Serine/Threonine Kinase AKT Is Frequently Activated in Human Bile Duct Cancer and Is Associated with Increased Radioresistance

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ABSTRACT

The prognosis for patients with bile duct cancer (BDC) remains poor. Although BDC cells are essentially radioresistant, recent reports have suggested that radiation therapy, in addition to its palliative role in the management of BDC, may improve patient survival. A better understanding of the mechanisms that lead to cellular radioresistance may assist in the development of more effective BDC therapies based on radiotherapy in combination with radiosensitizing agents. The serine/threonine kinase AKT/protein kinase B, a downstream effector of phosphatidylinositol 3'-kinase, is a well-characterized kinase that is known to play a critical role in antiapoptotic signaling pathways. In this investigation, we sought to clarify the role of AKT signaling in the radioresistance in BDC cells. First, to examine whether activated AKT is expressed in BDCs, tumor specimens were obtained from 19 consecutive BDC cases. Immunohistochemical staining using an anti-phosphorylated-AKT antibody showed that phosphorylated (activated) AKT was expressed in cancer cells but not in neighboring normal mucosa in 16 cases (84.2%). Next, to evaluate the role of AKT activation in the regulation of BDC cell radiosensitivity, clonogenic assays were performed using the phosphatidylinositol 3'-kinase inhibitor LY294002 with and without irradiation. LY294002 inhibited AKT activation in BDC cells and, on irradiation, decreased clonogenic survival in a radiation dose-dependent manner. Only a small decrease in cell viability was observed in cells exposed to LY294002. Expression of constitutively active AKT in BDC cells resulted in decreased radiosensitivity, whereas a dominant-negative AKT increased radiosensitivity. Furthermore, constitutively active AKT also inhibited radiation-induced apoptosis. Collectively, these results indicate that activated AKT in BDC cells is associated with radioresistance and suggest that pharmacological or genetic modulation of AKT activity may have important therapeutic implications in BDC patients treated with radiation.

INTRODUCTION

Despite recent advances in diagnosis and treatment, the prognosis of patients with bile duct cancer (BDC) remains poor. Surgical resection is possible in only a small proportion of patients (1, 2). In addition, the majority of BDC patients are >65 years of age, with the peak incidence occurring in the eighth decade of life (3). For these reasons, the use of radiotherapy has been advocated in the management of unresectable BDC. However, there are conflicting reports regarding the efficacy of radiotherapy in progressive cancers (4–6). Furthermore, BDC is most commonly an adenocarcinoma and is resistant to radiation therapy, and BDC cells are reportedly radioresistant in vitro (7). The molecular genetic factors that control the intrinsic cellular radiosensitivity of this tumor type are poorly understood.

AKT, also known as protein kinase B, is probably the best characterized kinase known to promote cellular survival. AKT is activated via phosphatidylinositol 3'-kinase (PI3k) by various extracellular stimuli that activate receptor tyrosine kinases. Activated AKT phosphorylates multiple downstream target proteins, including Bad, caspase-9, forkhead transcription factors, and inhibitor of nuclear factor κB kinase, and results in enhanced cell survival (8, 9). There is accumulating evidence that AKT is frequently overexpressed and overactivated in a variety of human tumors, including tumors of the ovary (10, 11), pancreas (12, 13), thyroid (14), prostate (15), breast (16), and myeloma cells (17). Although activated AKT appears to be involved in the growth of human malignant tumors, the activation status of AKT in BDC has not been reported to date.

Constitutively active AKT or PI3k has been shown to enhance protection against apoptotic-inducing insults, such as growth factor deprivation or UV irradiation (18, 19). Recent studies have shown that activated AKT signaling may enhance the radioresistance of cell lines derived from tumors of the breast, prostate, and head and neck (20–23). It has been reported also that the immunohistochemical evaluation of AKT phosphorylation status may be a prognostic marker for response to radiation therapy in head and neck cancer (24). Thus, the elevated AKT activity identified in human tumors may link not only to apoptosis suppression but also to sensitivity to radiation therapy.

In the present study, we examined whether activated AKT mediates radioresistance in human BDC cells. We also determined the expression status of phosphorylated (phospho) AKT in BDC by immunohistochemistry. Our data indicate that pharmacological or genetic modulation of AKT activity may have important therapeutic implications in BDC patients treated with radiation.

MATERIALS AND METHODS

Antibodies. Antibodies against AKT and phospho-AKT (Ser473 and Thr308) for Western blotting were purchased from New England Biolabs (Beverly, MA). Anti-HER2 antibody (HA) was obtained from Babco (Richmond, CA). For immunohistochemistry experiments, anti-phospho-AKT (Ser473) antibody was used on formalin-fixed, paraffin-embedded tissues. The selective PI3k inhibitor LY294002 was obtained from Sigma (Tokyo, Japan).

Tissue Samples and Immunohistochemical Analysis. Nineteen BDC tissues obtained by surgical resection were evaluated. None of the BDC patients had received radiation therapy before surgery. Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated through a graded alcohol series to distilled water. Sections were subjected to antigen retrieval by a domestic microwave oven in citrate buffer (pH 6.0) for 20 min. Immunohistochemical staining was performed using a deaminobenzadine-based detection method. Phosphospecific anti-AKT antibody, which recognizes phosphorylated (active) AKT, was used as the primary antibody. Negative controls consisted of omitting the primary anti-AKT antibody and were uniformly negative for immunostaining. Results of phospho-AKT immunostaining were divided into two groups according to staining intensity: the positive group was defined as having increased staining intensity compared with the corresponding normal tissues; and the negative group was defined as having no increase in staining over normal tissues. Immunohistochemical staining results were obtained by counting tumor cells from three different areas from each BDC patient sample.

Cell Culture. The human BDC cell lines HuCC-T1 and TKF-1 were provided by the RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air in DMEM (Life Technologies, Inc., Tokyo, Japan) supplemented with 10% fetal bovine serum. Both cell lines have mutant p53 (25).

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Expression Vector Construction. cDNA for a constitutively active form of HA epitope-tagged AKT (CA-AKT) was generated by adding a NH2-terminal myristoylation signal as described previously (26). cDNA for a dominant-negative form of HA epitope-tagged AKT (DN-AKT) was produced by converting the Lys179 codon to a Met codon. These cDNAs were subcloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Myr-HA-enhanced green fluorescent protein (EGFP)-AKT was also generated as described previously (26).

Transient Transfections and Detection of Apoptosis. HuCCT-1 and TFK-1 cell cultures at 70% confluency were transiently transfected with CA-AKT or DN-AKT expression plasmids using GenePorter2 (Gene Therapy System). Expression of the AKT plasmid was monitored by measuring the cellular levels of HA-tag and AKT proteins by Western blotting. At least 40% of the cells were transfected in all of the experiments. The relative number of transfected cells was determined by counting the number of EGFP-positive cells and assigning this number as a value of 1.0 for each experiment. Twenty random fields were counted for each assay. Each determination represents the average of three separate experiments. Apoptosis was evaluated by 4',6-diamidino-2-phenylindol (1 μg/ml) and analyzed by fluorescence microscopy to assess chromatin condensation.

Single-Dose Radiation Experiments and Clonogenic Survival Assays. Exponentially growing cholangiocarcinoma cells in monolayer culture were irradiated in 100-mm Petri dishes using a 137Cs GammaCell Irradiator (Atomic Energy of Canada, Ltd., Mississauga, Ontario, Canada) by single radiation exposure.

After exposure to ionizing irradiation, the cells were harvested for Western blot analysis and replated for clonogenic survival analysis. Survival after radiation exposure was defined as the ability of the cells to maintain their clonogenic capacity and to form colonies. Briefly, after exposure to radiation, cells were trypsinized, counted, and seeded for colony formation in 100-mm Petri dishes at 500-1000 cells/dish. After incubation intervals of 14–21 days, colonies were stained with crystal violet and counted manually. Colonies consisting of >50 cells were scored, and three to five replicate dishes containing 10–150 colonies/dish were counted for each treatment.

Protein Extraction and Western Blot Analysis. After treatment, cells were lysed with Tween 20 lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Tween 20, 10% glycerol, 2.5 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride, 25 μg/ml leupeptin, and 25 μg/ml aprotinin). Equal amounts of protein were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and incubated with specific primary antibodies against pan-AKT or phospho-AKT (Ser 473 and Thr308). Proteins were detected via incubation with horseradish peroxidase-conjugated secondary antibodies and visualized using the enhanced chemiluminescence detection system (NEN Life Science, Boston, MA).

RESULTS

Immunohistochemical Analysis of Phospho-AKT Expression in Human BDC. To elucidate the expression status and localization of phospho-AKT in BDC cells, we performed immunohistochemical analysis, using a phospho-AKT (Ser473) antibody, on 19 resected BDC samples with paired normal bile duct tissues used as controls. Fig. 1 shows a representative immunohistochemical staining of normal bile duct epithelium. Phospho-AKT staining was faintly detected in the nuclei and cytoplasm of nontumoral epithelial cells (Fig. 1A). In contrast, intense nuclear and cytoplasmic staining was observed in tumor cells (Fig. 1B), with strong phospho-AKT immunostaining observed in tumor cells invading along the nerves (Fig. 1, C and D). Fig. 1E shows a representative negative control in which the primary anti-AKT antibody was omitted. Negative controls were uniformly negative for immunostaining. Overall, 16 of 19 (84.2%) tumor specimens exhibited phospho-AKT staining. Statistical analysis showed that positive phospho-AKT staining did not correlate with any BDC pathological (histological differentiation, vascular invasion, or tumor depth) or clinical (tumor stage and patient age) features tested (data not shown).

Ionizing Radiation-Induced Phosphorylation of AKT. Although the AKT pathway has been shown to enhance survival after a variety of cellular stresses, the nature of its response to ionizing radiation has not been established in BDC cells. To investigate whether ionizing radiation affected the level of AKT phosphorylation, we examined the phosphorylation status of irradiated BDC cells by Western blotting.

Fig. 1. Immunohistochemical analysis of phosphorylated (phospho)-AKT expression in human bile duct cancer using an antibody specific for phospho-AKT (S473). A, representative immunohistochemical staining of normal bile duct epithelium (×400). B, intense nuclear and cytoplasmic staining observed in tumor cells (×40). C and D, tumor cells invading along nerves showing strong immunostaining for phospho-AKT (×100 and ×200). E, representative negative control in which the primary anti-AKT antibody was omitted. Negative controls were uniformly negative for immunostaining (×200).
AKT phosphorylation was apparent in TFK-1 and HuCC-T1 cells under basal conditions after serum deprivation, indicating that both cell lines have constitutive AKT activity. After exposure to 1 h of ionizing radiation, phospho-AKT levels were markedly elevated without changes in AKT protein levels in TFK-1 and HuCC-T1 cells (Fig. 2A). Phosphorylated patterns for phospho-AKT T308 correlated with the S473 pattern. No additional effect on phosphorylation level was observed in cells irradiated with doses >30 Gy. Then, we determined the effect of PI3k signaling inhibition on radiation-induced AKT phosphorylation. Pretreatment with LY294002, a chemical inhibitor of PI3K, abrogated irradiation-induced AKT phosphorylation in both TFK-1 and HuCC-T1 cells, consistent with the fact that both cell lines have constitutively active AKT (Fig. 2B). This result suggested that radiation-induced AKT phosphorylation in BDC cells is mediated via PI3k signaling. We confirmed the serial changes in AKT phosphorylation level using cells pretreated with LY294002 and then grown in the presence of serum after removal of LY294002. Cells pretreated with LY294002 showed reduced phospho-AKT levels at 0.5 h after treatment, with levels increasing later with the addition of serum, which indicated the reversible nature of AKT phosphorylation in the BDC cell lines (data not shown).

Clonogenic Survival Assays of LY294002-Treated BDC Cells. We performed colony formation assays in the presence of LY294002 with or without irradiation to assess the inhibitory effect of PI3k activity on cell survival. After serum deprivation, cells were pretreated with LY294002 for 2 h in 0.1% of serum-containing medium and then irradiated with doses of 0–5 Gy. After 2 h, medium was changed to remove LY294002. Treatment with LY294002 decreased clonogenic cell survival after irradiation for both cell lines, whereas no obvious toxicity was observed in nonirradiated cells (Fig. 3). It is noteworthy that both cell lines showed similar radiosensitization to LY294002, although HuCC-T1 cells had higher basal levels of AKT activity than TFK-1 cells. These results suggest that a threshold level of AKT phosphorylation, present in both cell lines, influences the subsequent sensitivity to radiation and that a transient decrease in phosphorylation-mediated endogenous AKT/PI3k signaling may be sufficient to enhance cellular radiosensitivity.

Effect of Dominant-Negative or Constitutively Active AKT on Radiation Sensitivity. In addition to abolishing PI3k activity, LY294002 has also been reported to inhibit DNA-dependent protein kinase and the ataxia telangiectasia mutated protein at higher doses (27, 28). To examine the precise role of phospho-AKT in radiosensitivity, we transfected the BDC cell lines with expression plasmids encoding DN- or CA-AKT. To assess transfection efficiency, expression levels of HA-tagged proteins were monitored by Western blotting or immunocytochemistry. Although survival fraction was influenced by the presence of untransfected cells, at least 40% of cells were transfected in all of the experiments. As shown in Fig. 4A, cells transiently transfected with CA-AKT showed increased expression of total and phospho-AKT compared with cells transfected with empty vector. In contrast, overexpression of DN-AKT decreased AKT phosphorylation levels of AKT but increased total AKT expression compared with empty vector. After transfection with the DN-AKT expression vector, BDC cells were irradiated with a dose of 0–5 Gy. As shown in Fig. 4B, DN-AKT expression resulted in decreased colony formation in both TFK-1 and HuCC-T1 cells, indicating enhanced radiosensitivity. In contrast, CA-AKT markedly decreased radiosensitivity.

To assess the effect of activated AKT on irradiated cell apoptosis, BDC cells were transiently transfected with an EGFP-containing vector encoding the myr-HA-AKT construct (CA-EGFP-AKT). BDC cells transfected with the EGFP-containing vector alone were used as a control. At 24 h after irradiation, 4′,6-diamidino-2-phenylindole nuclear staining was performed to confirm the apoptotic changes shown by the characteristic shrunken nucleus and apoptotic bodies. Transfected cells were easily distinguished from untransfected cells based on EGFP expression using a fluorescence microscope (Fig. 5A). Consistently, >15% of cells transfected with EGFP construct underwent apoptosis at 24 h after irradiation. As shown in Fig. 5B, the relative cell death of cells transfected with the EGFP construct was higher in irradiated cells than in nonirradiated cells. When the experiment was repeated using the CA-EGFP-AKT construct, transfected BDC cells showed a lower rate of cell death than cells transfected with

Fig. 2. Ionizing radiation-induced phosphorylation of AKT. A, at 1 h after irradiation, phosphorylated-AKT levels were markedly elevated in TFK-1 and HuCC-T1 cells. The endogenous phosphorylation of AKT was evaluated by monitoring the presence of phosphorylated-AKT using a specific antibody pS473 and pT308, respectively. B, the effect of inhibition of phosphatidylinositol 3′-kinase signaling on radiation-induced AKT phosphorylation.

Fig. 3. Clonogenic survival assays of LY294002-treated bile duct cancer cells. After serum deprivation, cells were pretreated with LY294002 for 2 h in 0.1% of serum-containing medium and irradiated. Medium was changed to remove LY294002 at 2 h after irradiation. The control (○) and the LY294002-treated (●) curve in A, TFK-1 cells; and B, HuCC-T1 cells. Bars, ±SD.
EGFP. These data demonstrate that AKT activation is sufficient to enhance BDC cell radioresistance.

**DISCUSSION**

There are several hypotheses as to why irradiated cell survival is increased in human tumor cell lines expressing mutant p53 (29–31). p53 status has been shown to correlate with radiosensitivity in human diploid fibroblasts and mouse hematopoietic cells, such that cells with wild-type p53 expression were more radiosensitive than cells with abnormal p53 expression. Wild-type p53 plays a central role in cell cycle regulation, presumably by specifically inhibiting cells from entering S phase before repair of DNA damage during the apoptotic response to radiation (32, 33). However, some investigators have shown evidence contrary to this hypothesis (34, 35). Furthermore, Moon et al. (7) reported that radioresistance of biliary cancer cells was similar to that of glioblastoma and melanoma cell lines, and did not correlate with p53 status. Because both BDC cell lines used in this study were negative for p53 tumor suppressor protein function (25), our data indicated that AKT status appeared to be a critical factor in radioresistance, at least in BDC cells lacking functional p53.

Activated AKT phosphorylates proteins that play significant roles in the control of apoptosis, cell growth, glucose uptake and utilization, and protein synthesis (9, 36). These proteins include Bad, a proapoptotic bcl-2 family member, which when phosphorylated by AKT releases Bcl-2, allowing it to perform its antiapoptotic function. Caspase-9, another effector of the intrinsic cell-death pathway, can also be activated after phosphorylation by AKT. The transcription factor FKHRL1 regulates the expression of genes encoding proapoptotic proteins, such as Fas-ligand, but is translocated to the cytoplasm after phosphorylation by AKT. AKT has been demonstrated also to inhibit UV-induced cell death by preventing the release of cytochrome c from mitochondria (37). In addition, it has been reported recently that AKT activation can overcome both the p53-independent G2-M cell-cycle checkpoint and apoptosis induced by γ irradiation (38). AKT has been reported also to play a role in PI3k-induced cyclin D1 expression through the activation of inhibitor of nuclear factor κB kinase complex α (39). Thus, it appears that AKT activation may have effects similar to p53 inactivation, such that it abolishes cell-cycle checkpoints and inhibits apoptosis. In fact, AKT has been shown to promote translocation of MDM2 to the nucleus, which leads to reduction of both p53 levels and transactivation (40).

The results shown in Fig. 2A indicate that ionizing radiation induces AKT phosphorylation without changes in AKT protein levels in BDC cells. A recent study has shown that ionizing radiation in the therapeutic dose range induces a reversible mitochondrial permeability transition and stimulates a transient cellular generation of reactive oxygen species (ROS; Ref. 41). Most of the experimental data support the role of ROS as second messengers in signal transduction, and it
has been reported that ROS can activate AKT (42). Although we did not examine the intracellular ROS levels in irradiated cells, AKT activation may be mediated by the ROS induction in irradiated BDC cells. For example, UV irradiation has been shown previously to induce phosphorylation and activation of AKT through the generation of ROS (43). Considering that ionizing radiation induces AKT phosphorylation in BDC cells, it is conceivable that AKT participates in a feedback loop whereby activation of AKT induced by ionizing radiation further increases the radiosensitivity of BDC cells. Previous studies have shown that the PI3K inhibitors wortmannin or LY294002 can radiosensitize other types of cancer cells (20, 21, 23). A recent study demonstrated that expression of constitutively active PI3k in cells resulted in increased radioresistance (44). In our study, clonogenic assays using a PI3k inhibitor or DN-AKT show that the level of AKT activation at the time of irradiation influences subsequent sensitivity to radiation and that a transient decrease in phosphorylation-mediated endogenous PI3k/AKT signaling may be sufficient to enhance cellular radioresistance. In addition, we demonstrated that CA-AKT markedly decreased BDC cell radioresistance. Thus, AKT is a potential target for enhancing response to radiotherapy in BDC patients.

Immunohistochemical data using an antibody specific for phospho-AKT showed nuclear and cytoplasmic staining of tumor cells. Intense staining was also observed in tumor cells migrating toward nerves (Fig. 1C). Perineural invasion is observed frequently in BDC (45), and the perineural space is thought to provide a suitable environment for tumor cell growth and chemotaxis through the release of soluble mitogenic and chemotactic factors (46). Intriguingly, interaction between BDC cells and peripheral neural tissues may increase AKT phosphorylation levels in a paracrine fashion, and perineural invasion is reported to be closely associated with BDC patient prognosis (45).

In conclusion, our findings indicate that AKT activation may have a critical role in BDC cell radiosensitivity. Selective inhibitors that specifically target AKT signaling may have important therapeutic implications when used in combination with radiation in the treatment of BDC patients.

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