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Blockade of Epidermal Growth Factor Receptor Signaling Leads to Inhibition of Renal Cell Carcinoma Growth in the Bone of Nude Mice¹

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ABSTRACT

Renal cell carcinoma (RCC) frequently produces metastases to the musculoskeletal system that are a major source of morbidity in the form of pain, immobilization, fractures, neurological compromise, and a decreased ability to perform activities of daily living. Patients with metastatic RCC therefore have a dismal prognosis because there is no effective adjuvant treatment for this disease. Because the epidermal growth factor receptor (EGF-R) signaling cascade is important in the growth and metastasis of RCC, its blockade has been hypothesized to inhibit tumor growth and hence prevent resultant bone destruction. We determined whether blockade of EGF-R by the tyrosine kinase inhibitor PKI 166 inhibited the growth of RCC in bone. We use a novel cell line, RBM1-IT4, established from a human RCC bone metastasis. Protein and mRNA expression of the ligands and receptors was assessed by Western and Northern blots. The stimulation of RBM1-IT4 cells with epidermal growth factor or transforming growth factor α resulted in increased cellular proliferation and tyrosine kinase autophosphorylation. PKI 166 prevented these effects. First, RBM1-IT4 cells were implanted into the tibia of nude mice, where they established lytic, progressively growing lesions, after which the mice were treated with PKI 166 alone or in combination with paclitaxel (Taxol). Immunohistochemical analysis revealed that tumor cells and tumor-associated endothelial cells in control mice expressed activated EGF-R. Treatment of mice with PKI 166 alone or in combination with Taxol produced a significant decrease in the incidence and size of bone lesions as compared with the results in control or Taxol-treated mice ($P < 0.001$). Treatment with PKI 166 also decreased the expression of phosphorylated EGF-R by tumor cells and tumor-associated endothelial cells, and this was even more pronounced with PKI 166 plus Taxol treatment. The PKI 166 plus Taxol combination produced apoptosis of tumor cells and tumor-associated endothelial cells. Tumor cell proliferation, shown by proliferating cell nuclear antigen positivity, was decreased in all treatment groups. In addition, the integrity of the bone was maintained in mice treated with PKI 166 or PKI 166 plus Taxol, whereas massive bone destruction was seen in control and Taxol-treated mice. These results suggest that blockade of EGF-R signaling inhibits growth of RCC in the bone by its effect on tumor cells and tumor-associated endothelial cells.

INTRODUCTION

RCC³ frequently involves systemic metastases (1). Indeed, metastases are diagnosed in one-third of patients at the time of diagnosis, and these are commonly in the bony skeleton (1, 2). In general,

metastases will develop in 50% of patients over the course of the disease, and despite earlier diagnosis and extensive efforts to develop more effective therapies consisting of systemic chemotherapy, hormonal therapy, and biological response modifiers, alone and in combination, the outcome remains poor, with <10% of patients with metastasis surviving 5 years (3, 4). The hindrance to effective treatment is the biological heterogeneity of RCC cells (5).

Patients with skeletal metastases have marked morbidity that includes varying degrees of pain and loss of function. The current adjuvant treatment for bone metastasis, radiation therapy, is often ineffective in slowing disease progression or decreasing pain. These patients also present a challenge to the orthopedic surgeon because lytic, destructive tumors are highly vascular (6–8). Surgical management of the bone lesions includes the prophylactic stabilization of impending fractures or management of actual fractures by fixation with hardware or prosthetic devices. Although the restoration of function and mobility coupled with pain relief can improve the patient's quality of life, nevertheless, it cannot slow the progression of the disease.

The EGF-R and its ligands, EGF and TGF- α , are overexpressed in human RCC as compared with normal renal tissue (9–14). The TGF- α produced by RCC cells causes autocrine- or paracrine-stimulated cell proliferation after it binds to EGF-R. These EGF-R signaling mechanisms have been associated with development and progression of RCC metastasis (15–17). Conversely, the blockade of EGF-R signaling decreases the proliferation of RCC cells *in vitro* (18–21). In particular, chimeric anti-EGF-R Mab C225 was found to decrease tumor growth and increase survival in ascites, s.c., and orthotopic RCC xenograft models (21).

A further consideration is that EGF and EGF-R are also found on dividing endothelial cells (19, 22). Because the activation of EGF-R induces angiogenic factors such as VEGF, its inhibition may therefore further inhibit tumor growth and angiogenesis.

The purpose of the present study was to determine whether the down-regulation of EGF-R signaling pathways by a novel oral EGF-R tyrosine kinase inhibitor, PKI 166, could inhibit the proliferation of RCC cells *in vitro* and the growth of human RCC cells implanted into the tibia of nude mice. We observed that PKI 166 markedly inhibited cell proliferation and tyrosine autophosphorylation of the EGF-R. In addition, PKI 166, alone or in combination with paclitaxel (Taxol), decreased the size and weight of the bone tumors and inhibited bone destruction in an orthotopic nude mouse model. The therapeutic effect resulted from both the direct inhibition of tumor cell proliferation and the apoptosis of tumor cells and tumor-associated endothelial cells.

MATERIALS AND METHODS

RCC Cell Lines and Culture Conditions. To study the biology of bone metastasis in RCC, we previously developed a relevant model using the RBM1 cell line, developed from a metastatic bone lesion in a patient with RCC (23). Chromosomal analysis of the RBM1 cell line revealed alterations typically noted in metastatic human RCC. Specifically, the RBM1 cells have a trisomy of chromosome 7 (the location of the EGF-R gene) and express high levels of EGF-R. The cells are also highly responsive to stimulation with EGF, as evidenced by proliferation assays and Western blotting (23). The cultured cells were then implanted into the tibia of nude mice, and the lytic lesions that

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³ The abbreviations used are: RCC, renal cell carcinoma; EGF, epidermal growth factor; EGF-R, EGF receptor; TGF- α , transforming growth factor α ; PCNA, proliferating cell nuclear antigen; Mab, monoclonal antibody; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; PECAM-1, platelet/endothelial cell adhesion molecule 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; TBS-T, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20; MVD, microvessel density.

formed correlated with the radiographic and histological appearance of human RCC bone metastasis. The resultant lytic lesion was harvested, and the cells were established in culture to yield the RBM1-IT4 line (23).

Cells were maintained in DMEM:Ham's F-12, supplemented with 10% fetal bovine serum with insulin-transferrin (Sigma, St. Louis, MO), sodium pyruvate, nonessential amino acids, L-glutamine, and a penicillin-streptomycin mixture (Life Technologies, Inc., Grand Island, NY). 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; min 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.

Reagents. PKI 166, a novel EGF-R tyrosine kinase inhibitor, was synthesized and provided by Novartis Pharma AG (Basel, Switzerland). For *in vitro* use, the powdered reagent was reconstituted in DMSO. For *in vivo* administration, PKI 166 was dissolved in DMSO and diluted 1:20 in HBSS. Taxol, obtained from Bristol-Meyers Squibb (Princeton, NJ), was diluted to a final solution of 1 mg/ml and used at a dose of 200 μl. All antibodies were purchased from the sources noted: rabbit anti-bFGF, Sigma; rabbit anti-VEGF/vascular permeability factor, Santa Cruz Biotechnology (Santa Cruz, CA); rabbit antihuman interleukin 8, Oncogene (Cambridge, MA); rat antimouse CD31/PECAM-1, PharMingen (San Diego, CA); peroxidase-conjugated rat antimouse IgG1, Jackson Research Laboratories (West Grove, CA); mouse anti-PCNA clone PC 10, DAKO (Carpinteria, CA); rabbit antihuman EGF, Santa Cruz Biotechnology; mouse antihuman TGF-α, Oncogene; bromodeoxyuridine, Becton Dickinson (San Jose, CA); rabbit antihuman EGF-R (activated form), Cell Signaling Technology Inc. (Beverly, MA); rabbit antihuman IgG1 EGF-R, Santa Cruz Biotechnology; peroxidase-conjugated F(ab')₂ fragment, Affinipure Fab fragment goat antimouse IgG, peroxidase-conjugated goat antirat IgG, Texas Red-conjugated goat antirat IgG, Jackson Research Laboratories; Alexa 488 FITC-conjugated goat antirabbit antibody and Alexa 594-conjugated goat antirat antibody, Molecular Probe (Eugene,

OR); monoclonal mouse antihuman EGF-R Mab3 for immunoprecipitation, Oncogene; Hoechst dye 3342 Mr 615.9 and Gill's hematoxylin, Sigma; stable 3,3'-diaminobenzidine tetrahydrochloride, Research Genetics (Huntsville, AL); protein A/G-agarose for immunoprecipitation, Santa Cruz Biotechnology; and pepsin, Fisher Scientific Inc. (Pittsburgh, PA). The nude mice tibial tumors were harvested, fixed in formalin, and embedded in paraffin. Sections were placed on ProbeOn slides (Fisher Scientific Inc.).

MTT was purchased from Sigma, and a stock solution was prepared by dissolving 2 mg of MTT in 1 ml of PBS and filtering the solution to remove particulates. The solution was protected from light, stored at 4°C, and used within a month. The electrochemiluminescence detection system was purchased from Amersham (Arlington Heights, IL), and the TUNEL assay was performed using a commercial apoptosis detection kit (Promega, Madison, WI) with modifications.

Proliferation Assays. The *in vitro* proliferation of RBM1 cells was determined using the MTT assay. In this experiment, 6 × 10³ cells were plated in a 96-well tray for 24 h. Cells were washed twice using serum-free medium and incubated for 72 h with PKI 166 and EGF in medium containing 1% fetal bovine serum. At the end, cells were incubated for 2 h in medium containing MTT and then lysed in DMSO. The conversion of MTT to formazan by metabolically viable cells was monitored by a 96-well microtiter plate reader at 570 nm (Dynatech, Inc., Chantilly, VA; Ref. 24).

Northern Blot Analysis of EGF-R, EGF, and TGF-α Expression in RBM1-IT4 Cells. Total cellular RNA was isolated from subconfluent cultures of the RBM1-IT4 cells. Poly(A)⁺ RNA isolated using oligo(dT) chromatography was fractionated on a 1% denaturing formaldehyde/agarose gel, electrotransferred at 0.8 ampere to a GeneScreen (New England Nuclear Dupont Biotechnology, Boston, MA) nylon membrane, and cross-linked under UV light. Blots were hybridized to radiolabeled cDNA probes, and autoradiography was used to visualize the results. Densitometry was performed to quantitate the steady-state levels of each transcript in comparison with control glyceraldehyde-3-phosphate dehydrogenase transcripts.

Western Blot Analysis of EGF-R Autophosphorylation. Serum-starved RBM1-IT4 cells were incubated for 15 min with or without 40 ng/ml recombinant human EGF or TGF-α (Life Technologies, Inc.). The cells were then washed with

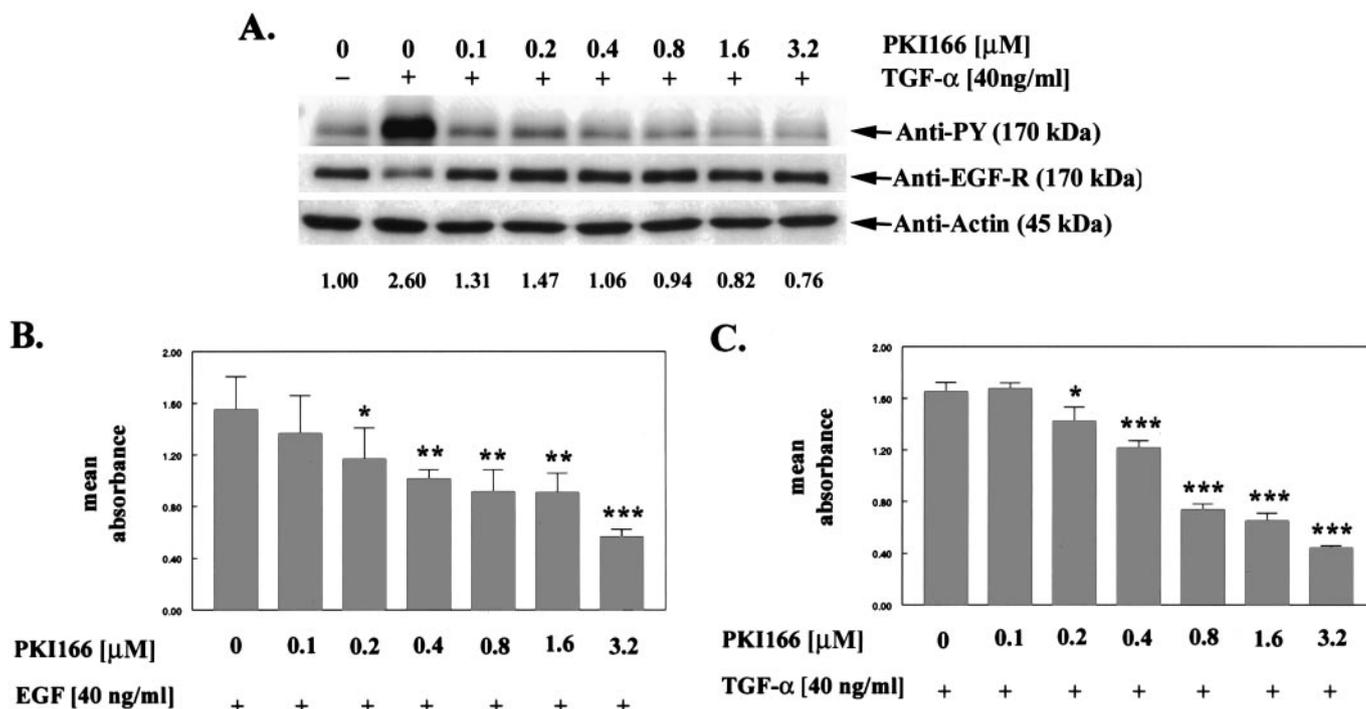


Fig. 1. The effect of EGF, TGF-α, and PKI 166 on RBM1-IT4 cells grown in culture. A, Western blot analysis with the addition of TGF-α and PKI 166 shows a 2.6-fold increase in tyrosine autophosphorylation when the RBM1-IT4 cells are stimulated with TGF-α for 15 min. Similar results are obtained using EGF. With increasing concentrations of PKI 166, there is a corresponding decrease in autophosphorylation. The *top blot* has been probed with anti-phosphotyrosine, the *middle blot* has been reprobed with anti-EGF-R, and the *bottom blot* has been reprobed with actin. Densitometric values were obtained after normalization to actin. B and C, a MTT assay was performed to measure the proliferation of RBM1-IT4 cells after the addition of (B) 40 ng/ml human recombinant EGF or (C) TGF-α alone or in combination with increasing amounts of PKI 166. Inhibition of proliferation increased with the dose of PKI 166. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

Table 1 Intratibial tumor growth in nude mice

Study group ^a	Incidence ^b (n)	Tumor weight (mg)		Bone lysis ^c	Body weight (g)
		Median	(range)		
Control	12/14	104	(45–356)	3.0	31 (27–40)
Taxol	8/10	15 ^d	(0–85)	2.7	30 (25–36)
PKI 166	5/7	21 ^d	(0–56)	1.3 ^e	29 (24–32)
PKI 166 + Taxol	5/10	1 ^d	(0–41)	0.9 ^f	28 (25–34)

^a RBM1-IT4 human RCC cells (4×10^5) were injected into the tibia of nude mice. Three days later, groups of mice were treated with i.p. injections of Taxol (200 μ g) alone (once a week), oral feedings of PKI 166 (100 mg/kg) alone (three times a week), Taxol in combination with PKI 166, or saline (control). All mice were killed on day 66.

^b Number of positive mice by H&E staining/number of mice injected.

^c The mean score on a numeric scale of 0–4.

^d Compared with controls: $P < 0.05$.

^e $P < 0.01$ compared with controls.

^f $P < 0.001$ compared with controls.

PBS containing 5 mM EDTA and 1 mM sodium orthovanadate, after which they were scraped on ice using lysis buffer [20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 20 μ M leupeptin, and 0.15 unit/ml aprotinin]. The lysate was incubated for 20 min on ice and then centrifuged to recover total protein. Immunoprecipitation was performed using Mab3 anti-EGF-R and protein A/G-agarose. Immunoprecipitates were analyzed by 7.5% SDS-PAGE and transferred onto 0.45- μ m nitrocellulose membranes. Nitrocellulose membranes were blocked with 3% BSA in TBS-T, probed with either polyclonal rabbit antihuman EGF-R (1:1000) or monoclonal anti-phosphotyrosine (Mab4G10; 1:2000; Upstate Biotechnology, Lake Placid, NY) in TBS-T, and incubated with horseradish peroxidase-conjugated donkey antirabbit IgG (1:2000; Sigma) or sheep anti-mouse IgG (1:2000), respectively, in TBS-T. Protein bands were visualized by the electrochemiluminescence detection system. Serum-starved RBM1-IT4 cells were also treated with PKI 166 (0.1–3.2 μ M) and incubated with or without EGF or TGF- α (40 ng/ml) for 15 min. Western blot analysis was then performed.

Intratibial Implantation of RBM1-IT4 Cells into Nude Mice. Male athymic nude mice were purchased from the animal production area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD) and maintained in specific pathogen-free-barrier animal facilities approved by the American Association for Accreditation of Laboratory Animal Care. They were used for experiments at 6 weeks of age. Cultured RBM1-IT4 cells (80% confluence) were harvested by a 2-min exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in medium containing 10% fetal bovine serum and resuspended in HBSS. Only suspensions consisting of single cells with >90% viability were used. The cells were injected, at a density of 4×10^5 , into the right tibia of nude mice anesthetized with i.p. sodium pentobarbital (Nembutal; 50 mg/kg). The mice were anesthetized weekly beginning 3 weeks after tumor cell injection, at which time they underwent digitized radiographic imaging with a Faxitron MX-20 X-ray unit (Wheeling, IL) to monitor the bone tumors with the images captured digitally.

Therapy of RCC Tumors in the Tibia of Nude Mice. Three days after the intratibial injection of RBM1-IT4 cells into the nude mice, the animals were randomized into one of the following treatment groups ($n = 10$ –15): (a) i.p. injection of Taxol (200 μ g) once per week; (b) oral administration of PKI 166

(100 mg/kg) three times per week (25, 26); or (c) treatment with PKI 166 and Taxol. Control mice received the vehicle solution for PKI 166 (DMSO diluted 1:20 in HBSS) p.o. and i.p. injections of HBSS.

Radiographic Grading Scheme. Radiographs of the tibia were first taken 3 weeks after injection and then taken once weekly until the end of the study. A grading system of bone lysis with numeric values ranging from 0 to 4+, based on multiple intratibial injection experiments, was used to determine the extent of bone destruction. A grade of 0 represents no lysis, 1+ is minimal but visible bone lysis within the medullary canal, 2+ is moderate bone lysis in the medullary canal with preservation of the cortex, 3+ is severe bone lysis with cortical disruption, and 4+ is massive destruction with soft tissue extension of the tumor.

Necropsy Procedures and Histological Studies. The experiment was ended when there was marked tibial bone destruction with or without soft tissue extension of the tumor in the control group. Mice were then euthanized with Nembutal and weighed. The right tibia was resected, weighed, and processed for histological studies. The weight of the uninvolved left tibia was recorded as an internal control. Histopathological analysis confirmed the nature of the disease. For immunohistochemistry studies and H&E staining, half of the tumors were fixed in formalin, decalcified, and embedded in paraffin. The other half were fixed using periodate-lysine-paraformaldehyde, decalcified, and embedded in OCT compound (Miles, Inc., Elkhart, IN). The bones were then decalcified by exposure for 10 days to PBS containing 10% EDTA. The lung and regional lymph nodes were examined, and the incidence of metastasis was confirmed histologically.

Immunohistochemical Determination of EGF, TGF- α , EGF-R, Activated EGF-R, PCNA, CD31, VEGF, and bFGF. Formalin-fixed, paraffin-embedded tissues were used for identification of EGF, TGF- α , EGF-R, activated-EGFR, VEGF, bFGF, and PCNA. In this experiment, tissue sections (4–6 μ m) were mounted on positively charged Superfrost slides (Fisher Scientific Co., Houston, TX) and dried overnight. Sections were deparaffinized in xylene, treated with a graded series of alcohol [100%, 95%, 80% ethanol/double distilled H₂O (v/v)], and then rehydrated in PBS (pH 7.5). Sections used for the PCNA analyses were microwaved for 5 min in water for “antigen retrieval.” Before staining with bFGF, the tissues were treated with pepsin for 20 min at 37°C and washed with PBS. Immunostaining for VEGF and CD31 was performed as described previously (19). Frozen tissues used for the identification of CD31/PECAM-1 were sectioned (8–10 μ m), mounted on positively charged Plus slides (Fisher Scientific Co.), and air-dried for 30 min, after which immunohistochemical studies were performed as described previously (27). Control samples exposed to secondary antibody alone showed no staining.

Immunofluorescence Double Staining for CD31/PECAM-1/TUNEL and CD31/PECAM-1/EGF-R. Before staining with immunofluorescence, sequential tissue sections were stained with H&E to confirm location within the tumor. Frozen periodate-lysine-paraformaldehyde-fixed tissues were sectioned (8–10 μ m), mounted on positively charged slides, and air-dried for 30 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 antimouse CD31 Mab (human cross-reactive) for 18 h at 4°C. After the samples were rinsed four times for 3 min each with PBS, the slides were incubated again with protein-blocking solution and incubated with the appropriate dilution (1:200) of secondary goat antirat antibody conjugated to Texas Red for 1 h

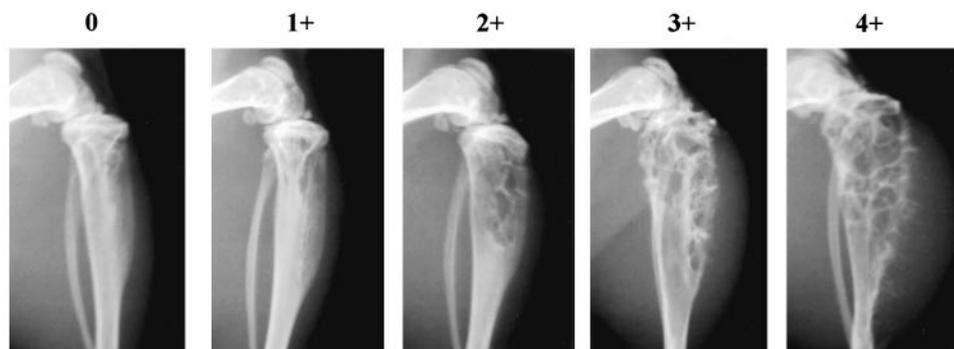


Fig. 2. Radiographic grading scheme. Varying degrees of bony lysis in the tibia of nude mice 9 weeks after injection of RBM1-IT4 cells. The destruction is graded from 0 to 4+ with increasing degrees of bone lysis. A grade of 0 represents no lysis, 1+ is minimal but visible bone lysis within the medullary canal, 2+ is moderate bone lysis in the medullary canal with preservation of the cortex, 3+ is severe bone lysis with cortical disruption, and 4+ is massive destruction with soft tissue extension of the tumor.

at room temperature in the dark. Samples were washed twice with PBS containing 0.1% Brij (Fisher Scientific Inc., Houston, TX) and then 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 preincubated with equilibration buffer (from the kit) for 20 min at room temperature. New buffer was added with the nucleotide mix and terminal deoxynucleotidyl transferase enzyme, and samples were 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 20 min. Samples were washed three times for 5 min with PBS to remove unincorporated fluorescein-dUTP.

Double staining for EGF-R and CD31/PECAM-1 was performed using a rabbit antihuman EGFR (dilution 1:100) with a goat antirabbit antibody conjugated to Alexia-488 (FITC) for the second antibody. A goat antirat antibody conjugated to Alexia-594 was used to detect CD31/PECAM-1.

To identify cell nuclei, the samples were incubated with 300 µg/ml Hoechst stain for 10 min at room temperature. Immunofluorescence microscopy was performed using a ×20 objective (Carl Zeiss, Inc., Thornwood, NY) 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 3CCD camera (Photometrics, Tucson, AZ) mounted on a Zeiss universal microscope (Carl Zeiss) and Optimus Image Analysis software

(Bioscan, Edmond, WA) installed on a Compaq computer with a Pentium chip, a frame grabber, an optical disk storage system, and a Sony Mavigraph UP-D7000 Digital Color Printer (Tokyo, Japan). Images were further processed using Adobe PhotoShop software (Adobe Systems, Mountain View, CA). For the CD31/PECAM-1 and TUNEL double staining, endothelial cells were identified by red fluorescence, and DNA fragmentation was shown by localized green and yellow fluorescence within the nucleus of apoptotic cells. The quantification of apoptotic endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to the 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. For the CD31-PECAM-1 and EGF-R double staining, endothelial cells were identified by green fluorescence, and the EGF-R was identified by red fluorescence. Cells with localized yellow fluorescence within the nucleus were considered EGF-R⁺ endothelial cells.

Quantification of MVD, VEGF, EGF-R (Activated), and PCNA. To quantify MVD, 10 random 0.159-mm² fields at ×100 were captured for each tumor, and the microvessels were quantified according to the method described previously (28). To quantify the intensity of the immunohistochemical reaction of activated EGF-R and VEGF, the absorbance of 40 positive cells/field in 10 random 0.039-mm² fields at ×200 of treated tumor tissues was measured using Optimus Image Analysis software (29). The samples were not counterstained; therefore, the absorbance was attributed solely to the product of the immunohistochemical reaction. VEGF and EGF-R-activated cytoplasmic immunoreactivity was evaluated by computer-assisted analysis and expressed as a density value. To quantify PCNA expression, the number of positive tumor

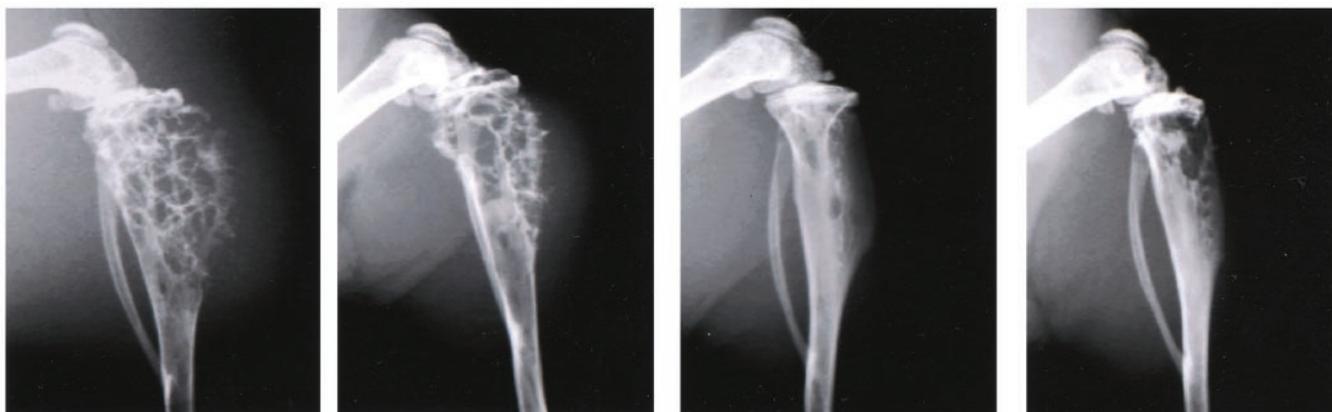
A.

CONTROL

TAXOL

PKI166

PKI166 / TAXOL



B.

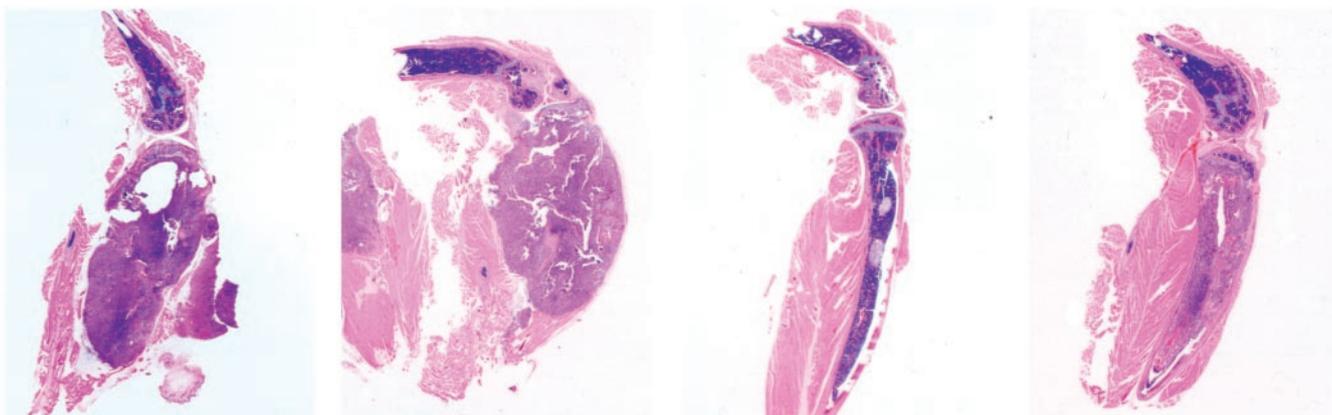
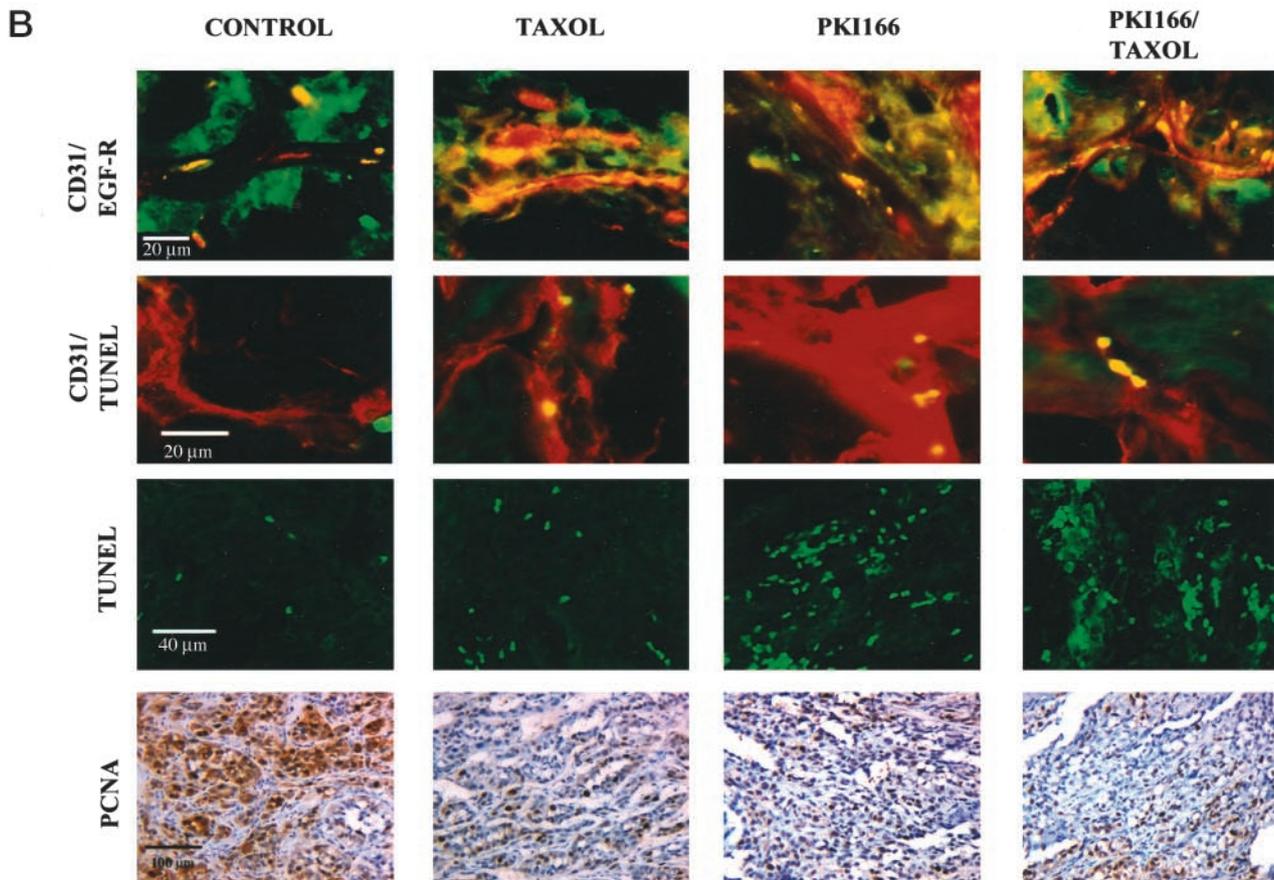
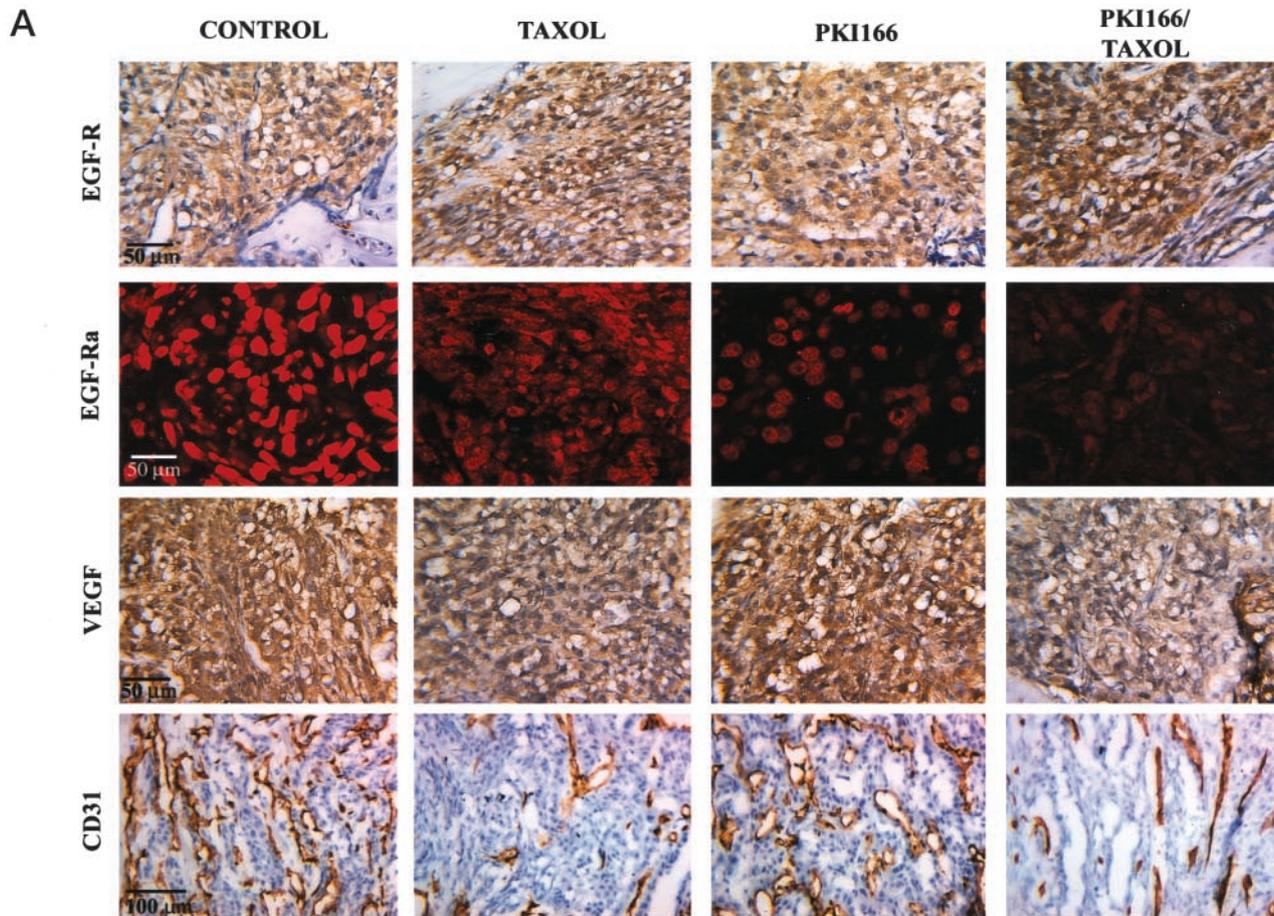


Fig. 3. Results after intratibial injection of RBM1-IT4 cells with or without EGF-R blockade. A, radiographs taken at 66 days in the four different groups of mice. Note the maintenance of the bony cortex in the mice treated with PKI 166 plus Taxol. B, H&E staining correlates well with the radiographic appearance. Large tumors were noted in the control and Taxol-treated mice, with extension into the surrounding soft tissues.



cells in 10 random 0.159-mm² fields at $\times 200$ was counted and expressed as a ratio of positive tumor cells to total tumor cells.

Statistical Analysis. Tibial tumor weight; mouse weight; expression of EGF-R (activated); quantification of PCNA, TUNEL, CD31, and VEGF results; and the percentage of apoptotic endothelial cells and EGF-R⁺ endothelial cells were compared by unpaired Student's *t* test. The number of mice with bone tumors between treatment groups was analyzed by the Mann-Whitney *U* test.

RESULTS

The Effects of EGF/TGF- α on Cell Division in Culture. The doubling time of the RBM1-IT4 cells *in vitro* is 32 h. The addition of 40 ng/ml recombinant human EGF or TGF- α to the cultures produced a 2–2.3-fold increase in proliferation ($P < 0.001$). The addition of PKI 166 at concentrations ranging from 0.2 to 3.2 μM produced a significant inhibition in the proliferation of cells stimulated with EGF or TGF- α ($P < 0.001$). A dose-response curve is shown in Fig. 1.

Inhibition of EGF-R Autophosphorylation in RBM1-IT4 Cells by PKI 166. Northern blot analysis revealed that TGF- α and EGF-R mRNA was expressed by RBM1-IT4 cells (data not shown). Western blot showed a high basal level of phosphorylation in RBM1-IT4 cells probed for anti-phosphotyrosine. Stimulation of the cells with 40 ng/ml EGF or TGF- α increased the tyrosine autophosphorylation of EGF-R 2.5-fold as compared with control cells. In contrast, the production of EGF-R protein was decreased by 87% in the cells treated with 40 ng/ml EGF and 2.5 μM PKI 166 (data not shown), compared with cells treated with EGF alone. PKI 166 (0.1–3.2 μM) inhibited the phosphorylation of EGF-R in a dose-dependent manner (Fig. 1). There is down-regulation of the EGF-R with the addition of EGF or TGF- α .

Inhibition of RCC Growth in the Bone of Nude Mice. RBM1-IT4 cells were injected in the tibia of athymic nude mice. Three days later, the mice were randomized into four treatment groups ($n = 10$ –15). All mice were killed on day 66, when the control mice were moribund. The experiment was repeated twice, and similar results were obtained. Representative data are summarized in Table 1.

Detailed necropsy revealed that 86% of the mice in the control group had intratibial tumors (Table 1). There was a marked decrease in the incidence of tumors in the PKI 166 plus Taxol group. In contrast, Taxol given as a single agent did not inhibit the development of bone tumors. The oral administration of PKI 166 alone significantly decreased the median tumor weight as compared with control mice (21 versus 104 mg; $P < 0.05$). However, the combination of oral PKI 166 and i.p. Taxol produced an even greater decrease in the median tumor volume (1 versus 104 mg; $P < 0.05$). Even Taxol alone decreased tumor weight (15 versus 104 mg; $P < 0.05$). Neither lung nor lymph node metastases were found in any of the mice. Treatments with PKI 166 alone or in combination with Taxol were well tolerated, with only a 10% or 4% decrease in body weight, respectively.

Radiographic Analysis of Intratibial Tumors. Data were collected on the radiographic appearance of each mouse tibia at regular intervals. Bone lysis was graded according to our scale with numeric values from 0 to 4+ (Fig. 2). There was a significant decrease in bone lysis in the mice treated with PKI 166 alone ($P < 0.01$) or in

combination with Taxol ($P < 0.001$; Table 1). Taxol alone did not inhibit bone destruction.

Histology and Immunohistochemistry Studies. Tibial tumors were processed for routine histology and immunohistochemical analyses. H&E staining revealed the presence of tumors within the medullary canal of the proximal tibia, the extent of bone destruction, and the extension of tumors into the surrounding soft tissues. The mice in the control and Taxol-treated groups had extensive tumors, destruction of the surrounding cortical bone, and extension of tumor cells into the soft tissues. In contrast, the tumors in mice treated with PKI 166 alone or in combination with Taxol were small, the cortical bone was preserved, and the tumor cells did not extend into the soft tissues (Fig. 3).

In vivo cell proliferation and apoptosis were evaluated using anti-PCNA antibodies and TUNEL, respectively (Fig. 4B). The percentage of PCNA⁺ tumor cells in the control group was $56 \pm 8\%$. It was $41 \pm 12\%$, $44 \pm 8\%$, and $31 \pm 12\%$ in the Taxol, PKI 166, and combination therapy groups, respectively ($P < 0.01$). The lowest percentage of PCNA⁺ cells was in the tumors of mice treated with the combination of PKI 166 and Taxol ($P < 0.001$; Fig. 4B).

The percentage of TUNEL⁺ cells in control and Taxol-treated tumors was $8 \pm 2\%$ and $13 \pm 6\%$, respectively. In mice treated with PKI 166 or PKI 166 plus Taxol, however, the percentage of apoptotic cells was significantly increased to $21 \pm 7\%$ and $34 \pm 10\%$, respectively ($P < 0.01$; Fig. 4B). The percentage of apoptotic cells was therefore inversely related to the percentage of dividing cells. The ratio of the percentage of PCNA⁺ to TUNEL⁺ cells in the control, Taxol, PKI 166, and PKI 166 plus Taxol groups was 7, 3.2, 2, and 0.9, respectively.

Immunohistochemistry studies using specific anti-EGF-R antibodies and antibodies specific to tyrosine-phosphorylated (activated) EGF-R demonstrated that tumors from all groups produced similar levels of EGF-R, whereas tumors from animals in the three treatment groups showed a significant decrease in the activated EGF-R staining ($P < 0.001$), with the greatest decrease seen in the tumors from the PKI 166 plus Taxol-treated animals. The staining was both nuclear and cytoplasmic for total EGF-R. It was primarily nuclear when a fluorescent antibody to activated EGF-R was used. The staining intensity for EGF, TGF- α , and bFGF was similar in all groups (Fig. 4A; data not shown). However, the production of VEGF by tumor cells was significantly decreased in all three treatment groups ($P < 0.05$ to < 0.001 ; Fig. 4A).

The MVD (measured by staining with antibodies against CD31) was 211 mm² in control tumors but was decreased to 135 and 158 mm², respectively ($P < 0.001$), in tumors from mice treated with Taxol or Taxol plus PKI 166 (Fig. 4A).

Double labeling immunohistochemistry using fluorescent antibodies against CD31 (*red*) and EGF-R (*green*) revealed that endothelial cells within the tumors expressed EGF-R (Fig. 4B). Endothelial cells in the contralateral normal leg in all groups also expressed EGF-R (data not shown), but there were more endothelial cells in the tumor tissue, as would be expected. In addition, the double labeling fluorescent technique used with CD31 (*red*) and TUNEL (*green*) revealed a progressive increase in the percentage of apoptotic endo-

Fig. 4. Immunohistochemical analysis after EGF-R blockade in nude mice. A, antibodies to EGF, TGF- α , EGF-R, EGF-R (activated), VEGF, CD31, and PCNA were used in our evaluation of the tibial tumors. Although treatment did not affect the expression of EGF (data not shown), TGF- α (data not shown), or EGF-R, note the marked decrease in the activated EGF-R in all groups. VEGF is decreased in all treatment groups, but the decrease is most pronounced in the Taxol group. A decrease in MVD (CD31 staining) was most pronounced in the Taxol- and combination-treated groups. B, antibodies to PCNA, CD31, TUNEL, and EGF-R were used to assess tumor and endothelial cell growth and apoptosis. EGF-R (*green*) and CD31 (*red*) staining was performed alone (data not shown) and shown superimposed. The latter showed the colocalization of EGF-R and CD31. EGF-R⁺ endothelial cells (*yellow staining*) were noted in all treatment groups. Double staining for CD31/TUNEL was performed with CD31 (*red staining*) and TUNEL (*green staining*). An increase in apoptotic endothelial cells (*yellow staining*) is seen in the tumors treated with PKI 166 or PKI 166/Taxol. TUNEL analysis revealed an increase in tumor cell apoptosis in all groups, with the most marked effect in the combination treatment group. There was also a decrease in tumor cell proliferation, as shown by PCNA staining, in all groups.

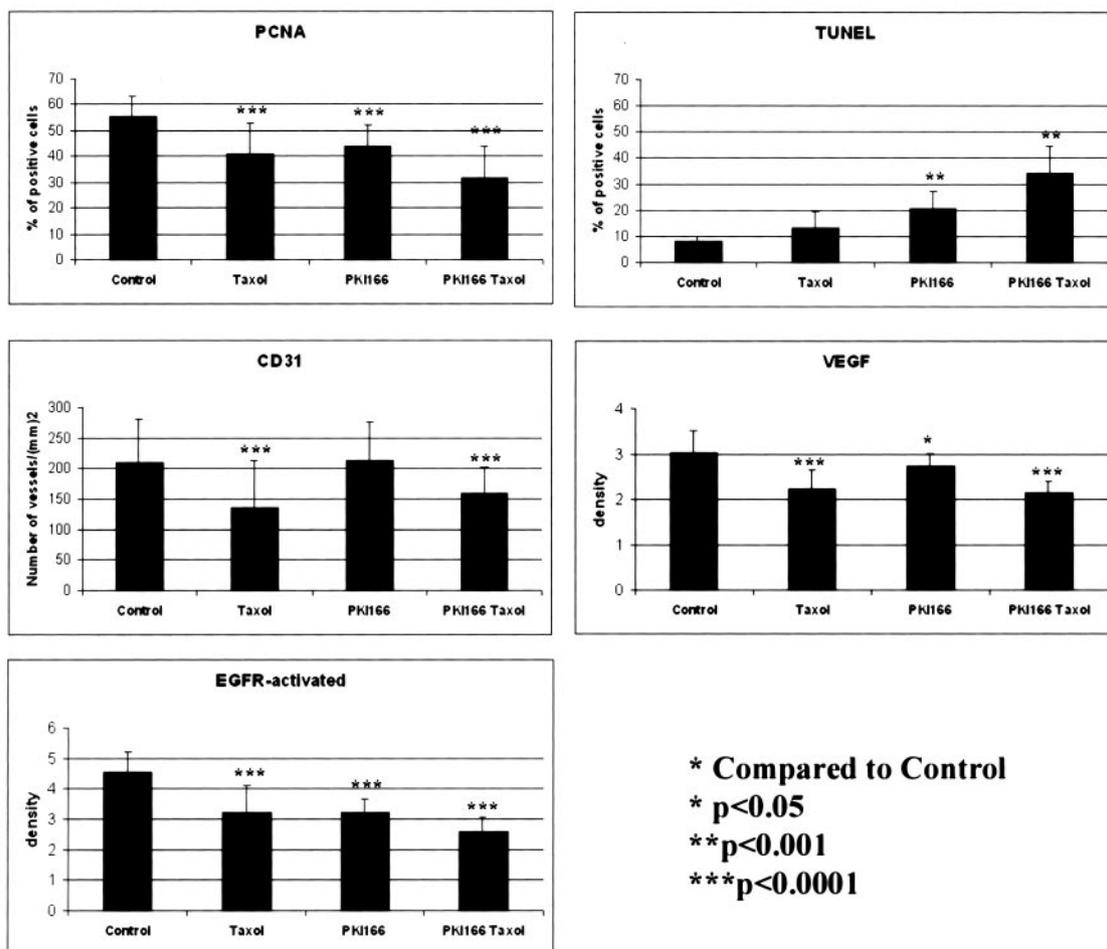


Fig. 5. Quantification of immunohistochemical data. Quantification of the tissue staining for PCNA, activated EGF-R, TUNEL, VEGF, and CD31. See "Materials and Methods" for details. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

thelial cells in the tumors treated with Taxol, PKI 166 alone, or PKI 166 plus Taxol (Fig. 4B). There was no evidence of apoptotic endothelial cells in the contralateral tibia of mice in any group, signifying a specificity of EGF-R blockade against tumor-associated endothelial cells.

Quantification of the immunohistochemical staining for PCNA, activated EGF-R, TUNEL, VEGF, and CD31 is documented in Fig. 5.

DISCUSSION

We observed that PKI 166 alone or in combination with Taxol significantly decreased the growth of human RCC cells implanted into the tibia of mice and also reduced bone lysis. These data also show that the blockade of the EGF-R signaling cascade can inhibit the proliferation of RCC cells *in vitro*, indicating a possible therapeutic strategy for this very difficult to treat tumor.

PKI 166 is a novel EGF-R tyrosine kinase inhibitor of the pyrrolo-pyrimidine class. Previous enzyme kinetic studies have shown that PKI 166 affects the EGF-R kinase by ATP-competitive inhibition (30). In addition, PKI 166 selectively inhibits the EGF-R intracellular domain over other tyrosine kinases or serine-threonine kinases. After oral administration, the drug is rapidly absorbed and occurs at high concentrations in the plasma for up to 8 h (30). The oral dosing of PKI 166 was well tolerated by the mice in our study.

Immunohistochemical analysis of the RCC lesions in the tibia of nude mice demonstrated the down-regulation of activated EGF-R in the mice treated with Taxol, PKI 166 alone, or PKI 166 in combina-

tion with Taxol, with the greatest effect noted in the combination treatment group. Endothelial cells in the bone lesions also expressed EGF-R, as determined by a double staining technique using antibodies against EGF-R and CD31. Thus, both tumor cells and endothelial cells in the tumors of mice treated with PKI 166 alone or PKI 166 and Taxol were induced to undergo apoptosis. This apoptosis, together with the decreased tumor cell proliferation, demonstrated the effectiveness of the EGF-R blockade. This treatment was specific against the tumor and tumor-associated endothelial cells and did not induce apoptosis in the endothelial cells in the contralateral tibia. The treatment also reduced MVD, shown by CD31 staining, and this was most prominent in the groups treated with Taxol, which has known anti-angiogenic effects. Our results mirror those obtained in mice with intratibial RCC tumors treated with the anti-EGF-R Mab C225.⁴ However, in the mice treated with C225, MVD was not affected because the antibody is active only against human cells and not mouse endothelial cells.

The progression and metastatic spread of human RCC has been convincingly associated with the expression of EGF-R. Because the tumors strongly express the ligands EGF and TGF- α , an autocrine loop has been implicated in rapid tumor growth (9, 31, 32). In particular, after the EGF-R binds to one of its ligands, the EGF-R is dimerized and activated by autophosphorylation and transphosphorylation of intracellular tyrosine residues. Therefore, because EGF-R

⁴ Unpublished observations.

regulates apoptosis, the inactivation of EGF-R PTK inhibits EGF-R-induced autophosphorylation, phosphatidylinositol 3'-kinase activation, mitogen-activated protein kinase activation, the entry of cells into the S phase, and the accumulation of cells in the G₁ phase. The decrease in tumor cell proliferation (PCNA⁺ cells) and increase in apoptosis of tumor cells (TUNEL⁺ cells) seen in response to PKI 166, Taxol, or the two in combination in our study may thus be due to cellular arrest at the G₁ restriction point as a consequence of EGF-R blockade. Because EGF-R is also expressed on dividing endothelial cells (22), it is postulated that the RCC cells stimulate EGF-R expression on the endothelial cells by a paracrine mechanism. The endothelial cells, in turn, may also stimulate tumor growth through a similar mechanism.

There was a decrease in VEGF but not bFGF staining in the treatment groups in these experiments, which differs from previous findings in a pancreas model in which PKI 166 inhibited the expression of both proangiogenic molecules (19). It has been postulated that blockade of the EGF-R signaling pathway reduces AP-1 activity and hence the transcription of VEGF (19). PKI 166 also directly inhibits the KDR and Flt-1 receptors for VEGF. In the current study, VEGF was inhibited by PKI 166 to a lesser degree than by Taxol or the drugs in combination. RCC is one of the most vascular tumors and has extremely high levels of VEGF in the untreated state. It is not known whether higher doses of PKI 166 would further inhibit VEGF.

Radiographically, there was decreased bone destruction in the mice treated with PKI 166 alone or in combination with Taxol. Because TGF- α and EGF increase the proliferation of osteoclast precursors, which, in turn, increase the number of osteoclasts and hence bone destruction, blockade of EGF-R signaling may therefore decrease the number and activity of osteoclasts (33).

In summary, PKI 166 was effective in decreasing tumor growth and bone lysis in mice with human RCC in the tibia. However, the addition of Taxol augmented the response by decreasing cell proliferation, activated EGF-R staining, and MVD and increasing the number of apoptotic tumor and tumor-associated endothelial cells. Whereas the antitumor effects of PKI 166 are thought to occur through reduction of EGF autophosphorylation, the *in vivo* phenotypic changes demonstrated are still theoretically thought to occur through this mechanism.

As we better understand the biological events that regulate RCC bone metastasis, it will be possible to design more effective therapy. Our demonstration that the blockade of EGF-R signaling can be effective in a murine model of this fatal human cancer is one outgrowth of such knowledge, and the next step is to translate this strategy into clinical reality.

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