

Inhibition of the DNA-Dependent Protein Kinase Catalytic Subunit Radiosensitizes Malignant Glioma Cells by Inducing Autophagy

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Abstract

DNA-dependent protein kinase (DNA-PK) plays a major role in the repair of DNA double-strand breaks induced by ionizing radiation (IR). Lack of DNA-PK causes defective DNA double-strand break repair and radiosensitization. In general, the cell death induced by IR is considered to be apoptotic. On the other hand, nonapoptotic cell death, autophagy, has recently attracted attention as a novel response of cancer cells to chemotherapy and IR. Autophagy is a protein degradation system characterized by a prominent formation of double-membrane vesicles in the cytoplasm. Little is known, however, regarding the relationship between DNA-PK and IR-induced autophagy. In the present study, we used human malignant glioma M059J and M059K cells to investigate the role of DNA-PK in IR-induced apoptotic and autophagic cell death. Low-dose IR induced massive autophagic cell death in M059J cells that lack the catalytic subunit of DNA-PK (DNA-PKcs). Most M059K cells, the counterpart of M059J cells in which DNA-PKcs are expressed at normal levels, survived, and proliferated although a small portion of the cells underwent apoptosis. Low-dose IR inhibited the phosphorylation of p70^{S6K}, a molecule downstream of the mammalian target of rapamycin associated with autophagy in M059J cells but not in M059K cells. The treatment of M059K cells with antisense oligonucleotides against DNA-PKcs caused radiation-induced autophagy and radiosensitized the cells. Furthermore, antisense oligonucleotides against DNA-PKcs radiosensitized other malignant glioma cell lines with DNA-PK activity, U373-MG and T98G, by inducing autophagy. The specific inhibition of DNA-PKcs may be promising as a new therapy to radiosensitize malignant glioma cells by inducing autophagy. (Cancer Res 2005; 65(10): 4368-75)

Introduction

Malignant gliomas are the most common primary brain tumors in adults. Despite advances in diagnosis and treatment, median survival is <1 year for patients with glioblastoma multiforme, the most malignant type (1). Radiotherapy, a standard adjuvant therapy, confers some survival advantage, but resistance of the tumor cells to the effects of radiation limits the success of the treatment. DNA repair pathways is one of the main reasons of the resistance to ionizing radiation (IR; ref. 2).

IR induces DNA double-strand breaks, which are the most lethal form of damage to DNA. In mammals, DNA double-strand breaks are generally repaired via nonhomologous end joining, in which DNA-dependent protein kinase (DNA-PK), a nuclear serine-threonine protein kinase, plays a key role (3–5). DNA-PK is composed of a catalytic subunit (DNA-PKcs) and a DNA-binding heterodimer consisting of Ku70 and Ku80. Ku binds to both ends of a double-strand break and recruits DNA-PKcs to the DNA end. Then, DNA-PK allows DNA ligase IV and X-ray cross-complementing 4 (XRCC4) to complete DNA repair (5).

In addition to its role in DNA repair, DNA-PK is also involved in apoptosis (4). When IR causes double-strand breaks, the DNA-PK complex senses and repairs them; however, when DNA damage is excessive, DNA-PK induces apoptosis. Typically, IR induces apoptosis by activating p53, Bax, and caspases, although p53-independent apoptosis has also been reported (6). Recently, another type of programmed cell death, autophagy, has been reported. Autophagic cell death is characterized by the prominent formation of double-membrane structures, called autophagosomes, in the cytoplasm, whereas the nucleus remains predominantly intact (7, 8). Autophagosomes are multiple-membrane structures often containing subcellular organelles, such as mitochondria (7). The cytoskeleton is well preserved until the late stage. On the other hand, in apoptotic cells, the nucleus is condensed or fragmented and the cytoskeleton is degraded from an early stage of cell death. We and others reported that IR at high doses induces autophagy in some types of cancer cells, including malignant glioma cells (9, 10). However, the relationship between DNA-PK and IR-induced autophagy is poorly defined.

In the present study, we used human malignant glioma M059J and M059K cells to assess the role of DNA-PK in IR-induced cell death. Although derived from the same malignant glioma specimen as M059K cells, M059J cells lack DNA-PKcs and are sensitive to radiation, whereas M059K cells express DNA-PKcs at a normal level and are resistant to the effects of radiation (11, 12). Therefore, M059J and M059K offer a useful model in which to study the role of DNA-PKcs in radiation-induced DNA damage. Reversal of the radiation-sensitive phenotype was confirmed by introducing into M059J cells a fragment of human chromosome 8, which contains a copy of the *DNA-PKcs* gene (13). We found that a low dose of IR induced massive autophagic cell death in M059J cells, but only occasional apoptotic cells were detected among M059K cells. Specific inhibition of DNA-PKcs in M059K cells induced autophagy and radiosensitized the cells. Next, we investigated whether the specific inhibition of DNA-PKcs radiosensitizes other malignant glioma cell lines expressing DNA-PKcs (such as U373-MG and T98G). Both tumor cells were sensitized to IR by inducing autophagy. To the best of our knowledge, this is

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the first study showing that the loss of DNA-PKcs plays an important role in IR-induced autophagy. Our results suggest that this novel form of radiosensitization induces autophagy in malignant gliomas.

Materials and Methods

Cell lines. Human malignant glioma M059J, M059K, U373-MG, and T98G cells and human fibroblast MRC5 cells were purchased from American Tissue Culture Collection (Rockville, MD). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 4 mmol/L glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂.

Ionizing radiation, clonogenic survival, and cell viability assay. Malignant glioma cells were irradiated with a ¹³⁷Cs-irradiator (Model E-0103, U.S. Nuclear Corp., Burbank, CA) at a dose rate of 3.312 Gy/min. For a clonogenic survival assay, cells irradiated with various doses were seeded in six-well plates, incubated at 37°C for 14 days, and then fixed with ethanol. Cells were stained with 0.5% crystal violet (Sigma, St. Louis, MO) and colonies that contained ≥50 cells were counted. The cytotoxic effect of IR for a short term was determined by a trypan blue dye exclusion assay as described previously (14). Tumor cells (1 × 10⁴-2 × 10⁴ cells/well) were seeded in 96-well, flat-bottomed plates after irradiation. One to three days after irradiation, cells were detached by trypsinization and the number of viable cells was counted. The viability of untreated cells was regarded as 100%.

Detection of apoptosis. Nuclei were stained with Hoechst 33258 (Sigma) to detect chromatin condensation or nuclear fragmentation, which are characteristic of apoptosis, as described previously (15). Treated tumor cells were fixed with 4% paraformaldehyde and stained with 0.5 µg/mL Hoechst 33258 for 15 minutes. Two hundred cells were counted under a fluorescence microscope and the incidence of apoptotic cells was scored.

Detection and quantification of acidic vesicular organelles with acridine orange staining. Acidic vesicular organelles were stained with acridine orange as described previously (9, 16). In acridine orange-stained cells, the cytoplasm and nucleus fluoresce bright green and dim red, whereas acidic compartments fluoresce bright red. The intensity of the red fluorescence is proportional to the degree of the acidity and volume of acidic vesicular organelles. Therefore, we can measure the extent of acidic vesicular organelles formation with red fluorescence of acridine orange staining. Tumor cells were stained with 1 µg/mL acridine orange for 15 minutes. Samples were then examined under a fluorescence microscope. Acridine orange labels acidic vesicular organelles, such as autophagosomes (9, 10). A typical acridine orange-positive cell exhibits granular distribution of acridine orange in the cytoplasm, indicative of autophagosome formation. To quantify the development of acidic vesicular organelles, cells were stained with acridine orange for 15 minutes, removed from the plate with trypsin-EDTA (Invitrogen), and collected for the FACSscan (Becton Dickinson, San Jose, CA) by using CellQuest software (Becton Dickinson).

Electron microscopy. Malignant glioma cells, grown on gelatinized plastic coverslips, were fixed for 2 hours with 2.5% glutaraldehyde (EM Science, Hatfield, PA) in 0.1 mol/L cacodylate buffer (pH 7.4), postfixed in 1% OsO₄ in the same buffer, and then analyzed by electron microscopy as described previously (16). Representative areas were chosen for ultrathin sectioning and viewed with a Hitachi 7600 electron microscope.

Transfection. The green fluorescent protein (GFP) and microtubule-associated protein 1 light chain 3 (LC3) fusion vector (GFP-LC3) was kindly provided by Drs. N. Mizushima and T. Yoshimori (National Institute for Basic Biology, Okazaki, Japan; ref. 17). LC3, the homologue of the yeast *Apg 8/Aut7p* gene, localizes on the autophagosomal membrane during autophagy (17, 18). We used the GFP-LC3 fused protein to detect autophagy. A β-galactosidase expression vector (Invitrogen) was used as the control. The sequence of antisenses against *DNA-PKcs* mRNA was 5'-ACACCGCTCCGAGCCCGCCAT-3' (19), and we used the sense of DNA-PKcs, 5'-ATGGCGGGCTCCGAGCCCGGTGT-3', as a control. FuGENE6 Transfection Reagent (Roche, Indianapolis, IN) was used according to manufacturer's instructions. The expression vector or antisense was transfected 24 hours before irradiation.

Western blotting. To detect the expression of ribosomal protein S6 kinase (p70^{S6K}) and p70^{S6K} that is phosphorylated at Thr³⁸⁹, soluble protein for Western blotting was harvested from treated tumor cells lysed in extraction buffer. Equal amounts of protein (40 µg) were separated by SDS-PAGE (7.5% gel; Bio-Rad, Richmond, CA) and transferred to a Hybond-P membrane (Amersham, Co., Piscataway, NJ). The membranes were treated with antibodies against p70^{S6K} and phosphorylated p70^{S6K} (Thr³⁸⁹; Cell Signaling, Beverly, MA) and subjected to Western blotting using an ECL-plus chemiluminescence reagent (Amersham).

DNA-dependent protein kinase activity assay. Whole-cell extracts were prepared using a modification of the method of Finnie et al. (20). Briefly, tumor cells were harvested and washed with PBS. Cell pellets were frozen at -80°C and resuspended in extraction buffer (20 mmol/L HEPES, 450 mmol/L NaCl, 50 mmol/L NaF, 25% v/v glycerol) with Complete protease inhibitor tablets (Roche). The swollen cells were disrupted by incubation alternatively on dry ice and at 30°C (three times) for 1 minute each. After microcentrifugation for 10 minutes at 4°C, supernatants were stored at -80°C before use. The SignaTECT DNA-PK assay system (Promega, Madison, WI) was used to measure DNA-PK activity according to the manufacturer's instructions. [γ-³²P]ATP was purchased from Perkin-Elmer (Boston, MA). Reactions were analyzed with a scintillation counter.

Senescence detection. Cells were stained with senescence-associated β-galactosidase by using the Senescence Detection kit (BioVision Research Products, Mountain View, CA).

Statistical analysis. The data were expressed as mean ± SD. Statistical analysis was done by using Student's *t* test (two-tailed). The criterion for statistical significance was taken as *P* < 0.05.

Results

Radiation effect on M059J and M059K cells. To compare the effects of radiation on the growth of M059J and M059K cells, we did a clonogenic survival assay. As expected, M059J cells were strikingly more sensitive to IR than M059K cells (Fig. 1A). Indeed, the killing effect of 2 Gy radiation was ~10 times higher on M059J cells than on M059K cells (the survival rates of the M059J and M059K cells were 3.9% and 39.1%, respectively). To assess the killing effect of 2 Gy radiation in the short term, we did a cell viability assay up to 3 days after irradiation. The viability of M059J and M059K cells decreased to 44.4% and 92.6%, respectively, after 3 days (Fig. 1B). These results are consistent with previous reports that M059J cells are ~10-fold more sensitive to IR than are M059K cells (11, 12).

Ionizing radiation induces autophagic cell death in M059J cells. To study whether IR induces apoptosis in M059J and M059K cells, we stained the cells with Hoechst 33258. In DNA-PK-deficient M059J cells, the nucleus of most cells was round and intact, and apoptotic cells were detected only in a very small fraction and did not increase in number (they ranged between 3.4% and 4.4% of the total number of cells) during the 3 days after 2 Gy irradiation (Fig. 2A-a, A-b, and B). In contrast, a small but significant increase in the number of apoptotic M059K cells (from 4.1% to 11.3%, *P* = 0.0016) was found (Fig. 2A-c, A-d and B), although viability was not affected (Fig. 1B). These results indicate that apoptosis was not induced in M059J cells after IR. They also suggest that only a small population of M059K cells undergoes apoptosis but that most cells continue to grow probably after DNA repair by DNA-PK.

Recently, autophagic cell death has been recognized as programmed cell death type II, in contrast to apoptosis which is programmed cell death type I (7, 8). We have reported that IR induces autophagy in malignant glioma cells (10). To determine whether IR induces autophagic cell death in M059J cells, we stained the cells with acridine orange. Acridine orange-positive

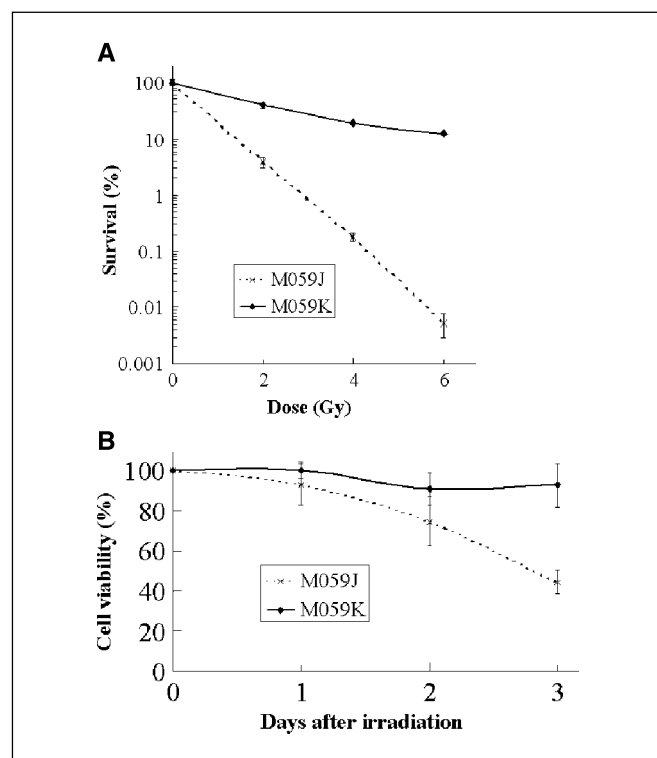


Figure 1. M059J cells are much more sensitive to radiation than M059K cells. *A*, radiation effect on clonogenic survival 14 days after irradiation. *Points*, mean of triplicate experiments; *bars*, SD. *B*, radiation effect on cell proliferation up to 3 days after 2 Gy irradiation. The viability of irradiated cells to nonirradiated cells was calculated. *Points*, mean of triplicate experiments; *bars*, SD.

cells with higher bright red fluorescence were frequently detected among M059J cells 3 days after 2 Gy irradiation (Fig. 3*A-b*), whereas they were scarce among nonirradiated cells (Fig. 3*A-a*). To quantify the incidence of acridine orange–positive cells, we did the fluorescence-activated cell sorting analysis. After 2 Gy irradiation, the proportion of acridine orange–positive cells increased from 4.5% to 41.9% among M059J cells, whereas there was no apparent increase among M059K cells (from 5.3% to 6.9%; Fig. 3*B*).

To confirm that IR induced autophagy in M059J cells, we observed changes in subcellular structure using electron microscopy. We observed autophagosomes to some extent even in untreated M059J cells (Fig. 3*C-a*). After 2 Gy irradiation, autophagosomes increased dramatically in number and contained cytoplasmic structures, lamellar structures, or residual digested material (Fig. 3*C-b*). In contrast, M059K cells showed few autophagosomes both before and after IR (data not shown).

To quantify autophagic vacuoles or autophagosomes, we transfected M059J cells with GFP-LC3 fusion plasmid and detected the distribution of LC3 (17). This is one of the specific methods for detecting autophagy (21). In nontreated cells, LC3 was homogeneously distributed in the cytoplasm (Fig. 3*D*). After 2 Gy irradiation, LC3 was distributed on punctate structures and GFP-LC3 dots were seen in some M059J cells (Fig. 3*D*). The percentage of M059J cells with GFP-LC3 dots increased significantly from 5.2% to 15.0% after IR ($P = 0.0048$; Fig. 3*E*).

Because autophagy is generally regulated by the mammalian target of rapamycin (mTOR) and its downstream p70^{S6K} (22), we examined whether IR affects this pathway in M059J cells. The

expression of phosphorylated p70^{S6K} at position Thr³⁸⁹ was strikingly reduced to an undetectable level 1 hour after 2 Gy irradiation in M059J cells but not in M059K cells (Fig. 3*F*). The expression level of total p70^{S6K} did not change in both cell types. These results collectively indicated that 2 Gy irradiation suppresses the mTOR pathway and induces autophagic cell death in M059J cells but not in M059K cells.

Inhibition of the catalytic subunit of DNA-dependent protein kinases radiosensitizes M059K cells by inducing autophagic cell death. Next, we investigated whether inhibition of DNA-PKcs radiosensitizes M059K cells by inducing autophagy. We used antisense oligonucleotides against DNA-PKcs (AS-DNA-PKcs) to inhibit DNA-PKcs specifically (19). A DNA-PK activity assay showed that treatment with AS-DNA-PKcs (300 nmol/L) for 24 hours inhibited 53% of DNA-PK activity in M059K cells, whereas no significant inhibition was detected in M059K cells treated with 300 nmol/L sense oligonucleotides against DNA-PKcs (S-DNA-PKcs) for 24 hours (Fig. 4*A*). A clonogenic survival assay revealed that the treatment with AS-DNA-PKcs sensitized M059K cells to IR (Fig. 4*B*). Moreover, a cell viability assay showed that AS-DNA-PKcs decreased the viability of M059K cells to ~60% of that of untreated or S-DNA-PKcs–treated cells 2 and 3 days after 2 Gy irradiation (Fig. 4*C*). We detected autophagic change with acridine orange staining. The proportion of acridine orange–positive cells increased from 7.7% to 16.3% among M059K cells treated with AS-DNA-PKcs

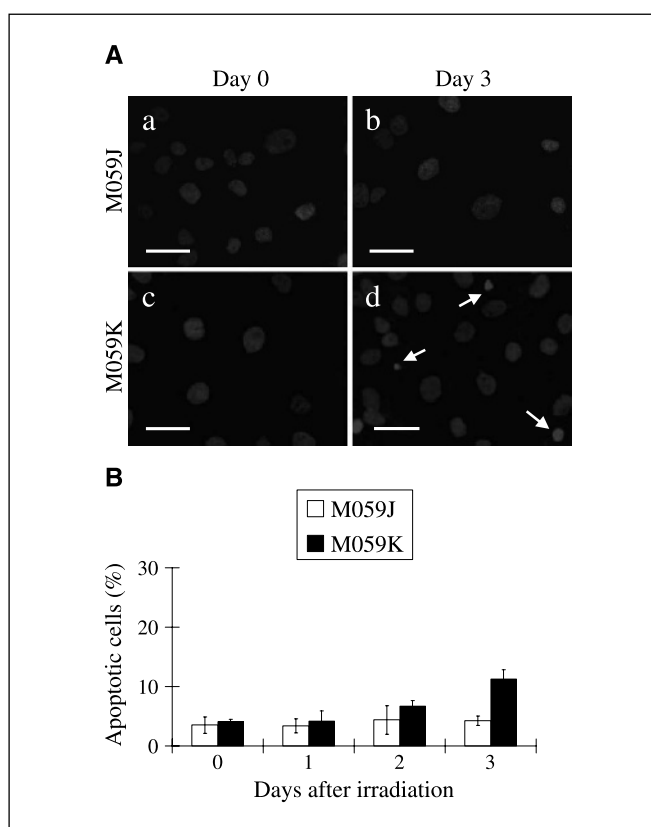
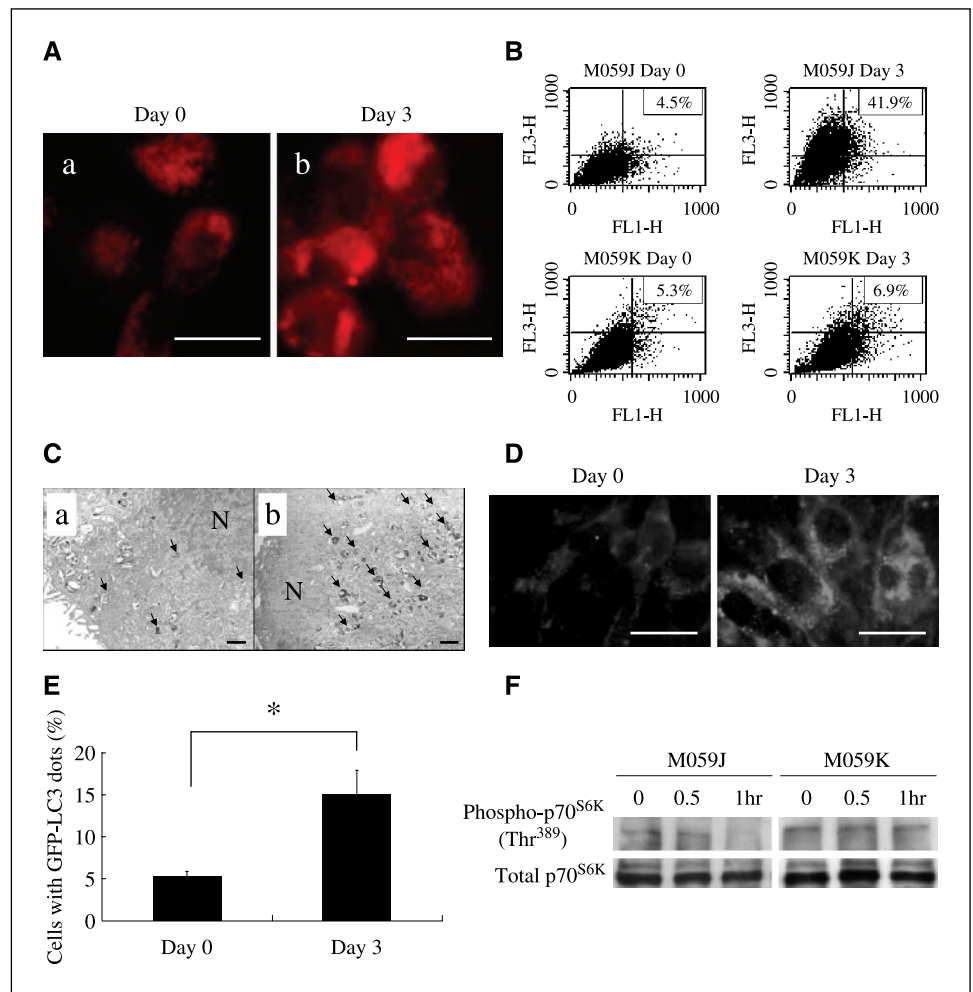


Figure 2. Low-dose irradiation does not induce apoptosis in M059J cells. M059J and M059K cells were stained with Hoechst 33258 from 0 to 3 days after 2 Gy irradiation. *A*, representative microphotographs of M059J and M059K cells before (*day 0*) and 3 days (*day 3*) after IR. *Arrows*, apoptotic nuclei. *Bars*, 50 μ m. *B*, quantification of cells with apoptotic nuclei. The percentage of apoptotic cells significantly increased only in M059K cells 3 days after irradiation ($P = 0.0016$). *Columns*, mean of triplicate experiments; *bars*, SD.

Figure 3. Low-dose irradiation induces autophagy and reduces the expression of p70^{S6K} in M059J cells. **A**, representative M059J cells stained with acridine orange before (a) and 3 days after (b) 2 Gy irradiation. Note prominent granular staining of acridine orange in the cytoplasm in (b). Bars, 10 μ m. **B**, quantification of acridine orange staining using flow cytometry before IR and 3 days after IR. M059J cells with prominent acridine orange staining increased after 2 Gy irradiation. Results shown are representative of three independent experiments. FL3-H indicates green color intensity and FL1-H shows red color intensity. **C**, representative electron micrographs of M059J cells before (a) and 3 days after (b) IR. Arrows, autophagic vacuoles. Bars, 1.0 μ m. N, nucleus. **D**, distribution of exogenous LC3 in M059J cells before (day 0) and 3 days after (day 3) IR. The GFP-LC3 cDNA vector was transfected to M059J cells and treated with 2 Gy irradiation. The cells were observed under a fluorescein microscope. **E**, quantification of the cells with characteristic GFP-LC3 dots that are indicative of autophagy. The proportion of the cells with GFP-LC3 dots was assessed in 100 GFP-positive cells in three different fields. Results shown are representative of three independent experiments. *, $P = 0.0048$. **F**, the expression of phosphorylated p70^{S6K} at position Thr³⁸⁹ and total p70^{S6K} in M059J and M059K cells. Proteins were isolated at indicated time points after 2 Gy irradiation and subjected to immunoblotting for phosphorylated p70^{S6K} and total p70^{S6K}.



(Fig. 4D). We did electron microscopy to further confirm the induction of autophagic changes. Three days after the treatment with radiation plus AS-DNA-PK, M059K cells showed increased formation of autophagic vacuoles that contained lamellar structures or residual digested material (Fig. 4E-b). These results indicate that AS-DNA-PK radiosensitizes M059K cells by increasing autophagic cell death.

Antisense oligonucleotides against the catalytic subunit of DNA-dependent protein kinases radiosensitize malignant glioma U373-MG and T98G cells. Our findings in M059K cells prompted us to determine whether other malignant glioma cells exhibiting DNA-PK activity would be radiosensitized by AS-DNA-PKcs. AS-DNA-PKcs inhibited 32.9% and 24.1% of DNA-PK activity in malignant glioma U373-MG and T98G cells, respectively (Fig. 5A). The extent of inhibition was smaller than that in M059K cells (53.0%; Fig. 4A) but was significant ($P < 0.05$). AS-DNA-PKcs radiosensitized both cell lines as detected with the clonogenic assay, whereas S-DNA-PKcs radiosensitized neither (Fig. 5B). Regarding the mechanism of radiosensitization, treatment with AS-DNA-PKcs increased the number of acridine orange-positive cells after 2 Gy irradiation from 5.1% to 11.7% in U373-MG cells and from 6.1% to 11.5% in T98G cells (Fig. 5C), whereas S-DNA-PKcs did not (data not shown). Because the increase in acridine orange-positive cells was smaller than that in M059K cells (from 7.7% to 16.3%; Fig. 4D), it raised a possibility

that other mechanisms, such as apoptosis or senescence, may have taken place in these cells. Hoechst 33258 staining showed no significant increase in apoptotic cells after 2 Gy irradiation in U373-MG cells and T98G cells pretreated with AS-DNA-PKcs compared with the cells pretreated with S-DNA-PKcs (Fig. 5D). Senescent cells were detected using the staining of senescence-associated β -galactosidase (23). Senescence-associated β -galactosidase staining was detected typically in the cytoplasm of confluent human fibroblast MRC5 cells (Fig. 5E, arrows; ref. 23). Although pale nuclear staining at a background level was detected in some U373-MG cells, clear positive staining of β -galactosidase in the cytoplasm was detected in <1% of U373-MG and T98G cells pretreated with AS-DNA-PKcs or S-DNA-PKcs and then irradiated at 2 Gy (Fig. 5E). These results collectively indicate that AS-DNA-PKcs radiosensitizes U373-MG and T98G cells by inducing autophagy at least in part and suggest that inhibition of DNA-PKcs represents a novel therapeutic approach to radiosensitize malignant glioma cells by inducing autophagic cell death.

Discussion

In the present study, we showed that low-dose IR induced autophagic cell death in DNA-PKcs-deficient M059J cells. The same low dose did not induce autophagy in M059K cells but induced apoptosis in only the small fraction of M059K cells that

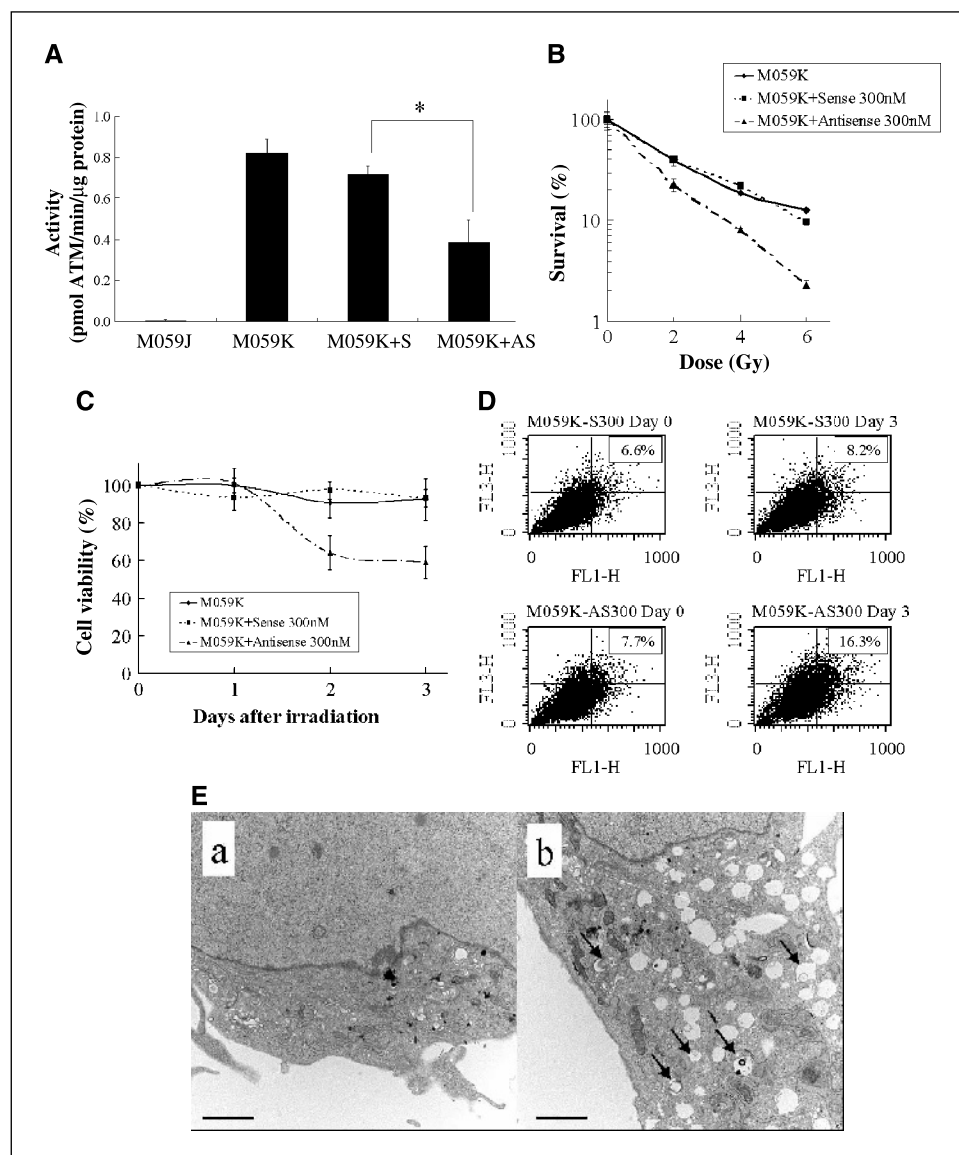


Figure 4. AS-DNA-PKcs radiosensitizes M059K cells by inducing autophagy. **A**, DNA-PK activity in nontreated M059J and M059K cells, and M059K cells treated with S-DNA-PKcs (300 nmol/L) or AS-DNA-PKcs (300 nmol/L) for 24 hours. Columns, mean of triplicate experiments; bars, SD. *, $P = 0.0044$. **B**, clonogenic survival 14 days after irradiation. M059K cells were treated with S-DNA-PKcs or AS-DNA-PKcs as described above and exposed to IR. Points, mean of triplicate experiments; bars, SD. **C**, cell viability up to 3 days after 2 Gy irradiation. M059K cells were treated with S-DNA-PKcs or AS-DNA-PKcs as described above and exposed to 2 Gy irradiation. Points, mean of triplicate experiments; bars, SD. **D**, quantification of acridine orange-positive cells using flow cytometry. M059K cells were treated with S-DNA-PKcs or AS-DNA-PKcs as described above, exposed to 2 Gy irradiation, and stained with acridine orange. Results shown are representative of three independent experiments. FL3-H indicates red color intensity and FL1-H shows green color intensity. **E**, representative electron microphotographs of M059K cells treated with S-DNA-PK (a) or AS-DNA-PK (b) as described above, exposed to 2 Gy IR, and fixed 3 days later. Arrows, autophagosomes. Bars, 2.0 μm.

have DNA-PKcs at a regular level, and most of the cells survived and proliferated. Importantly, the inhibition of DNA-PKcs produced by AS-DNA-PKcs radiosensitized not only the M059K cells but also other malignant glioma cells by inducing autophagy. This is the first report to show that the inhibition of DNA-PKcs induces autophagy.

Some types of cancer cells exhibit autophagic changes after treatments with IR and chemotherapeutic drugs (7, 9, 10, 24–26). Autophagy begins with the sequestering of cytosolic components, often including intracellular organelles within double-membrane structures. The vacuoles formed in this way are called autophagosomes (22, 27). As autophagosomes mature, their contents acidify. Finally, autophagosomes fuse with lysosomes and their contents are degraded by lysosomal hydrolases. The regulation of autophagy by molecular mechanisms and genes has been extensively studied in yeast. More than 10 autophagy-associated genes (*Aut/Apg*) that are essential in the autophagic process have been discovered (22). Homologues of some of these genes have been identified in mammals. For example, mTOR regulates many

APG genes, including *APG1*, *APG13*, and *APG17*, and inhibits autophagy. Beclin1, a Bcl-2-interacting protein structurally similar to Apg6, induces autophagy. Another group of *APG* genes is associated with the formation of autophagosomes. The Apg12-Apg5 conjugate localizes to the autophagosome precursors in an Apg7-dependent manner. As the membrane develops, LC3, a homologue of Apg8/Aut7p, is recruited to the membrane. Upon the complete formation of the autophagosome, the Apg12-Apg5 conjugate detaches from it, whereas LC3 remains associated with the membrane (28). Because the localization of LC3 to the autophagosomal membrane is highly specific to autophagy, it can be used to detect autophagy (17, 21).

In the present study, we used the GFP-LC3 fused protein to detect autophagy and showed its localization to the punctate structures (i.e., autophagosomes; Fig. 4). It has been proposed that autophagy leads cancer cells in one of two opposite directions (29). One direction leads to survival, by sequestering toxic materials or recycling proteins (9, 10). The other leads to death by responding to environmental stress. Recently, we identified that Bcl-2/adenovirus

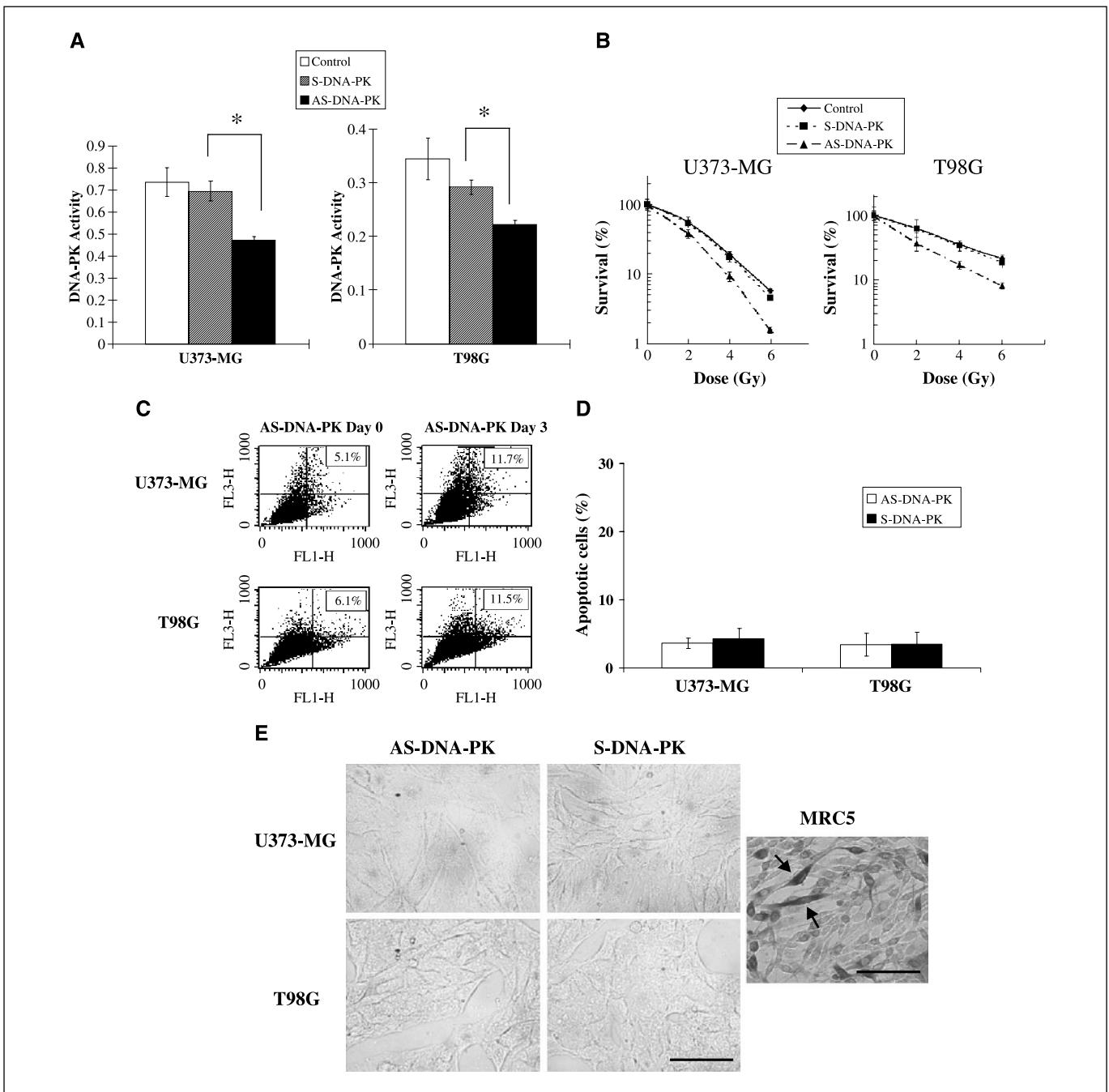


Figure 5. AS-DNA-PKcs induces autophagy and radiosensitizes U373-MG and T98G cells. **A**, DNA-PK activity in U373-MG and T98G cells treated with S-DNA-PKcs or AS-DNA-PKcs for 24 hours (500 nmol/L for U373-MG cells; 300 nmol/L for T98G cells). Columns, mean of triplicate experiments; bars, SD. * in U373-MG cells, $P = 0.0013$; * in T98G cells, $P = 0.0012$. **B**, clonogenic survival in U373-MG and T98G cells. Cells were treated with S-DNA-PKcs or AS-DNA-PKcs as described above and exposed to IR. **C**, quantification of acridine orange–positive cells in U373-MG and T98G cells treated with S-DNA-PKcs or AS-DNA-PKcs, exposed to 2 Gy irradiation, and stained 3 days later. Results shown are representative of three independent experiments. FL3-H indicates red color intensity and FL1-H shows green color intensity. **D**, quantification of cells with apoptotic nuclei stained with Hoechst 33258 dye in U373-MG and T98G cells treated with S-DNA-PKcs or AS-DNA-PKcs, exposed to 2 Gy irradiation, and stained 3 days later. Results shown are representative of three independent experiments. Columns, mean of triplicate experiments; bars, SD. **E**, senescence-associated β-galactosidase staining of U373-MG and T98G cells treated with S-DNA-PKcs or AS-DNA-PKcs, exposed to 2 Gy irradiation, and stained 3 days later. MRC cells are for positive control for senescence-associated β-galactosidase staining. Arrows, cells with positive staining in the cytoplasm. Bars, 20 μm.

E1B 19 kDa-interacting protein 3 (BNIP3) is associated with autophagic cell death caused by ceramide (24). We have more recently obtained evidence that BNIP3 is up-regulated in M059J cells after treatment with IR (data not shown), presumably mediating autophagic cell death.

DNA-PKcs is a member of the phosphatidylinositol 3-kinase–like family (3). The members of this family have a catalytic domain that has a homologue to phosphatidylinositol 3-kinase but do not phosphorylate lipids. Other members of the phosphatidylinositol 3-kinase–like family are ataxia-telangiectasia mutated (ATM),

ATM- and Rad3-related (ATR), and mTOR. ATM and ATR are associated with the control of cell cycle checkpoints in response to DNA damage (30). mTOR is a modulator of autophagy. The targets that DNA-PKcs phosphorylates include DNA-PKcs (31), both Ku subunits (32), XRCC4 (33), p53 (34), MDM2 (35), and c-Abl (36). The phosphorylation of DNA-PKcs, Ku subunits, and XRCC4 is associated with DNA repair, whereas that of p53, MDM2, and c-Abl induces apoptosis. The different roles played by these targets are, therefore, consistent with the notion that DNA-PK has dual roles in DNA damage: one is to sense DNA damage and repair it and the other is to induce apoptosis (4). Specifically, in response to DNA damage, the cell first tries to repair the damage and survive. However, if the cell cannot repair the damage, it undergoes apoptosis and avoids passing damaged DNA to its progeny cells. However, DNA damage does not induce apoptosis in DNA-PKcs^{-/-} cells (37, 38). In accordance with this theory, we did not detect apoptosis in M059J cells after treatment with IR. It is intriguing that the cell death in irradiated M059J cells was due to autophagy. In contrast, we detected some apoptotic cells in M059K cells after they received IR, but most of the cells survived. When DNA-PKcs was inhibited in M059K cells and other malignant glioma cells, autophagy instead of apoptosis was induced. Furthermore, we showed that the mTOR/p70^{S6K} pathway was suppressed by IR and autophagy was induced in M059J cells. Thus, it is tempting to speculate that DNA-PKcs plays a key role not only in the induction of apoptosis but also in the inhibition of autophagy. However, more studies are needed to support that conclusion.

Recently, several studies have focused on inhibiting DNA-PK specifically and sensitizing cancer cells to IR or anticancer drugs. Some investigators used antisense oligonucleotides and small interfering RNA designed to inhibit DNA-PKcs (19, 39, 40). Others used antisense oligonucleotides or peptide to inhibit Ku70 and Ku80

(41–43). Inhibiting one of these components of the DNA-PK complex resulted in the inhibition of DNA double-strand break repair and the radiosensitization of cells. These results are in accordance with the observations in DNA-PK knockout cells: DNA-PKcs^{-/-}, Ku70^{-/-}, and Ku80^{-/-} cells are all hypersensitive to IR (44–46). In the present study, we also showed that using AS-DNA-PKcs to specifically inhibit DNA-PKcs sensitized human malignant glioma M059K, U373-MG, and T98G cells to low-dose IR by inducing autophagy. The inhibition of DNA-PK activity was less in U373-MG and T98G cells than that in M059K cells. Interestingly, induction of autophagy paralleled to the extent of the inhibition of DNA-PK activity. Additionally, other mechanisms, such as apoptosis or senescence, was not detected in U373-MG and T98G cells, indicating that the induction of autophagy is only causative mechanism detected in this study for radiosensitization by AS-DNA-PKcs.

In summary, we showed that IR induces autophagic cell death in DNA-PKcs-deficient M059J cells. The inhibition of DNA-PKcs by using AS-DNA-PKcs induced autophagy and sensitized DNA-PKcs-proficient M059K cells to IR. AS-DNA-PKcs also radiosensitized other malignant glioma cells by inducing autophagy. Such radiation-induced autophagy may enhance the effect of cancer therapies.

Acknowledgments

Received 11/24/2004; revised 2/3/2005; accepted 2/25/2005.

Grant support: University of Texas M.D. Anderson Cancer Center (Y. Kondo), U.S. Public Health Service grants CA088936 and CA108558 (S. Kondo), and a generous donation from the Anthony D. Bullock III Foundation (S. Kondo and Y. Kondo).

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We thank Drs. Tamotsu Yoshimori and Noboru Mizushima for their kind gifts of vectors; Dr. Raymond Meyn for critical reading of this manuscript; and Dr. Anupama Munsch for her technical help.

References

- Laperriere N, Zuraw L, Cairncross G. Cancer Care Ontario Practice Guidelines Initiative Neuro-Oncology Disease Site Group. Radiotherapy for newly diagnosed malignant glioma in adults: a systematic review. *Radiother Oncol* 2002;64:259–73.
- Martin NM. DNA repair inhibition and cancer therapy. *J Photochem Photobiol B* 2001;63:162–70.
- Smith GC, Jackson SP. The DNA-dependent protein kinase. *Genes Dev* 1999;13:916–34.
- Bernstein C, Bernstein H, Payne CM, Garewal H. DNA repair/pro-apoptotic dual-role proteins in five major DNA repair pathways: fail-safe protection against carcinogenesis. *Mutat Res* 2002;511:145–78.
- Lees-Miller SP, Meek K. Repair of DNA double strand breaks by non-homologous end joining. *Biochimie* 2003; 85:1161–73.
- Verheij M, Bartelink H. Radiation-induced apoptosis. *Cell Tissue Res* 2000;301:133–42.
- Bursch W, Hochegger K, Torok L, Marian B, Ellinger A, Hermann RS. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. *J Cell Sci* 2000;113:1189–98.
- Bursch W, Ellinger A, Gerner C, Frohwein U, Schulte-Hermann R. Programmed cell death (PCD), Apoptosis, autophagic PCD, or others? *Ann N Y Acad Sci* 2000;926:1–12.
- Paglin S, Hollister T, Delohery T, et al. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Res* 2001;61: 439–44.
- Yao KC, Komata T, Kondo Y, Kanzawa T, Kondo S, Germano IM. Molecular response of human glioblastoma multiforme cells to ionizing radiation: cell cycle arrest, modulation of the expression of cyclin-dependent kinase inhibitors, and autophagy. *J Neurosurg* 2003;98: 378–84.
- Lees-Miller SP, Godbout R, Chan DW, et al. Absence of p350 subunit of DNA-activated protein kinase from a radiosensitive human cell line. *Science* 1995;267:1183–5.
- Allalunis-Turner MJ, Zia PK, Barron GM, Mirzayans R, Day RS III. Radiation-induced DNA damage and repair in cells of a radiosensitive human malignant glioma cell line. *Radiat Res* 1995;144:288–93.
- Hoppe BS, Jensen RB, Kirchgessner CU. Complementation of the radiosensitive M059J cell line. *Radiat Res* 2000;153:125–30.
- Mukai S, Kondo Y, Koga S, Komata T, Barna BP, Kondo S. 2-5A antisense telomerase RNA therapy for intracranial malignant gliomas. *Cancer Res* 2000;60: 4461–7.
- Kondo S, Barna BP, Morimura T, et al. Interleukin-1 β -converting enzyme mediates cisplatin-induced apoptosis in malignant glioma cells. *Cancer Res* 1995;55: 6166–71.
- Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. *Cancer Res* 2003;63:2103–8.
- Kabeya Y, Mizushima N, Ueno T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosomal membranes after processing. *EMBO J* 2000;19:5720–8.
- Kirisako T, Baba M, Ishihara N, et al. Formation process of autophagosome is traced with Apg8/Autp in yeast. *J Cell Biol* 1999;147:435–46.
- Sak A, Stuschke M, Wurm R, et al. Selective inactivation of DNA-dependent protein kinase with antisense oligodeoxynucleotides: consequences for the rejoining of radiation-induced DNA double-strand breaks and radiosensitivity of human cancer cell lines. *Cancer Res* 2002;62:6621–4.
- Finnie NJ, Gottlieb TM, Blunt T, Jeggo PA, Jackson SP. DNA-dependent protein kinase activity is absent in xrs-6 cells: implications for site-specific recombination and DNA double-strand break repair. *Proc Natl Acad Sci U S A* 1995;92:320–4.
- Kirkegaard K, Taylor MP, Jackson WT. Cellular autophagy: surrender, avoidance and subversion by microorganisms. *Nat Rev Microbiol* 2004;2:301–14.
- Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science* 2000;290: 1717–21.
- Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci U S A* 1995;92: 9363–7.
- Daido S, Kanzawa T, Yamamoto A, Takeuchi H, Kondo Y, Kondo S. Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. *Cancer Res* 2004;64:4286–93.
- Kanzawa T, Kondo Y, Ito H, Kondo S, Germano I. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. *Cancer Res* 2003;63:2103–8.
- Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S. Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ* 2004;11:448–57.
- Kim J, Klionsky DJ. Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. *Annu Rev Biochem* 2000;69:303–42.

28. Mizushima N, Yamamoto A, Hatano M, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol* 2001; 152:657-68.
29. Ogier-Denis E, Codogno P. Autophagy: a barrier or an adaptive response to cancer. *Biochim Biophys Acta* 2003;1603:113-28.
30. Iliakis G, Wang Y, Guan J, Wang H. DNA damage checkpoint control in cells exposed to ionizing radiation. *Oncogene* 2003;22:5834-47.
31. Douglas P, Sapkota GP, Morrice N, et al. Identification of *in vitro* and *in vivo* phosphorylation sites in the catalytic subunit of the DNA-dependent protein kinase. *Biochem J* 2002;368:243-51.
32. Chan DW, Ye R, Veillette CJ, Lees-Miller SP. DNA-dependent protein kinase phosphorylation sites in Ku 70/80 heterodimer. *Biochemistry* 1999;38:1819-28.
33. Yu Y, Wang W, Ding Q, et al. DNA-PK phosphorylation sites in XRCC4 are not required for survival after radiation or for V(D)J recombination. *DNA Repair* 2003; 2:1239-52.
34. Woo RA, McLure KG, Lees-Miller SP, Rancourt DE, Lee PW. DNA-dependent protein kinase acts upstream of p53 in response to DNA damage. *Nature* 1998;394: 700-4.
35. Mayo LD, Turchi JJ, Berberich SJ. Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res* 1997;57: 5013-6.
36. Kharbanda S, Pandey P, Jin S, et al. Functional interaction between DNA-PK and c-Abl in response to DNA damage. *Nature* 1997;386:732-5.
37. Wang S, Guo M, Ouyang H, et al. The catalytic subunit of DNA-dependent protein kinase selectively regulates p53-dependent apoptosis but not cell-cycle arrest. *Proc Natl Acad Sci U S A* 2000; 97:1584-8.
38. Woo RA, Jack MT, Xu Y, Burma S, Chen DJ, Lee PW. DNA damage-induced apoptosis requires the DNA-dependent protein kinase, and is mediated by the latent population of p53. *EMBO J* 2002;21: 3000-8.
39. Peng Y, Zhang Q, Nagasawa H, Okayasu R, Liber HL, Bedford JS. Silencing expression of the catalytic subunit of DNA-dependent protein kinase by small interfering RNA sensitizes human cells for radiation-induced chromosome damage, cell killing, and mutation. *Cancer Res* 2002;62:6400-4.
40. Collis SJ, Swartz MJ, Nelson WG, DeWeese TL. Enhanced radiation and chemotherapy-mediated cell killing of human cancer cells by small inhibitory RNA silencing of DNA repair factors. *Cancer Res* 2003;63: 1550-4.
41. Belenkov AI, Paiement JP, Panasci LC, Monia BP, Chow TY. An antisense oligonucleotide targeted to human Ku86 messenger RNA sensitizes M059K malignant glioma cells to ionizing radiation, bleomycin, and etoposide but not DNA cross-linking agents. *Cancer Res* 2002;62:5888-96.
42. Kim CH, Park SJ, Lee SH. A targeted inhibition of DNA-dependent protein kinase sensitizes breast cancer cells following ionizing radiation. *J Pharmacol Exp Ther* 2002;303:753-9.
43. Li GC, He F, Shao X, et al. Adenovirus-mediated heat-activated antisense Ku70 expression radiosensitizes tumor cells *in vitro* and *in vivo*. *Cancer Res* 2003;63: 3268-74.
44. Jhappan C, Morse HC III, Fleischmann RD, Gottesman MM, Merlino G. DNA-PKcs: a T-cell tumour suppressor encoded at the mouse scid locus. *Nat Genet* 1997;17: 483-6.
45. Gu Y, Jin S, Gao Y, Weaver DT, Alt FW. Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination. *Proc Natl Acad Sci U S A* 1997;94:8076-81.
46. Nussenzweig A, Sokol K, Burgman P, Li L, Li GC. Hypersensitivity of Ku80-deficient cell lines and mice to DNA damage: the effects of ionizing radiation on growth, survival, and development. *Proc Natl Acad Sci U S A* 1997;94:13588-93.

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Cancer Res 2005;65:4368-4375.

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