c-Met, and c-Kit were expressed in baculovirus and purified over glutathione-Sepharose. Kinase inhibition was measured by detecting the decrease in phosphorylation of poly(Glu/Tyr, 4:1) essentially as described previously for EGF-R. Each kinase was incubated under optimized buffer conditions in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 3–10 mM MgCl2, 10 μM NaN3, 1 mM DTT, 0.2 μCi/lassay (30 μl) [γ-32P]ATP, 8–10 μg/ml poly(Glu/Tyr, 4:1), and 1% DMSO in a total volume of 30 μl in the presence or absence of PKI166 for 10 min at ambient temperature. Reactions were terminated by adding 10 μl of 250 mM EDTA, and the reaction mixture was transferred onto an Immobilon-polyvinylidene difluoride membrane (Millipore, Bedford, MA). After being washed (0.5% H2PO4, soaked in ethanol, and dried), filters were counted in a liquid scintillation counter. IC50 for PKI166 were calculated by linear regression analysis of the percentage inhibition. Inhibition of Cdc2/cyclin B protein kinase (39, 40) and protein kinase C (41) was assayed as described previously.

Animals and Orthotopic Implantation of Tumor Cells. Male athymic nude mice (NCr-nu) were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U. S. Department of Agriculture, U. S. Department of Health and Human Services, and the NIH. The mice were used in accordance with institutional guidelines when they were 8–12 weeks of age.

Orthotopic Implantation of Tumor Cells. To produce tumors, L3.6pl cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed once in serum-free medium and resuspended in HBSS. Only suspensions consisting of single cells with >90% viability were used for the injections. Cells were injected into the pancreas as described previously (18). The mice were killed when moribund (at 5–6 weeks). The size and weight of the primary pancreatic tumors, the incidence of regional (celiac and paraaortic) lymph node metastasis, and the number of liver metastases were recorded. Histopathology confirmed the nature of the disease. For immunohistochemistry and histology staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin. Another part of the tumor was embedded in OCT compound (Miles, Inc., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C.

Therapy of Established Human Pancreatic Carcinoma Tumors Growing in the Pancreas of Athymic Nude Mice. Seven days after implantation of tumor cells into the pancreas, five mice were killed, and the presence of tumor lesions was determined. At this time, the median tumor volume was 18 mm3. Histological examination confirmed the lesions to be actively growing pancreatic cancer. The mice were randomized into the following treatment groups (n = 10): daily oral administration of PKI166 (100 mg/kg); twice-a-week i.p. injections of gemcitabine at 125 mg/kg; 10 daily oral administrations of PKI166 (50 or 100 mg/kg); and twice-a-week i.p. injections of gemcitabine (125 mg/kg). Control mice received oral vehicle solution for PKI166 (DMSO/0.5% Tween 80 diluted 1:20 in HBSS) and i.p. HBSS.

The therapy experiment was repeated to determine overall survival. To do so, mice were implanted with L3.6pl cells in the pancreas and randomized to the four treatment groups (n = 5) on day 7. The mice were killed and necropsied when they became moribund. The volume of pancreatic tumors and the incidence of lymph node and liver metastasis was recorded. Survival was evaluated by the Kaplan-Meier method.

To evaluate the therapeutic effect of gemcitabine in this animal model, we performed a preliminary dose-response experiment. Tumor cells (L3.6pl) were implanted into the pancreas, and 7 days later, groups of mice (n = 5) received twice weekly i.p. injections of 500, 250, 125, 62, 31, 15.5, and 7.5 mg/kg gemcitabine. All mice were killed on day 35. The volume of tumors and incidence of metastasis were determined.
Necropsy Procedures and Histological Studies. Mice were euthanized, and the body weight was determined. Primary tumors in the pancreas were excised and weighed. For immunohistochemistry and H&E staining procedures, one part of the tumor tissue was formalin fixed and paraffin embedded, and another part was embedded in OCT compound (Miles Inc.), rapidly frozen in liquid nitrogen, and stored at −70°C. Visible liver metastases were counted with the aid of a dissecting microscope and processed for H&E staining. All macroscopically enlarged regional (celiac and paraaortal) lymph nodes were harvested, and the presence of metastatic disease was confirmed by histology.

Immunohistochemical Determination of VEGF, IL-8, PCNA, CD31/PECAM-1, and EGF-R. Paraffin-embedded tissues were used for identification of VEGF, IL-8, EGF-R, and PCNA. Sections (4–6 μm thick) were mounted on positively charged Superfrost slides (Fisher Scientific, Co., Houston, TX) and dried overnight. Sections were deparaffinized in xylene, followed by treatment with a graded series of alcohol [100%, 95%, 80% ethanol/ddH2O (v/v)] and rehydrated in PBS (pH 7.5). Sections analyzed for PCNA were microwaved 5 min for “antigen retrieval” (42). All other paraffin-embedded tissues were treated with pepsin (Biomeda) for 15 min at 37°C and washed with PBS (43). Frozen tissues used for identification of CD31/PECAM-1, and activated EGF-R were sectioned (8–10 μm), mounted on positively charged Plus slides (Fisher Scientific), and air-dried for 30 min. Frozen sections were fixed in cold acetone (5 min), acetone/chloroform (v/v; 5 min), and acetone (5 min) and washed with PBS. Immunohistochemical procedures were performed as described previously (30). Positive reaction was visualized by incubating the slides with stable DAB for 10–20 min or 3-amino-9-ethylcarbazole after CD31 staining. The sections were rinsed with distilled water, counterstained with Gill’s hematoxylin for 1 min, and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining. Sections analyzed for activated EGF-R were pretreated with goat antiserum IgG F(ab); fragment (1:10 dilution in PBS) for 4–6 h before incubation with the primary antibody. The positive reaction after staining for activated EGF-R was enhanced with osmium tetraoxide (4% aqueous solution) at a 1:1000 dilution in double-distilled H2O after incubation with DAB.

Immunofluorescence Double Staining for CD31/PECAM-1 (Endothelial Cells) and TUNEL (Apoptotic Cells). Frozen tissues were sectioned (8–10 μm), mounted on positively charged slides, air-dried for 30 min, and fixed in cold acetone for 5 min, acetone + chloroform (1:1) for 5 min, and acetone for 5 min. Samples were washed three times with PBS, incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min at room temperature, and incubated with the appropriate dilution (1:400) of rat monoclonal antimouse CD31 antibody (human cross-reactive) over 18 h at 4°C. After the samples were rinsed four times for 3 min each with PBS, the slides were incubated with the appropriate dilution (1:200) of secondary goat antirat conjugated to Texas Red for 1 h at room temperature in the dark. Samples were washed twice with PBS containing 0.1% Brij and washed with PBS for 5 min.

TUNEL was performed using a commercially available apoptosis detection kit with the following modifications. Samples were fixed with 4% paraformaldehyde (methanol-free) for 10 min at room temperature, washed twice with PBS for 5 min, and then incubated with 0.2% Triton X-100 for 15 min at room temperature. After two washes of 5 min each with PBS, the samples were incubated with equilibration buffer (from kit) for 10 min at room temperature. The equilibrium buffer was drained, and reaction buffer containing equilibration buffer, nucleotide mix, and TdT enzyme was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h in the dark. The reaction was terminated by immersing the samples in 2× SSC for 15 min. Samples were washed three times for 5 min to remove unincorporated fluorescein-dUTP. For quantification of endothelial cells, the samples were incubated with 300 μg/ml of Hoechst stain for 10 min at room temperature. Fluorescent bleaching was minimized by treating slides with an enhancing reagent (ProLong solution). Immunofluorescence microscopy was performed using a ×40 objective (Zeiss Plan-Neofluar) on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured using a cooled CCD camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, England) on a Macintosh computer. Images were further processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nucleus of apoptotic cells. Quantification of apoptotic endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to total number of endothelial cells in 5–10 random 0.011-mm2 fields at ×400. For the quantification of total TUNEL expression, the number of apoptotic events was counted in 10 random 0.159-mm2 fields at ×100.

Quantification of MVD, PCNA, and Absorbance. To quantify MVD, 10 random 0.159-mm2 fields at ×100 were captured for each tumor, and microvessels were quantified according to the method described previously (44, 45). To quantify the immunohistochemical reaction intensity, the absorbance of 100 VEGF- and IL-8-positive cells in 10 random 0.033-mm2 fields at ×200 of treated tumor tissues was measured using Optimas Image Analysis software (24, 25, 31). The samples were not counterstained; therefore, the absorbance was attributable solely to the product of the immunohistochemical reaction. VEGF and IL-8 cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and expressed as a ratio of tumor cell expression to normal pancreatic gland expression multiplied by 100 (44, 45). To quantify PCNA expression, we counted the number of positive cells in 10 random 0.159-mm2 fields at ×100.

In Vitro Cytotoxicity Assay. In all assays, 700-1000 tumor cells were seeded into 38-mm2 wells of flat-bottomed 96-well plates in quadruplicate and allowed to adhere overnight. The cultures were then washed and refed with medium (negative control) or medium containing PKI166 with or without gemcitabine. After 6 days (control cultures did not reach confluence), the number of metabolically active cells was determined by MTT assay (46). After a 2–4-h incubation in medium containing 0.42 mg/ml of MTT, the cells were lysed in DMSO. The conversion of MTT to formazan by metabolically viable cells was monitored by an MR-3000 96-well microtiter plate reader at 570 nm (Dynatech, Inc., Chantilly, VA). Growth inhibition was calculated from the formula:

\[
\text{Cytostasis (\%)} = \left(1 - \frac{A}{B}\right) \times 100
\]

where A is the absorbance of treated cells and B is the absorbance of the control cells.

Western Blot Analysis of EGF-R Autophosphorylation after Treatment with PKI166. Serum-starved L3.6pl cells were treated with PKI166 (0.01, 0.05, and 0.5 μM) for 60 min and then incubated with or without 40 ng/ml recombinant human EGF for 10 min, washed, and scraped into PBS containing 5 mM EDTA and 1 mM sodium orthovanadate, and centrifuged, and the pellet was resuspended in lysis buffer [20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 0.15 unit/ml aprotinin], sonicated, and centrifuged to recover insoluble protein. Immunoprecipitation was performed using MAb anti-EGF-R (clone EGF-R) as described previously (31, 47). Immunoprecipitates were analyzed on a 12% PAGE and transferred onto 0.45-μm nitrocellulose membranes. The filters were blocked with 3% BSA in TDS [20 mM Tris-HCl (pH 7.5), 150 mM NaCl], probed with either polyclonal sheep antihuman EGF-R (1:1000) or monoclonal anti-phosphotyrosine (MAb 4G10; 1:200) in TTBS (0.1% Tween 20 in TBS), and incubated with or without recombinant human EGF (40 ng/ml) for 10 min. Western blot analysis was then carried out.

In Vitro Expression of VEGF and IL-8. L3.6pl cells (2000 cells/38 mm2well) were plated into 96-well plates in 200 μl of supplemented DMEM containing 5% FBS for 24 h and then treated with 0.05, 0.5, or 1 μM PKI166. Supplemented DMEM (5% FBS) and human IgG (10 μg/ml) served as controls. Seventy-two h later, the culture supernatants were collected, and the level of VEGF and IL-8 proteins (corrected for cell number) were determined by Quantikine ELISA kits.

Statistical Analysis. Pancreatic tumor volume, incidence of metastasis, expression of VEGF, IL-8, quantification of PCNA, TUNEL, CD31, and the percentage of apoptotic endothelial cells were compared by unpaired Student’s t test. Survival analysis was computed by the Kaplan-Meier method and compared by the log-rank test.
RESULTS

In Vitro Selectivity for Inhibition of Protein Kinases. PKI166, a novel EGF-R tyrosine kinase inhibitor of the pyrrolo-pyrimidine class, inhibited the ICD of the EGF-R kinase with an IC_{50} of 0.7 nM (Table 1). Enzyme kinetic studies support the idea that PKI166 affects the EGF-R kinase by ATP-competitive inhibition. PKI166 showed a high selectivity ratio of >3000 with respect to the inhibition of the serine/threonine kinases protein kinase C-α and Cdc2/cyclin B. With respect to other tyrosine kinases, PKI166 was also active against the c-Abl tyrosine kinase (IC_{50}, 28 nM) and showed some activity against c-Src (IC_{50}, 130 nM) and the VEGF receptor family tyrosine kinases KDR (IC_{50}, 327 nM) and Flt-1 (IC_{50}, 962 nM). However, there was a selectivity factor of >40 for inhibition of the EGF-R ICD (34). After a single oral administration of 100 mg/kg to mice, PKI166 is rapidly absorbed (t_{max}, 1 h) and high concentrations of PKI166 are detected in the plasma (C_{max}, 32.8 μM) and in s.c. A431 tumors (C_{max}, 53.0 μM). Sustained levels of unchanged PKI166 are found in the plasma of mice up to 8 h after oral administration (34).

Inhibition of EGF-R Autophosphorylation in Human Pancreatic Cancer Cells by PKI166. In the first set of experiments, we determined whether treatment of L3.6pl cells with PKI166 could inhibit EGF-stimulated tyrosine phosphorylation of the EGF-R. L3.6pl cells incubated 15 min with medium free of serum but containing EGF exhibited high levels of autophosphorylated EGF-R (M_{f} 170,000 band) as detected by antiphosphotyrosine antisera on Western blots of anti-EGF-R-immunoprecipitated cell lysates (Fig. 1). Pretreatment of the cells with PKI166 for 60 min, followed by a 15-min treatment with EGF, inhibited the autophosphorylation in a dose-dependent manner (0.01–0.5 μM). The M_{f} 170,000 band was confirmed as EGF-R by Western blot analysis using anti-EGF-R antisera (Fig. 1). Northern blot analysis demonstrated that the high levels of EGF-R-specific transcripts in the L3.6pl cells did not vary in response to the treatment. Gemcitabine at a concentration of 10 μM did not affect the autophosphorylation of EGF-R (data not shown).

In Vitro Cytotoxicity Mediated by Gemcitabine and PKI166. L3.6pl cells were incubated for 6 days in medium containing increasing concentrations of gemcitabine (0–10 μM) in the absence or presence of a noncytostatic concentration of PKI166 (0.03 μM). The cytotoxicity mediated by gemcitabine was enhanced by PKI166, the IC_{50} falling from 0.88 μM for gemcitabine to 0.52 μM (P < 0.05) in the presence of PKI166.

Inhibition of Pancreatic Cancer Growth and Metastasis. Athymic nude mice received injections in the pancreas with L3.6pl cells. Seven days later, the mice were randomized into four treatment groups of 10 mice each. The first group received twice weekly i.p. injections of gemcitabine at 125 mg/kg, the second group received daily oral administrations of PKI166 at 100 mg/kg, a third group received twice-weekly gemcitabine and daily PKI166, and the last group received HBSS as control. All mice were killed on day 35 because the control mice were moribund. Detailed necropsy revealed that all of the mice had tumors in the pancreas. The data summarized in Table 2 show that daily oral PKI166 or twice-weekly i.p. injections of gemcitabine significantly decreased median tumor volume as compared with control mice (220, 166, and 399 mm^3, respectively; P < 0.01). The combination of gemcitabine and PKI166 produced a still greater decrease in pancreatic median tumor volume (59 mm^3); P < 0.0001). Visible liver metastases (enumerated with the aid of a dissecting microscope) were present in 30% of control mice and 10% of the treatment groups (Table 2). Histologically positive regional lymph node metastases were found in 90% of control animals, in 90% of gemcitabine-treated animals, and 90% of PKI166-treated animals. However, only 60% of animals receiving both gemcitabine and PKI166 had histologically positive regional lymph node metastases (Table 2). Treatments with PKI166 alone or in combination with gemcitabine were well tolerated, as determined by maintenance of body weight (Table 2).

For a second survival study, we increased the group size to 15 mice. Mice were killed and necropsied when they became moribund, and the data are summarized in Table 3 and Fig. 2. The median survival time in the control group was 37 days. After treatment with gemcitabine alone, PKI166 alone, and combination therapy, the median survival time was 56, 70, and 75 days, respectively (control versus gemcitabine, P < 0.0002; control versus PKI166, P < 0.000001; control versus PKI166 and gemcitabine, P < 0.00001). Detailed necropsy revealed that all control mice or mice treated with only gemcitabine or only PKI166 had pancreatic tumors, whereas in mice receiving the combination regimen, tumor incidence was 75%. The incidence of liver metastasis in control mice, mice treated with gemcitabine alone, or PKI166 alone was 7 of 15, 11 of 15, and 5 of 15, respectively, but none of the mice treated with PKI166 and gemcitabine had visible liver metastases (P < 0.0001). The incidence of histologically confirmed lymph node metastasis was 14 of 15, 13 of 15, and 10 of 15 for control mice, gemcitabine-treated, or PKI166-treated mice, respectively, but in mice given the combination regimen, the incidence was 4 of 15 (P < 0.01). Increasing the dose of gemcitabine to 250 and 500 mg/kg did not further reduce pancreatic tumor growth and metastasis.

### Table 1 Relative inhibition of enzymatic activity by PKI166

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC_{50} [μM]</th>
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<tr>
<td>EGF-R-ICD</td>
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<tr>
<td>c-Src</td>
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<tr>
<td>c-Abl</td>
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<td>c-Kit</td>
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<tr>
<td>Serine/threonine kinases</td>
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<tr>
<td>PKC-α</td>
<td>&gt;100</td>
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<td>Cdc2/cyclin B</td>
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*Detailed descriptions are found in “Materials and Methods.”*
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Table 2. Therapy of human pancreatic carcinoma growing in the pancreas of nude mice

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Incidence</th>
<th>Median</th>
<th>Range</th>
<th>Tumor volume (mm³)</th>
<th>Liver</th>
<th>Lymph node</th>
<th>Median</th>
<th>Range</th>
<th>Body weight (g)</th>
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<tr>
<td>Saline control</td>
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<td>399</td>
<td>116-623</td>
<td>3/10²</td>
<td>9/10³</td>
<td>21</td>
<td>17-28</td>
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<td>Gemcitabine</td>
<td>10/10</td>
<td>166</td>
<td>61-273³</td>
<td>1/10</td>
<td>9/10</td>
<td>25</td>
<td>18-29</td>
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<td>25</td>
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<td>PKI166</td>
<td>10/10</td>
<td>220</td>
<td>61-361³</td>
<td>1/10</td>
<td>9/10</td>
<td>24</td>
<td>21-28</td>
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<td>24</td>
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<td>PKI166 + gemcitabine</td>
<td>10/10</td>
<td>59</td>
<td>9-119³</td>
<td>3/10</td>
<td>6/10</td>
<td>23</td>
<td>19-23</td>
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<td>23</td>
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</table>

³ L3.6pl human pancreatic cancer cells (1 × 10⁶) were injected into the pancreas of nude mice. Seven days later, groups of mice were treated with biweekly i.p. injections of gemcitabine (125 mg/kg) alone, daily oral feedings of PKI166 (50 mg/kg) alone, gemcitabine and PKI166, or saline (control). All mice were killed on day 35.

Table 3. Survival of nude mice with human pancreatic carcinoma by therapy with PKI166 and gemcitabine

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Incidence</th>
<th>Median</th>
<th>Range</th>
<th>Pancreatic tumors</th>
<th>Liver</th>
<th>Lymph node</th>
<th>Survival (days)</th>
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<td>Saline control</td>
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<td>15/15</td>
<td>5/15</td>
<td>10/15</td>
<td>70</td>
<td>34-90³</td>
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<tr>
<td>PKI166 + gemcitabine</td>
<td>10/15</td>
<td>0/15</td>
<td>4/15</td>
<td>75</td>
<td>30-100³</td>
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³ Mice received injections in the pancreas with 1 × 10⁶ L3.6pl cells. Seven days later, groups of mice were treated with biweekly i.p. injections of gemcitabine (125 mg/kg), daily oral feedings of PKI166 (50 mg/kg), gemcitabine and PKI166, or saline (control). Moribund mice were killed and necropsied.

MVD (measured by staining with antibodies against CD31) was directly proportional to expression of VEGF and IL-8, and EGF-R was expressed at levels similar to PKI166 and gemcitabine. The culture supernatants were harvested, and the levels of VEGF and IL-8 proteins were determined by ELISA. Control cells produced 1400 pg/ml VEGF and 1440 pg/ml IL-8. Gemcitabine-treated cells produced 1280 pg/ml VEGF and 2000 pg/ml IL-8. Cells treated with PKI166 produced 180 pg/ml VEGF and gemcitabine and PKI166 and saline (control). Moribund mice were killed and necropsied.

We next evaluated cell proliferation and apoptosis using anti-PCNA antibodies and the TUNEL method, respectively (Fig. 4). The mean number of PCNA-positive tumor cells in control tumors was 237 ± 39. After therapy with gemcitabine or PKI166, it was 237 ± 66 or 185 ± 33, respectively (Table 4). The lowest number of PCNA-positive cells (130 ± 45) was found in tumors of mice treated with both gemcitabine and PKI166 (P < 0.001).

The mean number of TUNEL-positive cells was inversely related to PCNA positivity. In control tumors, it was 66 ± 21; in gemcitabine-treated tumors, it was 225 ± 95; in PKI166-treated tumors, it was 287 ± 84; and in combination-treated tumors, it was 298 ± 80 (control versus gemcitabine, P < 0.04; control versus PKI166, P < 0.003; control versus PKI166 + gemcitabine, P < 0.001). There was no significant difference in the number of TUNEL-positive tumor cells in tumors treated with gemcitabine or PKI166 alone as compared with tumors in the combination therapy group. The calculated ratio of PCNA-positive over TUNEL-positive cells was 1.1, 0.64, 0.43, and 3.61 in mice treated with gemcitabine alone, PKI166 alone, combination therapy, and control mice, respectively.

Tumor cell production of VEGF and IL-8 was significantly reduced (P < 0.001) 35 days after initiation of treatment with PKI166 or PKI166 and gemcitabine as compared with gemcitabine alone or control mice (Table 4). To determine whether blockade of the EGF-R signaling pathway by PKI166 down-regulated expression of VEGF and IL-8 by L3.6pl cells, we plated the cells into wells and incubated the cells for 72 h in medium supplemented with 5% FBS with or without 1 μM PKI166 or 10 nm gemcitabine. The culture supernatants were harvested, and the levels of VEGF and IL-8 proteins were determined by ELISA. Control cells produced 1400 pg/ml VEGF and 1440 pg/ml IL-8. Gemcitabine-treated cells produced 1280 pg/ml VEGF and 2000 pg/ml IL-8. Cells treated with PKI166 produced 180 pg/ml VEGF (P < 0.001) and 640 pg/ml IL-8 (P < 0.01). Thus, blockade of the EGF-R signaling pathway decreased production of two important proangiogenic molecules. No effects were found for expression of basic fibroblast growth factor protein (data not shown).

Finally, the CD31/TUNEL fluorescent double-labeling technique revealed that many endothelial cells in tumors treated with PKI166 or combination therapy were undergoing apoptosis (yellow reaction, Fig. 5). A significant increase in the percentage of apoptotic endothelial...
cells over total endothelial cells was found in pancreatic tumors harvested 23 days after the initiation of treatment with PKI166 (20 mg/kg), twice-weekly i.p. injections of gemcitabine, and daily oral feedings of PKI166 (100 mg/kg), twice-weekly i.p. injections of gemcitabine, and daily oral feedings of PKI166 or HBSS control. Tissue sections were stained for expression of tyrosine-phosphorylated, activated EGF-R and total EGF-R as described previously (32–36). Tumors from all treatment groups stained positive for EGF-R. Only tumors from control mice or mice treated with gemcitabine alone stained positive for activated EGF-R.

DISCUSSION

Blockade of the EGF-R signaling pathway by oral administration of the novel EGF-R tyrosine kinase inhibitor PKI166 combined with i.p. injections of gemcitabine significantly inhibited growth and lymph node and liver metastasis of human pancreatic carcinoma cells implanted into the pancreas of nude mice. Treatment with PKI166 alone or gemcitabine alone reduced the growth of the primary neoplasms by 45%. However, the combination of PKI166 and gemcitabine significantly decreased the growth of primary pancreatic cancers (by 85%) and the incidence (production) of lymph node and liver metastasis. Consequently, this combination therapy led to significant prolongation of survival, leaving some mice free of the disease. The daily oral administration of PKI166 (50 or 100 mg/kg body weight) was well tolerated. Immunohistochemical analyses of the pancreatic cancers demonstrated down-regulation of activated EGF-R in lesions from mice treated with PKI166 alone or in combination with gemcitabine. This effect was accompanied by down-regulation in production of the proangiogenic molecules VEGF and IL-8 and a significant decrease in microvessel density. Moreover, double staining of endothelial cells with antibodies against CD31 and TUNEL suggested that the reduction in microvessel density was attributable to a significant increase of apoptosis in the endothelial cells.

The progressive growth of human pancreatic cancer has been associated with expression of EGF-R (13, 48), and coexpression of EGF-R with at least one of its ligands correlates with rapidly progressive disease (20). In addition to binding EGF and TGF-α, the EGF-R can be activated by heparin-binding EGF-like growth factor, β-cellulin, and amphiregulin (49). After ligand binding, EGF-R dimerizes and becomes activated through auto- and transphosphorylation on tyrosine residues within the intracellular domain (50). The EGF-R and its associated PTKs also regulate apoptosis (51, 52), and inactivation of EGF-R PTK has been shown to inhibit EGF-induced receptor

Fig. 3. Immunohistochemical determination of EGF-R and activated (act.) EGF-R in pancreatic cancers. Human pancreatic carcinomas from the pancreas of nude mice were harvested and processed for histology and immunohistochemical analyses 35 days after treatment with twice-weekly i.p. injections of gemcitabine (125 mg/kg), daily oral feedings of PKI166 (100 mg/kg), twice-weekly i.p. injections of gemcitabine, and daily oral feedings of PKI166 or HBSS control. Tissue sections were stained for expression of tyrosine-phosphorylated, activated EGF-R and total EGF-R as described previously (32–36). Tumors from all treatment groups stained positive for EGF-R. Only tumors from control mice or mice treated with gemcitabine alone stained positive for activated EGF-R.

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autophosphorylation, mitogen-activated protein kinase activation, phosphatidylinositol 3-kinase activation, entry of cells into S phase, cyclin E-associated kinase activity, and consequently accumulation of cells in the G1 phase of the cell cycle (53). Our present results closely agree with previous reports showing that targeting the EGF-R by an anti-EGF-R antibody (C225), in combination with radiation or chemotherapeutic agents, can significantly inhibit the growth of human tumors in nude mice (31, 54–59).

EGF-R is expressed not only on tumor cells (13, 17–21, 48, 49) but also on dividing endothelial cells (27, 28). Moreover, activation of EGF-R on tumor cells has been shown to induce production of the proangiogenic molecule VEGF (27). Our present data clearly show that in mice treated with PKI166 alone or PKI166 plus gemcitabine, cells in pancreatic tumors expressed the EGF-R but not the activated (phosphorylated) EGF-R. Treatment of mice with PKI166 alone or in combination with gemcitabine was associated with a decrease in tumor cell proliferation (PCNA<sup>+</sup>) and an increase in apoptosis of tumor cells (TUNEL<sup>+</sup>). Whether the increased apoptosis observed with the combination therapy was attributable to cellular arrest at the G<sub>1</sub> restriction point, a consequence of EGF-R blockade (55), was not established.

Our immunohistochemical analyses of tumor specimens revealed that the treatment of mice with PKI166 and gemcitabine produced a significant decrease in the number of tumor-associated blood vessels (MVD). This decrease could have been attributable to three nonexclusive mechanisms:

(a) Endothelial cells within many neoplasms have been shown to express EGF-R (27, 28, 60–62). Moreover, the binding of TGF-α to EGF-R on endothelial cells has been shown to stimulate their proliferation (27, 28). Because blockade of the EGF-R results in cellular arrest at the G<sub>1</sub> restriction point (48, 55), the decrease in MVD could have been attributable to a decrease in endothelial cell proliferation.

Table 4: Immunohistochemical analysis of human pancreatic carcinoma in the pancreas of control and treated nude mice

<table>
<thead>
<tr>
<th>Treatment group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tumor cells</th>
<th>Endothelial cells</th>
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<tbody>
<tr>
<td></td>
<td>PCNA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>TUNEL&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>Saline control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>237 ± 39&lt;sup&gt;c&lt;/sup&gt;</td>
<td>66 ± 21&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>PKI166</td>
<td>185 ± 33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>287 ± 84&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>Gemcitabine</td>
<td>237 ± 66&lt;sup&gt;c&lt;/sup&gt;</td>
<td>225 ± 95&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>PKI166 + gemcitabine</td>
<td>130 ± 45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>298 ± 82&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup>L3.6pl human pancreatic cancer cells (1 × 10<sup>6</sup>) were injected into the pancreas of nude mice. Seven days later, groups of mice were treated with biweekly i.p. injections of gemcitabine (125 mg/kg) alone, oral feedings of PKI166 (100 mg/kg) alone, gemcitabine and PKI166, or saline (control). All mice were killed on day 35.

<sup>b</sup>Mean ± SD positive cells/field determined from measurement of 10 random 0.159-mm<sup>2</sup> fields at ×100.

<sup>c</sup>Mean ± SD absorbance determined as described in “Materials and Methods.”

<sup>d</sup>Mean ± SD CD31/TUNEL-positive cells in 10 random 0.011-mm<sup>2</sup> fields at ×400. Fluorescent double labeling was performed on frozen tissue sections.

<sup>e</sup><sup>P</sup> < 0.001 as compared with controls.

Fig. 4. Immunohistochemical analyses. Tumors were harvested from control mice and mice treated with gemcitabine, or PKI166, or both gemcitabine and PKI166. The sections were immunostained for expression of PCNA (to show cell proliferation), TUNEL (fitc) (to show cell death), and VEGF, IL-8, and CD31/PECAM-1 (MVD). Tumors from mice treated with PKI166 plus gemcitabine had a decrease in PCNA<sup>+</sup> cells and an increase in TUNEL<sup>+</sup> cells. Tumors from mice treated with both compounds had a decrease in MVD (CD31<sup>+</sup> cells) and decreased immunoreactivity for VEGF and IL-8.
Our immunohistochemical analyses of tumor specimens clearly show that the decrease in activated EGF-R (in pancreatic tumors) was accompanied by a decrease in expression of the proangiogenic molecules VEGF and IL-8. Recent results have suggested that VEGF (63, 64) and IL-8 (65–67) can act as survival factors for immature blood vessel endothelial cells and that VEGF can protect endothelial cells from apoptosis induced by tumor necrosis factor-α or other stimuli (68–71).

Stimulation of the EGF-R signaling pathways is known to activate ras and raf, resulting in phosphorylation of c-fos and c-jun, leading to increased AP-1 transcriptional activity (72–75). Blockade of the EGF-R signaling pathways could therefore result in reduced activity of AP-1 and, hence, reduced transcription of VEGF and IL-8, which we indeed found to be the case. The decrease in VEGF production by tumor cells can prevent the recovery of dividing endothelial cells damaged by gemcitabine and hence lead to the pronounced enhancement of apoptosis in tumor-associated (presumably dividing) endothelial cells.

PKI166 can also inhibit the VEGF receptor KDR and Flt-1 activity (Table 1). Whether the antiangiogenic activity seen here was also attributable to a direct effect on the VEGF receptors needs further study.

In summary, we show that blockade of the EGF-R signaling pathway by the PTK inhibitor PKI166 in combination with gemcitabine produces significant therapy of human pancreatic carcinoma in nude mice. The inhibition of primary tumor growth and lymph node and liver metastasis is mediated by both direct antitumor effects and by antiangiogenesis effects. This combination therapy may therefore be an exciting new approach to the treatment of a devastating disease.

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EGF-R AND APOPTOSIS OF ENDOThelial CELls


Blockade of the Epidermal Growth Factor Receptor Signaling by a Novel Tyrosine Kinase Inhibitor Leads to Apoptosis of Endothelial Cells and Therapy of Human Pancreatic Carcinoma

Christiane J. Bruns, Carmen C. Solorzano, Matthew T. Harbison, et al.

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