The Phosphoinositide 3-Kinase/Akt Pathway: A New Target in Human Renal Cell Carcinoma Therapy

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Abstract

Metastatic renal cell carcinoma is resistant to current therapies. The phosphoinositide 3-kinase (PI3K)/Akt signaling cascade induces cell growth, cell transformation, and neovascularization. We evaluated whether targeting this pathway could be of therapeutic value against human renal cell carcinoma. The activation of the PI3K/Akt pathway and its role in renal cell carcinoma progression was evaluated in vitro in seven human cell lines by Western blot, cell counting, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, terminal deoxyribonucleotide transferase–mediated nick-end labeling assays, and fluorescence-activated cell sorting analysis, using two PI3K inhibitors, LY294002 and wortmannin, as well as by transfection with various Akt constructs and through Akt knockdown by small interfering RNA (siRNA). In vivo nude mice bearing human renal cell carcinoma tumor xenografts were treated with LY294002 (75 mg/kg/wk, 4 weeks, i.p.). Tumor growth was measured and tumors were subjected to Western blot and immunohistochemical analysis. Akt was constitutively activated in all cell lines. Constitutive phosphorylation of glycogen synthase kinase-3 (GSK-3) was observed in all cell lines, whereas forkhead transcription factor and mammalian target of rapamycin, although observed in all cell lines, were not constitutively phosphorylated. Exposure to LY294002 or wortmannin decreased Akt activation and GSK-3 phosphorylation and reduced cell growth by up to 70% through induction of cell apoptosis. These effects were confirmed by transfection experiments with Akt constructs or Akt siRNA. Importantly, LY294002 induced up to 50% tumor regression in mice through tumor cell apoptosis. Tumor neovascularization was significantly decreased by LY294002 treatment. Blood chemistries showed no adverse effects of the treatment. Our results suggest an important role of PI3K/Akt inhibitors as a potentially useful treatment for patients with renal cell carcinoma. (Cancer Res 2006; 66(10): 5130-42)

Introduction

Renal cell carcinoma is among the 10 leading causes of cancer-related deaths worldwide and its incidence is increasing steadily (1–3). Worldwide each year, 200,000 patients are diagnosed with this neoplasm, with an estimated 100,000 deaths (1, 3). At initial presentation, 30% of patients have metastases and another third will develop metastatic disease within 10 years. The 5-year survival rate is 70% at 5 years for localized renal cell carcinoma, but is only 5% in those with metastases. Indeed, metastatic renal cell carcinoma is also resistant to radiotherapy, and, despite extensive attempts, it also remains highly resistant to systemic therapy (4, 5). Thus, therapies for advanced renal cancer clearly remain inadequate and new therapeutic options for renal cell carcinoma are urgently needed.

Phosphatidylinositol 3,4,5-trisphosphate (PIP3) generated by class I phosphoinositide 3-kinases (PI3K) acts as a lipid second messenger essential for the translocation of protein kinase B or Akt to the plasma membrane (6–9). Akt is then phosphorylated at two sites, T308 in the kinase domain and S473 in the regulatory tail, by phosphoinositide-dependent kinase-1 (PDK-1) and other kinases known as PDK-2 (8, 9). Phosphorylation at T308 and S473 is essential for maximal Akt activation (8, 9). Phosphorylated Akt regulates the function of a broad array of intracellular proteins involved in fundamental processes, including cell proliferation, cell death, cell motility/adhesion, cell transformation, and neovascularization (8, 9). PIP3 levels, and thus Akt activation, are tightly regulated by the action of phosphatase and tensin homologue deleted from chromosome 10 (PTEN), initially identified as a tumor suppressor, and SH2-domain-containing inositol phosphatase, two phosphatases removing the phosphate moiety from the 3-OH and 5-OH positions, respectively. The PI3K/Akt pathway thus seems as an important cellular adaptation toward cell and tissue growth and transformation.

Biallelic inactivating mutations of the von Hippel-Lindau (VHL) tumor suppressor gene occur in patients with the VHL syndrome, an autosomal dominantly inherited syndrome associated with renal cell carcinoma and in up to 80% of patients with sporadic renal cell carcinoma (10–12). The VHL gene is believed to possess gatekeeper properties in renal proximal tubular cells from which conventional renal cell carcinoma originates (13, 14). At the molecular level, the VHL gene products are components of a ubiquitin-ligase complex involved in the down-regulation of several angiogenic and growth factors, such as vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β), that contribute to renal cell carcinoma development and growth (15, 16) and that have been shown to interact with the PI3K/Akt pathway in other cell or tissue systems (8, 17). Alterations of the PI3K/Akt pathway in human cancers have been documented (8, 9, 18). Recent data give clear evidence that the PI3K/Akt pathway is constitutively activated in various human cancers, including ovarian, prostate, and lung cancers, and plays a critical role in tumor progression (8, 9, 18). Crosstalk between the PI3K/Akt and other survival pathways, such as mitogen-activated protein kinase (MAPK) Erk1/2 pathway, has also been documented (19). Chemotherapeutic agents kill replicating tumor cells through induction of cell apoptosis. Recent studies in various types of
cancers show that activation of survival pathways plays a critical role in tumor resistance (20, 21). Synergistic effects on cell death by a combination of inhibitors of survival pathways and chemo- therapeutic drugs has been described for the PI3K/Akt and the MAPK Erk1/2 pathways (20, 22–24). Thus, there is now convincing evidence that the activation of the PI3K/Akt pathway is related not only to tumor progression but also to tumor resistance to radiation and systemic therapies.

Human renal cell carcinoma is sensitive to the apoptotic effect of a broad range of therapeutic drugs in vitro in cultured cells, but highly refractory to these compounds in in vivo models (25–27). Attempts to block multidrug resistance gene products in combination programs did not provide evidence of an involvement of these mechanisms in renal cell carcinoma resistance (4, 28). The involvement of cell survival pathways, such as the PI3K/Akt signaling pathway, in renal cell carcinoma progression, metastatic invasion, and resistance to therapies has not been investigated to date.

In the present study, our goal was to define whether targeting the PI3K/Akt pathway might be of therapeutic value against human renal cell carcinoma. Our results suggest an important role for PI3K/Akt inhibitors as a potentially useful treatment for patients with renal cell carcinoma.

Materials and Methods

Cells and Cell Culture

Human renal cell carcinoma cell lines 786-0, UOK-126, UOK-128, A498, ACHN, Caki-1, and Caki-2 were maintained in DMEM (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum. In some experiments, medium containing serum was replaced by DMEM without serum but supplemented with 0.1% bovine serum albumin (BSA). All renal cell carcinoma cell lines were obtained from American Type Culture Collection (Manassas, VA) except UOK-126 and UOK-128 that were generously given to us by Dr. P. Anglard (29). The cell lines are derived from human sporadic conventional renal cell carcinoma and are either expressing the VHL tumor suppressor gene (ACHN, Caki-1, and Caki-2 cell lines), which functions as a gatekeeper for conventional renal cell carcinoma, or VHL deficient (786-0, UOK-126, UOK-128, and A498 cell lines). Human hepatocytes were generously given by Dr. E. Alexandre (Laboratoire de Chirurgie Experimentale, Fondation Transplantation, Strasbourg, France) and immediately used for expression experiments. Unless otherwise specified, cells were used at 70% to 80% confluence.

Human Tumor Biopsies

Tumor and normal corresponding tissue of three patients with conventional renal cell carcinoma were obtained from the Urology Department of the University Hospital of Strasbourg. Patients had no history of hereditary VHL disease. Informed consent was given by all three patients. The tumors were staged pT3bN0M0 according to tumor-node-metastasis classification (5). At the time of radical nephrectomy, which was done with <30 minutes of renal ischemia, renal cell carcinoma tumors, and corresponding normal tissues were frozen and kept in liquid nitrogen until analysis by Western blot, as described below. Human non–small cell lung carcinoma of high stage (stage IV) biopsy was obtained from the Pathology Department of the University Hospital of Strasbourg. Informed consent was given by the patient.

Transient Transfection

786-0 cells (untransfected, 786-0 wt) and Caki-1 cells (untransfected, Caki-1 wt) were grown until 50% confluence. Cells were washed with serum-free medium and then transiently transfected using LipofectAMINE reagent (Invitrogen) according to the protocol of the manufacturer and as previously described (30), with 10 μg of one of the following Akt constructs in pcDNA 3.1/myc-His A, B, C vector: vector alone (786-0 V and Caki-1 V) or vector containing either wild-type full-length Akt cDNA (786-0 wtAkt and Caki-1 wtAkt), constitutively active Akt (aAkt, Thr308 and Ser473 were replaced by aspartate) cDNA (786-0 aAkt and Caki-1 aAkt), or inactive dominant-negative Akt (dnAkt, Thr308 and Ser473 were replaced by alanine) cDNA (786-0 dnAkt and Caki-1 dnAkt). Akt wt, aAkt, and dnAkt constructs were generous gifts from Drs. J. Haendeler and S. Dimmeler (Molecular Cardiology, Department of Internal Medicine III, University of Frankfurt, Frankfurt, Germany). Following transfections, cells were subjected to Akt expression analysis at the time indicated in Results or in the figures or figure legends by Western blot according to the protocol described below. Untransfected and transfected cells were used for further experiments at the day of maximal Akt expression.

Akt Knockdown by Small Interfering RNA Transient Transfection

Small interfering RNA (siRNA) duplexes specific for human Akt1 and control nonsilencing siRNA were obtained from Ozyme (Cell Signaling local distributor, St Quentin Yvelines, France). Transient transfection of 786-0 and Caki-1 cells was done according to the instructions of the manufacturer. Briefly, 786-0 and Caki-1 cells were seeded in six-well plates or in 25 cm² plates (20,000/mL), grown for 24 hours (∼50% confluence), and then transiently transfected with 100 nmol/L Akt-specific siRNA or control siRNA using the transfection reagent provided by the manufacturer, which also served as control without siRNA. Medium was replaced 24 hours later by fresh medium and cells were grown for an additional 24-hour period (48 hours posttransfection) before Western blot analysis of Akt expression as described below. In other sets of experiments, cells were subjected to flow cytometry analysis as described below.

Western Blot Analysis

Whole cell lysates or tumor tissue were prepared in lysis buffer as described (30, 31). Protein concentrations were determined according to the method of Lowry et al. (32) with BSA as standard. Equivalent amounts of protein (10-30 μg) were resolved by SDS-polyacrylamide gels (10%) and transferred to enhanced chemiluminescence (ECL) nitrocellulose membranes (Roche Diagnostics, Meylan, France). The membranes were blocked for 1 hour in TBS containing 0.1% Tween 20 (TBST) and 5% (w/v) nonfat dry milk at room temperature under regular shaking. After at least three washes in the same buffer, membranes were incubated overnight at 4°C under regular shaking with the appropriate dilution of the following primary antibodies: polyclonal rabbit anti-PI3K p110α antibody at 1/500 dilution; polyclonal rabbit anti-Akt antibody at 1/250 dilution; polyclonal rabbit anti-phospho-Akt (T308) antibody at 1/125 dilution; polyclonal rabbit anti-phospho-Akt (S473) at 1/250 dilution and monoclonal mouse anti-PTEN antibody at 1/500 dilution; polyclonal rabbit anti-FKHR (forkhead transcription factor) antibody diluted at 1/250; polyclonal rabbit anti-phospho-FKHR (S256) antibody diluted at 1/250; polyclonal rabbit anti-mammalian target of rapamycin (mTOR) antibody diluted at 1/500 and polyclonal rabbit anti-phospho-mTOR (S2448) antibody diluted at 1/500; polyclonal rabbit anti-phospho-glycogen synthase kinase-3 (GSK-3) (S9) dilated at 1/250 and polyclonal rabbit anti-poly-ADP-ribose/polymerase (PARP) antibody diluted at 1/500. All primary antibodies recognize human protein and were purchased from Ozyme. A monoclonal mouse anti-β-actin antibody (Sigma-Aldrich, St Quentin Fallavier, France) was used at 1/5,000 dilution for visualization of protein gel loading. The membranes were then washed at least thrice in TBST buffer and incubated for 1 hour at room temperature with the appropriate horseradish peroxidase (HRP)–conjugated secondary antibody [i.e., donkey anti-rabbit antibody at 1/1,000 dilution (Amersham, Courtaboeuf, France) or sheep anti-mouse antibody 1/2,000 dilution (Amersham)]. The protein–antibody complexes were detected by ECL (Amersham) according to the protocol of the manufacturer. For each multiple staining, membranes were debotted using ReBlot Plus reagent according to the protocol of the manufacturer (Euromedex, Mundolsheim, France) and rebotted with the corresponding primary antibody specified above and described in the figures or figure legends.

Cell Proliferation Measurements

Renal cell carcinoma cell proliferation was assessed by counting adherent cells, as described (30). Renal cell carcinoma cells were seeded

in 24-well or 6-well plates (20,000/mL), grown for 48 hours and then treated for 48 hours with LY294002 (Sigma-Aldrich) or wortmannin (VWR International, Strasbourg, France) at the concentrations indicated in Results or in the figures or figure legends. LY294002 and wortmannin were dissolved in DMSO. DMSO alone served as control for these experiments. To avoid any nonspecific toxic effects of DMSO on cell growth, DMSO concentrations were kept below 2% (v/v) in all experiments. Initial experiments showed that DMSO decreases cell growth only if used at concentrations higher than 5% (v/v) in culture medium.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Cell Viability Measurements

This assay is based on the conversion of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to pure formazan crystals by metabolically active cells and provides a quantitative estimate of viable cells (33). Renal cell carcinoma cells were seeded in 96-, 24-, or 6-well plates (20,000/mL), grown for 48 hours, and then treated for 48 hours with LY294002, wortmannin, or DMSO (control) at the concentrations indicated in Results or in the figures or figure legends. After this period, MTT (Sigma-Aldrich) at 0.5 mg/mL (final concentration) was added to each well for 4 hours at 37°C. Then, 100% (v/v) of a solubilization solution (10% SDS in 50 mM HCl) was added to each well and plates were incubated for 24 hours at 37°C. Spectrophotometric absorbance was measured at 570 nm (reference 690 nm) using a multplate reader (µQuant apparatus; Fisher Bioblock Scientific, Illkirch, France).

Assessment of Cell Death

Terminal deoxynucleotidyl transferase–mediated nick-end labeling analysis. Untransfected renal cell carcinoma cells were seeded in four-well Tissue-Tek chamber slides (Daitcher, Brumath, France) at 20,000 per well, grown for 48 hours, and then treated for 24 or 48 hours with LY294002, wortmannin, or DMSO (control) at the concentrations indicated in Results or in the figures or figure legends. For 786-0 and Caki-1 cells transiently transfected with the various Akt constructs, cells were treated with the PI3K inhibitors at the time of maximal Akt expression for the duration indicated in Results or in the figures or figure legends. Cell death was measured using the cell death detection kit based on the terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) method, according to the protocol of the manufacturer (Roche Diagnostics) and as previously described (30). Total and stained cells in 10 fields (0.25 mm² each) were counted for cell death, which was then expressed as a percentage of stained cells to total cells.

Flow cytometry analysis. 786-0 and Caki-1 cells were seeded in 25 cm² plates (20,000/mL), grown for 48 hours, and then treated for 48 hours with LY294002, wortmannin, or DMSO (control) at the concentrations indicated in Results or in the figures or figure legends. For 786-0 and Caki-1 cells transiently transfected with Akt-specific siRNA or control siRNA, cells were treated or not with the PI3K inhibitors 24 hours posttransfection (i.e., 48 hours after plating) for 48 hours. Floating and adherent cells harvested by trypsinization were resuspended, after washing in PBS, in 100 mL of the incubation buffer (140 mmol/L NaCl, 5 mmol/L CaCl₂, and 10 mmol/L HEPES buffer) containing Annexin V–FITC and propidium iodide (1 µg/mL) and incubated in dark chamber at 4°C for 10 minutes. After centrifugation, the supernatant was withdrawn and cells fixed in dark chamber in 200 mL of formol 1% at 4°C for 10 minutes. After centrifugation, cells were resuspended in 200 mL of incubation buffer and subjected to fluorescence-activated cell sorter (FACS) Scan (Becton Dickinson, Franklin Lakes, NJ) analysis using the corresponding software. This method with nonpermeabilized cells allows to concomitantly assess cell necrosis and cell apoptosis.

Renal Cell Carcinoma Tumor Model

Tumor implantation and growth. All animal studies were in compliance with French animal use regulations. Seven-week-old male Swiss Nu/Nu nude mice (Ifa-Credco, St Germain sur l’Arbresle, France) were given s.c. injections of 10 million 786-0 cells into the skin of the back (n = 12). The tumor rate was 100%. Tumor size was measured using calipers and tumor volume was calculated as length × width × height × 0.5236 (30, 34). Four weeks after injection, tumors had grown to a volume of 426.6 ± 67.4 mm³. The tumor-bearing mice were then separated into two groups of six animals. The two groups had comparable mean tumor volume of 423.2 ± 93.7 and 430.0 ± 105.8 mm³ (not significant). One group received an ip. injection thrice per week of 25 mg/kg LY294002 dissolved in DMSO/1 × PBS buffer (diluent, 1:1; v/v) whereas the other received the diluent only (control group). The two groups were treated for 28 days. Tumor size was measured using calipers twice per week and tumor volume calculated as above. Mice were euthanized at the end of the treatment period with ether and blood was harvested from the heart. The plasma was separated for electrolytes (calcium, sodium, phosphate, and potassium), creatinine, albumin, and urea analysis (Laboratoire de Biochimie Générale et Spécialisée, Hospices Civils, Strasbourg, France). Tumors were then removed, cleaned from adjacent tissues, and cut into two pieces. One piece was snap-frozen and stored at −80°C for subsequent Western blot evaluation of activated Akt with the anti-phospho-Akt antibodies. The other piece was fixed in formalin, paraffin-embedded, and 4-µm-thick sections were prepared. Some sections were stained with H&E as previously described (30) and the others were used for subsequent immunohistochemistry analysis.

Immunohistochemistry. Paraffin-embedded sections were dried on polylysine-coated slides overnight at 37°C. The slides were then deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and incubated with 3% hydrogen peroxide in PBS buffer for 5 minutes to block endogenous peroxidase activity. Immunohistochemical studies were done with different primary antibodies as described below.

To detect phosphorylated Akt, tumor sections were stained with a polyclonal rabbit anti-phospho-Akt (S473) antibody specific for immunohistochemistry (Ozyme). After heat-induced epitope retrieval [i.e., immersion in citrate-phosphate buffer (pH 6) followed by incubation for 15 minutes at 95°C], slides were incubated with the primary antibody at 1/100 dilution in PBS buffer containing 0.1% Triton X-100 (PBST) overnight at 4°C. After several washes in PBST buffer, staining was detected using the LSAB2-HRP system (DAKO, Trappes, France), according to the protocol of the manufacturer, followed by a quick (30 seconds) H&E counterstaining.

The proliferative index of renal cell carcinoma tumors was determined by staining tumor sections with a mouse monoclonal anti-human Ki67 antibody (Mib-1; DAKO). After heat-induced epitope retrieval, slides were incubated with the primary antibody at 1/100 dilution in PBST for 4 hours at room temperature. Identical procedure than for phospho-Akt staining described above was then used for visualization of Ki67 expression. To determine the proliferative index, the number of total and stained cells in 15 fields (0.25 mm² each) were counted (15 fields for 0.25 mm² each) in a blinded fashion by an experienced urologic histopathologist (Dr. Véronique Lindner, Department of Pathology, University Hospital, Strasbourg, France). The proliferative index was expressed as a percentage of stained cells to total cells.

The apoptotic index of renal cell carcinoma tumors was measured using an apoptosis detection kit based on the TUNEL method (Roche Diagnostics) according to the protocol of the manufacturer. Briefly, after deparaffinization and rehydration, slides were incubated with the TUNEL reaction mixture for 60 minutes at 37°C, in a humidified atmosphere in the dark. The TUNEL reaction mixture was prepared immediately before use and kept on ice. The slides were then incubated with converter-HPR mixture for 30 minutes at 37°C in a humidified atmosphere in the dark. The reaction was visualized by incubating the slides with 3,5-diaminobenzidine for 10 minutes at room temperature. The apoptotic index was determined and quantified as described above for the proliferative index.

To measure the effect of the treatments on microvascular density, tumor sections were stained for endothelial cells using a mouse monoclonal anti-human CD31 antibody (DAKO). The slides were deparaffinized and rehydrated as described above. After enzymatic digestion using proteinase K, slides were then incubated with the primary antibody at 1/40 dilution for 30 minutes at room temperature. Immunoperoxidase procedures as described above for phospho-Akt or Ki67 stainings were used for visualization. Microvessel density was determined in a blinded manner by counting, for each tumor, both vessel intersecting points and the total number of vessels in four to five fields (0.25 mm² each) showing the highest vascular density.
Statistical Analysis
All values are expressed as mean ± SE. Values were compared using multifactorial ANOVA followed by the Student-Newman-Keul’s test for multiple comparisons. P < 0.05 was considered significant.

Results
PI3K/Akt signaling pathway in renal cell carcinoma cell lines. To determine whether the PI3K/Akt pathway is activated in renal cell carcinoma cells, we examined the expression of total and phospho-Akt (T308 and S473) in a panel of human renal cell carcinoma cell lines expressing or not the VHL tumor suppressor gene. Akt is expressed in all human renal cell carcinoma cell lines tested (Fig. 1A). Using phosphospecific antibodies, Akt was found to be phosphorylated at both T308 and S473 residues in all human renal cell carcinoma cell lines, although at variable levels from one cell line to another (Fig. 1A). Identical results were obtained in 48-hour serum-starved cells showing that Akt is constitutively activated in these cells (data not shown). In these experiments, human hepatocytes as well as Akt control cell extracts (Ozyme) were used as control to ensure that the phosphospecific antibodies were working properly (data not shown).

To study whether the activation of the PI3K/Akt was tumor specific, we analyzed total Akt expression and the phosphorylation status of Akt in tumor biopsies obtained from three renal cell carcinoma patients and in corresponding normal renal tissues present in the biopsies. Akt is expressed in both tumor and corresponding normal renal tissues at a similar level (Fig. 1B). High amount of Akt was found to be phosphorylated in tumor samples compared with corresponding normal tissues in which phospho-Akt was only barely detectable (Fig. 1B).

In our panel of cell lines, we further examined the expression of PI3K and PTEN, two upstream regulators of Akt activation, as well as the phosphorylation status of three downstream targets of activated Akt (i.e., the FKHR transcription factors, mTOR and GSK-3β). In addition, the expression of PARP and of its apoptosis-specific cleavage products were also measured.

PI3K and PTEN are expressed in all human renal cell carcinoma cell lines at variable levels from one cell line to the other (Fig. 2A). In all cell lines, a positive relation was observed between the intensity of PI3K and the level of Akt phosphorylation (Figs. 1A and 2A). In all cell lines, except Caki-1, a negative relation was observed between the intensity of PTEN expression and the level of Akt phosphorylation (Figs. 1A and 2A). The FKHR transcription factor as well as mTOR are expressed in all renal cell carcinoma cell lines tested but were not found to be present in a phosphorylated state in any cell lines (Fig. 2B). Because mTOR and FKHR were not found in a phosphorylated state in our panel of human renal cell carcinoma cell lines, we used human non–small cell lung carcinoma of high stage (IV) biopsy was used as a positive control. A, B, and C, representative radiographs of at least three independent experiments. The VHL status of the cell lines is shown below the blots.
of phospho-Akt (i.e., 786-0, UOK-126, A498, and Caki-1 cells), there was higher levels of phospho-GSK-3β (Fig. 2C). PARP is expressed in all renal cell carcinoma cell lines but none of its cleavage fragments were detected in the cell lines tested (Fig. 2C).

These results suggest that the PI3K/Akt signaling pathway is constitutively activated in human renal cell carcinoma cells in vitro regardless of the VHL status and that the activation of this pathway is tumor specific. Interestingly, GSK-3 seems as one of the downstream targets regulated by constitutively activated Akt.

**Effect of PI3K inhibitors on renal cell carcinoma cell growth and death.** Because the PI3K/Akt pathway is constitutively activated in renal cell carcinoma cells expressing or not expressing the VHL gene, we used 786-0 and Caki-1 cells, deficient and expressing the VHL gene, respectively, for further experiments. To determine whether the PI3K/Akt signaling pathway is involved in the growth of renal cell carcinoma cells, we examined the effect of LY294002 and wortmannin, two known specific inhibitors of PI3K, on cell proliferation, viability, and death. The density of adherent cells was significantly decreased by both inhibitors in a dose-dependent manner in the two cell lines (Fig. 3A). Cell density was maximally decreased by around 60% in both cell lines. The maximal inhibition was observed for 10 to 20 μmol/L LY294002 and 10 to 20 nmol/L wortmannin. The effects on cell viability of both inhibitors were similar to the effects on cell density in the two cell lines (Fig. 3B). After testing a range of concentrations from 2.0 to 30 μmol/L and from 0.5 to 30 nmol/L for LY294002 and wortmannin, respectively, the IC50 for cell density and viability was found to be around 2 to 4 μmol/L for LY294002 and 1 to 2 nmol/L for wortmannin in both cell types. We assessed how LY294002 and wortmannin affected cell density and viability by determining their effects on cell apoptosis. Two methods were used to assess the nature of cell death—TUNEL staining and FACS analysis. TUNEL staining was significant increase in 786-0 (Fig. 4A) treated with 20 μmol/L LY294002 or 20 nmol/L wortmannin for

![Figure 3. Effect of PI3K blockade on renal cell carcinoma cell growth in vitro. A, effects of LY294002 (top left and right) and wortmannin (bottom left and right) at various concentrations on cell density in 786-0 cells (top and bottom left) and Caki-1 cells (top and bottom). Points, mean [n = 7-15 (top left), n = 4 (top right), n = 8 (bottom left), and n = 8 (bottom right)]; bars, SE. *, P < 0.05; **, P < 0.01 from no inhibitor. B, effects of LY294002 (top left and right) and wortmannin (bottom left and right) at various concentrations on cell viability in 786-0 cells (top and bottom left) and Caki-1 cells (top and bottom right). Points, mean [n = 6 (top left), n = 6 (top right), n = 4 (bottom left), and n = 4 (bottom right)]; bars, SE. *, P < 0.05; **, P < 0.01 from no inhibitor.](https://www.aacrjournals.org/content/cancerres/66/10/5134/F5.large.jpg)
24 hours compared with control treatments and the effect was maximal after 48 hours. Identical results were obtained in Caki-1 cells (data not shown). FACS analysis in 786-0 cells confirmed the apoptotic nature of the cell death induced by both PI3K inhibitors (Fig. 4B). Indeed, in cells treated with 20 μmol/L LY294002 or 20 nmol/L wortmannin, 46.4 ± 1.5% and 44.3 ± 6.5% were in an apoptotic state, respectively, compared with 5.9 ± 1.4% in control-treated cells, and no evidence of cell necrosis was observed (Fig. 4B, right). Again, identical results were obtained in Caki-1 cells treated with either inhibitor (data not shown).

Thus, in both cell types, PI3K inhibition decreases cell number and cell viability by specifically inducing cell apoptosis.

Effect of PI3K inhibitors on Akt phosphorylation. The phosphorylation status of Akt at both T308 and S473 was investigated in 786-0 and Caki-1 cells treated with a maximally effective concentration of LY294002 (20 μmol/L) or wortmannin (20 nmol/L) for 0 to 48 hours. In 786-0 cells, both inhibitors led to a rapid (within 30 minutes) and complete decrease in Akt phosphorylation at both sites (Fig. 5A). These effects were transient, with a progressive return to baseline level after 48 hours (Fig. 5A). Similar expression levels of phospho-Akt were obtained in Caki-1 cells treated with either inhibitor (data not shown). Because constitutive phosphorylation of the direct Akt substrate, GSK-3, was observed in our panel of cell lines, we also assessed the expression level of Akt-specific phospho-GSK-3 (S9) in cells treated with either PI3K inhibitor. 786-0 and Caki-1 cells were treated with a maximally effective concentration of LY294002 (20 μmol/L) or wortmannin (20 nmol/L) for 0 or 48 hours. As expected, in 786-0 cells, the expression profile of phospho-GSK-3 (S9) was quite identical to that of phospho-Akt (T308 and S473; Fig. 5B). Similar results were obtained in Caki-1 cells (data not shown).

These results strongly suggest that the PI3K inhibitors target Akt activation.

Specificity toward Akt of PI3K inhibition-induced renal cell carcinoma cell death. To show that the effects of LY294002 and wortmannin on renal cell carcinoma apoptosis were specific for inhibiting Akt activity, we used two experimental approaches. First, we transiently transfected tumor cells with various Akt constructs or vector alone and, as for the results depicted in Fig. 4A, we assessed apoptosis by TUNEL analysis in cells treated with either PI3K inhibitor. 786-0 and Caki-1 cells were treated with a maximally effective concentration of LY294002 (20 μmol/L) or wortmannin (20 nmol/L) for 0 or 48 hours. As expected, in 786-0 cells, the expression profile of phospho-GSK-3 (S9) was quite identical to that of phospho-Akt (T308 and S473; Fig. 5B). Similar results were obtained in Caki-1 cells (data not shown).
LY294002 (20 μmol/L) or wortmannin (20 nmol/L). For the first experimental approach, the expression of each Akt construct was monitored every day from day 3 to day 7 posttransfection by Western blot analysis of Akt expression. In 786-0 cells, the expression was shown to be maximal between day 3 and 5 (Fig. 6A). Similar time-dependent expression of Akt was observed in Caki-1 cells (data not shown). Cells were treated at day 3 with either LY294002 or wortmannin for 48 hours and subjected to apoptosis analysis. In 786-0 cells, similar results were obtained with LY294002 and wortmannin in vector alone–transfected cells (Fig. 6B) compared with nontransfected cells (see Fig. 4A and B). In 786-0 cells transfected with either the wtAkt or aAkt constructs, LY294002 and wortmannin were unable to induce cell apoptosis in both cell types (Fig. 6B). In 786-0 cells transfected with the dnAkt construct, apoptosis was significantly increased compared with vector alone–transfected cells in the absence of treatment and no further apoptosis was observed in response to either PI3K inhibitors (Fig. 6B). Similar results were obtained in Caki-1 cells (data not shown). In 786-0 cells transfected with Akt-specific siRNA, Akt expression was significantly reduced (Fig. 6C, inset), whereas no difference were noted between untransfected and cells transfected with control nonspecific siRNA. These results show the high efficiency of the siRNA used in knocking down Akt. Similar efficiency was obtained in Caki-1 cells (data not shown). In 786-0 cells treated with either inhibitor, similar results as the ones depicted in Fig. 4B were obtained (Fig. 6C). Interestingly, apoptosis was induced in cells transfected with Akt-specific siRNA compared with nontransfected or control siRNA-transfected cells (Fig. 6C), further arguing the critical role played by Akt in tumor cell death.

Figure 5. Effects of PI3K inhibitors on PI3K/Akt pathway activation in renal cell carcinoma cells in vitro. Western blots of 786-0 cell lysates incubated with antibodies against phospho-Akt (T308), phospho-Akt (S473), and β-actin (A) and phospho-GSK-3β (S9) and corresponding β-actin (B) in cells treated with 20 μmol/L LY294002 or 20 nmol/L wortmannin for the indicated period of time. Representative radiographs of at least three independent experiments.

Figure 6. Specificity toward Akt of PI3K inhibition–induced renal cell carcinoma cell death in vitro. A, Western blots of 786-0 cell lysates incubated with antibodies against Akt and β-actin in cells transiently transfected with the various Akt constructs (wtAkt, aAkt, and dnAkt) for the indicated period of time. Akt expression was shown to be maximal between 3 and 5 days posttransfection. Representative radiographs of at least three independent experiments. B, effects of the treatment of 786-0 cells transfected with the vector alone (vector) or the various Akt constructs (wtAkt, aAkt, and dnAkt) with 20 μmol/L LY294002 (open columns) or 20 nmol/L wortmannin (gray columns) or no inhibitor (black columns) for 48 hours on cell death, as measured by TUNEL staining. Columns, mean (95% confidence intervals); bars, SE. **, P < 0.01 from control with no inhibitor at the corresponding transfection condition; #, P < 0.01 from control of vector alone–transfected cells. C, 786-0 cells transfected or not with Akt-specific siRNA or control siRNA (siCtl) were treated with 20 μmol/L LY294002 or 20 nmol/L wortmannin or no inhibitor for 48 hours and subjected to flow cytometry analysis as detailed in Materials and Methods. Columns, mean (95% confidence intervals); bars, SE. **, P < 0.01 from control with no PI3K inhibitor or control siRNA; #, P < 0.05; ##, P < 0.01 from the corresponding PI3K inhibitor. No evidence of cell necrosis was observed. Inset, Western blots of 786-0 cell lysates incubated with antibodies against Akt and β-actin in cells untransfected or transiently transfected with control siRNA or Akt-specific siRNA. Representative radiograph of at least three independent experiments.
survival. In 786-0 cells transfected with Akt-specific siRNA, the efficiency of both PI3K inhibitors in inducing apoptosis was substantially decreased. Again, similar results were obtained in Caki-1 cells (data not shown).

These results show that the apoptotic effects of PI3K inhibitors were attributable to Akt inhibition.

**Effects of PI3K inhibition on renal cell carcinoma tumor growth in vivo.** To evaluate the biological significance of PI3K inhibition in renal cell carcinoma, we used the xenograft athymic mouse model. In severe combined immunodeficient or athymic mice, LY294002 at high dose (>100 mg/kg) has been shown to induce respiratory depression, lethargy, and hyperkeratosis resulting in dry and scaly skin (35, 36). To avoid such adverse effects of LY294002, mice were treated with the PI3K inhibitor at a moderate dose (i.e., 25 mg/kg/wk in three to four injections). In the first 15 days of treatment, LY294002 inhibited renal cell carcinoma tumor growth by >80% compared with control-treated mice. This effect was followed by significant tumor regression ranging from 10% to 50% at the end of the treatment period compared with tumor volumes at the onset of treatment (Fig. 7A and B). All of the mice tolerated LY294002 at the dose level selected and maintained body weight (Table 1). Plasma concentrations of electrolytes, creatinine, albumin, and urea showed no difference between animals treated in control or with LY294002 over the period of treatment (Table 1).

Tumors harvested from the two groups of mice were processed for histology, immunohistohistochemistry, and Western blot analysis. Microscopically, all of the tumors displayed areas of necrosis and no difference were noted between groups (data not shown). To confirm that LY294002 blocked the PI3K/Akt pathway in vivo, Akt phosphorylation status was measured by immunohistochemistry with a specific anti-phospho-Akt (S473) antibody and by Western blot. The results show that, as in vitro in culture cells, Akt is present in a phosphorylated, activated state in renal cell carcinoma tumors in vivo. Immunoblot analysis clearly showed a reduction in phosphorylated Akt levels in tumors of LY294002-treated mice compared with tumors of control-treated mice, confirming appropriate drug targeting in vivo (Fig. 7C). The proliferative index was not different between tumors obtained from control- and LY294002-treated mice (28.9 ± 2.6% versus 25.6 ± 2.0% of Ki67-stained cells in tumors of control- and LY294002-treated mice, respectively; n = 6, not significant; Fig. 8A). In marked contrast, however, treatment with LY294002 increased significantly by 40% (P < 0.05) the apoptotic index (5.5 ± 0.4% versus 7.7 ± 0.6% of TUNEL-stained cells in tumors of control- and LY294002-treated mice, respectively; n = 6, P < 0.05; Fig. 8B). The neovascularization,
as measured by the number of vessel intersecting points, was not statistically different between both groups of animals \((P = 0.1; n = 6; \text{Fig.} 8C)\). However, when the total number of vessels was measured as a function of surface area, a significant increase in vessel density was observed in tumors from LY294002-treated mice \((P < 0.01; n = 6)\).

These results suggest an important role for the PI3K/Akt signaling pathway in regulating renal cell carcinoma tumor growth through inhibition of tumor cell apoptosis \textit{in vivo}. In addition, the treatment with the PI3K inhibitor seems to be safe, at least at the dose selected.

### Discussion

Several lines of evidence suggest that the PI3K/Akt signaling pathway is implicated in a wide array of biological and pathophysiologic responses. Among these are inhibition of cell apoptosis and stimulation of cell proliferation \((8, 9)\). We found that all selected human renal cell carcinoma cell lines expressed Akt and displayed elevated levels of phosphorylated Akt when grown either in the absence or presence of serum, indicating a constitutive activation of the Ser/Thr kinase Akt in human renal cell carcinoma. Our results also show that activation of Akt is tumor specific. We choose to use normal renal tissue instead of human proximal tubular cells to check for Akt activation in normal conditions because these cells have been shown to express active Akt in culture conditions \((37)\). Alterations of the PI3K/Akt signaling pathway in human cancer might occur upstream of PI3K, directly at the level of PI3K and/or downstream of PI3K.

The regulation of the PI3K/Akt signaling pathway upstream of PI3K is the result of the ligand-dependent activation of tyrosine kinase receptors or G protein-coupled receptors \((18, 38)\). Epidermal growth factor (EGF), VEGF, and TGF-\(\beta\) are important factors involved in human renal cell carcinoma carcinogenesis and all of them have been described to activate the PI3K/Akt signaling pathway in other cell and tissue systems \((39–41)\). Although no data are available concerning the involvement of the PI3K/Akt signaling cascade in the cellular responses to these factors in human renal cell carcinoma, it is possible that the constitutive activation of Akt results from the effects on their respective receptors of various growth factors that are secreted by renal cell carcinoma cells.

The major alteration described to occur in the PI3K gene in human tumors is amplification \((8)\). The PI3K \(p110\alpha\) catalytic subunit is encoded by the PI3KCA gene located on chromosome 3q26, a region frequently duplicated or amplified in various human cancers \((42–46)\). Interestingly, Samuels et al. \((47)\) described gene mutations of \(PI3KCA\) gene in a fraction \((4-32\%)\) of human colon, gastric, breast, and lung cancers and in glioblastomas, and reported that these mutations increased kinase activity. Thus, PI3KCA mutants are likely to act as oncogenes in human cancers. Our results suggest that the \(PI3KCA\) gene is expressed at various levels in human renal cell carcinoma and that this expression correlates with the extent of Akt phosphorylation. However, we did not investigate whether the \(PI3KCA\) gene is genetically altered in human renal cell carcinoma. Whether such mutations, if any, could also affect its activity in renal cell carcinoma remains an open and interesting question.

The human tumor suppressor gene \(PTEN\) is located on chromosome 10q23 and is involved in the regulation of various signal transduction pathways \((48)\). In addition to its function as a protein phosphatase, \(PTEN\) acts as a phospholipid phosphatase with PIP3, the upstream regulator of Akt activity, as a substrate \((48)\). Akt has been shown to be continuously activated in cells lacking functional \(PTEN\), an effect that can be reversed by reintroduction of the \(PTEN\) gene \((49)\). The tumor-suppressive properties of \(PTEN\) might thus relate, at least in part, to the down-regulation of Akt activity. Loss of \(PTEN\) through mutation and/or deletion has been linked to the carcinogenesis of various cancers, including prostate, bladder, and breast cancers and melanoma \((50, 51)\). Although \(PTEN\) mutation may be a rare event in human renal cell carcinoma \((52–55)\), \(PTEN\) deletion has been shown to correlate with poor prognosis by some authors \((56)\) but not by others \((57, 58)\). Thus, the role \(PTEN\) might play in renal cell carcinoma carcinogenesis is still controversial. In accord with the PIP3 phosphatase activity of \(PTEN\), we found a clear negative relationship between the level of \(PTEN\) expression and the level of phospho-Akt, at both T308 and S473, in all cell lines. Interestingly, although \(PTEN\) protein was present in Caki-1 cell line, these cells have lower phospho-Akt levels compared with 786-0, UOK-126, or A498 cell lines, while expressing a comparable total Akt level. The reason of this apparent discrepancy is not known but could be explained by the origin of the tumor cells (i.e., the patient) or by a more complex regulation of Akt phosphorylation in this cell line.

No gene mutation of Akt has been reported in human cancer \((38)\). However, other studies have found \(Akt1\), \(Akt2\), and/or \(Akt3\) gene amplification in some tumors, including prostate, ovarian, and breast cancers \((38)\). In human renal cell carcinoma, the mutation status of Akt has not yet been investigated. In a former study by Schmoll et al. \((59)\) using reverse transcription-PCR, no significant evidence of an overexpression of Akt1, Akt2, and Akt3

### Table 1. Treatments of nude mice with LY294002 do not affect plasma variables

<table>
<thead>
<tr>
<th>Group</th>
<th>Calcium (mmol/L)</th>
<th>Sodium (mmol/L)</th>
<th>Phosphate (mmol/L)</th>
<th>Potassium (mmol/L)</th>
<th>Chloride (mmol/L)</th>
<th>Creatinine ((\mu)mol/L)</th>
<th>Albumin (g/L)</th>
<th>Urea (mmol/L)</th>
<th>Weight g (day 0)</th>
<th>Weight g (day 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.90 ± 0.27</td>
<td>154.00 ± 1.48</td>
<td>1.15 ± 0.07</td>
<td>5.30 ± 0.12</td>
<td>114.33 ± 2.38</td>
<td>28.17 ± 0.83</td>
<td>27.47 ± 0.65</td>
<td>9.00 ± 0.67</td>
<td>32.15 ± 0.71</td>
<td>32.61 ± 0.9</td>
</tr>
<tr>
<td>LY294002</td>
<td>4.14 ± 0.26</td>
<td>155.83 ± 1.30</td>
<td>1.47 ± 0.14</td>
<td>5.52 ± 0.35</td>
<td>115.67 ± 1.76</td>
<td>28.33 ± 0.80</td>
<td>27.32 ± 0.52</td>
<td>9.70 ± 0.69</td>
<td>31.61 ± 0.56</td>
<td>31.84 ± 0.44</td>
</tr>
</tbody>
</table>

NOTE: Mice treated for 28 days with control or with LY294002 were anesthetized, and the blood was collected. Concentrations of calcium, sodium, phosphate, potassium, chloride, creatinine, albumin, and urea in plasma were measured. Animal weights were measured at the beginning (day 0) and at the end (day 28) of each treatment.
transcripts was found in 16 samples of renal cell carcinoma tumor compared with the nontumor tissue from the same patient, suggesting that Akt genes are not amplified in human renal cell carcinoma. More recently, Horiguchi et al. (60) evaluated Akt activation by immunohistochemistry in 48 human renal cell carcinoma biopsies and investigated its association with pathologic features and clinical outcome. They found that elevated Akt activation could be a common finding, especially in high-grade tumors and metastatic disease, and thus suggested that Akt might have an important role in the pathogenesis and progression of renal cell carcinoma. Our results are in agreement with those reported by Schmoll et al. (59) and Horiguchi et al. (60) and suggest that genetic alterations of Akt, if any, are not involved in the constitutive activation of Akt in human renal cell carcinoma.

Phosphorylation of the forkhead family of transcription factors, to which FKHR belongs, by Akt inhibits transcription of pro-apoptotic genes such as Fas ligand, insulin-like growth factor binding protein-1, and Bim (8, 9, 18, 38). Akt also phosphorylates and activates mTOR, and, although the downstream target of mTOR is controversial in the literature, activated mTOR promotes translation of members of the cyclin D family, including cyclin D1, which, in turn, stimulates cell progression through phosphorylation and inactivation of the retinoblastoma protein pRb (8, 9, 18, 38). Phosphorylation of GSK-3 on S9 by Akt inhibits its activity, thus preventing the phosphorylation of the cytoplasmic signaling molecule β-catenin that impedes its degradation. β-catenin can then translocate to the nucleus to induce the expression of various genes, such as cyclin D1, and stimulates cell progression (8, 9, 18, 38). In addition to induction of cell progression, additional studies also show that Akt-dependent phosphorylation of mTOR and GSK-3 promotes cell survival through inhibition of cell apoptosis (61, 62). PARP is a 116 kDa nuclear polymerase involved in DNA repair in response to environmental stress (63). This protein can be cleaved by many caspases in vitro (64), and is one of the main cleavage targets of caspase-3 in vivo (64). The cleavage of human PARP separates the NH2-terminal DNA binding domain of PARP (24 kDa) from its COOH-terminal catalytic domain (89 kDa; ref. 64). PARP is important for cells to maintain their viability and its

**Figure 8.** Histopathologic analysis of tumors from control- and LY294002-treated mice. A, tumor sections of control-treated (left) or LY294002-treated (right) mice immunostained with an antibody against Ki67 (black nuclei; magnification, ×400). Right, quantification of Ki67 staining (proliferative index). Columns, mean (n = 6); bars, SE. B, tumor sections of control-treated (left) or LY294002-treated (right) mice immunostained for DNA fragmentation (TUNEL; black nuclei; magnification, ×400). Right, quantification of TUNEL staining (apoptotic index). Columns, mean (n = 6); bars, SE. *, P < 0.05 from control-treated mice. The treatment with LY294002 induced cell apoptosis. C, top, tumor sections of control-treated (left) or LY294002-treated (right) mice immunostained for CD31 (magnification, ×400); Bottom left, quantification of crossing vessels intersecting points per surface area (0.25 mm²). Columns, mean (n = 6); bars, SE. Bottom right, quantification of vessels per surface area (0.25 mm²). Columns, mean (n = 6); bars, SE. **, P < 0.05 from control-treated mice.
Our results show that GSK-3β is constitutively phosphorylated in all human renal cell carcinoma cell lines tested, whereas FKHR and mTOR are not found in a phosphorylated state in these cell lines. In addition, PARP was found in its native, uncleaved form in all renal cell carcinoma cell lines tested. Taken together, these results indicate that GSK-3β is one of the downstream Akt targets whose activity is regulated by the PI3K/Akt signaling pathway in human renal cell carcinoma. Thus, phosphorylation of GSK-3β by Akt might contribute to the intrinsic resistance of renal cell carcinoma.

The VHL/hypoxia-inducible factor (HIF) system has been shown to interact with the PI3K/Akt signaling pathways (8, 17). Among oncogenic signaling pathways that have been shown to activate HIF-1α through either protein synthesis or translation is Akt and its downstream targets, including FKHR, GSK-3, or mTOR (8, 17). Our results did not show any difference in the expression patterns of the various molecular components of the PI3K/Akt pathway depending on the VHL status of the cells, suggesting that the constitutive activation of this pathway is independent on the VHL status of renal cell carcinoma.

Our studies support this tenet and indicate that LY294002 and wortmannin markedly reduced in a concentration-dependent manner human renal cell carcinoma cell growth in vitro and regardless of the VHL status of the cells. The flavonoid derivative LY294002 is a potent, competitive and reversible inhibitor of the ATP binding site of PI3K, and is highly specific for PI3K (18, 66). Wortmannin is a fungal metabolite that also interacts with the ATP binding site of PI3K, but in an irreversible manner (18, 66). The specificity of the effect of the inhibitors toward the PI3K/Akt pathway was clearly evidenced by the rapid and almost complete inhibition of Akt phosphorylation at both T308 and S473, and confirmed by the use of various Akt constructs and Akt knockdown through gene silencing. In addition, the IC50 for tumor cell growth inhibition obtained in our study is in the range of the reported IC50 for inhibition of PI3K reported for both inhibitors (18, 66). Our results also indicate that the effect of both inhibitors was obtained through induction of cell apoptosis. Together with our other results, it is tempting to speculate that the phosphorylation of GSK-3β by Akt is involved in tumor cell survival.

Another noteworthy finding in our study is the regression of human renal cell carcinoma tumors by LY294002 in vivo. We chose to use LY294002 instead of wortmannin in these experiments in nude mice mainly for two reasons: First, this compound has reversible effects on PI3K activity and, second, its effectiveness toward PI3K inhibition has been shown for other tumor types in vivo in nude mice (35, 36). The dose and injection schedule of LY294002 used was apparently well tolerated by all animals. The specificity of the effect of LY294002 toward the PI3K/Akt axis was also confirmed in vivo by both immunohistochemistry and Western blot analysis of phospho-Akt (S473). Consistent with the results obtained in vitro in cultured cells, the effect of LY294002 was shown to be the consequence of an induction of cell death by the PI3K inhibitor. These results suggest that the PI3K/Akt signaling pathway is constitutively activated in the in vivo environment as well, and participates in the overall growth of human renal cell carcinoma. These effects of PI3K inhibition both in vitro and in vivo on human renal cell carcinoma growth have never been reported previously. Our results clearly indicate that the PI3K/Akt signaling pathway is one of the main molecular pathway involved in the intrinsic resistance of human renal cell carcinoma. It is important to note that growth inhibition by both inhibitors was not complete even at high concentrations. Therefore, these results also indicate not only the possibility that tumor cell growth signals from growth factors might pass mainly through the PI3K/Akt pathway but also the possibility that other growth/survival pathways may exist in human renal cell carcinoma, such as, for example, the MAPK Erk1/2 pathway. Whether these pathways are part of a common or distinct cellular signaling network and whether they also participate in the therapy-induced tumor resistance, as observed in other tumors, is at present unknown.

Another significant finding in the current study was the increased microvessel density in renal cell carcinoma tumors grown in nude mice in response to PI3K inhibition. This is a quite surprising result in light of the proangiogenic activity of the PI3K/Akt cascade that is consistently reported in the literature in other cell and tissue systems (8, 17). In addition, the stimulatory effect on VEGF expression has been shown to be obtained through the transcriptional activity of HIFs in ovarian cancer cells (66). As presented above, HIFs are stabilized due to VHL deficiency in the majority of human renal cell carcinoma regardless of oxygen tension. Thus, one might have expected to observe an inhibitory effect on neovascularization by PI3K blockade. However, our results suggest that the PI3K/Akt pathway has antiangiogenic activity in human renal cell carcinoma. Clarification of the molecular mechanisms of this effect awaits additional investigation. It should be stressed that most, but not all, studies show that renal cell carcinoma is a highly vascularized tumor (67, 68). Indeed, it has been proposed that in renal cell carcinoma tumors with poor prognosis, decreased microvessel density is associated with the development of large vessels, which facilitate metastatic spread (68). We previously reported tumor regression in vivo in nude mice bearing human renal cell carcinoma tumors in response to blockade of parathyroid hormone–related protein by specific antibodies (30). In this study, we also observed an increase in neovascularization in tumors of mice treated with the PTHrP-neutralizing antibody. Thus, the angiogenic effect (i.e., increased microvessel density) in response to the PI3K blockade might be beneficial therapeutically, together with the induction of apoptosis. One explanation for our results may come from the very interesting recent studies of Chen et al. (69). Indeed, in their report, they show that angiogenic responses were increased in Akt1 knockout mice in three different experimental models and that these responses were linked to reduced expression of thrombospondin-1 and thrombospondin-2, two well-known vascular regulators, in these mice. The angiogenic effects we observed with LY294002 in tumors grown in nude mice thus also argument the specificity toward Akt of the actions of the PI3K inhibitor. Thrombospondins have been shown to be expressed in >75% of renal cell carcinoma in humans (70). Thus, it will be interesting in future works to determine whether the angiogenic effects we observed with LY294002 in tumors grown in nude mice might be correlated to thrombospondin expression.

The molecular basis of the inherent and broad-spectrum drug resistance in human renal cell carcinoma is largely unknown. The results reported in the present study indicate that activation of the PI3K/Akt pathway in renal cell carcinoma might be one of the resistance mechanisms. Others have shown that PI3K inhibition by LY294002 or wortmannin enhanced chemotherapy-induced tumor cell apoptosis in other cancers. Additional studies are required to determine the extent to which inhibition of the PI3K/Akt signaling pathway restores drug sensitivity in human renal cell carcinoma. Our results also indicate that each of the molecular entities of the...
PI3K/Akt pathway merits investigation as prognostic factor for renal cell carcinoma patients. The current studies argue that PI3K inhibition is a potentially useful treatment for renal cell carcinoma patients.

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References


69. Chen J, Somanath PR, Razorenova O, et al. Akt1 regulates pathological angiogenesis, vascular matura-

The Phosphoinositide 3-Kinase/Akt Pathway: A New Target in Human Renal Cell Carcinoma Therapy

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