Autophagy for Cancer Therapy through Inhibition of Pro-apoptotic Proteins and Mammalian Target of Rapamycin Signaling*

Kwang Woon Kim, Robert W. Mutter, Carolyn Cao, Jeffrey M. Albert, Michael Freeman, Dennis E. Hallahan, and Bo Lu

From the Department of Radiation Oncology, Vanderbilt Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Autophagy is an alternative cell death pathway that is induced by mammalian target of rapamycin (mTOR) inhibitors and up-regulated when apoptosis is defective. We investigated radiation-induced autophagy in the presence or absence of Bax/Bak with or without an mTOR inhibitor, Rad001. Two isogenic cell lines, wild type (WT) and Bak/Bak−/− mouse embryonic fibroblasts and tumor cell lines were used for this study. Irradiated Bak/Bak−/− cells had a decrease of Akt/mTOR signaling and a significant increase of pro-autophagic proteins ATG5–ATG12 COMPLEX and Beclin-1. These molecular events resulted in an up-regulation of autophagy. Bax/Bak−/− cells were defective in undergoing apoptosis but were more radiosensitive than the WT cells in autophagy. Both autophagy and sensitization of Bak/Bax−/− cells were further enhanced in the presence of Rad001. In contrast, inhibitors of autophagy rendered the Bak/Bax−/− cells radioresistant, whereas overexpression of ATG5 and Beclin-1 made the WT cells radiosensitive. When this novel concept of radiosensitization was tested in cancer models, small interfering RNAs against Bak/Bax also led to increased autophagy and sensitization of human breast and lung cancer cells to gamma radiation, which was further enhanced by Rad001. This is the first report to demonstrate that inhibition of pro-apoptotic proteins and induction of autophagy sensitizes cancer cells to therapy. Therapeutically targeting this novel pathway may yield significant benefits for cancer patients.

The Bcl-2 family of proteins are well characterized as regulators of apoptosis. The multidomain pro-apoptotic members of this family, such as Bax and Bak, act as a gateway for caspase-mediated cell death (1–4). Many reports have shown that pro-apoptotic approaches sensitize cells to DNA-damaging agents (5). Furthermore, several studies have demonstrated that apoptosis-permissive cells are more sensitive to ionizing radiation than apoptosis-refractory cells (6, 7). There is also contradictory data, however, showing that overexpression of the anti-apoptotic protein Bcl2 makes tumor xenografts more sensitive to ionizing radiation (8).

Autophagic cell death is morphologically characterized by a cell with an intact nucleus (9, 10) and an accumulation of cytoplasmic double-membrane autophagic vacuoles called autophagosomes. This process can be initiated in the presence of mammalian target of rapamycin (mTOR) inhibitors or starvation. Ionizing radiation has also been shown to induce autophagy (11). Autophagosomes contain subcellular organelles, such as mitochondria, needed for survival (12, 13) and contain high concentrations of microtubule-associated protein light chain 3 (LC3). Etoposide (an inhibitor of topoisomerase II and a common apoptotic reagent) has been shown to induce autophagy in Bax/Bak−/− double knock-out (DKO) mouse embryonic fibroblast (MEF) cells (6, 14). Radiosensitivity of Bax/Bak−/− DKO MEF cells has never been tested by the gold standard clonogenic assay, although cell viability of irradiated Bax/Bak−/− thymocytes per propidium iodide staining was not significantly different at 24 h after irradiation (15).

Although radiation-induced apoptosis mediated by Bax/Bak has been extensively studied over the past decade, it is not a major form of cell death (believed to account for <20%) (16). Other forms of non-apoptotic cell death have been described, which include mitotic catastrophe, necrosis, autophagy, and senescence. The relative contributions of the apoptotic and non-apoptotic death pathways to radiation sensitivity are poorly understood. Using the Bax/Bak−/− DKO MEF and cancer cell models, we showed that the radiosensitization in the presence of Bak and Bax results from increased autophagy, which is blocked by an autophagic inhibitor, 3-MA, or small interfering RNAs (siRNAs) against pro-autophagic proteins such as ATG5 and Beclin-1. Further enhancement was induced in the presence of an mTOR inhibitor, Rad001.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary MEFs derived from wild type and Bax-Bak DKO mice and immortalized by transfection with a plasmid containing SV40 genomic DNA were generously provided by Dr. Stanley J. Korsmeyer. (Dana-Farber Cancer Insti-
tute, Boston, MA) MEFs were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.1 μmol/liter 2-mercaptoethanol. MDA-MB-231 breast cancer cells and H460 lung carcinoma cells (obtained from the American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium and RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin respectively. All cells were incubated at 37 °C and humidified 5% CO₂. These cells were treated with either Me₂SO or Rad001. Alternatively, they were transfected with either expression plasmids or siRNA of ATG5 or Beclin-1.

**Immunoblotting**—Cells (0.5 × 10⁶) were collected at various time points after treatment and then washed with ice-cold phosphate-buffered saline twice before the addition of lysis buffer (20 mm Tris-HCl, pH 7.4, 150 mm NaCl, 20 mm EDTA, 1% Nonidet P-40, 50 mm NaF, 1 mm Na₃VO₄, 1 mm NaMO₄, and mixture inhibitor (5 μl/ml) (Sigma)). Protein concentration was quantified by the Bio-Rad method. Equal amounts of protein were loaded into each well and separated by 12.5% SDS-PAGE gel followed by transfer onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked using 5% nonfat dry milk in phosphate-buffered saline-Tween 20 for 1 h at room temperature. The blots were then incubated overnight at 4 °C with antibodies to caspase-3 (Cell Signaling Technology), ATG-5 (a gift from Dr. Mizushima), Beclin-1 (Santa Cruz Biotechnology), mTOR, phospho-mTOR (Ser-2448), Akt, phospho-Akt (Ser-473), S6 ribosomal protein, or phospho-S6 ribosomal protein (Ser-240/244) (Cell Signaling Technology), and actin. Goat anti-rabbit IgG secondary antibody (1:5000; Santa Cruz Biotechnology), mTOR, phospho-mTOR (Ser-2448), Akt, phospho-Akt (Ser-473), S6 ribosomal protein, or phospho-S6 ribosomal protein (Ser-240/244) (Cell Signaling Technology), and actin. Goat anti-rabbit IgG secondary antibody (1:5000; Santa Cruz Biotechnology) was incubated for 45 min at room temperature. Immunoblots were analyzed using the chemiluminescence detection system (PerkinElmer Life Sciences) according to the manufacturer’s protocol and autoradiography.

**Clonogenic Assay**—Cells were fixed for 15 min with 3:1 methanol-acetic acid and stained for 15 min with 0.5% crystal violet (Sigma) in methanol. The surviving colonies were counted, and their fractions were calculated as mean colony counts/cell inoculated × plating efficiency (or PE), where PE was defined as mean colony counts/cell inoculated for irradiated controls.

**Measurement of Apoptosis**—Percent of apoptosis was measured using the Annexin V-fluorescein isothiocyanate apoptosis detection kit I (Pharmlingen) with flow cytometry. Cells were plated into 100-mm dishes for each data point. After 24 h of 37 °C incubation, the groups were irradiated with 0.5, or 10 Gy. Cells were then incubated for 24 h. Thereafter, the cells were trypsinized (keeping all floating cells) and counted for each sample. The cells were washed twice with cold phosphate-buffered saline and then resuspended in 1 × binding buffer. 100 μl of the solution (5 × 10⁵ cells) were then transferred to a 5 ml culture tube. 3 μl of Annexin V-fluorescein isothiocyanate and 3 μl of propidium iodide were added. After 15 min of incubation at room temperature in the dark, 400 μl of 1 × binding buffer was added to each tube and analyzed by FACSScan.

**Autophagy Assay**—Cells were transfected with 2.5 μg of GFP-LC3 expression plasmid (a gift from Dr. Mizushima) using the Lipofectamine reagent (Invitrogen). After 24 h, the cells were treated with 5 Gy. After 24 or 48 h, the fluorescence of GFP-LC3 was observed under a confocal fluorescence microscope.

**Electron Microscopy**—Cells were washed with serum-free Dulbecco’s modified Eagle’s medium with a brief wash in pre-warmed 0.1 M cacodylate buffer and then fixed with 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer for 1 h. The cells were washed, post-fixed with 2% osmium tetroxide, dehydrated with ascending grades of ethanol and propylene oxide, and embedded in LX-112 medium (Ladd). After polymerization, ultra-thin (90 nm) sections were cut with a diamond knife, collected on uncoated copper grids and stained with uranyl acetate (1%) and lead citrate (0.2%). The samples were examined with a JEOL-1010 electron microscope (JEOL) operated at 80 kV.

**siRNA Transfection**—siRNA Bak (mouse), siRNA Bak (mouse), siRNA Bax (human), siRNA Bak (human), and siRNA Beclin (mouse) were purchased from Santa Cruz Biotechnology. siRNA ATG5 (mouse) was synthesized by Dharmacon Research. Cells were transfected with 25 nm siRNAs using Lipofectamine 2000. The transfected cells were used for subsequent experiments 24 h later.

**RESULTS**

**Radiation Sensitization When the Bax/Bak-mediated Apoptosis Is Attenuated**—We used DKO MEF cells that were deficient for Bak and Bax to determine the extent of apoptosis and radiation sensitivity as a consequence of lacking Bak and Bax. We examined proteolytic cleavage of caspase-3 as a marker for apoptosis in both WT and DKO cells in a dose- and time-dependent manner. Caspase-3 cleavage was not detected in the DKO cells at any time point or dose level, whereas it was apparent in the irradiated WT cells (Fig. 1A). A corroborative assay, Annexin V–fluorescein isothiocyanate staining was performed to measure the percent of apoptosis in the irradiated WT and DKO cells (Fig. 1B). 24 h following 5 Gy, the percentage of apoptotic cells was increased by 6.5-fold in WT cells, and 10 Gy induced an 8.2-fold increase. As expected, in the irradiated Bak/Bax⁻/⁻ MEF cells there was minimal change in the percentage of apoptotic cells after 5 or 10 Gy.

To determine whether inhibited apoptosis affects radiation sensitivity, clonogenic survival assay was performed in WT, DKO, and WT cells transfected with siRNAs against Bak and Bax to exclude potential clonal variation between MEF cells. As shown in Fig. 1C, Bak and Bax were attenuated in WT cells transfected with siRNAs against Bak and Bax. Unexpectedly, radiation sensitivity was found greatly enhanced in Bak/Bax⁻/⁻ MEF cells compared with WT MEFs. Dose enhancement ratio (DER) is defined as the ratio of doses required to decrease survival to 20%. The DER in Bak/Bax⁻/⁻ MEF cells relative to their WT counterparts, was 1.7 (p < 0.05, Student’s t test, n = 3). Clonogenic survival represents an integral of cell death measured over several days or weeks. Consistently, WT cells transfected with Bax/Bax siRNAs produced a DER of 1.4 compared with the cells transfected with a control siRNA (p < 0.05, n = 3, Student’s t test). Taken together, the results in Fig. 1 suggest that radiation sensitivity is increased in MEF cells that are defective in the Bax/Bak-mediated apoptosis.
Radiosensitization in the Absence of Bax/Bak

Increased Autophagy in the Irradiated Bax and Bak\(^{-/-}\) Cells—
To determine whether radiosensitivity of DKO cells is mediated by non-apoptotic programmed cell death, such as autophagy, we transfected WT and DKO cells with GFP-tagged LC3 plasmid (17) and detected the distribution of GFP. As shown in Fig. 2A, diffuse cytoplasmic localization of GFP-LC3 was observed in the transfected WT MEF cells and Bax/Bak\(^{-/-}\) MEF cells without undergoing irradiation, whereas the irradiated Bax/Bak\(^{-/-}\) MEF cells showed significantly increased punctate fluorescence or lysosomal localization of LC3 protein in 24 h. However, there was little increase in punctate fluorescence seen in the irradiated WT MEF cells. As shown in Fig. 2B, the number of cells with punctate GFP-LC3 fluorescence significantly increased in a time-dependent manner in Bax/Bak\(^{-/-}\) MEF cells, from 33 (24 h after irradiation) to 60% (48 h after irradiation), but only a slight increase was seen in WT cells. To confirm the induction of autophagic changes in Bax/Bak\(^{-/-}\) MEF cells, we examined these cells using electron microscopy. As shown in Fig. 2C, irradiated WT MEF cells did not show appreciable autophagosomes (Fig. 2C, ii), whereas Bax/Bak\(^{-/-}\) MEF cells showed numerous autophagosomes containing recognizable cellular material (Fig. 2C, iv and vi). The fusion of late autophagosomes and lysosomes is another characteristic of autophagy that was observed in these cells (Fig. 2C, v).

Induced Pro-autophagic Signaling in the Irradiated Bax and Bak\(^{-/-}\) MEF Cells—Because the mTOR pathway is known to regulate initiation of autophagy, we examined Akt/mTOR signaling by determining the levels of phospho-Akt, phospho-mTOR, and phospho-S6 in the irradiated WT and Bax/Bak\(^{-/-}\) MEF cells. As shown in Fig. 3A, the phosphoproteins were increased in the irradiated WT cells, whereas they were decreased in the irradiated Bax/Bak\(^{-/-}\) MEF cells. To determine whether radiation up-regulated the expression of autophagic proteins in the Bax/Bak\(^{-/-}\) MEF cells, we used immunoblotting to examine the expression of ATG-5 and Beclin-1 at 0, 24, 48, and 72 h after 5 Gy irradiation. There was considerable increase of both the ATG5-ATG12 complex and Beclin-1 (Fig. 3B) in the irradiated Bax/Bak\(^{-/-}\) MEF cells. In contrast, there was minimal change in these proteins seen in the irradiated WT MEF cells. These data suggest that pro-autophagic signaling is up-regulated in the irradiated DKO cells.

Inhibition of Autophagic Signaling Induces Radiation Resistance—3-MA, an inhibitor of class III phosphatidylinositol 3-kinase, has been shown to inhibit autophagy (18). To determine whether the 3-MA-treated DKO cells had decreased autophagic signaling, ATG5-ATG12 COMPLEX and Beclin-1 were examined by Western blotting. As shown in Fig. 4A, we found that 3-MA attenuated the induction of both ATG5-ATG12 COMPLEX and Beclin-1 in the irradiated DKO cells. To determine whether inhibiting autophagy by 3-MA affects the radiation sensitivity of DKO cells, clonogenic survival was assayed. As shown in Fig. 4B, DKO cells in the presence of 3-MA became more radiation-resistant by a factor of 1.8 when calculated at a survival value of 50% (\(p < 0.004\), Student’s s t test). This result suggests that 3-MA increased radiosensitivity by attenuating autophagic cell death in Bax/Bak\(^{-/-}\) MEF cells.
To confirm that inhibition of autophagic signaling induces radioresistance of DKO MEF cells, the Bax/Bak^−/− MEF cells were transfected with siRNAs against ATG5 and Beclin-1 or a siRNA control. As shown in Fig. 4C, both proteins were decreased following the transfection of siRNAs against these proteins. The clonogenic assay shown in Fig. 4D indicates that DKO cells transfected with siRNA directed against both gene products of ATG5 and Beclin-1 became more radioresistant by a factor of 1.4 when calculated at a survival value of 50%, compared with cells transfected with a control siRNA (p < 0.006, Student’s t test, n = 3). DKO cells transfected with siRNA directed against either ATG5 or Beclin-1 alone showed increased radioresistance, but the effect was slightly less than that seen with the cells transfected with siRNAs against both gene products (data not shown).

**Induction of Autophagy Increases Radiation Sensitivity**—To determine whether induction of autophagy through inhibition of mTOR enhances the radiation sensitivity of MEF cells, WT and DKO MEF cells were treated with 10 nM Rad001, an inhibitor of mTOR. The clonogenic assay shown in Fig. 5A indicates that DKO cells became more sensitive in the presence of Rad001 by a DER of 1.37 (p < 0.001, Student’s t test, n = 3), whereas the effect on WT cells was slight (DER = 1.06, p < 0.006, Student’s t test, n = 3).

**FIGURE 2. Increased autophagy in the irradiated Bax and Bak^−/− MEF cells.** A, GFP-LC3-transfected cells were treated with and without 5 Gy and then examined by fluorescence microscopy after 24 h. B, the percentage of cells with punctate GFP-LC3 fluorescence was calculated relative to all GFP-positive cells. Error bars are shown as mean ± S.D. C, electron micrograph of WT (i) or Bax/Bak^−/− MEF cells (ii) cells without radiation and WT (iii) or Bax/Bak^−/− MEF cells treated with 5 Gy (iv–vi). Arrows depict autophagosomes in cells containing recognizable cellular material (iv, ×66,000) or a late autophagosome fusing with a lysosome (v, ×25,000). Numerous autophagosomes were observed, and the mitochondria were also affected (vi, arrow, ×11,500).

**FIGURE 3. Induced pro-autophagic signaling in the irradiated Bax and Bak^−/− MEF cells.** WT and DKO MEF cells were irradiated with 0 or 5 Gy. Cells were collected at various time points, and protein extracts were made for Western blotting. A, shown are immunoblots of phospho-Akt, phospho-mTOR, and p-S6 using the lysates from WT and DKO MEF cells treated with 0 or 5 Gy after 30 min. B, also shown are immunoblots of ATG5-ATG12 complex and Beclin-1 using the lysates from WT and DKO MEF cells treated with 0 or 5 Gy after 24, 48, and 72 h. Actin was probed to demonstrate equal loading.

To confirm that inhibition of autophagic signaling induces radioresistance of DKO MEF cells, the Bax/Bak^−/− MEF cells were transfected with siRNAs against ATG5 and Beclin-1 or a siRNA control. As shown in Fig. 4C, both proteins were decreased following the transfection of siRNAs against these proteins. The clonogenic assay shown in Fig. 4D indicates that DKO cells transfected with siRNA directed against both gene products of ATG5 and Beclin-1 became more radioresistant by a factor of 1.4 when calculated at a survival value of 50%, compared with cells transfected with a control siRNA (p < 0.006, Student’s t test, n = 3). DKO cells transfected with siRNA directed against either ATG5 or Beclin-1 alone showed increased radioresistance, but the effect was slightly less than that seen with the cells transfected with siRNAs against both gene products (data not shown).

**Induction of Autophagy Increases Radiation Sensitivity**—To determine whether induction of autophagy through inhibition of mTOR enhances the radiation sensitivity of MEF cells, WT and Bax/Bak^−/− MEF cells were treated with 10 nM Rad001, an inhibitor of mTOR. The clonogenic assay shown in Fig. 5A indicates that DKO cells became more sensitive in the presence of Rad001 by a DER of 1.37 (p < 0.001, Student’s t test, n = 3), whereas the effect on WT cells was slight (DER = 1.06, p < 0.006, Student’s t test, n = 3).
Bak and Bax, which significantly decreased their expression (Fig. 6A). Clonogenic assay showed that the reduction of Bak and Bax increased the radiation sensitivity of both cell lines. As shown in Fig. 6B, DER is 1.23, \( p < 0.003 \) (Student’s \( t \) test) for the breast cancer model and is 1.62, \( p < 0.003 \) (Student’s \( t \) test) for the lung cancer model as shown in Fig. 6C. Similar findings were noted in prostate cancer cell lines, PC3, and DU145 cells (data not shown). We also analyzed whether inhibition of mTOR using Rad001 would further enhance the radiation sensitivity of cancer cells. Both cancer cell lines were first transfected with siRNAs against Bak and Bax to block apoptosis. 24 h later, these cells were incubated with Rad001 to induce autophagy. Subsequently, they were irradiated with 0–6 Gy, and clonogenic assay was performed. As shown in Fig. 6, B and C, the combined treatment of Rad001 and siRNAs against Bak and Bax resulted in even greater radiosensitization (breast cancer: DER = 1.50, \( p < 0.002 \); lung cancer: DER = 1.98, \( p < 0.001 \)).

To determine whether siRNAs directed against Bak and Bax and inhibition of mTOR with Rad001 induced autophagy in irradiated MDA-MB-231 breast cancer cells and H460 lung cancer cells, both cancer cells were transfected with GFP-LC3. Increased punctate fluorescence, suggestive of autophagy, was seen after radiation treatment in both cancer cell lines. The greatest amount of autophagy was seen in the combination treatment (Fig. 6, D and E). These results also suggest that autophagy mediates the increased radiosensitivity in cancer models.

**DISCUSSION**

This study showed evidence that autophagy is induced as an alternative form of cell death in irradiated cells that are unable to undergo apoptosis. The extent of autophagy was found to determine radiation sensitivity. Inducing autophagy through the inhibition of apoptosis or directly triggering autophagic signaling by mTOR inhibitors are viable strategies that can be applied to cancer therapy. Because strategies aimed at enhancing apoptosis have for many years been a focus of cancer therapeutic research, this is a novel concept that could affect the direction of cancer drug development.

---

**FIGURE 4. Inhibition of autophagic signaling induces radiation resistance.** A, wild type (WT) and Bax/Bak \(^{-/-} \) MEF (DKO) cells were cultured in the absence (−) or presence (+) of 100 or 200 μM 3-MA, an inhibitor of type III phosphatidylinositol 3-kinase, and irradiated with 0 or 5 Gy. Western analyses of ATG5-ATG12 COMPLEX and Beclin-1 in the irradiated cells with or without pre-treatment of 3-MA. B, WT and Bax/Bak \(^{-/-} \) MEF (DKO) cells were cultured in the absence or presence of 10 or 20 μM 3-MA and irradiated with 0–6 Gy. After 8 days, the colonies were stained and scored. Data are based upon three separate repeated experiments and shown as the mean ± S.D. C, WT and DKO MEF cells were transfected with 25 nM siRNAs directed against ATG5 and Beclin-1. The protein levels of ATG5-ATG12 COMPLEX and Beclin-1 were detected by Western analysis 48 h following transfection. D, DKO cells were transfected with siRNAs against ATG5 and Beclin-1 or a control siRNA. They were then irradiated with 0–6 Gy. After 8 days, colonies were stained and scored. Shown are the mean ± S.D. of three separate repeated experiments.

We also determined whether overexpression of pro-autophagic proteins ATG5 or Beclin-1 enhances radiation sensitivity. WT and DKO cells were transfected with expression plasmids of ATG5 and Beclin-1 or a vector control. The clonogenic assay shown in Fig. 5B indicates that WT and DKO cells overexpressing ATG5 and Beclin-1 became more radiosensitive (WT: DER = 1.25, \( p < 0.002 \); DKO: DER = 1.29, \( p < 0.008 \)).

siRNA against Bak/Bax and Rad001 Sensitize Cancer Cells to Ionizing Radiation—To apply the above concept, inducing autophagy as a potential strategy to enhance cell death following irradiation, we determined whether reduction of pro-apoptotic proteins Bak and Bax alters the radiation sensitivity of cancer cells. MDA-MB-231 breast cancer cells and H460 lung cancer cells were transfected with siRNAs directed against the combination treatment (Fig. 6, D and E). These results also suggest that autophagy mediates the increased radiosensitivity in cancer models.
We found that radiation induced the cleavage of caspase-3 in WT and not in Bax/Bak^{-/-} MEF cells. This is consistent with the fact that radiation can induce apoptosis through activation of caspases (19–22). WT MEF cells stained by Annexin-V were also significantly higher than the DKO cells. These results suggest that radiation induces apoptotic cell death through activation of caspases in the presence of Bax and Bak. We expected that apoptosis-permissive cells would show decreased clonogenicity after irradiation, because previous publications have shown that apoptosis-permissive cells were more radiosensitive as compared with apoptosis-refractory cells (6, 7). However, we found increased radiation sensitivity of Bax/Bak^{-/-} MEF cells by clonogenic assay. This concept was confirmed by attenuating levels of Bax and Bak in the WT cells. We speculated that alternative cell death, such as autophagy, in the absence of caspase activation may explain this phenomenon (19, 20). Recent papers report that cell death still occurs in Bax/Bak^{-/-} mice, implying that alternative mechanisms for cell death exist in these animals (14). When radiation effects on Bax/Bak^{-/-} MEF cells were examined 24 h after irradiation using propidium iodide staining (13), the sensitizing effect was not observed, because propidium iodide staining simply does not catch non-apoptotic cell deaths occurring 24 h following irradiation. In this study, we investigated radiation sensitivity using the classic clonogenic survival assay, which sums up all forms of cell death over a period of 8–10 days. Our data supports the idea that alternative cell death pathways, such as autophagy, may play a more dominant role in radiation-induced cell death than previously thought.

Recent studies report that treatment with radiation and chemotherapeutic drugs can induce autophagy in cancer cells (23, 24). Autophagy is a dynamic process consisting of the formation and fusion of membrane compartments. For the development of membranes during the autophagic process, LC3, a homologue of Apg8/Aut7p, is recruited to the membrane for the formation of autophagosomes (25). After forming a complete spherical autophagosome, LC3 remains associated with the membrane (17). At least 16 autophagy-associated genes (Atg/Apg) that are essential in the autophagic process have been identified (26). Among these, Atg5 and Atg12 are two known important genes (27), and the protein Atg5 induces autophagy by covalently binding to Atg12. Beclin-1 interacts with Bcl-2/Bcl-xL and forms part of a class III phosphatidylinositol 3-kinase complex that is crucial for autophagy, and which can be inhibited by 3-MA (28, 29). Here we demonstrated upregulation of Atg5/Atg12 and Beclin-1 in Bax/Bak^{-/-} MEF cells following irradiation and increased radioresistance when DKO cells were treated with 3-MA, findings consistent with these known molecular events of autophagy.

We have shown significantly increased autophagy (over 60% at 48 h following irradiation) in the irradiated Bax/Bak^{-/-} MEF cells. This was examined by morphology per electron microscopy and by characteristic autophagosomes per LC3 location using GFP-LC3 fusion protein. Induction of autophagy was also found in the irradiated WT cells but was kept <20%. This suggests that the absence of Bax and Bak hyperactivated autophagy under radiation-induced stress. We then examined the pro-autophagic signaling by detecting Akt/mTOR activity in the irradiated Bax/Bak^{-/-} MEF cells following irradiation and increased radioresistance when DKO cells were treated with 3-MA, findings consistent with these known molecular events of autophagy.

We have shown significantly increased autophagy (over 60% at 48 h following irradiation) in the irradiated Bax/Bak^{-/-} MEF cells. This was examined by morphology per electron microscopy and by characteristic autophagosomes per LC3 location using GFP-LC3 fusion protein. Induction of autophagy was also found in the irradiated WT cells but was kept <20%. This suggests that the absence of Bax and Bak hyperactivated autophagy under radiation-induced stress. We then examined the pro-autophagic signaling by detecting Akt/mTOR activity in the irradiated Bax/Bak^{-/-} MEF cells. Attenuated phosphorylation of Akt, mTOR, and S6 proteins and increased levels of ATG-5 and Beclin-1 were detected in the irradiated Bax/Bak^{-/-} MEF cells. All of these molecular changes could potentiate autophagy. Much is left to be understood in terms of the molecular
relationship of Bax/Bak expression and the Akt-mTOR-S6K pathway, and we are currently conducting experiments to further explore their interaction. Nevertheless, our work and that of others indicates that apoptosis and autophagy are likely associated through complementary activation pathways still yet to be completely delineated.

The role of autophagy in radiation sensitivity has not previously been established. In this study, we clearly showed for the first time that inhibition of autophagy with 3-MA or attenuating key players of autophagy, such as Atg5 and Beclin-1, results in radiation resistance. On the other hand, we showed that mTOR inhibitor, Rad001, or overexpression of pro-autophagic proteins, such as Atg5 and Beclin-1, led to increased radiation sensitivity, supporting the importance of autophagy in regulating cell sensitivity to ionizing radiation.

Finally, we tested the concept of inhibiting Bak and Bax to enhance radiation sensitization in cancer cells. This has not previously been tested before, largely because overexpression of these proteins has previously been shown to increase apoptosis and therefore enhance radiation effects. Indeed, enhancement of
of apoptosis has been a main focus of cancer therapy (30–33). Here, we found that attenuation of Bak and Bak in either H460 lung cancer cells or MDA-MB-231 breast cancers using siRNA against these proteins induced autophagy and led to increased radiation sensitivity. Inhibition of mTOR with Rad001 added further sensitization of these cancers by further up-regulating autophagy. This enhancement of autophagy through mTOR inhibition is consistent with prior studies that demonstrate that inhibition of mTOR initiates autophagy. Because most cancers have certain defects in their apoptotic machinery, therapeutically targeting the non-apoptotic pathway, such as autophagy, may yield better clinical outcomes for patients undergoing cytotoxic cancer therapy.

In summary, the present study provides compelling experimental evidence that the Bcl-2-family pro-apoptotic proteins Bak and Bak play a critical role in mediating the mechanism of cell death following irradiation. Our data support the hypothesis that irradiation up-regulates non-apoptotic programmed cell death (autophagy) in cells that are unable to undergo Bax/Bak-mediated apoptotic cell death and that radiosensitivity can be increased by attenuating Bak and Bak in several cancer cells. This can be further enhanced by the direct promotion of autophagy using the now clinically available mTOR inhibitor Rad001. These strategies targeting autophagic cell death deserve further investigation in animal models of cancer and eventually in cancer patients requiring radiotherapy.

Acknowledgments—We thank Dr. Jay Jerome for technical help with the electron microscopy. We are grateful to Drs. Stanley Korsmeyer, Tamotsu Yoshimori, and Noboru Mizushima for providing reagents.

REFERENCES

1. Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. (2001) Science 292, 727–730
2. Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. (2003) Science 300, 135–139
3. Oakes, S. A., Scorrano, L., Opferman, J. T., Bassik, M. C., Nishino, M., Pozzan, T., and Korsmeyer, S. J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 105–110
4. Ruiz-Vela, A., Opferman, J. T., Cheng, E. H., and Korsmeyer, S. I. (2005) EMBO Rep. 6, 379–385
5. Brown, J. M., and Attardi, L. D. (2005) Nat. Rev. Cancer 5, 231–237
6. Story, M. D., Voehringer, D. W., Malone, C. G., Hobbs, M. L., and Meyn, R. E. (1994) Int. J. Radiat. Biol. 66, 659–668
7. Held, K. D. (1997) Apoptosis 2, 265–282
8. Wouters, B. G., Denko, N. C., Giaccia, A. J., and Brown, J. M. (1999) Oncogene 18, 6540–6545
9. Baehrecke, E. H. (2002) Nat. Rev. Mol. Cell Biol. 3, 779–787
10. Reggiori, F., and Klionsky, D. J. (2005) Curr. Opin. Cell Biol. 17, 415–422
11. Yao, K. C., Komata, T., Kondo, Y., Kanzawa, T., Kondo, S., and Germano, I. M. (2003) J. Neurosurg. 98, 378–384
12. Bursch, W., Hochegger, K., Torok, L., Marian, B., Ellinger, A., and Hermann, R. S. (2000) J. Cell Sci. 113, 1189–1198
13. Bursch, W. (2001) Cell Death Differ. 8, 569–581
14. Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C. B., and Tsujimoto, Y. (2004) Nat. Cell Biol. 6, 1221–1228
15. Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Schwartz, K., Chen, Y., Wei, M., Eng, V. M., Adelman, D. M., Simon, M. C., Ma, A., Golden, J. A., Evan, G., Korsmeyer, S. J., MacGregor, G. R., and Thompson, C. B. (2000) Mol. Cell 6, 1389–1399
16. Verheij, M., and Bartelink, H. (2000) Cell Tissue Res. 310, 133–142
17. Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y., and Yoshimori, T. (2001) J. Cell Biol. 152, 657–668
18. Shintani, T., and Klionsky, D. J. (2004) Science 306, 990–995
19. Gozuacik, D., and Kimchi, A. (2004) Oncogene 23, 2891–2906
20. Yu, L., Alva, A., Su, H., Dutt, P., Freundt, E., Welsh, S., Baehrecke, E. H., and Lenardo, M. J. (2004) Science 304, 1500–1502
21. Yu, L., Lenardo, M. J., and Baehrecke, E. H. (2004) Cell Cycle 3, 1124–1126
22. Ding, H. F., and Fisher, D. E. (2001) J. Pediatr. Hematol. Oncol. 23, 185–188
23. Paglin, S., Hollister, T., Delohery, T., Hackett, N., McMahlil, M., Spichaks, E., Domingo, D., and Yahalom, J. (2001) Cancer Res. 61, 439–444
24. Kanzawa, T., Germano, I. M., Komata, T., Ito, H., Kondo, Y., and Kondo, S. (2004) Cell Death Differ. 11, 448–457
25. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Komniani, E., Oheumi, Y., and Yoshimori, T. (2000) EMBO J. 19, 5720–5728
26. Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2003) Int. J. Biochem. Cell Biol. 35, 553–561
27. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M., and Ohsumi, Y. (1998) Nature 395, 395–398
28. Liang, X. H., Kleeman, L. K., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B., and Levine, B. (1998) J. Virol. 72, 8586–8596
29. Yue, Z., Jin, S., Yang, C., Levine, A. J., and Heintz, N. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15077–15082
30. Pataer, A., Fang, B., Yu, R., Kagawa, S., Hunt, K. T., McDonnell, T. J., Roth, J. A., and Swisher, S. G. (2000) Cancer Res. 60, 788–792
31. Shinoura, N., Saito, K., Yoshida, Y., Hashimoto, M., Asai, A., and Kirino T. (2000) Cancer Gene Ther. 7, 739–748
32. Arafat, W. O., Gomez-Navarro, J., Xiang, J., Barnes, M. N., Mahasrethi, P., and Alvarez, R. D. (2000) Mol. Ther. 1, 545–554
33. Arafat, W. O., Buchsbaum, D. J., Gomez-Navarro, J., Tawil, S. A., Olsen, C., and Xiang, J. (2003) Int. J. Radiat. Oncol. Biol. Phys. 55, 1037–1050