Targeting the Phosphatidylinositol 3-Kinase/Akt Pathway for Enhancing Breast Cancer Cells to Radiotherapy[1]

Ke Liang, Weidong Jin, Christiane Knuefermann, Mathias Schmidt,[2] Gordon B. Mills, K. Kian Ang, Luka Milas, and Zhen Fan[3]

Departments of Experimental Therapeutics [K. L., W. J., C. K., M. S., Z. F.], Molecular Therapeutics [G. B. M., Z. F.], Radiation Oncology [K. K. A.], and Experimental Radiation Oncology [L. M.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

The phosphatidylinositol 3-kinase (PI-3K)/Akt pathway, regulated by its upstream growth factor receptor tyrosine kinases, plays a critical role in promoting cell proliferation and inhibiting cell death. The aim of this study was to determine whether the PI-3K/Akt activity contributes to the resistance of human breast cancer cells to ionizing radiation and whether inhibition of the PI-3K/Akt pathway could sensitize human breast cancer cells to radiotherapy. To determine a causal relationship between the activity of Akt and radioresistance in human breast cancer cells, MCF7 cells, transfected with constitutively active H-Ras (RadG12V) or constitutively active Akt, were chosen for analysis of the cell clonogenic survival fraction and induction of apoptosis after ionizing radiation. The PI-3K-specific inhibitor LY294002 was used to examine whether inhibition of PI-3K could sensitize these cells to radiation treatment. Our results indicate that the expression of constitutively active Ras (which activated Akt in a PI-3K-dependent manner) and the expression of constitutively active Akt (which caused a PI-3K-independent activation of Akt) each increased cellular resistance to radiation. Inhibition of PI-3K with LY294002 reverted the constitutively active Ras-mediated radioresistance but not the constitutively active Akt-mediated radioresistance. Our data suggest that Akt may be a potential target for enhancing the response to radiotherapy in patients with breast cancer.

Introduction

Breast cancer remains a major cause of cancer-related deaths among American women. Despite continued progress, 211,300 new breast cancer cases are expected in 2003 in the United States alone, and 39,800 women in the United States will die this year of breast cancer.[4] Innovative molecule-targeted therapies are urgently needed to improve the prognosis of women with this disease. Radiotherapy, as an integral part of the current comprehensive breast cancer treatment regimen, may be used to eradicate remaining cancer cells in the breast, chest wall, or axilla after surgery or to reduce the size of an advanced tumor before surgery. The ability to deliver radiation therapy accurately to cancer sites has increased dramatically over the past decades, and this has greatly diminished side effects. There also have been extensive studies for new approaches to sensitize cancer cells to ionizing-radiation treatment, in the hopes of improving treatment outcome.

PI-3K,[5] a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit, plays a central role in cell growth regulation and possibly in tumorigenesis. It generates specific inositol lipids (PI-3,4,5-P_3 and PI-3,4-P_2) that have been implicated in the regulation of cell proliferation, differentiation, senescence, cytoskeletal changes, motility, metastasis, invasion, angiogenesis, and survival (1–3). Previous studies have shown that several members of the PIKK family, which share significant sequence homology at their COOH terminus, play a key role in regulating the cellular response to ionizing radiation-induced DNA damage (4–7). Cells defective in ataxia telangiectasia mutated protein (ATM), ataxia- and Rad3-related protein (ATR), or DNA-dependent protein kinase (DNA-PK) are known to be extremely sensitive to ionizing radiation and are defective in the repair of DNA damage (8–10). Radiosensitization may be induced in cancer cells by the irreversible PI-3K inhibitor wortmannin (11–14) or the reversible PI-3K inhibitor LY294002 (12). Wortmannin or LY294002 inhibits certain mammalian PI-3Ks by covalent or noncovalent modification of a critical lysine residue in their phosphotransferase domains (15). The logic for these studies was that, because of the presence of the COOH-terminal sequence homology among the PIKK family members, these PIKKs may also be sensitive to inhibition by wortmannin or LY294002.

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[2] Present address: Department of RPR/P3 Oncology Research, ALTANA Pharma AG, D-78467 Konstanz, Germany.
[3] To whom requests for reprints should be addressed, at Unit 36, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. E-mail: zfan@mdanderson.org.

[4] Source from American Cancer Society at www.cancer.org.
[5] The abbreviations used are: PI-3K, phosphatidylinositol 3-kinase; PIKK, PI-3K-related kinase family; PH, pleckstrin homology; ΔPH-Akt1-farn, Flag-tagged farnesylated Akt1 lacking the PH domain; PI-3,4,5-P_3, phosphatidylinositol 3,4,5-triphosphate; PI-3,4-P2, phosphatidylinositol 3,4-diphosphate; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PE, plating efficiency; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP-X nick end labeling; IκK, IκB kinase; COX-2, cyclooxygenase-2; SF2, survival fraction of 2 Gy.

Recent evidence suggests that PI-3K may also be a target for sensitizing cancer cells to ionizing radiation. A recent study (16) found that the PI-3K pathway plays a critical role in mediating enhanced radioresistance by the *Ras* oncogene. The study showed that the PI-3K inhibitor LY294002 radiosensitized cells bearing a constitutively active Ras mutant but did not affect the survival of cells with wild-type Ras. Inhibition of the PI-3K downstream target p70S6K by rapamycin, of the Raf-MEK-MAP-kinase pathway by PD98059, or of the Ras-MEK kinase-p38 pathway by SB203580 had no effect on radiation survival in cells with oncogenic *Ras* (16). Furthermore, expression of active PI-3K in cells with wild-type Ras resulted in increased radiation resistance that could be inhibited by LY294002 (16).

One of the best characterized downstream targets for the PI-3K lipid products is Akt1, which was discovered about a decade ago (17–19) and which is now known to be a member of a family of closely related, highly conserved cellular homologues that consists of at least two additional members (Akt2 and Akt3; Refs. 20, 21). Each Akt protein consists of a NH_2-terminal PH domain, a serine-threonine kinase domain, and a COOH-terminal regulatory tail (22). Akt has been shown to be a critical player in oncogenesis (23). The increased Akt kinase activity may result from increased activity of the PI-3K attributable either to the binding of its p85 subunit to the activated epidermal growth factor receptor kinase family proteins, particularly HER2 (24), or to mutational inactivation or deletion of the PTEN tumor suppressor gene, which inhibits Akt activity by dephosphorylating the PI-3,4,5 P_3 and PI-3,4 P_2 produced by PI-3K (25, 26).

There is increasing evidence indicating that PI-3K/Akt plays an important role in breast cancer tumorigenesis. Increased kinase activity of Akt2 was found in ~40% of breast cancer specimens in one recent study (27). Another recent study found that PTEN expression is frequently reduced in advanced breast cancers (28). The study reported reduced PTEN protein expression in 38% of invasive cancers and in 11% of *in situ* cancers (28). Akt activity was found to be constitutive in breast cancer cell lines with either HER2 overexpression or PTEN mutation (29).

An elevated level of Akt activity was associated with increased cellular resistance to the treatment with doxorubicin, transtuzumab, or tamoxifen in breast cancer cell lines (29). Similarly, increased cellular resistance to cisplatin, paclitaxel, VP16, or ionizing radiation was found in non-small cell lung cancer cell lines with high levels of PI-3K/Akt activity (30). Transient expression of a constitutively active Akt in non-small cell lung cancer cells with low Akt activity conferred on these cells resistance to chemotherapy- or radiotherapy-induced apoptosis; treatment of the cells with LY294002 sensitized the cells to treatment with chemotherapy or radiotherapy (30). The results of these studies demonstrated that modulation of PI-3K/Akt activity in cancer cells may alter the sensitivity of the cells to conventional therapies.

The aim of the present study was to dissect the upstream signals of Akt activation and to determine whether expression of a constitutively active Akt would alter the sensitivity of breast cancer cells to radiotherapy. We chose MCF7 breast cancer cell transfectant clones expressing a constitutively active Ras (G12V) or expressing a constitutively active Akt (farnesylated Δ PHAkt1; Ref. 31). Compared with parental MCF7 or control-vector transfected MCF7 cells, MCF7RasG12V or MCF7Akt1-farn clones all showed enhanced survival after ionizing radiation and exhibited increased resistance to ionizing radiation-induced apoptosis. In contrast to the MCF7RasG12V cells, which exhibit a PI-3K-dependent (LY294002-sensitive) increase in resistance to ionizing radiation, the constitutively active status of Akt1 in the MCF7Akt1-farn clones conferred a PI-3K-independent (LY294002-insensitive) resistance to ionizing radiation, indicating that Akt1 alone can causally induce cellular resistance to ionizing radiation and, thus, might be a potential target for radiosensitization of cancer cells.

Materials and Methods

Antibodies and Reagents. Antibodies directed against total Akt, and ser473-phosphorylated Akt1 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-Ras antibody was from B. D. Bioscience Transduction Laboratory (San Jose, CA). Anti-β-actin antibody was from Sigma Chemical Co. (St. Louis, MO). LY294002 was obtained from CalBiochem Corp. (San Diego, CA).

Cell Lines and Culture. All of the breast cancer cell lines (MCF7, SKBR3, MDA468, T47D, ZR75B) were grown and routinely maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and were incubated in a 37°C humidified atmosphere (95% air and 5% CO_2).

Establishment of MCF7 Cell Clones Expressing Constitutively Active Ras or Constitutively Active Akt1. Transfection was performed with the Fugene-6 transfection kit (Roche Diagnostic, Indianapolis, IN). MCF7 cells were transfected with the pcDNA3.1 backbone vector or the pcDNA3.1 vector containing a PCR-generated ΔPH-Akt1-farn that contains a PH domain-deleted Akt1 cDNA sequence along with a NH_2-terminal Flag-tag sequence and a COOH-terminal farnesylation sequence, as we reported recently (31) or the pcDNA3.1 vector containing the H-RasG12V that was subcloned from the plasmid pSRα-H-RasV12 (kindly provided by Dr. Richard A. J. Janssen at the Center for Biologics Evaluation and Research, United States Food and Drug Administration, Bethesda, MD). Stable clones for each type of transfection were selected by neomycin (400 μg/ml) for Ras transfectants and by hygromycin (200 μg/ml) for Akt transfectants. Surviving clones were evaluated for expression by Western blot analysis with the appropriate antibodies.

Ionizing Radiation. Cells grown on Petri dishes were irradiated with γ rays from a high-dose rate ^{137}Cs unit (4.5 Gy/min) at room temperature. After irradiation at various doses, the cells were harvested by trypsinization for various studies as described below.

Clonogenic Assay. Cells were plated in triplicate into 6-cm dishes with densities varying from 300 to 1200 cells/dish (to yield 50–200 colonies/dish) depending on the radiation dose that the cells received. The cells were then cultured in a 37°C, 5% CO_2 incubator for 10 days. Individual

colonies (>50 cells/colony) were fixed and stained with a solution containing 0.25% Gentian violet and 10% ethanol for 10 min. The colonies were counted with the FluorChem 8800 Imaging System (Alpha Innotech Corporation, San Leandro, CA) using a visible light source. The PE represents the percentage of cells seeded that grow into colonies under a specific culture condition of a given cell line. The clonogenic survival is PE-normalized percentage of irradiated cells seeded that grow into colonies. The survival fraction, expressed as a function of irradiation, was calculated as follows: survival fraction = colonies counted/(cells seeded × PE/100). SF2 stands for a specific survival fraction of the cells that received 2 Gy irradiation.

Quantification of Apoptosis by ELISA. An apoptosis ELISA kit (Roche Diagnostics Corp.) was used to quantitatively measure cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes), as we reported previously (32). This photometric enzyme immunoassay was performed exactly according to the manufacturer's instructions.

TUNEL Assay. The TUNEL assay was performed as described previously (32). Briefly, after fixation with 1% formaldehyde on ice for 1 h, the cells were washed once with PBS and then post-fixed in ice-cold 70% ethanol overnight. The next day, the cells were washed once with PBS before incubation with 50 μl of TdT reaction solution containing 5 units of TdT, 0.5 nmol of biotin-dUTP, and 2.5 mM $CoCl_2$ in TdT buffer (Roche Diagnostics Corp.) at 37°C for 1 h. After the reaction, the cells were stained with a buffer containing 2.5 μg/ml FITC-avidin, 0.1% Triton X-100, and 5% dried low fat milk in 4× SSC [0.6 M sodium chloride-60 mM sodium citrate (pH 7)] in the dark at room temperature for 1 h. Before flow cytometric analysis with a FACScan flow cytometer (Becton Dickinson, San Jose, CA), the cells were counterstained with a solution containing 5 μg/ml propidium iodide and 10 μg/ml RNase A in PBS. The FITC signal was analyzed with Epics Elite software (Coulter Corp., Miami, FL).

Western Blot Analysis. The cells in culture dishes were washed with cold PBS two times and then harvested with a rubber scraper. Cell pellets were lysed with a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotinin. The lysates were cleared by centrifugation, and the supernatants were collected. Equal amounts of lysate protein were used for the Western blot analyses with the indicated antibodies (33). Specific signals were visualized using the enhanced chemoluminescence (ECL) detection kit (Amersham, Arlington Heights, IL).

Results

Effect of Constitutively Active Ras(G12V) or Constitutively Active Akt on the Sensitivity of MCF7 Cells to Ionizing Radiation. To determine whether Akt plays a causal role in conferring increased resistance of cells to ionizing radiation, we compared two experimental approaches to achieve constitutively active status of Akt in MCF7 breast cancer cells. One approach was to transfect MCF7 cells with a constitutively active Ras expression vector. The constitutively active Ras, by directly binding and activating the catalytic p110 subunit of PI-3K (34), generates PI-3,4-P_2 and PI-3,4,5-P_3; the latter two will then recruit Akt to the cell membrane, in which Akt is activated by the phosphoinositide-dependent kinases (35). The other approach was to transfect MCF7 cells with a constitutively active Akt expression vector. We transfected MCF7 cells with an expression vector that contains a PH domain-deleted Akt1 cDNA sequence along with a NH_2-terminal Flag-tag sequence and a COOH-terminal farnesylation sequence (ΔPH-Akt1-farn), which we demonstrated is constitutively active (PI-3K-independent; Ref. 31). As shown in Fig. 1*A*, MCF7RasG12V cells expressing a constitutively active Ras (*gel c, Lane 3*) showed a higher level of serine 473-phosphorylated (activated) Akt, compared with

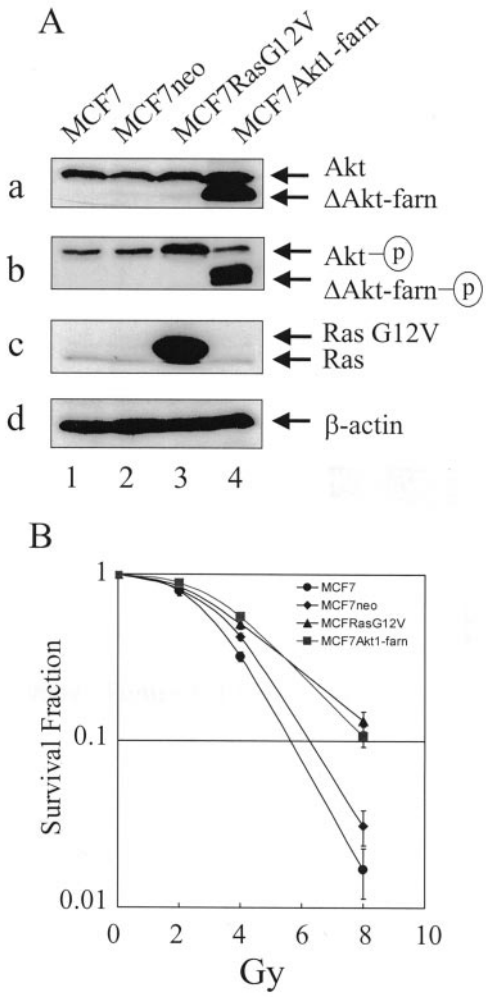

Fig. 1. Effect of the expression of constitutively active Ras or constitutively active Akt on the radiosensitivity of MCF7 cells. In *A*, the expression levels of total Akt, serine 473 phosphorylated Akt, Ras protein, and β-actin (serving as loading control) were determined by Western blot analyses with their respective antibodies in MCF7 parental cells, MCF7neo cells, MCF7RasG12V, and MCF7Akt1-farn cells. In *B*, clonogenic survival fractions (determined by normalizing the colony efficiencies to the respective control values) are shown for MCF7 parental cells, MCF7neo cells, MCF7RasG12V, and MCF7Akt1-farn cells after 0, 2-, 4-, and 8-Gy irradiation.

the levels of phosphorylated endogenous Akt in parental MCF7 cells, control-vector transfected cells, or the MCF7Akt-farn cells. In contrast, MCF7Akt1-farn cells expressing a farnesylated PH domain-deleted Akt (*gel a, Lane 4*) showed a markedly increased level of phosphorylated ΔPH-Akt1. Clonogenic survival study indicated that MCF7 cells expressing constitutively active Ras or constitutively active Akt both showed increased resistance to ionizing radiation (Fig. 1*B*). At the 8-Gy dose, the survival rate increased from 3% in the control-vector-transfected MCF7 clone to 13% in constitutively active Ras-transfected cells and 11% in constitutively active Akt-transfected cells.

Effect of Inhibition of PI-3K on the Sensitivity to Ionizing Radiation of MCF7 Cells Expressing Constitutively Active Ras(G12V) or Constitutively Active Akt. We next examined the effect of the PI-3K-specific inhibitor LY294002 on sensitizing the MCF7 cells to ionizing radiation. We first investigated the timing of LY294002 treatment with ionizing radiation in MCF7 cells by comparing the clonogenic survival of MCF7 cells after a 4-Gy single-dose irradiation. Exposure of the cells to LY294002 was conducted by three different schedules: 1 h preradiation exposure, 10 days postradiation exposure starting 24 h after ionizing radiation, and a full-course exposure (*i.e.,* 1 h preradiation exposure plus 10 days postradiation exposure starting 24 h after ionizing radiation). A 1-h preexposure of the MCF7 cells to 5 μM LY294002 had minimal effect on clonogenic survival of the MCF7 cells (44 *versus* 41%). Treatment of the cells with 5 μM LY294002, started 24 h after ionizing radiation, more effectively reduced the clonogenic survival of MCF7 from 44 to 34%. However, a combination of preexposure of 5 μM LY294002, and the presence of 5 μM LY294002 started 24 h after ionizing radiation achieved the best result. The clonogenic survival of MCF7 cells was reduced from 44 to 15.9% (Fig. 2).

We next compared the survival fraction of the cells with escalating doses of irradiation (Fig. 3). Compared with ionizing radiation alone, a full-course exposure to LY294002 (as described in Fig. 2) enhanced the radiosensitivity of parental MCF7 cells (Fig. 3*A*), MCF7neo cells (Fig. 3*B*), and MCF7RasG12V cells (Fig, 3*C*) but not MCF7Akt1-farn cells (Fig. 3*D*). A point that needs to be clarified is that, when we calculated the survival fraction, the collective effects of radiation and LY294002 treatment were normalized to the effect caused by treatment of the cells with LY294002 alone in each of the individual cell lines.

The effect of LY294002 on inhibiting the activities of Akt in the MCF7 cells was demonstrated by reduced phosphorylation of Akt on serine 473 (Fig. 4). Western blot analysis showed that exposure of the cells to LY294002, either before (Fig. 4, *Lanes 1 versus Lanes 2*) or after radiation (*Lanes 3–5 versus Lanes 4–6*), inhibited the basal phosphorylation levels of Akt in parental MCF7 and control vector-transfected MCF7 cells and inhibited elevated phosphorylation level of Akt in MCF7RasG12V cells but not in MCF7Akt1-farn cells. Whereas the phosphorylation level of endogenous Akt was reduced as expected in MCF7Akt1-farn cells (Fig. 4*D*, *Lane 1 versus Lane 2*), the phosphorylation level of the constitutively active Δ PH-Akt-farn was not sensitive to the treatment

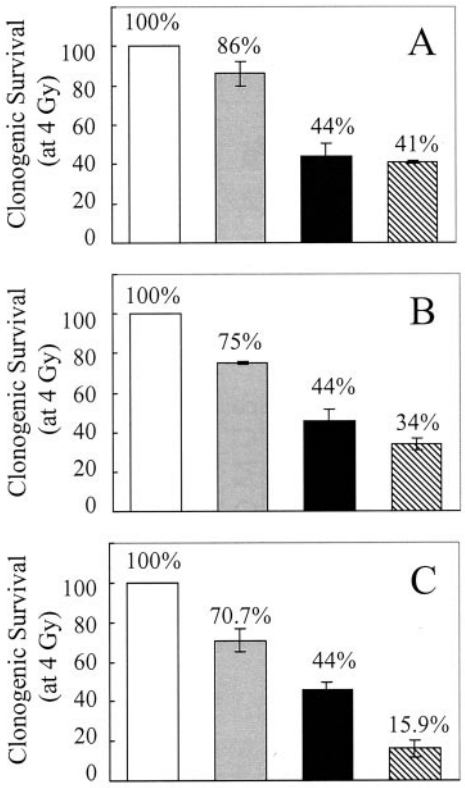

Fig. 2. Comparison of radiation sensitization and the timing of PI-3K inhibition by LY294002 in MCF7 cells. MCF7 cells had either a 1-h preradiation exposure to 5 μM LY294002 (*A*), a 10-day postradiation exposure to 5 μM LY294002 started 24 h after radiation (*B*), or a combination of 1-h preradiation exposure and 10-days postradiation exposure to 5 μM LY294002 started 24 h after radiation (*C*). Individual clone numbers were counted on day 10 after irradiation. Colony efficiencies (determined by dividing the number of survival colonies by the number of cells seeded) were plotted as a function of each individual treatment. □, Carrier; ▨, LY294002; ■, XRT; ▧, LY294002 + XRT.

with LY294002, neither before nor after ionizing radiation (*Lanes 3–5 versus Lanes 4–6*).

It has been well demonstrated that DNA damage caused by ionizing radiation induces apoptosis in many cell types. To demonstrate that the increased postradiation survival of MCF7 cells expressing constitutively active Ras or Akt and the radiosensitization by the PI-3K inhibitor LY294002 were associated with the induction and inhibition of apoptosis, we used an apoptosis ELISA to quantify apoptosis after radiation. We found that, compared with MCF7 parental or control vector-transfected cells, MCF7RasG12V and MCF7Akt-farn cells both showed increased resistance to ionizing radiation-induced apoptosis (Fig. 5). LY294002 alone had a modest effect on MCF7 parental cells or control vector-transfected cells in inducting apoptosis, being slightly more effective on MCF7RasG12V and less effective on MCF7Akt-farn cells. The combination of LY294002 with radiation enhanced the induction of apoptosis in all of the groups. Of note, LY294002 markedly sensitized radiation-induced apoptosis in MCF7RasG12V cells, whereas it only minimally sensitized radiation-induced apoptosis in MCF7Akt-farn cells. This observation is consistent with the cell survival data. The in-

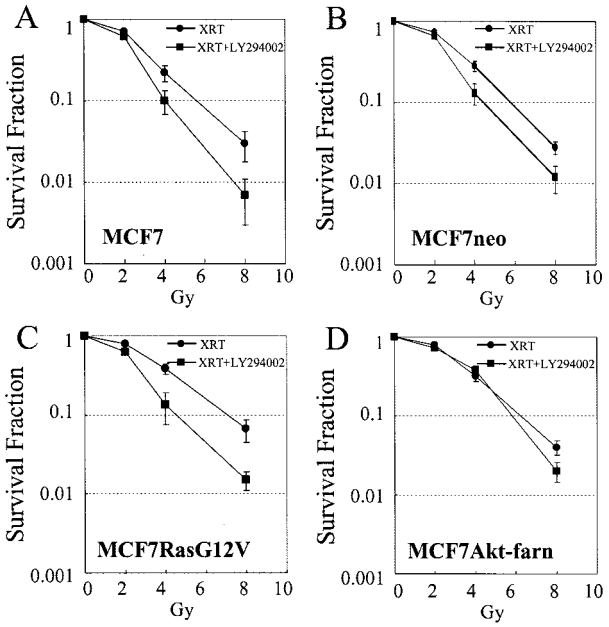

Fig. 3. Effect of the PI-3K inhibitor LY294002 on the clonogenic survival in MCF7 parental cells, MCF7neo cells, MCF7RasG12V, and MCF7Akt1-farn cells. The four cell lines received various radiation doses (0, 2, 4 and 8 Gy) with and without 1-h preradiation exposure and 10-days postradiation exposure to 5 μM LY294002 started 24 h after the irradiation. Clonogenic survival fractions were calculated by normalizing the colony efficiencies to their respective control values (DMSO or LY294002 treatment alone).

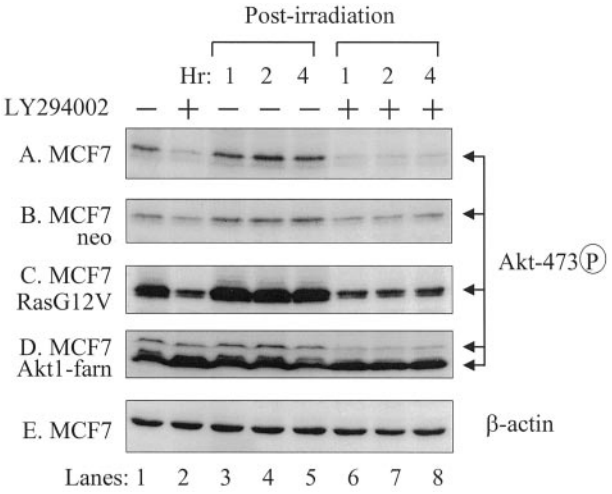

Fig. 4. Effect of the PI-3K inhibitor LY294002 on the levels of phosphorylated Akt in MCF7, MCF7neo, MCF7RasG12V, and MCF7Akt-farn cells before and after radiation. Parental MCF7, MCF7neo, MCF7RasG12V and MCF7Akt-farn cells (*Lanes 3–8*) were exposed to 20 μM LY294002 1 h before or 1, 2, or 4 h after radiation. Equal amounts of cells lysates were prepared for Western blot analysis with antibodies directed against serine-473 phosphorylated Akt and β-actin (showing equal loading of protein samples in each lane). Equal loading was seen in each lane of all four groups of cells, but only the data with MCF7 parental cells are shown as representative.

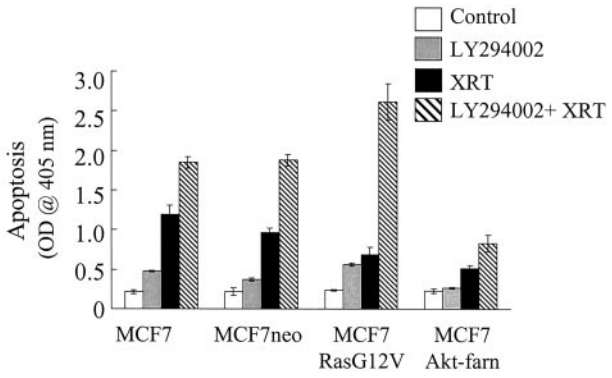

Fig. 5. Effect of the PI-3K inhibitor LY294002 on radiation-induced apoptosis in MCF7 parental cells, MCF7neo cells, MCF7RasG12V, and MCF7Akt1-farn cells. The four groups of cells were treated with 5 μM LY294002 alone overnight (12 h), a 10-Gy irradiation alone, or 5 μM LY294002 overnight followed by 10-Gy irradiation. The cells were cultured for an additional 24 h after radiation or LY294002 treatment and then were collected for apoptosis ELISA analyses as described in "Materials and Methods."

creased resistance to radiation-induced apoptosis conferred by constitutively active Akt is PI-3K independent (LY294002 insensitive). The modest radiosensitization could be attributed to the inhibition of endogenous Akt, which is PI-3K-dependent and, thus, LY294002-sensitive. The increased and PI-3K-independent radioresistance by constitutively active Akt was further confirmed by TUNEL apoptosis assay (shown in Fig. 6). After irradiation, 19% of parental MCF7 cells were TUNEL-positive (Fig. 6), compared with only 3% of the MCF7Akt1-farn cells. Treatment of the parental MCF7 cells with LY294002 increased the TUNEL-positive rate from 19 to 56.7%. In contrast, treatment of MCF7Akt1-farn cells with LY294002 increased the TUNEL-positive rate from 3 to only 21%, a percentage similar to the rate for parental MCF7 cells irradiated without LY294002 treatment.

Expression of Akt in Human Breast Cancer Cells and Radiosensitization by LY294002 Treatment. To explore the generality of the radiosensitization induced by the inhibition of the PI-3K/Akt pathway, we examined the levels of total and phosphorylated Akt protein and the radiosensitivity after LY294002 treatment in four additional human breast cancer cell lines (SKBR3, MDA468, T47D, and ZR75B). Akt was commonly expressed in all of the cell lines tested, whereas the levels of phosphorylated Akt varied (Fig. 7*A*), apparently because of the differences in signal transduction in individual cell lines. Regardless of the levels of Akt phosphorylation, exposure to LY294002 sensitized all of the cell lines to ionizing radiation. SF2 (survival at 2 Gy) assays showed reductions in the numbers of colonies in all four of the cell lines tested. For the plotting of the SF2, the reduction in the numbers of colonies by the combination treatment of 2 Gy radiation and LY294002 was normalized to the effects caused by parallel treatment of the cells with LY294002 alone in each of the individual cell lines (Fig. 7*B*). A caveat (addressed in the "Discussion" section) is that, although each cell line showed different radiosensitivity, and the radiosensitivity did not seem to correlate directly with the ser-473-phosphorylation level of Akt, exposure to LY294002 sensitized all of the cell lines to ionizing radiation.

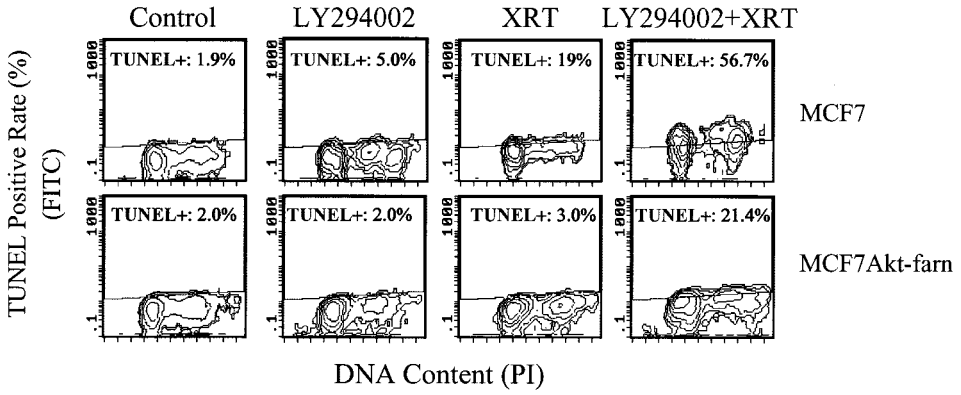

Fig. 6. TUNEL flow cytometric analysis on the effect of the PI-3K inhibitor LY294002 on radiation-induced apoptosis in MCF7 parental cells and MCF7Akt1-farn cells. The cells were cultured for overnight with or without 5 μM LY294002, followed by 10-Gy irradiation or no radiation. The cells were harvested 24 h after radiation or LY294002 treatment, and subjected to TUNEL flow cytometric analysis as described in "Materials and Methods."

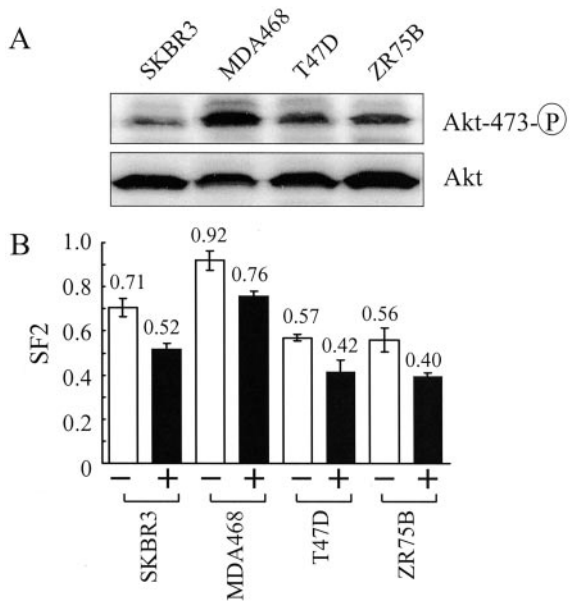

Fig. 7. Expression and phosphorylation levels of Akt in a panel of human breast cancer cell lines and the effect of the PI-3K inhibitor LY294002 on the clonogenic survival of the cell lines after radiation. In *A*, equal amounts of protein lysates from a panel of the four indicated breast cancer cell lines were examined for total and phosphorylated levels of Akt by Western blot analysis with appropriate antibodies. In *B*, SF2 (Survival fraction at 2 Gy) of the four breast cancer cell lines with or without 5-μM LY294002 postradiation treatment are shown. The SF2 with or without LY294002 treatment was determined by normalizing the colony efficiencies to their respective control values (DMSO vehicle control or LY294002 alone) as described in "Materials and Methods."

Discussion

Earlier work has shown that the PI-3K/Akt pathway plays a central role in conferring on cancer cells, resistance to various cytotoxic and therapeutic agents (36). Several recent studies have also shown a potential role of the PI-3K/Akt pathway in mediating radioresistance in cancer cells (37–39). Most of the studies to date, however, have focused on a higher level of the pathway. The novel observation reported in our present studies is that we dissociated Ras activated-Akt from the constitutively activated Akt, with reference to their differential response to the intervention by PI-3K-specific inhibitors, and, thus, demonstrated a definitive role of Akt in altering the sensitivity of breast cancer cells to radiotherapy.

The results shown in Fig. 7 indicate that there might be other molecular targets in addition to the PI-3K/Akt that LY294002 sensitizes to radiation. In fact, the radiosensitization by LY294002 or another PI-3K inhibitor, wortmannin, was attributed to the inhibition of some members of the PIKK family such as DNA-PK (12–14) and ATM (11–14), which are well known to regulate cellular radiosensitivity. In the present study, we demonstrated that this LY294002-induced radiosensitization may also involve Akt, one of the best-characterized downstream targets of the phospholipids generated by PI-3K after its activation. Using MCF7 transfectants expressing constitutively active Ras or constitutively active Akt, we demonstrated that both types of the transfectants exhibited increased resistance to ionizing radiation, with the MCF7RasG12V clones being PI-3K dependent and MCF7Akt1-farn being PI-3K independent. Our data indicate that Akt plays a causal role in conferring cellular resistance to radiotherapy, which is mediated primarily by inhibiting ionizing radiation-induced apoptosis. Akt may be one of major downstream mediators of the Ras-mediated radioresistance. Our observation supports a recent study demonstrating the important role of PI-3K in mediating Ras-induced radioresistance (16) and justifies Akt as a potential target for specific radiosensitization of human cancers.

Recent studies have clearly demonstrated that the activity of Akt is critical for providing cell-survival signals triggered by growth factors, extracellular matrix, and other stimuli (22). Although there has been a rapid expansion in the number of identified physiological Akt substrates that are involved in various aspects of cellular function, there are clearly candidates that are directly involved in the regulation of apoptosis (40). Akt can suppress apoptosis by directly interacting with and phosphorylating these proapoptotic proteins. For example, Akt phosphorylates the proapoptotic Bcl-2 partner Bad, thereby preventing Bad from binding to and blocking the activity of Bcl-x, a cell survival factor (41). Akt phosphorylates and inactivates caspase-9, an important initiation caspase of the mitochondria pathway-mediated apoptosis (42). Akt is also involved in transcriptional regulation of proapoptotic and antiapoptotic genes. Akt can phosphorylate

the forkhead family transcription factors (FKHR, FKHRL-1, and AFX; Refs. 43–45), resulting in reduced expression of proapoptotic genes such as the apoptosis-inducing Fas ligand (43) and the Bcl-2 interacting mediator of cell death (Bim; Ref. 46). Akt may also phosphorylate IκK (47) and cyclic AMP-response element binding protein (CREB; Ref. 48). IκK promotes degradation of IκB and thereby increases the activity of NFκB (49), a well-known cell survival factor that activates prosurvival genes such as inhibitor of apoptosis-1 (IAP-1) and IAP-2, whereas CREB has been shown to activate Bcl-2 promoter. Thus, it would be interesting to determine whether some or all of these Akt substrates are involved in the radioresistance conferred by Akt.

Another potential linker between the Ras/Akt signaling and radiation resistance is COX-2. We have previously demonstrated that treatment of tumor cells with a selective inhibitor of COX-2 greatly enhanced the tumor radioresponse (50, 51). It has been shown that the expression of COX-2 is regulated via the Ras signaling pathway. Induction of a mutated Ras increased the levels of COX-2 in certain intestinal epithelial cells (52, 53). Akt is involved in Ras-induced expression of COX-2 and the stabilization of COX-2 mRNA in some types of cell lines (54). The results of our present study raise an interesting question as to whether COX-2 plays a role in Ras- and/or Akt-mediated radiation resistance. We are currently investigating this possibility.

Other potential downstream molecules involved in PI-3K/Akt-mediated resistance to ionizing radiation could be the candidates that activate DNA repair. There was an enhanced activity of DNA polymerase-β, one of the repair enzymes, concomitant to the activation of the PI-3K/Akt pathway, that delivered a survival signal in Friend erythroleukemia cells exposed to 15 Gy of radiation (55). In that study, preincubation of the cells with the PI-3 kinase inhibitor wortmannin or LY294002 inhibited the activation of the DNA polymerase-β and sensitized the cells to ionizing radiation. However, the inhibition of repair from radiation damage is less likely to be a significant mechanism of the LY294002-induced cellular radiosensitization in our study, because the degree of sensitization was greater when LY294002 was given 24 h after radiation rather than 1 h before radiation delivery. *In vitro* repair from radiation damage in mammalian cells is generally considered to be complete within 4 h after radiation (56).

In summary, our results indicate that the activity of Akt is one of the determinants that play a causal role in the sensitivity to radiation. This increased cell survival of cell clones with a high level of Akt is associated with decreased radiation-induced apoptosis in the cells, and, thus, Akt may be a rational target to improve the response of cancer cells to radiotherapy. A future direction of our studies is to extend our work to animal models. The observation that Akt is a critical player in the PI-3K-mediated radioresistance may support a novel molecular-targeted approach for sensitizing human cancer cells to radiotherapy with novel inhibitors that specifically inhibit Akt.

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Molecular Cancer Therapeutics

Targeting the Phosphatidylinositol 3-Kinase/Akt Pathway for Enhancing Breast Cancer Cells to Radiotherapy [1]

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