Blockade of Epidermal Growth Factor Receptor Signaling on Tumor Cells and Tumor-Associated Endothelial Cells for Therapy of Human Carcinomas

Cheryl H. Baker, Daniel Kedar, Marya F. McCarty,
Rachel Tsan, Kristen L. Weber,
Corazon D. Bucana, and Isaiah J. Fidler

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

The purpose of this study was to determine whether the expression of epidermal growth factor receptor (EGF-R) and activated EGF-R by tumor-associated endothelial cells is influenced by interaction with specific growth factors in the microenvironment. Different human carcinoma cell lines expressing EGF-R with low or high levels of EGF/transforming growth factor (TGF)- α were implanted into orthotopic organs of nude mice. In the EGF/TGF- α -positive bladder cancer (253J-BV), pancreatic cancer (L3.6pl), and renal cancer (RBM1-IT) but not in the EGF/TGF- α -negative renal cancer SN12-PM6, tumor-associated endothelial cells expressed EGF-R and activated EGF-R. Mice were implanted with human 253J-BV bladder tumors (EGF+) or human SN12-PM6 renal tumors (EGF-). Treatment with oral PKI 166 (a specific inhibitor of EGF-R phosphorylation) alone, intraperitoneal paclitaxel alone (253J-BV), gemcitabine alone (SN12-PM6), or combination of PKI 166 and chemotherapy produced a 60%, 32%, or 81% reduction in the volume of 253J-BV bladder tumors, respectively, and 26%, 23%, or 51% reduction in the volume of SN12-PM6 kidney tumors, respectively. Immunohistochemical analyses demonstrated down-regulation of activated EGF-R in EGF/TGF- α -positive and EGF/TGF- α -negative lesions from mice treated with PKI 166, although apoptosis of tumor-associated endothelial cells was found only in EGF/TGF- α -positive tumors. Collectively, these data suggest that expression of activated EGF-R by tumor-associated endothelial cells provides an important target for therapy. (*Am J Pathol* 2002, 161:929-938)

We recently reported that the oral administration of an epidermal growth factor receptor (EGF-R) tyrosine kinase inhibitor, PKI 166,¹ inhibited progressive growth and metastasis of human pancreatic cancer implanted orthotopically in nude mice.^{2,3} Therapy with PKI 166 alone or in combination with gemcitabine induced sig-

nificant apoptosis in tumor cells and in tumor-associated endothelial cells.^{2,3} Whether the induction of apoptosis in tumor-associated endothelial cells was mediated by direct or indirect mechanisms remained unclear. The indirect mechanism suggested by immunohistochemical analyses of the experimental pancreatic tumors showed that the decrease in activated EGF-R was accompanied by a decrease in expression of vascular endothelial growth factor (VEGF) and interleukin (IL)-8, which function as survival factors for immature blood vessel endothelial cells⁴⁻⁸ by protecting the cells from apoptosis induced by different biological agents such as tumor necrosis factor.⁹⁻¹² The direct mechanism for induction of apoptosis in endothelial cells exposed to PKI 166 depends on their expression of EGF-R. Some dividing endothelial cells have been shown to express EGF-R,¹³⁻¹⁶ and blockade of the EGF-R, which results in cellular arrest at the G₁ restriction point,¹⁷ can lead to apoptosis of these cells.^{2,3} However, whether the expression of EGF-R by endothelial cells is conditioned by their microenvironment (and thus whether the direct mechanism is more likely) is unknown.

The purpose of the present study was therefore to determine whether the expression of EGF-R and activated EGF-R by tumor-associated endothelial cells is influenced by interaction with specific growth factors in the microenvironment. Our data suggest that endothelial cells in tumors that produce high levels of EGF or transforming growth factor (TGF)- α express EGF-R and activated EGF-R. In the presence of chemotherapeutic agents, these endothelial cells are highly susceptible to induction of apoptosis by a specific inhibitor of EGF-R protein tyrosine kinase.

Supported in part by Cancer Center Support Core grant CA16672, SPORE in Prostate Cancer grant CA90270, and SPORE in Ovarian Cancer grant CA93639 from the National Cancer Institute, the National Institutes of Health.

Accepted for publication June 4, 2002.

Address reprint requests to Dr. Isaiah J. Fidler, Department of Cancer Biology-173, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. E-mail: ifidler@mdanderson.org.

Materials and Methods

Animals

Male athymic nude mice (NCI-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 United States Department of Agriculture, United States Department of Health and Human Services, and the National Institutes of Health. The mice were used in accordance with institutional guidelines when they were 8 to 12 weeks old.

Endothelial Cells and Culture Conditions

Primary human microvascular endothelial cells isolated from the dermis (HMVEC-D) (Cascade Biologics, Portland, OR) were cultured in modified MCDB 131 medium, containing microvascular growth supplement (Cascade Biologics). For *in vitro* assays, HMVEC-D were treated in Eagle's minimal essential medium supplemented with 0 to 2.5% fetal bovine serum (FBS), sodium pyruvate, non-essential amino acids, L-glutamine, a twofold vitamin solution (Life Technologies, Inc., Grand Island, NY) and penicillin-streptomycin mixture (Flow Laboratories, Rockville, MD). Adherent monolayer cultures of endothelial cells were incubated on plastic at 37°C in a mixture of 5% CO₂ and 95% air. The cultures were maintained for no longer than eight passages after recovery from frozen stocks.

Western Blot Analysis of Endothelial Cell EGF-R and Activated EGF-R

Adherent serum-starved HMVEC-D were incubated for 15 minutes with or without 10 ng/ml of basic fibroblast growth factor (bFGF) or 40 ng/ml of EGF (R&D Systems, Minneapolis, MN). EGF-R and activated EGF-R proteins were detected using polyclonal rabbit anti-human EGF-R (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal anti-phosphotyrosine monoclonal antibody 4G10 (1:2000; Upstate Biotechnology, Lake Placid, NY), respectively. Immunoblotting was performed as previously described.^{2,3}

In Vitro Cytotoxicity Assay

HMVEC-D (50,000 cells/well) were seeded into 1% gelatin-coated 96-well plates in triplicate. Sixteen hours later, the medium was replaced with medium alone (negative control) or medium containing increasing concentrations of PKI 166 with or without bFGF (10 ng/ml) or EGF (40 ng/ml) (R&D Systems). After 3 days (control cultures did not reach confluence), the number of metabolically active cells was determined by the MTT assay.¹⁸

Apoptosis

HMVEC-D were incubated for 16 hours on 1% gelatin-coated glass chamber slides in MCDB 131 medium containing 10% FBS. The medium was removed and the cells were incubated in 2.5% FBS Eagle's minimal essential medium. After an additional 16 to 18 hours, 10 ng/ml of bFGF (or 10 ng/ml VEGF) or 40 ng/ml of EGF (or 100 ng/ml of TGF- α) (R&D Systems) were added to the medium. Medium alone served as a control. After a 30-minute incubation, 2 μ mol/L of PKI 166 was added to the cultures for 15 minutes. The cells were then washed with phosphate-buffered saline (PBS) and the terminal dUTP nick-end labeling (TUNEL)^{2,3} assay was performed using a commercially available apoptosis detection kit (Promega, Madison, WI). Immunofluorescence microscopy was performed using a $\times 20$ objective to individually select for green (TUNEL) and red (propidium iodide) fluorescence. The results were analyzed and images were captured as previously described.^{2,3}

Northern Blot Analysis

Polyadenylated mRNA was extracted from 10⁸ cells of different tumor cell lines growing in culture using the FastTrack mRNA isolation kit (Invitrogen Co., San Diego, CA). The mRNA was electrophoresed onto 1% denatured formaldehyde agarose gel, electrotransferred to Gene-screens nylon membranes (DuPont Co., Boston, MA), and cross-linked with an UV Stratalinker 1800 (Stratagene, La Jolla, CA) at 120,000 mJ/cm². Filters were prehybridized with rapid hybridization buffer [30 mmol/L NaCl, 3 mmol/L sodium citrate, and 0.1% sodium dodecyl sulfate (w/v)] (Amersham, Piscataway, NJ) at 65°C for 1 hour. The membranes were then hybridized and probed for TGF- α by using the Rediprime random labeling kit (Amersham); the presence of GAPDH was used to control for loading. The cDNA probe used was a 0.9-kb *EcoRI* endonuclease cDNA fragment from the PMI-TGF1 plasmid corresponding to the human TGF- α gene.¹⁹ The steady-state expression of TGF- α mRNA transcript was quantified by densitometry of autoradiographs with the use of the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA); each sample measurement was calculated as the ratio of the average areas of the specific mRNA transcript to the 1.3-kb GAPDH mRNA transcript in the linear range of the film.

Carcinoma Cell Lines and Culture Conditions

The highly tumorigenic and metastatic human pancreatic cancer (L3.6pl),²⁰ human bladder cancer (253J-BV),²¹ human renal cancer (SN12-PM6),²² and the human renal cancer (RBM1-IT)²³ cell lines were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 5% FBS, sodium pyruvate, nonessential amino acids, L-glutamine, a twofold vitamin solution (Life Technologies) and penicillin-streptomycin mixture (Flow Laboratories). Adherent monolayer cultures were maintained on plastic and incubated at 37°C in a mixture of 5% CO₂ and 95% air. The cultures were free of *Mycoplasma* and the

following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 12 weeks after recovery from frozen stocks.

Orthotopic Implantation of Tumor Cells

To produce tumors, L3.6pl,²⁰ 253J-BV,²¹ SN12-PM6,²² and RBM1-IT,²³ cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed once in serum-free medium and resuspended in Hanks' balanced salt solution (HBSS). Only suspensions consisting of single cells with greater than 90% viability were used for the injections. Injection of cells into the pancreas, bladder, and renal subcapsule was performed as previously described.^{20–22}

Therapy of 253J-BV and SN12-PM6 Growing Orthotopically in Nude Mice

Seven days after implantation of tumor cells into the bladder or renal subcapsule, the mice were randomized into four groups as follows ($n = 10$): 1) oral vehicle solution for PKI 166 (dimethyl sulfoxide/0.5% Tween 80 diluted 1:20 in water) and intraperitoneal HBSS (control group); 2) weekly (Tuesday) intraperitoneal injections of a 200 μ g/dose of paclitaxel alone or twice weekly (Tuesday, Thursday) intraperitoneal injections of 125 mg/kg of gemcitabine alone; 3) three times per week (Monday, Wednesday, Friday) oral administration of 100 mg/kg of PKI 166 alone; and 4) three times per week oral administrations of 100 mg/kg of PKI 166 combined with weekly intraperitoneal injections of a 200 μ g/dose of paclitaxel or twice weekly intraperitoneal injections of 125 mg/kg of gemcitabine.

Necropsy Procedures and Histological Studies

Mice were euthanized and body weight determined. Primary tumors in the pancreas, bladder, or kidney were excised and weighed. For immunohistochemistry and hematoxylin and eosin-staining procedures, one part of the tumor tissue was formalin-fixed and paraffin-embedded and another part was embedded in OCT compound (Miles, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen, and stored at -70°C .

Immunohistochemical Determination of TGF- α , EGF-R, Activated EGF-R, CD31/EGF-R, and CD31/Act.EGF-R

Frozen tissues of L3.6pl, 253J-BV, SN12-PM6, and RBM1-IT cell lines growing orthotopically in nude mice

were sectioned (8 to 10 μ m), mounted on positively charged Plus slides (Fisher Scientific, Pittsburgh, PA), and air-dried for 30 minutes. The sections were fixed in cold acetone for 5 minutes, followed by 1:1 acetone:chloroform (v:v) for 5 minutes, and then acetone for 5 minutes. Sections analyzed for TGF- α were incubated at 4°C for 18 hours with a 1:100 dilution of polyclonal rabbit anti-human TGF- α (Santa Cruz Biotechnology). A positive reaction was visualized by incubating the slides for 1 hour with a 1:200 dilution of Alexa Fluor 594-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) at room temperature for 1 hour avoiding exposure to light. Samples were then stained for EGF-R and activated EGF-R. Sections analyzed for activated EGF-R were pretreated with goat anti-mouse IgG1 F(ab')₂ fragment (1:10 dilution in PBS; Jackson Research Laboratories, West Grove, CA) for 8 to 12 hours before incubation with a primary antibody. The samples were then incubated at 4°C for 18 hours with a 1:200 dilution of polyclonal rabbit anti-human EGF-R antibody (Santa Cruz Biotechnology) and a 1:200 dilution of monoclonal mouse anti-human antibody for the activated EGF-R (Chemicon, Temecula, CA). A positive reaction was visualized by incubating the slides for 1 hour with a 1:200 dilution of Alexa Fluor 594-conjugated goat anti-rabbit or Alexa Fluor 594-conjugated goat anti-mouse (Molecular Probes) at room temperature for 1 hour avoiding exposure to light. Fluorescent bleaching was minimized by covering the slides with 90% glycerol and 10% PBS.

Sections analyzed for CD31/EGF-R and CD31/Act.EGF-R were fixed and washed as described above. Samples were then incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 minutes at room temperature and then incubated with a 1:200 dilution of polyclonal rabbit anti-human EGF-R antibody (Santa Cruz Biotechnology) or 1:200 dilution of mouse anti-human activated EGF-R (Chemicon) (after pretreatment with goat anti-mouse IgG1 fragment; Jackson Research Laboratories) for 18 hours at 4°C . After the samples were rinsed four times for 3 minutes each with PBS, they were incubated with a 1:400 dilution of secondary Alexa 594-conjugated goat anti-rabbit or Alexa 594-conjugated goat anti-mouse (Molecular Probes) for 1 hour at room temperature in the dark. Samples were then washed twice with PBS, incubated with protein-blocking solution in PBS for 20 minutes at room temperature, and then incubated with a 1:400 dilution of monoclonal rat anti-mouse CD31/PECAM-1 antibody (PharMingen, San Diego, CA) for 18 hours at 4°C . After the samples were rinsed four times for 3 minutes each with PBS, they were incubated with a 1:200 dilution of secondary Alexa 488-conjugated goat anti-rat (Molecular Probes) for 1 hour at room temperature in the dark. The samples were then rinsed with double-distilled H_2O . Fluorescent bleaching was minimized by covering the slides with 90% glycerol and 10% PBS. Immunofluorescence microscopy was performed using a $\times 20$ objective. The results were analyzed and images were captured as previously described.^{2,3}

Statistical Analysis

Bladder and kidney tumor volume was analyzed by the unpaired Student's *t*-test.

Results

Expression of EGF-R and Activated EGF-R Protein

In the first set of studies, we determined whether the *in vitro* exposure of human dermal endothelial cells to EGF or TGF- α would enhance protein expression of EGF-R and activated EGF-R. Western blot analysis was performed on serum-starved human dermal endothelial cells subsequent to 15 minutes of incubation in medium (control) or medium containing 10 ng/ml of bFGF, 10 ng/ml of VEGF, or 40 ng/ml of EGF (or 100 ng/ml of TGF- α). Human endothelial cells incubated with bFGF, VEGF, or EGF exhibited similar levels of EGF-R protein (170-kd band). In contrast, high levels of autophosphorylated

Table 1. Induction of Apoptosis in Human Dermal Endothelial Cells by PKI 166

Mitogen*	TUNEL ⁺ (%)
Control	0 [†]
bFGF (10 ng/ml)	0
VEGF (10 ng/ml)	0
EGF (40 ng/ml)	85
TGF- α (100 ng/ml)	89

*Human dermal endothelial cells (HMVEC-D) (50,000/chamber) were incubated for 16 hours on 1% gelatin-coated glass chamber slides in MCDB 131 medium containing 10% FBS. The medium was removed and the cells were incubated in 2.5% FBS Eagle's minimal essential medium. After an additional 16 to 18 hours 10 nm/ml of bFGF, 10 ng/ml of VEGF, 40 ng/ml of EGF, or 100 ng/ml of TGF- α were added to the medium. Medium alone served as the control. Following a 30-minute incubation, 2 μ mol/L PKI 166 was added to the cultures for 15 minutes. The cells were then washed with PBS and the TUNEL assay was performed.

[†]The percentage of TUNEL⁺ endothelial cells in 10 random 0.159-mm² fields at $\times 100$ magnification.

EGF-R protein (170-kd) were found only in endothelial cells incubated for 15 minutes with EGF, indicating that EGF-R in endothelial cells is activated when stimulated by EGF (Figure 1A).

Functional Assessment of EGF-R on Microvascular Endothelial Cells

To determine whether the EGF-R expressed on endothelial cells is functional, we performed a cytotoxicity assay using the EGF-R tyrosine kinase inhibitor PKI 166.¹ HMVEC-D (50,000 per 38-mm² well) were plated on 1% gelatin-coated 96-well plates and incubated for 3 days in medium containing different concentrations (0 to 4.0 μ mol/L) of PKI 166 in the absence or presence of 10 ng/ml of bFGF (negative control) or 40 ng/ml of EGF (active group). The data shown in Figure 1B demonstrate that medium supplemented with 10 ng/ml of bFGF or 40

Figure 1. A: Protein level of EGF-R and activated EGF-R. Serum-starved HMVEC-D cultures were incubated for 15 minutes in medium (control) or medium with 10 ng/ml of bFGF, 10 ng/ml of VEGF, or 40 ng/ml of EGF. The levels of EGF-R protein (170 kd) and phosphorylated EGF-R (170 kd) were determined by Western blotting. Expression of EGF-R did not vary among the groups. In contrast, only endothelial cells incubated for 15 minutes with EGF expressed high levels of activated EGF-R. Densitometric quantification of the ratio between the Mr 170,000 phosphotyrosine-specific and the Mr 170,000 EGF-R-specific bands was compared in each case with the untreated cells whose ratio was defined as 1.0. **B:** Growth inhibition of human dermal endothelial cells treated *in vitro* with PKI 166. Cultures of HMVEC-D were incubated in control medium or in medium containing increasing concentrations (0 to 4 μ mol/L) of PKI 166 in the presence or absence of bFGF (10 ng/ml) or EGF (10 ng/ml). The cells were incubated for 72 hours at 37°C when the number of metabolically active cells was determined using the tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium assay. Bars, SD; *, *P* < 0.001.

Figure 2. Northern blot analysis of mRNA for TGF- α in pancreatic cancer L3.6pl, bladder cancer 253J-BV, kidney cancer SN12-PM6, and kidney cancer RBM1-IT cell lines. GAPDH served as control for loading. L3.6pl, 253J-BV, and RBM1-IT expressed high levels of TGF- α -specific mRNA, whereas SN12-PM6 did not express detectable levels of TGF- α -specific mRNA. This is one representative experiment of three.

Figure 3. Immunohistochemical determination of TGF- α , EGF-R, activated EGF-R, CD31/EGF-R, and CD31/Act.EGF-R in the pancreatic, bladder, and kidney cancers growing in nude mice. Human cancers from the respective organs of nude mice were harvested and processed for histology and immunohistochemical analyses. Note the positive staining for CD31/EGF-R and CD31/Act.EGF-R only in TGF- α -positive tumors.

ng/ml of EGF stimulated cell proliferation. Cytotoxicity mediated by PKI 166, however, was only observed in cultures incubated with EGF. Similarly, endothelial cells incubated in medium containing VEGF were not sensitive to PKI 166, whereas endothelial cells incubated in medium containing TGF- α were (data not shown).

The TUNEL assay^{2,3} (Table 1) demonstrated that in endothelial cells incubated with EGF (or TGF- α), PKI 166 mediated 80 to 90% apoptosis. In endothelial cells incubated with bFGF or VEGF, the addition of PKI 166 did not produce apoptosis (0%). These data suggest that cell-surface EGF-R expressed by endothelial cells exposed to

EGF is functional because its specific inhibition by PKI 166 leads to apoptosis.

Expression of TGF- α

Northern blot analysis for TGF- α steady-state gene expression by the human pancreatic cancer cell line L3.6pl, the human bladder cancer cell line 253J-BV, the human kidney cancer cell line SN12-PM6, and the human kidney cancer cell line RBM1-IT is shown in Figure 2. We observed differences in the expression of TGF- α -specific

Table 2. Therapy of Human Bladder Cancer Growing in the Bladder of Nude Mice

Treatment group*	Bladder tumors			Body weight (g)	
	Incidence	Tumor volume (mm ³)		Median	Range
		Median	Range		
Saline control	10/10 [†]	422	221–683	22	18–30
Paclitaxel	10/10	278 [‡]	110–325	24	17–26
PKI 166	10/10	180 [‡]	69–385	24	21–31
PKI 166 + paclitaxel	10/10	82 [§]	35–156	22	15–25

*253J-BV human bladder cancer cells (1×10^6) were injected into the bladder of nude mice. Seven days later, groups of mice were treated with weekly intravenous injections of paclitaxel (200 μ g/dose) alone, thrice-weekly oral feedings of PKI 166 (100 mg/kg) alone, PKI 166 in combination with paclitaxel, or saline (control). All mice were killed on day 35.

[†]Number of positive mice/number of mice injected.

[‡] $P < 0.005$ as compared to controls.

[§] $P < 0.0001$ as compared to controls.

mRNA transcripts among the various human carcinoma cell lines. The L3.6pl, 253J-BV, and RBM1-IT cancer cell lines expressed high levels of TGF- α mRNA, whereas there were undetectable levels of the TGF- α mRNA transcript in the SN12-PM6 cell line.

EGF-R Expression on Tumor-Associated Endothelial Cells in TGF- α High and Low Expressing Tumors

To determine whether the expression of EGF-R (total and activated) on tumor-associated endothelial cells was dependent on the expression of EGF/TGF- α by the tumors, immunohistochemical analyses were performed on the human carcinomas (expressing high or low levels of EGF/TGF- α) growing orthotopically in nude mice. The data shown in Figure 3 show that, independent of site of growth, tumor-associated endothelial cells expressed EGF-R and activated EGF-R in tumors producing TGF- α (L3.6pl pancreatic cancer, 253J-BV bladder cancer, and RBM1-IT renal cancer) but not in tumors that do not produce TGF- α (SN12-PM6).

Therapy of 253J-BV Bladder Cancer and SN12-PM6 Kidney Cancer Growth by PKI 166 and Chemotherapy

Seven days after implantation of 253J-BV cells into the bladder and SN12-PM6 cells into the kidney, nude mice

were randomized into four treatment groups ($n = 10$). The first group received weekly intraperitoneal injections of paclitaxel at 200 μ g/dose or twice-weekly intraperitoneal injections of gemcitabine at 125 mg/kg, respectively. The second group received thrice-weekly oral administrations of PKI 166 at 100 mg/kg, a third group received weekly paclitaxel and thrice-weekly oral PKI 166 (bladder) or twice-weekly gemcitabine and thrice-weekly oral PKI 166 (kidney), and the last group served as control and received HBSS intraperitoneal or oral dimethyl sulfoxide/0.5% Tween 80 diluted 1:20 in water. All mice were euthanized on day 35 because the control mice were moribund. Detailed necropsy revealed that all of the mice had tumors in the bladder or kidney. The data summarized in Table 2 shows that weekly injections of paclitaxel or thrice-weekly oral PKI 166 significantly decreased median tumor volume as compared with control mice (278, 180, and 422 mm³, respectively; $P < 0.005$). The combination of paclitaxel and PKI 166 produced a still greater decrease in bladder median tumor volume (82 mm³; $P < 0.0001$). In sharp contrast, the data in Table 3 show that a significant decrease in median kidney tumor volume as compared to control was only observed in mice treated with combination of twice-weekly injections of gemcitabine and thrice-weekly oral PKI 166 (136 and 349 mm³; $P < 0.001$).

Histology and Immunohistochemical Analyses

Tumors harvested from the different treatment groups were processed for routine histology and immunohisto-

Table 3. Therapy of Human Renal Cancer Growing in the Kidney of Nude Mice

Treatment group*	Kidney tumors			Body weight (g)	
	Incidence	Tumor weight (mg)		Median	Range
		Median	Range		
Saline control	10/10 [†]	349	260–968	26	23–31
Gemcitabine	10/10	270	199–683	27	20–32
PKI 166	10/10	258	245–717	26	20–31
PKI 166 + Gemcitabine	10/10	136 [‡]	208–489	27	21–32

*SN12-PM6 human renal cancer cells (1×10^6) were injected into the kidney of nude mice. Seven days later, groups of mice were treated with weekly intravenous injections of gemcitabine (125 mg/kg) alone, thrice-weekly oral feedings of PKI 166 (100 mg/kg) alone, PKI 166 in combination with gemcitabine, or saline (control). All mice were killed on day 35.

[†]Number of positive mice/number of mice injected.

[‡] $P < 0.001$ as compared to controls.

253J-BV Bladder Cancer

SN12-PM6 Renal Cancer

Figure 4. Immunohistochemical analysis of EGF-R, activated EGF-R, and CD31/PECAM-1 (microvessel density) in bladder or kidney tumors harvested from control mice and mice treated with paclitaxel, or gemcitabine, respectively, or PKI 166, or combination therapy. Tumors from all treatment groups stained positive for EGF-R. Only tumors from control mice or mice treated with paclitaxel or gemcitabine alone stained positive for activated EGF-R. Tumors from mice treated with PKI 166 plus paclitaxel or gemcitabine had a significant decrease in CD31⁺ cells.

Figure 5. Immunofluorescent CD31 (endothelial cells) and TUNEL (apoptosis) double labeling of human bladder and human renal cancer growing in the respective organs of nude mice. Bladder cancers (253J-BV cells) or kidney cancers (SN12-PM6 cells) from mice treated with HBSS (control), paclitaxel, or gemcitabine, respectively, PKI 166, or combination therapy were harvested on day 35 of the treatment. The tissues were processed for immunohistochemistry using anti-CD31 antibodies (Texas Red) and TUNEL (FITC-green). A representative sample (original magnification, $\times 400$) of this CD31/TUNEL fluorescent double-labeling staining is shown. Fluorescent red, CD31-positive endothelial cells; fluorescent green, TUNEL-positive cells; fluorescent yellow, TUNEL-positive endothelial cells.

chemical analyses. Immunohistochemistry using specific anti-EGF-R antibodies and antibodies against tyrosine phosphorylated (activated) EGF-R demonstrated that tumors from all treatment groups expressed similar levels

of EGF-R, whereas only tumors from control mice or mice treated with paclitaxel or gemcitabine stained positive for activated EGF-R. (Figure 4). We next evaluated microvessel density by staining with antibodies against CD31

(Figure 4). In the bladder and kidney tumors, we found a significant reduction in tumor microvessel density per field after treatment with PKI 166 or combination therapy.

Finally, the CD31/TUNEL fluorescent double-labeling technique revealed an increase in apoptosis of tumor-associated endothelial cells only in the EGF/TGF- α -positive bladder tumors. There were no apoptotic endothelial cells detected in the EGF/TGF- α -negative kidney tumors (Figure 5). These data suggest that the expression of EGF-R (total and activated) on tumor-associated endothelial cells is indeed dependent on EGF/TGF- α expression by the tumors and provides another important target for therapy.

Discussion

The present results demonstrate that endothelial cells express EGF-R and it is activated EGF-R in response to EGF or TGF- α . Although our results agree with previous reports that dividing endothelial cells can express EGF-R,^{13,14} mere proliferation is not sufficient for expression of EGF-R nor its activation. The finding that the expression of growth factor receptors on endothelial cells is conditioned by the organ microenvironment is a clear example that supports the venerable "seed and soil" hypothesis.^{24,25}

To determine the biological specificity and consequence of EGF-R expression by the human endothelial cells, we incubated the cells with PKI 166, a specific inhibitor of EGF-R phosphorylation.¹ EGF-R and its associated PTKs are known to regulate apoptosis,^{26,27} and inactivation of EGF-R PTK has been shown to inhibit EGF-induced receptor autophosphorylation, mitogen-activated protein kinase activation, phosphatidylinositol 3-kinase activity, entry into S phase, and cyclin E-associated kinase activity leading to accumulation of cells in the G₁ phase of the cell cycle.¹⁷ Induction of endothelial cell apoptosis was found only in cells stimulated with EGF (or TGF- α), ie, cells that expressed the activated EGF-R.

EGF signaling has previously been shown to play a role in angiogenesis. For example, under *in vitro* conditions, the proliferative and migratory responses of endothelial cells can be stimulated by EGF or TGF- α ,^{13,14,28,29} and these effects have been associated with increased levels of the Ets-1 transcription factor.³⁰ Similarly, several *in vivo* models show that the EGF peptide can stimulate angiogenesis.^{14,31} This biological effect of EGF may be coupled to the immediate early gene Egr-1 and a downstream cascade of Egr-1-responsive genes.³² Moreover, stimulation of the EGF-R signaling pathways is known to activate *ras* and *raf*, resulting in phosphorylation of *c-fos* and *c-jun*, and hence increased AP-1 transcriptional activity.³³⁻³⁶

Angiogenesis and EGF-R signaling have been independently evaluated as targets for therapy.^{2,3,21,37-39} EGF-R and its ligands (EGF and TGF- α) are commonly expressed in many human carcinomas, and their expression is associated with progressive disease.⁴⁰ The co-expression of both the ligand and its receptor on tumor cells have led to the hypothesis that an EGF-R-dependent autocrine loop contributes to the malignant pheno-

type.⁴¹ The paracrine effect of these ligands is less well understood. These current studies show that in carcinomas expressing high levels of EGF or TGF- α , ie, human pancreatic (L3.6pl), transitional cell (253J-BV), and renal cell (RBM1-IT) carcinomas, both tumor cells and tumor-associated endothelial cells express EGF-R and it is activated. In contrast, in carcinomas expressing low levels of the ligand, ie, human renal cell carcinoma (SN12-PM6), endothelial cells do not express detectable levels of EGF-R and activated EGF-R. Immunohistochemistry analyses of the bladder (TGF- α -positive) and kidney (TGF- α -negative) tumors demonstrated down-regulation of activated EGF-R in lesions from mice treated with PKI 166 (alone or in combination with chemotherapy), illustrating that the tumor cells expressing activated EGF-R serve as one target for treatment with PKI 166. Induction of apoptosis of endothelial cells was found only in tumors expressing the activated EGF-R. We hypothesize that EGF-R-expressing endothelial cells are another major target for therapy with PKI 166.

In the SN12PM6 renal carcinoma, the expression of EGF-R was independent of expression of TGF- α . In this tumor, the endothelial cells did not express activated EGF-R and were not induced to undergo apoptosis by PKI 166. The reduction in microvessel density in this tumor could be because of either direct apoptosis mediated by gemcitabine or by a decrease in viable tumor cells and hence a decrease in vascularity.

In summary, we report that the interaction of endothelial cells with EGF or TGF- α regulates the expression of EGF-R, leading to the phosphorylation/activation of the receptor, and that this activation can be directly blocked by a tyrosine kinase inhibitor specific to EGF-R. The clinical use of PTK inhibitors of EGF-R should therefore be more effective against neoplasms that express high levels of EGF or TGF- α . In these tumors, both the tumor cells and the tumor-associated endothelial cells can express high levels of activated EGF-R. Induction of apoptosis in tumor cells and in tumor-associated endothelial cells should therefore produce additive or synergistic therapeutic effects.

Acknowledgments

We thank Walter Pagel for critical editorial review and Lola López for expert assistance in the preparation of this manuscript.

References

1. Traxler P, Buchdunger E, Furet P, Gschwind H-P, Ho P, Mett H, O'Reilly T, Pfaar V, Thomas H: Preclinical profile of PKI 166—a novel and potent EGF-R tyrosine kinase inhibitor for clinical development. *Clin Cancer Res* 1999, 5:3750s
2. Bruns CJ, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, Abbruzzese J, Traxler P, Buchdunger E, Radinsky R, Fidler IJ: 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. *Cancer Res* 2000, 60:2926–2935
3. Solorzano CC, Baker CH, Tsan R, Traxler P, Cohen P, Buchdunger E, Killion JJ, Fidler IJ: Optimization for the blockade of the epidermal growth factor receptor signaling for therapy of human pancreatic carcinoma. *Clin Cancer Res* 2001, 8:2563–2572

4. Benjamin LE, Golijanin D, Itin A, Podes D, Keshet E: Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 1999, 103:159–165
5. Benjamin LE, Keshet E: Conditioned switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. *Proc Natl Acad Sci USA* 1997, 94:8761–8766
6. Kumar R, Yoneda J, Bucana CD, Fidler IJ: Regulation of distinct steps of angiogenesis by different angiogenic molecules. *Int J Oncol* 1998, 12:749–757
7. Kitadai Y, Haruma K, Sumii K, Yamamoto S, Ue T, Yokozaki H, Yasui W, Ohmoto Y, Fidler IJ, Tahara E, Kajiyama G: Expression of interleukin-8 correlates with vascularity in human gastric carcinomas. *Am J Pathol* 1998, 152:93–100
8. Xu L, Xie K, Mukaida N, Matsushima K, Fidler IJ: Hypoxia-induced elevation in interleukin-8 expression by human ovarian carcinoma cells. *Cancer Res* 1999, 59:5822–5829
9. Syridopoulos I, Brogi E, Kearney M, Sullivan AB, Cetrulo C, Isner M, Losordo DW: Vascular endothelial growth factor inhibits endothelial cell apoptosis induced by tumor necrosis factor- α : balance between growth and death signals. *J Mol Cell Cardiol* 1997, 29:1321–1330
10. Gerber HP, Dixit V, Ferrara N: Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* 1998, 273:13313–13316
11. Nor JE, Christensen J, Mooney DJ, Polverini PJ: Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. *Am J Pathol* 1999, 154:375–384
12. Watanabe Y, Dvorak HV: Vascular permeability factor/vascular endothelial growth factor inhibits anchorage-disruption-induced apoptosis in microvessel endothelial cells by inducing scaffold formation. *Exp Cell Res* 1997, 233:340–349
13. Goldman CK, Kim J, Wong WL, King V, Brock T, Gillespie GY: Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol Biol Cell* 1993, 4:121–133
14. Schreiber AB, Winkler ME, Derynck R: Transforming growth factor- α : a more potent angiogenic mediator than epidermal growth factor. *Science* 1986, 232:1250–1253
15. Bohling T, Hauta E, Kujala M, Claesson-Welsh L, Alitalo K, Haltia M: Expression of growth factors and growth factor receptors in capillary hemangioblastoma. *J Neuropathol Exp Neurol* 1996, 55:522–527
16. Rockwell P, O'Connor WJ, King K, Goldstein NI, Zhang LM, Stein CA: Cell-surface perturbations of the epidermal growth factor and vascular endothelial growth factors by phosphorothioate oligodeoxynucleotides. *Proc Natl Acad Sci USA* 1997, 94:6523–6528
17. Mendelsohn J: Epidermal growth factor receptor inhibition by a monoclonal antibody as anticancer therapy. *Clin Cancer Res* 1997, 3:2703–2707
18. Fan D, Poste G, O'Brian CA, Seid C, Ward NE, Earnest LE, Fidler IJ: Chemosensitization of murine fibrosarcoma cells to drugs affected by the multidrug resistance phenotype by the antidepressant trazodone: an experimental model for the reversal of intrinsic drug resistance. *Int J Oncol* 1992, 1:735–742
19. Clarke R, Bruner N, Katz D, Glanz P, Dickson RB, Lippman ME, Kern FG: The effects of a constitutive expression of transforming growth factor- α on the growth of MCF-7 human breast cancer cells in vitro and in vivo. *Mol Endocrinol* 1989, 3:372–380
20. Bruns CJ, Harbison MT, Kuniyasu H, Eue I, Fidler IJ: In vivo selection and characterization of metastatic variants from human pancreatic adenocarcinoma by using orthotopic implantation in nude mice. *Neoplasia* 1999, 1:50–62
21. Perrotte P, Matsumoto T, Inoue K, Kuniyasu H, Eve BY, Hicklin DT, Radinsky R, Dinney CPN: Antiepidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* 1999, 5:257–265
22. Naito S, Walker SM, Fidler IJ: In vivo selection of human renal cell carcinoma cells with high metastatic potential in nude mice. *Clin Exp Metastasis* 1989, 7:381–389
23. Weber KL, Price JE, Doucet M, Hicklin D, Radinsky R: Regulation of renal cell bone metastasis by EGF-R signaling: a novel therapeutic target. *Proc Am Assoc Cancer Res* 2001, 42:772
24. Paget S: The distribution of secondary growths in the cancer of the breast. *Lancet* 1889, 1:571–573
25. Fidler IJ: Modulation of the organ microenvironment for the treatment of cancer metastasis. *J Natl Cancer Inst* 1995, 84:1588–1592
26. Uckun FM, Narla RK, Jun X, Zeren T, Venkatachalam T, Waddick KG, Rostostev A, Myers DE: Cytotoxic activity of epidermal growth factor Genistein against breast cancer cells. *Clin Cancer Res* 1998, 4:901–912
27. Uckun FM, Narla RK, Zeren T, Yanishevski Y, Myers DE, Waurzyniak B, Ek O, Schneider E, Messinger Y, Chelstrom LM, Gunther R, Evans W: In vivo toxicity, pharmacokinetics, and anticancer activity for Genistein linked to recombinant human epidermal growth factor. *Clin Cancer Res* 1988, 4:1125–1134
28. Azuma M, Tamatani T, Fukui K, Yoki T, Yoshida H, Bando T, Hogue MO, Kamogashira T, Ogina K, Nishino N, Suzuki T, Sato M: Identification of EGF as an angiogenic factor present in conditioned medium from human salivary gland adenocarcinoma cell clones with varying degrees of metastatic potential. *Cancer Lett* 1994, 84:189–198
29. Zheng J, Bird IM, Melsaether AN, Magness RR: Activation of the mitogen-activated protein kinase cascade is necessary but not sufficient for basic fibroblast growth factor- and epidermal growth factor-stimulated expression of endothelial nitric oxide synthase in ovine fetoplacental artery endothelial cells. *Endocrinology* 1999, 140:1399–1407
30. Iwasaka C, Tanaka K, Abe M, Sato Y: Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells. *J Cell Physiol* 1996, 169:522–531
31. Stewart R, Nelson J, Wilson DJ: Epidermal growth factor promotes chick embryonic angiogenesis. *Clin Biol Int Rep* 1989, 13:957–965
32. Tsai JC, Liu L, Guan J, Aird WC: The Egr-1 gene is induced by epidermal growth factor in ECV304 cells and primary endothelial cells. *Am J Physiol* 2000, 279:C1414–C1424
33. Kerbel RS, Vitoria-Petit A, Okada F, Rak J: Establishing a link between oncogenes and tumor angiogenesis. *Mol Med* 1998, 4:286–295
34. Grugel S, Finkenzeller G, Weindel K, Barleon B, Marme D: Both v-Ha-Ras and v-Raf stimulate expression of the vascular endothelial growth factor in NIH 3T3 cells. *J Biol Chem* 1995, 270:25915–25919
35. Rozakis-Adcock M, Fernely R, Wade J, Pawson T, Bowtell D: The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature* 1993, 363:83–85
36. Rak J, Mitsuhashi Y, Bayko L, Filmus J, Shirasawa S, Sasazuki T, Kerbel RS: Mutant ras oncogene upregulated VEGF/PlGF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res* 1995, 55:4575–4580
37. Radinsky R, Risin S, Fan D, Dong Z, Bielenberg D, Bucana CD, Fidler IJ: Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. *Clin Cancer Res* 1995, 1:19–31
38. Petit AM, Rak J, Hung MC, Rockwell P, Goldstein N, Fendly B, Kerbel RS: Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases downregulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 1997, 151:1523–1530
39. Ciardiello F, Damiano V, Bianco R, Bianco C, Fontanini G, DeLaurentis M, DePlacido S, Mendelsohn J, Bianco AR, Tortora G: Antitumor activity of combined blockade of epidermal growth factor receptor and protein kinase. *Am J Cancer Inst* 1996, 88:1770–1776
40. Wells A: EGF receptor. *Int J Biochem Cell Biol* 1999, 6:637–643
41. Smith JJ, Derynck R, Korc M: Production of transforming growth factor- α in human pancreatic cancer cells: evidence for a superagonist autocrine cycle. *Proc Natl Acad Sci USA* 1987, 84:7567–7570