Inhibition of Mammalian Target of Rapamycin or Apoptotic Pathway Induces Autophagy and Radiosensitizes PTEN Null Prostate Cancer Cells

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Abstract

The phosphatidylinositol 3-kinase/Akt pathway plays a critical role in oncogenesis, and dysregulation of this pathway through loss of PTEN suppression is a particularly common phenomenon in aggressive prostate cancers. The mammalian target of rapamycin (mTOR) is a downstream signaling kinase in this pathway, exerting prosurvival influence on cells through the activation of factors involved in protein synthesis. The mTOR inhibitor rapamycin and its derivatives are cytotoxic to a number of cell lines. Recently, mTOR inhibition has also been shown to radiosensitize endothelial and breast cancer cells in vitro. Because radiation is an important modality in the treatment of prostate cancer, we tested the ability of the mTOR inhibitor RAD001 (everolimus) to enhance the cytotoxic effects of radiation on two prostate cancer cell lines, PC-3 and DU145. We found that both cell lines became more vulnerable to irradiation after treatment with RAD001, with the PTEN-deficient PC-3 cell line showing the greater sensitivity. This increased susceptibility to radiation is associated with induction of autophagy. Furthermore, we show that blocking apoptosis with caspase inhibition and Bax/Bak small interfering RNA in these cell lines enhances radiation-induced mortality and induces autophagy. Together, these data highlight the emerging importance of mTOR as a molecular target for therapeutic intervention, and lend support to the idea that nonapoptotic modes of cell death may play a crucial role in improving tumor cell kill.

Introduction

Prostate cancer is the most common cancer in men. Radiotherapy is a mainstay in managing early-stage or inoperable locally advanced disease. Recent innovations in three-dimensional conformal and intensity-modulated radiation therapy have allowed for maximal delivery of radiation while limiting toxicity (1, 2). Finding agents that sensitize malignant cells to radiation would increase tumor response while minimizing toxicity to surrounding organs by lowering effective therapeutic doses.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway promotes normal cell growth and proliferation (3), and its constitutive activation has been implicated in many human cancers, including the pancreas, ovary, and stomach (4, 5). Although direct amplification of PI3K/Akt has not been found in prostate cancer (5), mutations in the suppressor of this pathway, PTEN, have been described with high frequency, especially in more advanced and aggressive neoplasms (5, 6).

The mammalian target of rapamycin (mTOR), also known as RAFT1, RAPT1, and FRAP, is a 289 kDa serine-threonine kinase downstream of Akt (7). mTOR phosphorylates and inactivates the translation suppressor eukaryotic initiation factor 4E-binding protein 1 (4E-BP1, PHAS1), and also activates ribosomal p70 S6 kinase (S6K1). Principally, through these two factors, the normal activation of mTOR results in an increase in global protein synthesis (8, 9). Recently, it has been suggested that dysregulation of mTOR contributes to oncogenesis in a broad range of cancers (10). Although precise mechanisms are unknown, mTOR-mediated alterations in protein synthesis, aberrant cell cycle signaling, and inhibition of apoptosis may all play causal roles (11, 12). The ability of mTOR inhibitors to attenuate progrowth, proliferative, and prosurvival actions of mTOR has therefore generated much interest (10).

mTOR presents an attractive therapeutic target in the PI3K/Akt pathway because it acts downstream of broader function upstream proteins (13). PI3K inhibitors, such as wortmannin and LY294002, have limited clinical potential due to lack of kinase specificity and metabolic instability (14). However, when the immunosuppressant rapamycin binds its intracellular target FKBP12, this complex specifically blocks mTOR-mediated phosphorylation of S6K1 and 4E-BP1, providing more effective pathway targeting (9). In vitro studies have established the ability of rapamycin to inhibit cellular transformation, and potent rapamycin derivatives, such as CCI-779, AP 23573, and RAD001, have been developed for use in cancer therapy (for review, see ref. 15). Transgenic mice with activation of Akt or deficiencies in PTEN have shown sensitivity to these agents (16), and RAD001 specifically has been shown to reverse prostate neoplastic phenotypes in mice expressing human Akt (17). Although mTOR inhibitors have been shown to increase the radiosensitivity of endothelial and breast cancer cells (18–20), their potential radiosensitizing effects have not been specifically examined in prostate cancer cells. In this study, we examined the combined effects of RAD001 and irradiation on cell survival in both PTEN−/− PC-3 and PTEN+/+ DU145 cell lines. Because we found that mTOR inhibition confers radiosensitivity and induces the nonapoptotic cell death pathway of autophagy, we also examined induction of autophagy with apoptosis inhibitors as a way to enhance radiation cytotoxicity. Because solid tumors are only variably sensitive to apoptotic death, our results support the notion that alternatives to apoptosis, such as autophagy, may be exploited to improve tumor cell killing (21).
Materials and Methods

Cell culture and drug treatment. PC-3 and DU145 prostate carcinoma cells (American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were incubated at 37°C and humidified 5% CO₂. Irradiation was given by use of a 137Cs irradiator (J.L. Shepherd and Associates, Glendale, CA). RAD001 was obtained from Novartis Pharmaceutical (East Hanover, NJ), Z-VAD from Axxora (San Diego, CA), and Bak small interfering RNA (siRNA) from Santa Cruz Biotechnologies (Santa Cruz, CA).

In vitro clonogenic assay. PC-3 and DU145 cells were treated with RAD001 (10 nmol/L, 1 hour), Z-VAD (10 μmol/L, overnight), or DMSO control. Transfection with siRNA Bak (20 nmol/L) and siRNA Bax (20 nmol/L) or siRNA ATG5 (20 nmol/L) and siRNA Beclin-1 (20 nmol/L) or M5 control (40 nmol/L) was carried out by use of a mixture of 20 μL LipofectAMINE 2000/mL (Life Technologies, Gaithersburg, MD). Cells were irradiated with 0 to 6 Gy as indicated, at a dose rate of 1.8 Gy/min. After irradiation, the medium was changed, and cells were incubated at 37°C for 8 days. Cells were then fixed for 15 minutes with 3:1 methanol/acetic acid and stained for 15 minutes with 0.5% crystal violet (Sigma). Cells were counted with a dissecting microscope. Colonies were identified as discrete groups of cells of more than 50 cells, and surviving fraction was calculated as the mean colonies counted / [(cells plated) × (plating efficiency)], where plating efficiency was defined as (mean colonies counted) / (cells plated) for nonirradiated controls. Experiments were conducted in triplicate with mean, SD, and P values (Student’s t test) calculated.

Western immunoblots. Cells were treated (RAD001 50 nmol/L, 1 hour) or irradiated (5 Gy) according to the individual study. Two hours postirradiation, cells were washed with ice-cold PBS twice before the addition of lysis buffer. Equal amounts of protein were loaded into each well and separated by 6.5%, 10%, or 15% SDS-PAGE gel, followed by transfer onto nitrocellulose membranes. Membranes were blocked by use of 5% nonfat dry milk or 1% bovine serum albumin in TBS-T buffer for 1 hour at room temperature. The blot was then incubated overnight at 4°C with rabbit anti-phospho-mTOR, total mTOR, phospho-AKT, total AKT, phospho-S6, and total S6 antibodies, all at 1:1,000 and purchased from Cell Signaling Technology (Beverly, MA); rabbit anti-p-actin antibody (1:2,000) was purchased from Sigma-Aldrich, St. Louis, MO in methanol. After staining, colonies were visually counted with a cutoff of 50 viable cells. Surviving fraction was calculated as (mean colonies counted) / [(cells plated) × (plating efficiency)], where plating efficiency was defined as (mean colonies counted) / (cells plated) for nonirradiated controls. Experiments were conducted in triplicate with mean, SD, and P values (Student’s t test) calculated.

Measurement of apoptosis. Annexin V-FITC is a sensitive probe for identifying apoptotic cells. Propidium iodide is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Apoptosis was measured using Annexin V-FITC Apoptosis Detection kit 1 (BD Pharmingen, San Diego, CA) with flow cytometry. PC3 and DU145 cells were plated into 100 mm dishes for each data point. After 24 hours of 37°C incubation, the cells were treated with RAD001 for 1 hour. Cells were then irradiated with 3 Gy as indicated. Medium was then changed and cells were further incubated for 24 hours at 37°C. Cells were then trypsinized (keeping all floating cells) and counted. Cells were washed twice with cold PBS and then resuspended in 1 × binding buffer. One hundred microliters of the solution (5 × 10⁶ cells) were transferred to a culture tube to which 3 μL Annexin V-FITC and 3 μL propidium iodide were added. After 15 minutes of incubation at room temperature in the dark, 400 μL of 1 × binding buffer was added to each tube and samples were analyzed by FACScan.

Results

Radiosensitization of prostate cancer cells by RAD001. To determine whether the mTOR inhibitor RAD001 radiosensitizes prostate cancer cells, we used clonogenic assays to assess cell survival. First, we examined drug treatment alone and found that 10 nmol/L RAD001 decreased cell survival in both PC-3 cells and DU145 cells compared with controls (Fig. 1A). PC-3 cells were more sensitive to mTOR inhibition, as the survival of treated PC-3 cells was 63% that of untreated cells. The survival of treated DU145 cells was 76% that for untreated cells, suggesting a slight relative resistance of DU145 to drug treatment compared with PC-3 (Fig. 1A).

Based on a previously developed protocol, cells were treated with RAD001 (10 nmol/L) or DMSO control for 1 hour before irradiation (18). One hour provides sufficient time for mTOR inhibition to occur without producing significant cytotoxicity, ensuring that any changes in cell survival are attributable to RAD001-induced radiosensitivity (20). Clonogenic assays showed decreased survival in both cell lines (Fig. 1B). Importantly, RAD001 exerts a more potent radiosensitizing effect on PC-3 cells compared with DU145 cells, as evidenced by the dose-enhancing ratio (DER) of 1.2 seen in treated PC-3 cells, compared with 1.02 in DU145 cells (Fig. 1B). These results suggest that mTOR inhibition with RAD001 enhanced the radiosensitivity of PC-3 cells by ~20%, 10 times higher than that attained in DU145 cells.

Effect of radiation and RAD001 on mTOR signaling in prostate cancer cells. To establish the efficacy of RAD001 in inhibiting mTOR signaling and examine the effects of radiation and RAD001 combination on mTOR signaling, PC-3 and DU145 cells were treated with 10 nmol/L RAD001 alone, 5 Gy alone, or in combination. Levels of p-Akt, p-mTOR, and p-S6 (mTOR downstream target) were measured with Western immunoblots 2 hours after treatment (Fig. 2). As expected, PTEN-competent DU145 cells expressed lower levels of p-Akt compared with PC-3 cells at baseline. Additionally, radiation alone did not increase levels of p-Akt, p-mTOR, or p-S6. Previous experiments showed that neither cell line exhibited appreciable postirradiation induction of these proteins at 15 minutes, 30 minutes, or 1 hour postirradiation (data not shown).

Our results showed dramatic attenuation of p-S6 expression after RAD001 and radiation (Fig. 2), confirming the ability of the drug to block mTOR signaling. Although low levels of baseline p-S6 were detected in DU145 cells despite the presence of p-mTOR, RAD001 completely eliminated this limited expression of p-S6. This indicates total blockade of downstream mTOR signaling in these cells (Fig. 2). Combination treatment with both RAD001 and radiation also resulted in the inhibition of phosphorylation of S6, demonstrating successful attenuation of mTOR activity in both cell lines.
Radiation induces cell death in prostate cancer cells treated with RAD001. To elucidate the mechanism responsible for the RAD001-induced radiosensitization of PC-3 cells, flow cytometry was used to determine the relative contribution of apoptosis to increased cell death following irradiation. Figure 3A shows that in PC3 cells, apoptosis was not up-regulated in response to mTOR inhibition alone. Additionally, adding RAD001 to irradiated cells only mildly increased apoptosis above the levels achieved by 3 Gy alone. Similarly, in DU145 cells, mTOR inhibition alone did not increase levels of apoptosis, but combination treatment with radiation resulted in higher levels of apoptosis than that of radiation alone (Fig. 3A). Importantly, the basal level of apoptosis observed in untreated cells was demonstrably higher in the DU145 cell line, reinforcing the assumption that unchecked PI3K/Akt signaling promotes survival in PTEN−/− PC-3 cells.

Indirect immunofluorescence with cotransfected GFP-LC3 plasmid was used in PC-3 cells to investigate whether autophagy was induced as an alternative cell death pathway. Microtubule-associated protein-1 LC3 is an important component of mammalian autophagosomes, and thus the GFP-LC3 fusion protein has been used as a reliable marker for their presence (19, 22). In PC-3 cells, the subcellular localization of LC3 changed in response to radiation from a diffuse scattering throughout the cytosol to a characteristic punctate pattern indicative of autophagosome formation (Fig. 3B). As shown in Fig. 3C, quantitative analysis of this effect revealed that RAD001 alone fails to enhance this process above baseline, although when combined with radiation it produces a synergistic increase, with nearly 60% of transfected cells displaying the signature fluorescence pattern of autophagy. DU145 cells did show a quantitative increase in autophagosome formation following treatment with RAD001 alone, as well as with radiation alone. Furthermore, combining RAD001 with radiation resulted in the highest level of autophagy.

Blocking apoptosis promotes autophagy and enhances radiosensitivity in PC3 and DU145 cells. Our unpublished data suggested that by inhibiting apoptosis, cells can be radiosensitized through alternative cell death pathways. To further investigate this phenomenon, we examined the effects of blocking caspase-dependent apoptosis with Z-VAD on autophagosome formation and clonogenic survival. Our results show significant increase of autophagosome formation achieved with 10 μmol/L Z-VAD (Fig. 4A), with >2-fold increases seen in both cell lines. We then analyzed both PC3 and DU145 cells with clonogenic assays to determine the extent to which this effect modulates postradiation cell mortality, and whether mTOR inhibition further enhances this process. In the PC-3 cell line, the results indicate a large survival difference between controls and those cells treated with RAD001, Z-VAD, or a combination of both (Fig. 4B). Treatment with 10 μmol/L Z-VAD alone yielded a DER of 1.24, and in
combination with RAD001 the DER increased to 1.36. In DU145 cells, Z-VAD alone only minimally affected survival with a DER of 1.05, and Z-VAD/RAD001 combination yielded a modest DER of 1.08 (Fig. 4C).

An in vitro Bax/Bak knockout mimic was generated by using siRNAs against Bax and Bak to confirm the effects of apoptosis inhibition on radiosensitization. It was determined that 20 nmol/L each of Bax/Bak siRNA was sufficient to block expression of these proapoptotic factors in both cell lines (Fig. 5A). Clonogenic assays then done using this concentration of Bax/Bak siRNA showed that although RAD001 alone induces little autophagy, mTOR inhibition works synergistically with radiation in driving cells to autophagy in prostate cancer cells. Notably, of GFP-transfected cells, a higher percentage undergo autophagy at baseline in DU145 cells compared with PC-3 cells, again reflecting loss of PTEN suppression of PI3K/Akt prosurvival signals in PC-3 cells.

determine whether the observed radiosensitization is partly dependent on autophagic process, we attenuated autophagy by transfecting siRNAs against ATG5 and Beclin-1 into the prostate cancer cells. Figure 5E shows the attenuation of ATG5 and Beclin-1 levels seen 24 hours after transfection of the respective siRNAs into the prostate cancer cells. As shown in Fig. 5F, cells transfected with ATG5 and Beclin-1 siRNA became more resistant to radiation as their survival curves shifted upwards (DER for siRNA control versus siRNA ATG5 and Beclin1 is 1.31, P = 0.008 for PC3 cells; and 1.19, P = 0.004 for DU145 cells).

Discussion

In the present study, we found that both mTOR and apoptosis inhibition sensitize PTEN−/− PC-3, but not PTEN+/+ DU145, cells to the cytotoxic effects of radiation. This is associated with the promotion of autophagy. Our data did not show increased mTOR signaling in the prostate cancer cell line DU145 after irradiation; however, the high basal activity of the unsuppressed Akt pathway in PTEN−/− PC-3 cells make it difficult to show further increases in mTOR signaling after irradiation. By providing more substrate on which RAD001 could act, this heightened baseline expression may account for the difference in how these cell lines responded to drug treatment.

Radiation-induced activation of the PI3K/Akt/mTOR prosurvival pathway is counterproductive to the killing of malignant
cells, and this induction can occur within 15 minutes after irradiation (23, 24). Specifically, events following mTOR-mediated phosphorylation of translational machinery enhance expression of the protective kinase TLK1B, which confers cellular radioresistance (25). Indeed, the capacity to achieve radiosensitization through mTOR inhibition has been shown before (18–20). Our results similarly indicate that RAD001 radiosensitizes prostate cancer cells, specifically PTEN-deficient PC-3 cells. PTEN dephosphorylates the second messenger PIP3, interrupting PI3K activation of Akt and decreasing overall flux through the PI3K pathway (26). Mice that are PTEN heterozygous (+/-) have been observed to develop prostatic intraepithelial neoplasia with nearly 100% frequency, although the animals may succumb to other cancers before these lesions can evolve into macroinvasive carcinoma (27, 28). More recently, mice in which a prostate-specific PTEN deletion has been achieved through use of prostate-restricted expression of Cre-recombinase have gone on to develop invasive and metastatic prostate carcinomas with very high penetrance (29, 30). The relevance of these murine models is verified by studies of human prostate cancer that have shown that loss of PTEN is strongly associated with more aggressive cancers (6, 31). Hence, our data, which show that the PTEN-deficient prostate cancer cell line PC-3 is particularly susceptible to radiosensitization via mTOR inhibition, have important clinical implications.

Despite the considerable evidence supporting the oncogenic potential of mTOR, the precise mechanism by which mTOR activation leads to cell transformation has not yet been clearly defined. Dysregulation of critical components of protein synthesis machinery may allow aberrant cell cycle signals to facilitate cell immortality (11). Overexpression of cyclin D1 may play a particularly crucial role, as recent studies have suggested that this molecule can restore proliferative potential after rapamycin treatment (32). Alternatively, mTOR exerts prosurvival influence through hypoxia-inducible factor 1α, which targets many essential glycolytic enzymes and protects a variety of solid tumors from their relatively poor oxygenation status (5, 17, 33). mTOR is also a point of convergence in balancing the cellular response to growth factors, such as insulin (12) and multiple stressors, including hypoxia (34) and nutrient starvation (7). mTOR inhibition with rapamycin has been shown to induce a starvation-like state in mammalian cells (35). Eukaryotic cells without a sufficient nutrient source may undergo autophagy as a self-limited survival strategy, as the breakdown of intracellular structures provides the organism with a supply of ATP (36). However, this starvation response will ultimately lead to cell demise if no rescue can be secured with additional nutrients.

mTOR may be the critical link in mediating PI3K/Akt prosurvival signals with the suppression of autophagy. It has been shown that dysfunctional Akt signaling promotes autophagy in mammalian cells (37), and that the phosphorylation of p70S6 kinase by mTOR (or PDK1) prevents autophagy (38). Although autophagy has been documented as a form of programmed cell death enacted in response to various noxious stimuli, including...
viruses, toxins, and chemotherapy (36, 39), there is accumulating evidence that mTOR inactivation also initiates autophagy (19, 40). These observations lie in accordance with our findings that suggest mTOR inhibition with RAD001 sensitizes prostate cancer cells to radiation via the promotion of autophagy.

Our experiments using Z-VAD and Bax/Bak siRNA show that by blocking caspase-dependent apoptosis, cells can be further sensitized to the cytotoxic effects of radiation. Similar to RAD001, Z-VAD also seems to achieve radiosensitization through the promotion of autophagy. The shunting of cell death through the autophagic pathway in a caspase-deficient context has been observed before in MCF-7 breast cancer cells (19). Here, we have shown that inhibiting apoptosis dramatically induces autophagy and effectively radiosensitizes prostate cancer cells.

Figure 5. Blocking Bax/Bak–dependent apoptosis also enhances radiation-induced cell killing. A, 20 nmol/L Bax/Bak siRNA blocks production of Bax/Bak proteins. B, in PC-3 cells, treatment with Bax and Bak antisense RNA (siRNA, 20 nmol/L) produces equivalent radiosensitivity (DER 1.18) to RAD001 (DER 1.18), but combination treatment results in a synergistic increase in radiosensitivity [DER (versus MS control) 1.26]. C, treating DU145 cells with Bax/Bak siRNA also results in radiosensitization (DER 1.14). RAD001, although incapable of inducing significant radiosensitization alone, enhances the radiosensitivity obtained with Bax/Bak siRNA (DER 1.26). D, quantitative analysis of GFP-LC3 transfection shows increased autophagosome formation after radiation and RAD001 independently in both cell lines, with combination treatment resulting in the highest fraction of transfected cells undergoing autophagy for both PC-3 (0.69 ± 0.01) and DU145 (0.48 ± 0.06) cells. E, levels of ATG5 complex and Beclin at 24 hours after being transfected with ATG5 and Beclin siRNA. Note the levels of these proteins in the cells transfected with control siRNA were set to 1 for comparison. F, treating PC3 or DU145 cells with ATG5/Beclin siRNA results in decreased radiosensitivity.
Establishing that autophagy, in the absence of apoptosis, was partially responsible for the heightened radiosensitivity conferred by mTOR inhibition lends further credence to the idea that the traditional tenets of cancer treatment may exaggerate the role of p53-dependent cell death. Brown and Wouters (41) have argued that neither p53 status nor the ability to undergo apoptosis significantly affects the sensitivity of solid tumor cells to DNA-damaging agents. For example, many malignant cells have lost the capacity to undergo normal apoptosis, so it is reasonable to expect alternative modes of cell death to play a more prominent role in determining the sensitivity of a particular tumor to genotoxic agents (21). With the emergence of this new paradigm questioning the apoptotic sensitivity of solid tumors, our results showing the autophagy secondary to mTOR and apoptosis inhibition underscore the need to further investigate how to augment alternative mechanisms of cell death, such as autophagy, programmed necrosis that can occur after prolonged or massive autophagy, or mitotic catastrophe. The genetic constitution of a cancer cell may determine which mode of death to pursue.

Although this study was conducted in vitro, there is evidence to suggest that RAD001 efficacy may be improved under in vivo conditions. For instance, rapamycin is known to impede angiogenesis (42), and our group has previously shown decreased tumor vascular density in murine models and sensitized vascular endothelium when mTOR inhibition is coupled with radiation (18). This raises the possibility of an additional antitumor effect in the stroma, and suggests that a greater in vivo response to mTOR inhibition may be observed than would be predicted by in vitro studies.

In summary, we believe that this is the first article to show that mTOR inhibition with RAD001 is capable of radiosensitizing these cells by amplifying the autophagic pathway of cell death. This effect is more pronounced in the clinically relevant PTEN-deficient cell line. As more in vivo studies show the feasibility and efficacy of mTOR inhibition, methods of monitoring mTOR levels in vivo will become increasingly important. The duration of S6K1 inactivation in peripheral blood mononuclear cells correlates with tumor response to mTOR inhibition in rats, and thus potentially offers a valuable biomarker to assay RAD001 efficacy in patients (43). Additionally, because mTOR induces GLUT1 expression, preclinical studies have shown the viability of assaying reduced glucose uptake using 18FDG-potent positron tomography as a way of imaging areas of mTOR inhibition in vivo (44). These preclinical results provide valuable guidance in designing clinical trials using mTOR inhibitors.

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