Adenovirus-Mediated Expression of a Dominant Negative Ku70 Fragment Radiosensitizes Human Tumor Cells under Aerobic and Hypoxic Conditions

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Abstract

Ku70 is one component of a protein complex, the Ku70/Ku80 heterodimer, which binds to DNA double-strand breaks and activates DNA-dependent protein kinase (DNA-PK), leading to DNA damage repair. Our previous work has confirmed that Ku70 is important for DNA damage repair in that Ku70 deficiency compromises the ability of cells to repair DNA double-strand breaks, increases the radiosensitivity of cells, and enhances radiation-induced apoptosis. Because of the radioresistance of some human cancers, particularly glioblastoma, we examined the use of a radio-gene therapy paradigm to sensitize cells to ionizing radiation. Based on the analysis of the structure-function of Ku70 and the crystal structure of Ku70/Ku80 heterodimer, we designed and identified a candidate dominant negative fragment involving an NH_2-terminal deletion, and designated it as DNKu70. We generated this mutant construct, stably overexpressed it in Rat-1 cells, and showed that it has a dominant negative effect (i.e., DNKu70 overexpression results in decreased Ku-DNA end-binding activity, and increases radiosensitivity). We then constructed and generated recombinant replication-defective adenovirus, with DNKu70 controlled by the cytomegalovirus promoter, and infected human glioma U-87 MG cells and human colorectal tumor HCT-8 cells. We show that the infected cells significantly express DNKu70 and are greatly radiosensitized under both aerobic and hypoxic conditions. The functional ramification of DNKu70 was further shown *in vivo*: expression of DNKu70 inhibits radiation-induced DNA-PK catalytic subunit autophosphorylation and prolongs the persistence of γ-H2AX foci. If radiation-resistant tumor cells could be sensitized by down-regulating the cellular level/activity of Ku/DNA-PK, this approach could be evaluated as an adjuvant to radiation therapy. [Cancer Res 2007;67(2):634–42]

Introduction

For the survival of irradiated mammalian cells, the repair of radiation-induced double-strand break is essential (1–6), and such repair apparently involves two mechanistically distinct, but sometimes overlapping, pathways: homologous recombination and nonhomologous end joining. Recent evidence indicates that the DNA-dependent protein kinase (DNA-PK), activated by DNA ends, is a central component in the nonhomologous end joining pathway. DNA-PK is a serine/threonine kinase that consists of a large catalytic subunit of M_r 465,000 (DNA-PKcs) and a DNA-targeting component Ku, which itself is a heterodimer of M_r 70,000 and M_r 86,000 polypeptides (Ku70 and Ku80, respectively; refs. 7, 8). To understand the mechanistic and functional aspects of Ku and DNA-PKcs in modulating stress responses *in vitro* and *in vivo*, we and others have established $Ku80^{-/-}$, $Ku70^{-/-}$, and $DNA\text{-}PKcs^{-/-}$ cell lines and generated $Ku80^{-/-}$, $Ku70^{-/-}$, and $DNA\text{-}PKcs^{-/-}$ homozygous mutant mice (9–11). These studies clearly show that the absence of Ku protein results in radiation hypersensitivity, deficiencies in DNA double-strand break repair and V(D)J recombination, and impaired lymphocyte development. The use of the Ku- and DNA-PKcs–deficient cell lines has yielded much information on the roles of Ku and DNA-PKcs in transcriptional regulation, mammalian stress response, and recombination processes.

Given the crucial role of the DNA-PK complex in determining the response of cells to radiation, targeting the components of this complex represents an appealing opportunity to increase the radiosensitivity of mammalian cells (12, 13). The purpose of this study was to identify a dominant negative construct of Ku70 (designated as DNKu70) and to examine the feasibility of using adenovirus-mediated expression of DNKu70 in a radio-gene therapy paradigm to sensitize radioresistant human tumor cells to ionizing radiation. Based on data obtained from structure-function analyses of Ku70 and Ku80 (14–17), we hypothesized that a construct with a deletion of the NH_2-terminal region of Ku70 might be a potential candidate. Thus, we generated an NH_2-terminal deleted mutant construct, stably overexpressed it in Rat-1 cells, and showed that it has a dominant negative effect (i.e., overexpression of DNKu70 in Rat-1 cells resulted in decreased Ku-DNA end-binding activity and increased radiosensitivity). We then constructed recombinant replication-defective virus rAd(CMV-DNKu70) in which the DNKu70 gene fragment is placed under the control of the cytomegalovirus (CMV) promoter and infected human glioma U-87 MG cells and human colorectal tumor HCT-8 cells with them. Our data show that DNKu70 protein is significantly expressed in the infected cells and that their radiosensitivity is greatly increased. The functional ramification for the role of DNKu70 in modifying DNA damage repair was further shown *in vivo*; that is, expression of DNKu70 inhibits ionizing radiation–induced DNA-PKcs autophosphorylation and prolongs the persistence of γ-H2AX foci formation after ionizing radiation.

Hypoxic regions are known to exist in many human cancers, and hypoxia-induced radioresistance has been postulated to be an obstacle in achieving local control in tumors with sizable hypoxic fraction. Thus, we investigated the effect of rAd(CMV-DNKu70) on the radiosensitivity of hypoxic U-87 MG cells as well as hypoxic

Note: We note with regret the passing of Dr. Ligeng Li on May 13, 2006.

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HCT-8 cells, and observed that overexpression of DNKu70 protein led to significant radiosensitization. If tumor cells in general and radiation-resistant (due to hypoxia or other factors) tumor cells in particular could be sensitized by modulating the cellular level/ activity of Ku/DNA-PK, this approach could be developed as adjuvant to targeted radiation therapy.

Materials and Methods

Cell culture. For transfection experiments, we used Ku70-deficient mouse embryo fibroblasts (*Ku70*$^{-/-}$ MEF; ref. 11) and Rat-1 rat fibroblast cells, routinely cultured in DMEM (Life Technologies, Inc., Rockville, MD), 10% fetal bovine serum, and antibiotics (18). To evaluate the efficiency of adenovirus-mediated gene transfer and to test whether adenovirus-mediated expression of DNKu70 radiosensitizes tumor cells, we used human glioma U-87 MG and human colorectal carcinoma HCT-8 cells, routinely cultured in Eagle's MEM (for U-87 MG) or RPMI 1640 (for HCT-8), 10% fetal bovine serum, 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, and antibiotics.

Generation of stable cell lines expressing various domains of Ku70. To determine the functional significance of various domains of Ku70 on radiosensitivity, we generated stable cell lines in which the various domains of human Ku70 (14, 15) were constitutively and stably expressed in *Ku70*$^{-/-}$ MEFs. In brief, the human *Ku70* cDNA mutant fragment encoding either an NH_2-terminal deletion protein (ΔN, with amino acid residues 1–61 deleted) or a COOH-terminal deletion protein (ΔC, with amino acid residues 601–609 deleted) was cloned into the expression vector pMV12 (19) containing a hygromycin resistance gene. The resulting pMV12-hKu70ΔN (or pMV12-hKu70ΔC) construct was used to transfect (*i*) *Ku70*$^{-/-}$ MEFs for complementation study in Ku70-deficient cells and (*ii*) Rat-1 cells for assessing the effect of mutant Ku70 overexpression in Ku70-expressing cells (18, 19). Drug-resistant cells were selected in medium containing hygromycin (300 μg/mL) for 2 to 3 weeks and individual colonies were isolated and grown in monolayers.

Radiation and cell survival. Cells were irradiated with a Cs-137 unit (Mark 1 model 68, JL Shephard and Associate, San Fernando CA) at ~2.5 Gy/min, and survival was determined using colony formation assay (20). Cells were trypsinized immediately after irradiation, counted, serially diluted, and plated in 60-mm Petri dishes. After incubation at 37°C for 7 to 8 days (for *Ku70*$^{-/-}$ MEFs and Rat-1 cells) or 2 to 3 weeks (for U-87 MG cells and HCT-8 cells), colonies were fixed, stained, and counted. Surviving fractions were normalized by the plating efficiency of unirradiated controls (~10–30% for U-87 MG and 30–50% for the others). All experiments were done at least thrice.

Antibodies and Western blot analysis. To examine the expression of DNKu70, protein samples (20 μg) were analyzed by Western blotting (19). The affinity-purified mouse monoclonal antibody N3H10 specific to the Ku70 protein was from NeoMarkers (Fremont, CA) (1:200). Secondary antibodies were from Pierce (Rockford, IL; ref. 1:20,000).

To examine the autophosphorylation of DNA-PKcs, nuclear extracts were prepared with a Nuclear Extraction Kit (Imgenex Corp., Port Coquitlam, British Columbia, Canada). Forty micrograms of protein from each sample were analyzed by Western blotting (21, 22). Anti–phospho-DNA-PKcs antibodies (rabbit polyclonal pT2609 and pS2056) were a generous gift from Dr. D. Chen (UT Southwestern Medical Center, The University of Texas, Dallas, TX; refs. 21, 22) and DNA-PKcs 18-2 monoclonal antibody was purchased from NeoMarkers.

Preparation of cell extracts and gel mobility shift assay. Preparation of cell extracts and the gel mobility shift assays were done as described (19). Equal amounts of cellular proteins (30–50 μg) from various cell extracts were incubated with a ^{32}P-labeled double-stranded oligonucleotide, 5′-GGGCCAAGAATCTTCCAGCAGTTTCGGG-3′, containing the heat shock element from the rat heat shock protein 70 promoter (19). The protein-bound and free oligonucleotides were electrophorically separated on 4.5% native polyacrylamide gels. The gel slabs were dried and autoradiographed with Kodak X-OMAT film and a Dupont Cronex Lightning Plus intensifying screen at −80°C.

Fluorescent immunostaining. Fluorescent immunostaining was done as described (23). Cells grown on eight-well chamber slides were exposed to 0 or 2 Gy of γ-radiation and returned to 37°C incubation for 1 or 12 h. The cells were fixed with 4% paraformaldehyde, stained with mouse monoclonal anti–γ-H2AX (Upstate Biotech, Chicago, IL) antibody, and then with Cy5 goat anti-mouse secondary antibody (Biomeda, Foster City, CA). Cells were washed in PBS and mounted using Vectashield mounting medium with 4,6 diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). All the cells were processed simultaneously and all the images were obtained using the same parameters (23).

Generation of recombinant replication-defective adenovirus construct containing the DNKu70 fragment. We have constructed the replication-defective adenovirus (Ad5-ΔE1/E3) in which the DNKu70 fragment was placed under the control of the CMV promoter using AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA). First, the 1,647-bp cDNA fragment encoding the COOH-terminal human *Ku70* gene (amino acid residues 62–609) was cloned in the pShuttle-CMV vector, and the presence of this insert was confirmed by restriction enzyme digestion and sequence analysis. To produce the recombinant adenovirus (rAd) plasmid, the bacteria BJ5183 cells were cotransformed with linearized pShuttle vector (containing *DNKu70* gene fragment under the control of CMV promoter) and the pAdEasy-1 vector. The individual positive recombinant adenovirus plasmid is then used to transform XL10-Gold Ultracompetent cells, so that the recombinant adenovirus plasmid DNA can be amplified. The amplified recombinant adenovirus plasmid DNA was purified, confirmed by *Pac*I digestion and sequence analysis, and used to transfect HEK 293 cells.

Generation of primary adenovirus stock with recombinant adenovirus plasmid, virus amplification, and titer determination. To prepare the primary adenovirus stock, the recombinant adenovirus plasmid DNA was linearized by *Pac*I digestion and used to transfect HEK 293 cells using the Stratagene MBS Mammalian Transfection Kit with a modified calcium phosphate transfection protocol. The transfected cells were incubated at 37°C for 7 to 10 days, then harvested and subjected to four freeze/thaw cycles using, alternately, liquid nitrogen and a 37°C water bath. Cell lysates were centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant (primary virus stock) was transferred to a fresh screw-cap mini-centrifuge tube and stored at −80°C.

Recombinant adenoviruses were further amplified to produce sufficient viruses for this study. This was achieved by infecting larger quantities of exponentially growing low-passage HEK 293 cells with the virus stock. Infected cells were harvested and, after four freeze/thaw cycles, the cell lysates were centrifuged on cesium chloride step gradients at 60,000 × *g* at 4°C for 2 h to separate viruses from defective particles and empty capsids. Recovered virus bands were dialyzed thrice against PBS. Viruses were aliquoted and stored at −80°C. Titers of the virus were determined in terms of plaque-forming units and 50% tissue culture infectious dose as previously described (24).

Adenovirus infection of experimental cells. Exponentially growing cells were infected with the appropriate amount of replication-defective adenoviruses and incubated at 37°C for 2 h with gentle shaking. Afterwards, fresh growth medium was added to each dish. To examine the kinetics of expression of DNKu70, the infected cells were further incubated at 37°C for 24 to 120 h and DNKu70 expression was determined by Western blot analysis. To assess radiosensitivity, the infected cells were incubated at 37°C for 48 h before γ-ray irradiation and colony formation assay was done. Surviving fractions were normalized by the plating efficiency of unirradiated, infected cells. All experiments were done at least thrice.

The radiosensitivity of the infected cells under hypoxic condition was also determined. The infected cells were incubated at 37°C for 24 h (normoxic), then transferred to an In Vivo$_2$ 400 Hypoxic workstation (Ruskinn, Inc., Cincinnati, OH), and equilibrated for 24 h in a gas mixture of 0.5% O_2, 5% CO_2, and 94.5% N_2 at 37°C and 90% humidity. The hypoxic cells were then irradiated and their survival was determined.

Results

The NH_2-terminal region of Ku70 is essential for radioresistance. To determine the functional significance of the various

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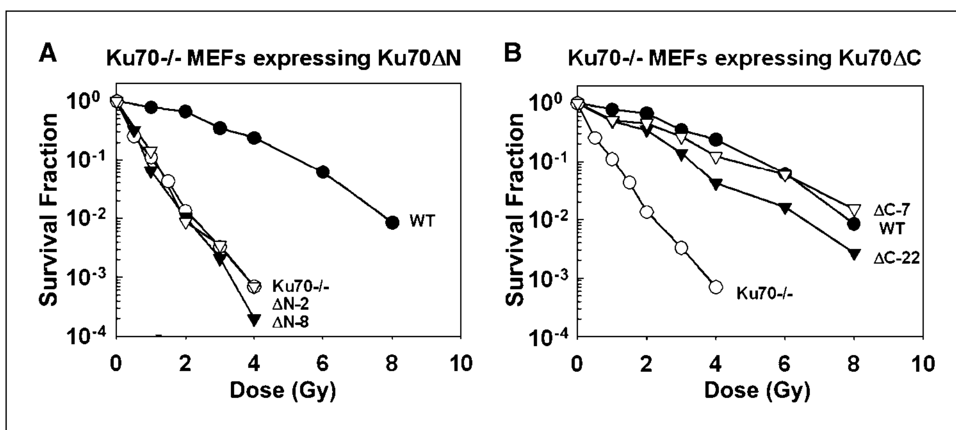

Figure 1. γ-ray survival curves of $Ku70^{-/-}$ MEFs constitutively expressing deletion mutants of human Ku70. SV40-immortalized $Ku70^{-/-}$ MEFs were stably transfected with deletion mutants of human Ku70, missing either the NH_2-terminal 61 amino acid residues (ΔN) or the COOH-terminal 9 amino acids (ΔC), and individual colonies were isolated (e.g., ΔN-2, ΔN-8, ΔC-7, and ΔC-22). These cells were exposed to graded doses of γ-ray and the survivals determined by colony formation assay. *A, $Ku70^{-/-}$* cells expressing the Ku70ΔN were as sensitive as the $Ku70^{-/-}$ cells; *B, $Ku70^{-/-}$* cells expressing Ku70ΔC were as radiation resistant as the wild-type controls. Experiments have been reported thrice and yielded similar results (±10%); for clarity, error bars are not shown.

domains of Ku70 on radiosensitivity, we generated stable cell lines in which the various domains of Ku70 (14, 15) were constitutively expressed in the knockout $Ku70^{-/-}$ MEFs. In brief, SV40-immortalized $Ku70^{-/-}$ MEFs were stably transduced with expression vectors containing the deletion mutants of human Ku70, missing either the NH_2-terminal 61 amino acid residues (designated as ΔN) or the COOH-terminal 9 amino acids (designated as ΔC), and individual stable colonies were isolated (designated as ΔN-2, ΔN-8, ΔC-7, and ΔC-22). These cells, which were in exponential growth phase, were exposed to graded doses of γ-ray and the survivals were determined by colony formation assay (Fig. 1). Our results showed that (*i*) $Ku70^{-/-}$ cells were much more sensitive to γ-ray than their wild-type cells; (*ii*) $Ku70^{-/-}$ cells expressing the NH_2-terminal deletion mutant Ku70ΔN were as sensitive as the knockout $Ku70^{-/-}$ cells; and (*iii*) $Ku70^{-/-}$ cells expressing the COOH-terminal deletion mutant Ku70ΔC were as radiation resistant as the wild-type controls and $Ku70^{-/-}$ cells expressing the intact human Ku70 protein. Therefore, our data showed that the NH_2-terminal region (amino acid residues 1–61) of Ku70 is essential, but the COOH-terminal 9 amino acids (amino acids 601–609) are not needed for conferring radiation resistance (i.e., restoring the radiation resistance of $Ku70^{-/-}$ MEFs to that of the wild-type MEFs). This provided the baseline data for the design and construction of the DNKu70 expression vector described below.

Expression of DNKu70 sensitizes Rat-1 cells to ionizing radiation. Based on the functional analysis of Ku70 described above and elsewhere (14, 15) and the crystal structure of Ku70/Ku80 heterodimer (16, 17), we identified a candidate DNKu70 fragment involving an NH_2-terminal deletion of the amino acid residues 1 to 61 of human Ku70 (designated as DNKu70). We generated the expression vectors containing this DNKu70 fragment (e.g., the amino acid residues 62–609 of human Ku70) and stably transfected Rat-1 cells with the plasmids. Individual colonies were isolated and designated as DN-7, DN-9, or DN-11, DN-13, or DN-14. The expression of the 60 kDa mutant protein (DNKu70) was examined by Western blot analysis with antibodies specifically directed toward

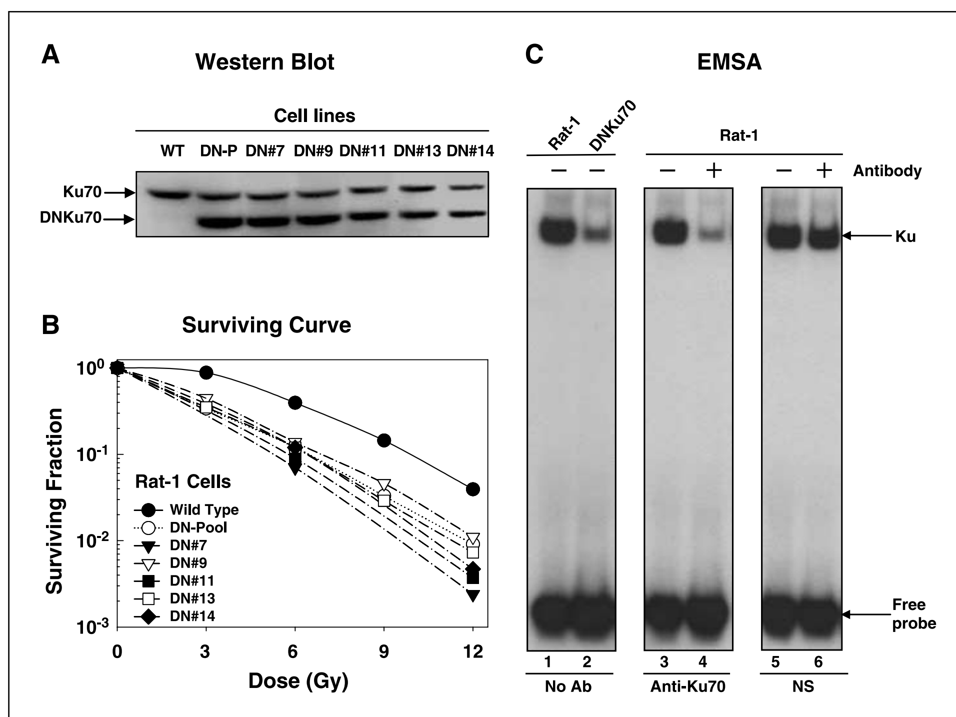

Figure 2. Overexpression of DNKu70 sensitizes Rat-1 cells to ionizing radiation. Rat-1 cells were stably transfected with a plasmid containing the NH_2-terminal deletion mutant of human Ku70 (designated as *DNKu70*) and individual colonies were isolated. *A,* Western blot analysis with Ku70-specific antibody. *WT,* parental Rat-1 cells; *DN#7, DN#9, DN#12, DN#13,* and *DN#14,* individually isolated clones of Rat-1 cells stably expressing DNKu70. *B,* the radiation response, measured using clonogenic survival assay, shows that this DNKu70 fragment has dominant negative activity, increasing the radiosensitivity of Rat-1 cells; for clarity, error bars are not shown. *C,* the Ku-DNA end-binding activity for Rat-1 parental cells (*Rat-1*) and Rat-1 cells overexpressing DNKu70 (*DNKu70*) was determined by electrophoresis mobility shift assay.

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the COOH-terminal fragment of Ku70. Figure 2*A* shows that DNKu70 of the appropriate size was expressed in DN-7, DN-9, or DN-11, DN-13, and DN-14, but not in the parental Rat-1 cells. Furthermore, the expression of DNKu70 does not impair the expression of full-length Ku70.

To examine whether expression of the DNKu70 affected radiosensitivity, the response of Rat-1, DN-7, DN-9, DN-11, DN-13, and DN-14 cell lines to ionizing radiation was measured. Figure 2*B* clearly shows that overexpression of DNKu70 radiosensitizes Rat-1 cells. Repeated evaluations showed that the radiosensitivity of these cells remains the same for >6 months after clonal isolation (data not shown). Thus, the expression of DNKu70 can have a stable dominant negative effect, radiosensitizing Rat-1 cells.

To gain insight into the basis for such radiosensitization, we examined the double-strand DNA end-binding activity in parental and Rat-1 cells overexpressing DNKu70 by the electrophoresis mobility shift assay (Fig. 2*C*). It has previously been shown that Ku (Ku70/Ku80 heterodimer complex) represents the major DNA end-binding activity in whole-cell extracts. Thus, there is a band representing the Ku-dependent DNA end-binding activity in Rat-1 cells (Fig. 2*C, lanes 1, 3*, and *5*), which was absent in our previous study with $Ku70^{-/-}$ cells (11). In addition, this band seems to be Ku70 specific, as its intensity was significantly reduced by the addition of anti-Ku70 antibodies (Fig. 2*C, lane 4*) but unaffected by antibodies not specific to Ku70 (Fig. 2*C, lane 6*). Importantly, the intensity of this band was decreased in Rat-1 cells overexpressing DNKu70 (Fig. 2*C, lane 2*) relative to the parental Rat-1 cells. Furthermore, there was no evidence for a smaller DNA end-binding product in DNKu70-expressing cells, suggesting that neither DNKu70 alone nor a putative DNKu70-Ku80 heterodimer has DNA end-binding activity. These results show that DNKu70-expressing cells have decreased Ku-DNA end-binding activity relative to Rat-1 cells, providing an explanation for their increased radiosensitivity. Taken together, these data suggest that DNKu70 confers a dominant negative effect and provide an important mechanism to sensitize radioresistant cells.

Adenovirus-mediated DNKu70 expression radiosensitizes human glioma U-87 MG cells and human colorectal carcinoma HCT-8 cells under both aerobic and hypoxic conditions. To test the hypothesis that overexpression of DNKu70 can sensitize human tumor cells, we generated large quantities of rAd(CMV-DNKu70) in 293 cells, purified and determined the titers in plaque-forming units, and confirmed with 50% tissue culture infectious dose. We first examined the efficiency and efficacy of the adenovirus-mediated expression of DNKu70 in U-87 MG cells as a function of multiplicity of infection (MOI). Exponentially growing U-87 MG cells were infected with rAd(CMV-DNKu70) at MOIs of 1, 5, 25, 50, and 100; 72 h after infection, the expression of DNKu70 was examined by Western blot analysis (Fig. 3*A*). As shown in Fig. 3*A*, DNKu70 is abundantly expressed in infected U-87 MG cells at an MOI of ≥5. We then examined the kinetics of DNKu70 expression as a function of time after infection. Figure 3*B* shows that DNKu70 expression is apparent at 12 h after infection, with its level reaching a maximum at 48 to 72 h and remaining high at 120 h.

To examine the effect of adenovirus-mediated constitutive expression of DNKu70 on radiosensitivity, U-87 MG cells were infected with these viruses at an MOI of 50, and the DNKu70 protein level and the radiosensitivity under aerobic condition were then determined. The data in Fig. 4*A* and *B* clearly showed that, 48 h after infection, U-87 MG cells significantly expressed DNKu70 and were greatly radiosensitized. For example, at a dose of 6 Gy, their surviving fraction in air is ~30% (*solid circle*), as compared with ~5% (*open circle*) when infected with rAd(CMV-DNKu70). A baseline study of three experiments comparing parental and rAd(CMV-DNKu70)–infected U-87 MG cells yielded respective plating efficiencies of 14% and 22%, 11% and 7.3%, and 12% and 14%, respectively (i.e., 12.3 ± 1.5% for parental and 14.4 ± 7% for recombinant adenovirus–infected cells). These data show that the knockdown of Ku70 activity did not affect the viability of U-87 MG cells.

The radiosensitization effect of DNKu70 was also examined in human colorectal tumor HCT-8 cells. Figure 4*C* and *D* clearly shows that, 48 h after infection, the DNKu70 is significantly expressed in HCT-8 cells, and the radiosensitivity is greatly increased. For example, at a dose of 6 Gy, the surviving fraction of control cells is ~10% (*solid circle*), as compared with ~3% (*open circle*) for the HCT-8 cells infected with rAd(CMV-DNKu70).

Tumor hypoxia, present in many human cancers, is associated with radioresistance and treatment failure. Thus, we evaluated the radiosensitivity of rAd(CMV-DNKu70)–infected cells under hypoxic condition (0.5% O_2). Figure 4*A* and *C* clearly shows that adenovirus-mediated expression of DNKu70 radiosensitizes both hypoxic U-87 MG and HCT-8 cells.

Expression of DNKu70 inhibits ionizing radiation–induced Ser^{2056} and Thr^{2609} phosphorylation of DNA-PKcs *in vivo*. To examine the functional ramification of the inhibition of Ku-DNA binding activity by DNKu70 expression in Fig. 2*C*, we studied the

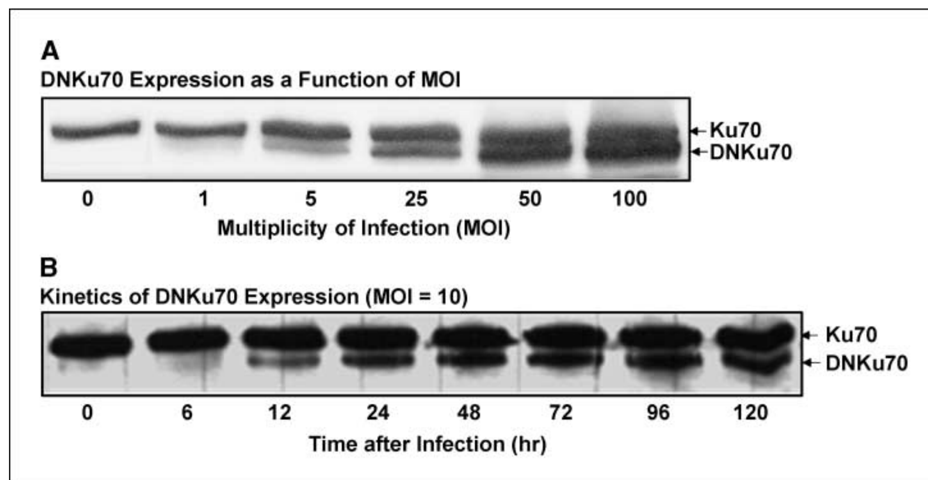

Figure 3. Adenovirus-mediated expression of a DNKu70 fragment in U-87 MG cells. *A,* DNKu70 expression as a function of MOI. Exponentially growing U-87 MG cells were infected with rAd(CMV-DNKu70) at MOIs of 1, 5, 25, 50, and 100; 72 h after infection, the expression of DNKu70 was examined by Western blot analysis with anti-Ku70 antibody. *B,* kinetics of DNKu70 expression. U-87 MG cells were infected with rAd(CMV-DNKu70) at an MOI of 10, and the expression of DNKu70 at 6, 12, 24, 48, 72, 96, and 120 h after infection was examined by Western blot analysis with anti-Ku70 antibody. An equal amount of protein (20 μg) from each sample was used for our analysis.

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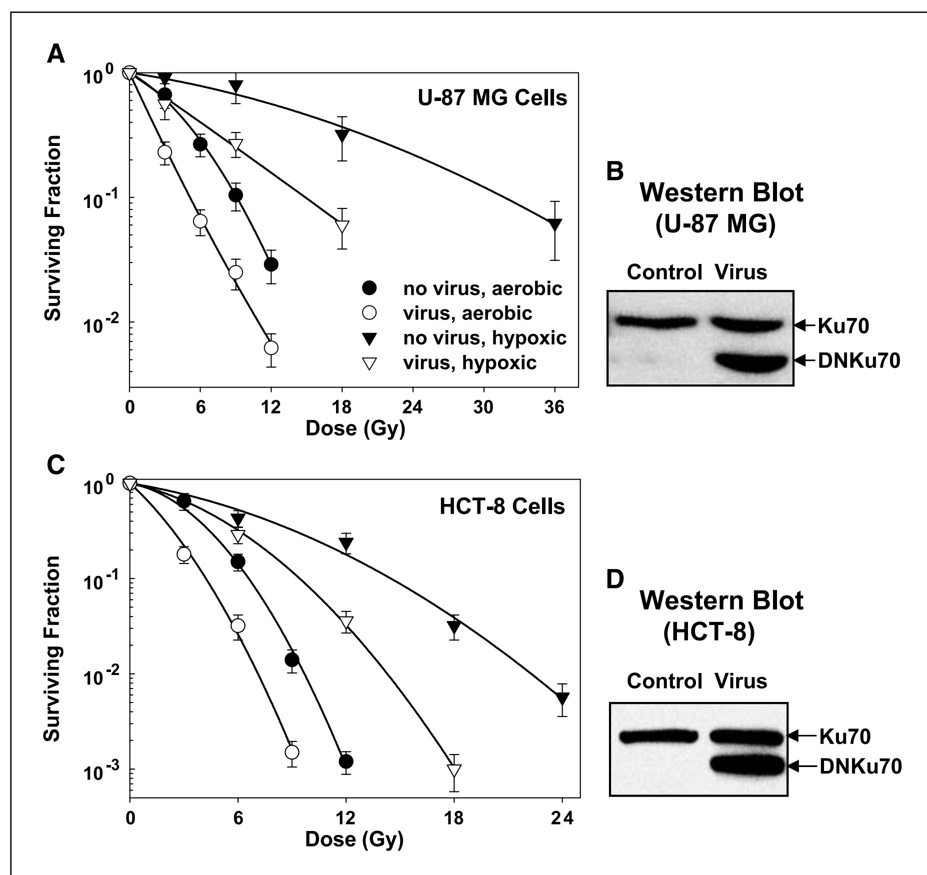

Figure 4. Adenovirus-mediated constitutive expression of DNKu70 radiosensitizes U-87 MG cells and HCT-8 cells under aerobic and hypoxic conditions. Cells were infected with rAd(CMV-DNKu70) at an MOI of 50 and, 48 h after infection, one group of cells was exposed to graded doses of γ-ray under aerobic condition and survival was determined by colony formation assay (*A* and *C*), and the second group of cells was used to determine DNKu70 expression by Western blot analysis with Ku70-specific antibody (*B* and *D*; control, uninfected cells). To examine the radiosensitivity of infected cells under hypoxic condition, cells were infected with rAd(CMV-DNKu70) at an MOI of 50, incubated at 37°C for 24 h (normoxic), then transferred to an In Vivo$_2$ 400 Hypoxic workstation (Biotrace) and equilibrated for 24 h in a gas mixture of 0.5% O_2, 5% CO_2, and 94.5% N_2 at 37°C and 90% humidity. The hypoxic cells were then irradiated and their survival was determined by colony formation assay (*A* and *C*). *A* and *B*, U-87 MG cells; *C* and *D*, HCT-8 cells. ●, no virus, cells irradiated under aerobic condition; ○, cell infected with virus and irradiated under aerobic condition; ▲, no virus, cells irradiated under hypoxic condition; △, cells infected with virus and irradiated under hypoxic condition.

effect of DNKu70 expression on the activation of DNA-PK *in vivo* after ionizing radiation treatment. We infected U-87 MG and HCT-8 cells with rAd(CMV-DNKu70) and, 72 h later, irradiated them (10 Gy). After 30 or 60 min at 37°C, cells were harvested and nuclear extracts were prepared. Western blotting analyses were done using anti-pS2056 phosphospecific antibodies or anti-pT2609 phosphospecific antibodies, which recognize ionizing radiation–induced Ser^{2056} or Thr^{2609} phosphorylation of DNA-PKcs, respectively. Figure 5 clearly shows that in control U-87 MG (*right*) and HCT-8 cells (*left*), the DNA-PKcs are autophosphorylated at Ser^{2056} and Thr^{2609} in response to ionizing radiation (*lanes 2, 3, 6*, and *7*), a result consistent with the findings by other investigators (21, 22). In contrast, in rAd(CMV-DNKu70)–infected U-87 MG and HCT-8 cells, this autophosphorylation of Ser^{2056} and Thr^{2609} in response to

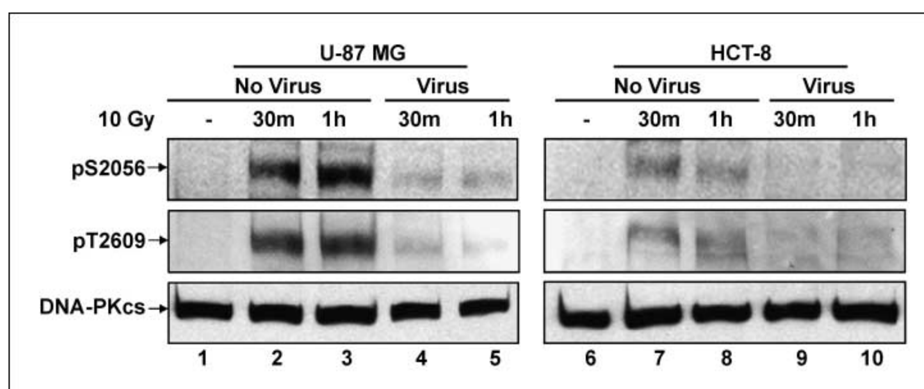

Figure 5. Radiation-induced Ser^{2056} and Thr^{2609} phosphorylation of DNA-PKcs *in vivo* is inhibited by DNKu70 expression. Human U-87 MG glioma cells and colorectal cancer HCT-8 cells were mock infected (*lanes 1–3* and *lanes 6–8*) or infected with replication-defective adenovirus rAd(CMV-DNKu70) containing a CMV promoter–driven DNKu70 (*lanes 4, 5, 9*, and *10*). Seventy-two hours after infection, the infected (or control mock infected) cells were irradiated (10 Gy) and then incubated at 37°C for 30 or 60 min. Cells were then harvested and nuclear extracts were prepared. Ser^{2056} and Thr^{2609} phosphorylation of DNK-PKcs in response to ionizing radiation were analyzed by Western blotting using anti-pS2056 phosphospecific antibodies (*top*) or anti-pT2609 phosphospecific antibodies (*middle*), or anti–DNA-PKcs antibody (*bottom*). Forty micrograms of nuclear extracts were loaded for each lane, for U-87 MG cells (*lanes 1–5*) and for HCT-8 cells (*lanes 6–10*). *Lanes 1* and *6,* no virus, no ionizing radiation; *lanes 2, 3, 7,* and *8,* no virus, cells irradiated at 10 Gy and allowed to recover for 30 min or 1 h; *lanes 4, 5, 9,* and *10,* virus-infected cells, irradiated at 10 Gy and allowed to recover for the indicated times (30 min or 1 h). In unirradiated, virus-infected cells, Ser^{2056} and Thr^{2609} of DNA-PKcs are not phosphorylated (data not shown).

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ionizing radiation is significantly reduced (*lanes 4, 5, 9*, and *10*), whereas the DNA-PKcs protein levels in control and in virus-infected U-87 MG and HCT-8 cells are comparable (Fig. 5).

Effect of DNKu70 expression on ionizing radiation–induced γ-H2AX foci formation in HCT-8 and U-87 MG cells. To determine whether DNKu70 expression–mediated radiosensitization is due to inhibition of DNA double-strand break repair *in vivo*, we monitor the fate of double-strand breaks using histone γ-H2AX as a marker. Phosphorylation of H2AX, which creates γ-H2AX foci, occurs *in situ* within a megabase domain of chromatin flanking each double-strand break (25), and γ-H2AX foci serve as useful surrogate for unrepaired, residual double-strand breaks over a wide range of radiation doses (25–27). Analysis of the kinetics of γ-H2AX clearance after ionizing radiation reveals a correlation between increased γ-H2AX persistence and unrepaired DNA damage and cell death (28). Recent genetic and biochemical studies suggest that phosphorylated H2AX (γ-H2AX) plays an important role in the recruitment and/or retention of DNA repair and checkpoint proteins (28). *H2AX*$^{-/-}$ mouse embryonic fibroblasts are radiation sensitive and show deficiency in DNA damage repair compared with their wild-type counterparts (29).

Control and rAd(CMV-DNKu70)–infected U-87 MG and HCT-8 cells were irradiated with 0 or 2 Gy and, 1 or 12 h later, were fixed and stained with antibodies specific to γ-H2AX (Fig. 6*A* and *B*, HCT-8 and U-87 MG, respectively). In control HCT-8 and U-87 MG cells (Fig. 6*A* and *B, top*), γ-H2AX foci formation is evident and abundant at 1 h after 2 Gy, and decreased significantly by 12 h post-ionizing radiation. By comparison, the adenovirus-mediated DNKu70 expression significantly reduces γ-H2AX foci formation at 1 h post-ionizing radiation treatment in both cell types.

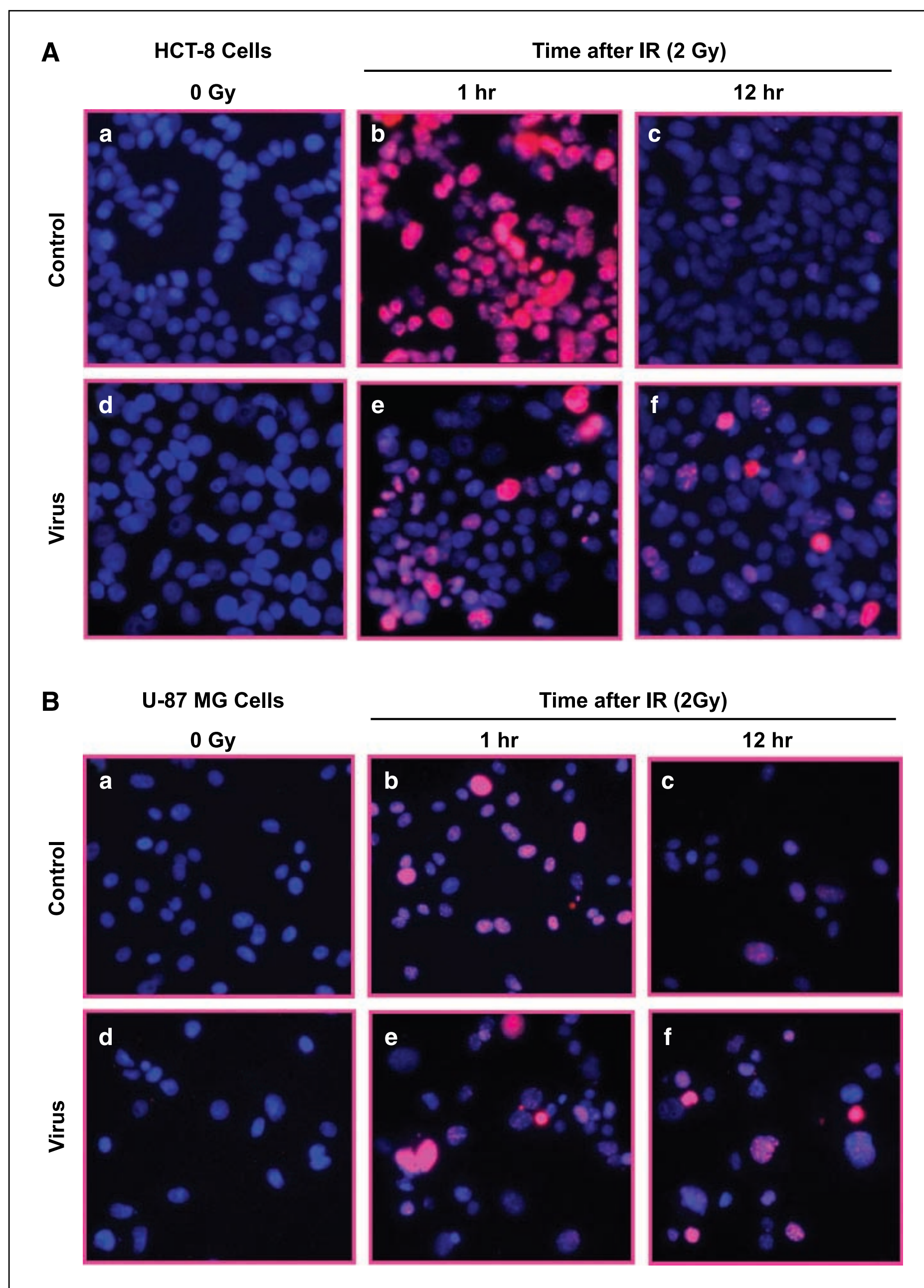

Figure 6. Effect of adenovirus-mediated expression of DNKu70 on ionizing radiation–induced γ-H2AX foci formation in HCT-8 and U-87 MG cells. Control and virus-infected U-87 MG and HCT-8 cells were irradiated with 2 Gy. Cells were fixed at 1 or 12 h after irradiation and stained with 4′,6-diamidino-2-phenylindole (*blue*) and anti–γ-H2AX antibody (*red*). Results of γ-H2AX foci formation (*merged image*) were shown for HCT-8 cells (*A*) and U-87 MG cells (*B*). *Top,* control uninfected cells; *bottom,* adenovirus-infected cells expressing DNKu70. In the control HCT-8 and U-87 MG cells, γ-H2AX foci formation is abundant at 1 h after 2 Gy and decreased significantly by 12 h post-ionizing radiation (*IR*; compare *b* and *c* in both *A* and *B*). By comparison, the adenovirus-mediated DNKu70 expression in HCT-8 and U-87 MG cells significantly reduces γ-H2AX foci formation (compare *b* and *e* in both *A* and *B*), and prolongs the persistence of γ-H2AX foci even at 12 h post-ionizing radiation (compare *e* and *f* in both *A* and *B*).

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Furthermore, DNKu70 expression prolongs the persistence of γ-H2AX foci at relatively high levels at 12 h post-ionizing radiation in the infected cells (Fig. 6*A* and *B, bottom*).

Discussion

Ionizing radiation induces several types of DNA lesions, such as base damage, DNA single-strand breaks, and DNA double-strand breaks. Nonhomologous end joining represents the major pathway for the repair of double-strand breaks in mammalian cells and is essential for the survival of irradiated cells (1–6). A major participant in this pathway is DNA-PK, which consists of DNA-PKcs and the heterodimeric Ku. Ku is composed of two tightly associated, but different, polypeptides of 70 and 80 kDa (Ku70 and Ku80) and has double-strand DNA end-binding activity, thereby targeting the complex to DNA ends. In the past decades, studies using various mutant cell lines and knockout mice and their respective MEFs have shown that defects in Ku70, Ku80, or DNA-PKcs led not only to hyperradiosensitivity and deficiency in DNA damage repair but also to altered V(D)J recombination. Given the crucial role of DNA-PK complex in determining the response of cells to ionizing radiation, targeting the components of this complex represents an appealing approach to radiosensitize mammalian cells. In this study, we have provided the proof of principle of using adenovirus-mediated gene transfer of a DNKu70 fragment as a strategy to radiosensitize human glioma U-87 MG tumor and human colorectal cancer HCT-8 cells.

Previously, we have derived $Ku70^{-/-}$ MEFs and showed that they are much more radiosensitive than the wild-type cells (Fig. 1). To determine the significance of the various domains of Ku70 relative to radiosensitivity, we generated stable cell lines in which specific domains of Ku70 were constitutively expressed in $Ku70^{-/-}$ MEFs and found that the NH_2-terminal region (amino acid residues 1–61) of Ku70 is essential, but that the COOH-terminal is not needed, for conferring radioresistance (Fig. 1). In this study, based on our own data in Fig. 1 and analyses of the structure-function of Ku70/Ku80 heterodimer by others (14–17), we hypothesized that a construct with a deletion of the NH_2-terminal region of Ku70 might be a potential candidate. Thus, we generated an expression vector containing a candidate DNKu70 (62–609 amino acids, involving an NH_2-terminal deletion), stably overexpressed it in Rat-1 cells, and showed that its overexpression resulted in decreased Ku-DNA end-binding activity and increased radiosensitivity (Fig. 2). These data suggest that this DNKu70 confers a dominant negative effect and may be used to sensitize radioresistant cells.

Encouraged by the results described above (Fig. 2), we generated replication-defective adenovirus rAd(CMV-DNKu70). Our data show that U-87 MG cells can be efficiently transduced by these viruses (Fig. 3*A*), with an abundant DNKu70 expression that reaches a maximum at ∼48 h and remains high at 120 h after infection (Fig. 3*B*). As a result of the adenovirus-mediated expression of DNKu70, the radiosensitivity of the infected U-87 MG cells is greatly increased (Fig. 4). We have extended our study to other human tumor cells and showed that DNKu70 expression also radiosensitizes human colorectal tumor HCT-8 cells. As discussed in a subsequent paragraph, these *in vitro* findings are directly extensible to *in vivo* systems, as was shown in our previous studies with antisense Ku70 following the same strategy (12).

Malignant glioma represents the majority of primary brain tumors. Many clinical trials, conducted by the Brain Tumor Study Group and others, have shown that external radiation therapy after surgery is the best treatment for malignant glioma (30). Analysis of the data from these trials evinced a dose response, with better survival at 60 Gy than at 50 Gy (31). However, the maximum tolerated dose with conventional fractionated radiotherapy is 60 to 65 Gy, with an unacceptable incidence of radiation injury to the brain at higher doses (32). Due to this constraint on dose to normal brain tissue, local control of glioblastoma has been difficult to achieve. Because glioblastoma is among the most radioresistant of tumors, there has been much interest and research in improving its treatment outcome. However, despite the use of many adjunctive treatment strategies in addition to surgery, chemical radiosensitizers, stereotactic irradiation, and variation in dose fractionation, the prospect of cure or long-term survival is poor. This is partly due to the radiation sensitivity of normal brain, but more so to the intrinsic radioresistance of glioblastomas.

Another factor that could negatively affect the radiocurability of glioblastoma is tumor hypoxia, which is known to exist in many human cancers. Thus, we tested the applicability of our approach to radiosensitize hypoxic U-87 MG cells. As shown in Fig. 4, our experimental results convincingly show that adenovirus-mediated expression of DNKu70 increases the radiosensitivity of hypoxic U-87 MG cells.

This study is an extension of our previous investigation that showed that adenovirus-mediated, heat-activated antisense Ku70 expression can modulate Ku70 protein level and radiosensitize tumor cells *in vitro* (12). As described in the article (12), we also conducted *in vivo* studies, injecting into FSa-II tumors the recombinant adenovirus vectors containing an antisense Ku70 under the control of a heat shock inducible promoter. Our data showed that 42.5°C heat shock induced antisense Ku70 expression and significantly attenuated Ku70 protein in FSa-II tumors. Importantly, adenovirus-mediated, heat-activated antisense Ku70 expression sensitized the tumors to ionizing radiation, as assessed by both *in vivo/in vitro* colony formation assay and tumor growth delay. Thus, given the findings of the present study, it is expected that Ku70 modulation by adenovirus-mediated DNKu70 induction in brain tumors will lead to radiosensitization *in vivo*.

Data from this study indicate that the adenovirus-mediated DNKu70 induction approach may have advantages relative to our previous strategy using adenovirus-mediated, heat-activated antisense Ku70 expression (12). First, the expression of DNKu70 is more stable, reaching a maximal at ∼48 h and remaining high even at 120 h after viral infection (Fig. 3). In comparison, heat-activated antisense Ku70 RNA expression reached a maximum between 8 and 12 h after heat shock and is almost undetectable by 24 h. Second, the degree of radiosensitization achieved in virus-infected DNKu70-expressing cells is higher than that observed in virus-infected antisense Ku70–expressing cells. For example, the surviving fraction at 3 Gy is decreased by 3.5-fold in virus-infected DNKu70-expressing U-87 MG cells, relative to control, whereas the decrease was only 2-fold for U-87 MG cells infected with adenovirus containing heat shock protein 70 promoter–driven antisense Ku70 and heat shocked at 42.5°C for 30 min (data not shown).

Recently, several groups are exploring the combination of gene and radiation therapy with the goal of using gene therapy approaches to increase the radiosensitivity of various tumors (1, 33, 34). The emphasis of this strategy is to further improve on the local control of cancers by high-precision, high-dose radiotherapy, and this is being evaluated in clinical trials (1, 34–42). In this context, our study suggests the potential of significantly radiosensitizing tumor cells under aerobic and hypoxic conditions by

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reducing the cellular activity of Ku/DNA-PK with viral vector–mediated gene delivery. The adequacy of target coverage is an important issue in gene therapy and in gene therapy–mediated radiosensitization. In our laboratory, we are developing strategies and methods to improve target coverage. We believe that improvements will result by combining several approaches: technical innovations for precise targeting, the use of mild hyperthermia to increase vector delivery, and the use of conditionally replicative adenovirus to increase infection efficiency. To achieve precise targeting to the most radioresistant (hypoxic) part of the tumor, we are conducting research on hypoxia image–guided adenovirus delivery based on positron emission tomography imaging with hypoxic cell–specific radiotracers (e.g., ^{18}F-misonidazole). In addition, to improve the uniformity of delivery, we are developing an image-guided robotic system which, under computer control, injects the adenovirus at a matrix of locations within the target. In addition, we are investigating the use of mild hyperthermia to increase vector delivery (43) and the use of conditionally replicative adenovirus to increase infection efficiency (44, 45).

Given that tumor hypoxia is an important factor in treatment outcome and that the hypoxic cells are the most radioresistant, we hypothesize that targeting the hypoxic fraction of the tumor may be an efficacious approach for radio-gene therapy. To test this hypothesis, we are currently generating adenoviruses in which the radiosensitizing effector DNKu70 gene is placed under the control of a hypoxia-inducible promoter. Several laboratories, including ours, have investigated the potential detrimental effect to normal biological processes due to the down-regulation of DNA-PK (10, 11, 46). However, by selecting the appropriate adenoviral vector, the probability of its integration into the host chromosomes is extremely low. In addition, the expression of the effector gene is efficient, but transient, and unlikely to have harmful results for normal tissues.

In this study, the functional ramification for a role of DNKu70 in modifying DNA damage repair was examined. Our results show that the expression of DNKu70 inhibits ionizing radiation–induced DNA-PKcs autophosphorylation and prolongs the persistence of γ-H2AX foci after ionizing radiation. The interplay of these molecular events is not clearly understood at present. However, the ability of DNKu70 to modify the radiation response *in vivo* may provide a link between biochemical, genetic, and cytologic approaches to study double-strand break repair intermediates and pathways. Recently, many studies have suggested γ-H2AX as a therapeutic target for improving the efficacy of radiation therapy (28). Therapies or agents that block γ-H2AX foci formation by inhibiting upstream kinase activity, directly inhibit γ-H2AX function, or increase the persistence of γ-H2AX after ionizing radiation (28, 29, 47–49) all deserve further investigation as potential radiosensitizing modalities.

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